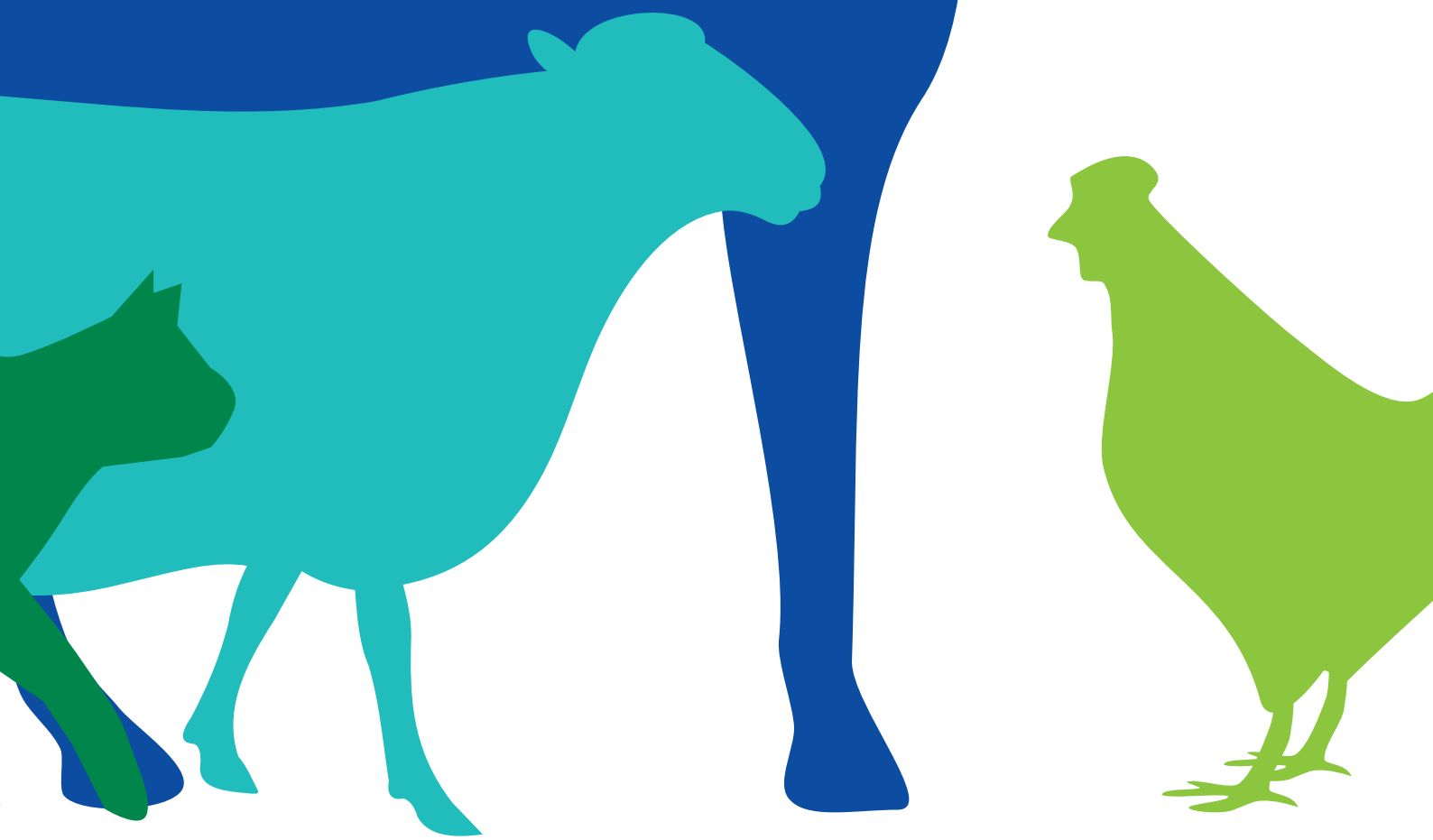
REPRODUCTION IN SOUTH AMERICAN CAMELIDS

EDITED BY: Marcelo Horacio Miragaya and Marcelo H. Ratto
PUBLISHED IN: Frontiers in Veterinary Science



frontiers Research Topics

ISSN 1664-8714
ISBN 978-2-88971-426-1
DOI 10.3389/978-2-88971-426-1

About Frontiers

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers Journal Series

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to Quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.
Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area! Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: frontiersin.org/about/contact

REPRODUCTION IN SOUTH AMERICAN CAMELIDS

Topic Editors:
Marcelo Horacio Miragaya, University of Buenos Aires, Argentina
Marcelo H. Ratto, Austral University of Chile, Chile

Citation: Miragaya, M. H., Ratto, M. H., eds. (2021). Reproduction in South American Camelids. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88971-426-1

Table of Contents

frontiers in Veterinary Science

EDITORIAL
published: 10 August 2021
doi: 10.3389/fvets.2021.722949

Editorial: Reproduction in South American Camelids

Marcelo Ratto[1]* *and Marcelo Horacio Miragaya*[2]

[1] *Instituto de Ciencia Animal, Facultad de Ciencias Veterinarias, Universidad Austral de Chile, Valdivia, Chile,* [2] *Facultad de Ciencias Veterinarias, Instituto de Investigación y Tecnología en Reproducción Animal, Universidad de Buenos Aires, Buenos Aires, Argentina*

Keywords: Llama (Lama glama), alpaca, reproductive biotechnology, theriogenology, andrology

Editorial on the Research Topic

Reproduction in South American Camelids

When we proposed this Research Topic to Frontiers in Veterinary Science, we aimed to invite and congregate the best of the ongoing research that expands our current understanding of reproductive biology in South American camelids (SACs). We did not pursue this only as a way to communicate among the scientific community researching in the field but also to share the fascinating aspects of reproduction in these species to a broad audience, including practitioners and colleagues working on education, conservation, and applied reproduction. In this Research Topic, we received contributions that include a mini-review and several original research articles encompassing fundamental aspects of reproductive physiology and applied reproductive biotechnologies.

SACs are species displaying unique reproductive features, many of which still are not understood; examples of these are their induced ovulatory mechanism and laterality of gestation. In this Research Topic, Berland et al. report neuroanatomical aspects of brain GnRH and kisspeptin neurons in female llamas; these neuropeptide-synthesizing cells orchestrate the release of gonadotrophic hormones that control follicle development and ovulation. A second contribution by Gallelli et al. characterize the temporal association between follicular waves and circulating concentrations of estradiol and insulin grow factor-1 in llamas, showing that the rise of both hormones parallels the development of follicular waves. In another study, Bianchi et al. report evidence of dose-dependent estradiol-induced ovulation in llamas, resignifying the potential role of estradiol in the ovulatory mechanism of SACs. In another piece of original research, Norambuena et al. report that alpacas under moderate energy restriction negatively affect body condition score and CL size, but this does not result in significant changes in CL vascularization and progesterone concentrations. Finally, a study by Ratto et al. show that the ovulation laterality and intrauterine embryo location do not induce asymmetrical differences of the mesometrial and endometrial vascularization area of uterine horns during the first 30 days of gestation.

Several pieces of evidence have shown that the beta-nerve growth factor (β-NGF) –which is also implicated in ovulation in SACs– exerts pivotal roles in the reproductive biology of SACs. In this line, Valderrama et al. show that β-NGF enhances the expression of genes involved in angiogenesis and progesterone synthesis as well as progesterone output in preovulatory llama granulosa cells *in vitro*. After conception, more than 90% of the gestations occur in the left uterine horn in SACs; a study conducted by Barraza et al. characterize the bilateral horn gene and immunohistochemical spatial expression of β-NGF and its receptor, TrkA, in the endometrium of non-pregnant and early pregnant alpacas; the gene expression of the angiogenic factor VEGFA and number of vessels was also assessed.

Closing the set of physiological aspects of reproduction in SACs, El Zawam et al. determine serum testosterone concentrations in response to administration of human chorionic gonadotropin (hCG) and its correlation with testicular weight in alpacas of different age.

OPEN ACCESS

Edited and reviewed by:
Ahmed Tibary,
Washington State University,
United States

***Correspondence:**
Marcelo Ratto
marceloratto@uach.cl

Specialty section:
This article was submitted to Animal Reproduction - Theriogenology, a section of the journal Frontiers in Veterinary Science

Received: *09 June 2021*
Accepted: *15 July 2021*
Published: *10 August 2021*

Citation:
Ratto M and Miragaya MH (2021) Editorial: Reproduction in South American Camelids. Front. Vet. Sci. 8:722949. doi: 10.3389/fvets.2021.722949

The lack of understanding of the peculiar reproductive characteristics of SACs has impeded to make substantial improvement of their low reproductive rates. It is essential to improve and develop new strategies that increase reproductive efficiency, and so the development of biotechnologies is necessary to fulfill these objectives. For example, semen cryopreservation has low efficiency, and when artificial insemination is performed with it, poor results are obtained, affecting the advances in genetic progress and selection. Here, we received a set of different studies related to reproductive biotechnologies in female and male SACs. This set opens with a review by Morrell and Abraham about semen collection and handling in SACs. Original research conducted by Zampini, Castro-González et al. report electron microscopy ultrastructural alterations of llama sperm during cooling and freezing, using a conventional camelid semen cryopreservation protocol. Another study by Carretero et al. shows that the air-drying process has a negative effect on llama sperm DNA. Finally, Valdivia et al. report cryopreservation of alpaca testicular tissue and isolated testicular cells.

Several contributions aimed to develop strategies to enhance the performance of cryopreserved semen were received in this Research Topic. In one of these, Sari et al. report that the addition of β-NGF significantly increases the percentage of total motility and vigor of post-refrigerated sperm. Another study by Guillén Palomino et al. evaluate the efficiency of Androcoll-E^{TM} to separate llama sperm from seminal plasma and freezing extender in frozen-thawed semen, demonstrating their separation while preserving the viability, membrane function, and acrosome integrity. In the same area, Bertuzzi et al. compare a non-commercial extender with egg yolk and the commercial extender Andromed® with and without egg yolk for cooling alpaca sperm obtained from diverted deferent ducts. A study by Aisen et al. investigated the effect of whole seminal plasma addition to alpaca spermatozoa after the freezing-thawing-process on dynamic and morphological parameters and an artificial insemination trial. Closing the set of contributions focused on semen cryopreservation, a study conducted by Lutz et al. report a field-efficient technique for cryopreservation of alpaca preimplantation embryos using a modified horse vitrification protocol.

In the subject of female reproductive biotechnology, Zampini, Veiga et al. report the development of a synchronization and superstimulation protocol for embryo donors in llamas showing that a protocol based on GnRHa, PGF2α, and eCG allows a fixed-timed mating without the use of ultrasonography. In another contribution of Zampini, Gallelli et al. using color-Doppler ultrasonography, they show that the uterine blood flow (UBF), but not corpus luteum blood flow (CLBF), may be a useful predictor for early pregnancy diagnosis in llamas 8 days post-mating.

The papers presented in this Research Topic provide new insights into different aspects of reproductive biology of SACs and also demonstrate the efforts of the current research to provide a better understanding of their peculiarities that, in turn, will allow overcoming challenges in applied reproduction in SACs.

We would like to thank all the authors who contributed to this Research Topic and express our gratitude to the editors, reviewers, and staff of Frontiers in Veterinary Science that made possible this Research Topic in Reproduction in South American Camelids. Finally, we also extend our thanks to Frontiers Media for the fee waivers provided which supported some of the contributions presented in this Research Topic.

AUTHOR CONTRIBUTIONS

MR and MHM wrote the editorial together. Both authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 15 October 2020
doi: 10.3389/fvets.2020.576204

Estradiol-17β Injection Induces Ovulation in Llamas

Carolina P. Bianchi[1,2]*, *Micaela A. Benavente*[1,2], *Florencia Viviani*[1], *María F. Gallelli*[3] and *Marcelo A. Aba*[1,2]

[1] Laboratorio de Endocrinología, Facultad de Ciencias Veterinarias, Universidad Nacional del Centro de la Provincia de Buenos Aires (UNCPBA), Tandil, Argentina, [2] Centro de Investigación Veterinaria Tandil, Consejo Nacional de Investigaciones Científicas y Técnicas de Argentina, Tandil, Argentina, [3] Instituto de Investigación y Tecnología en Reproducción Animal, Facultad de Ciencias Veterinarias, Universidad de Buenos Aires, Consejo Nacional de Investigaciones Científicas y Técnicas de Argentina, Tandil, Argentina

This study aimed to investigate the effect of three different doses of estradiol-17β on ovulation and subsequent luteal development and function in llamas. Twenty-three llamas were examined daily by transrectal ultrasonography until the detection of an ovulatory follicle (≥8 mm). Thereafter, animals were divided into five groups: Control ($n = 3$; treated with 1.6 ml of saline solution), GnRH group ($n = 6$, treated with an intravenous injection of 8.4 μg Buserelin), and estradiol groups that received 0.6 mg (E1, $n = 4$), 1 mg (E2, $n = 4$), or 1.6 mg (E3, $n = 6$) of estradiol-17β intravenously. Detection of ovulation was based on ultrasonographic visualization of disappearance of the largest follicle and subsequent presence of a newly formed corpus luteum (CL) and progesterone concentration exceeding 1 ng ml^{-1}. Daily blood samples were collected to determine plasma progesterone concentration. Ovulation rate was 0% for control and E1 groups, 25% for E2 group, and 100% for GnRH and E3 groups. Differences in the mean CL diameter between GnRH and E3 groups were not statistically significant. Plasma progesterone concentration was similar between groups during the different days in ovulated animals. However, the day that the plasma progesterone concentration was above 1 ng ml^{-1} and the day that the highest plasma progesterone concentration was achieved differed among E3 and GnRH groups, occurring later in females treated with estradiol. In conclusion, an injection of estradiol-17β is capable of inducing ovulation in llamas and the response depends on the dose used. Most of the animals required the highest tested dose (1.6 mg) to induce the ovulatory process. Although the CL diameter in females induced to ovulate with estradiol was similar to that in llamas induced to ovulate with a GnRH analog, the rise in plasma progesterone concentration above 1 ng ml^{-1} and the peak progesterone concentration were attained 1 day later in the estradiol treated females.

Keywords: estradiol-17β, ovulation, corpus luteum, progesterone, llamas

OPEN ACCESS

Edited by:
Marcelo H. Ratto,
Austral University of Chile, Chile

Reviewed by:
Firdous Ahmad Khan,
St. George's University, Grenada
Martin G. Maquivar,
Washington State University,
United States

***Correspondence:**
Carolina P. Bianchi
cbianchi@vet.unicen.edu.ar

Specialty section:
This article was submitted to Animal Reproduction - Theriogenology, a section of the journal Frontiers in Veterinary Science

Received: *25 June 2020*
Accepted: *01 September 2020*
Published: *15 October 2020*

Citation:
Bianchi CP, Benavente MA, Viviani F, Gallelli MF and Aba MA (2020) Estradiol-17β Injection Induces Ovulation in Llamas. Front. Vet. Sci. 7:576204. doi: 10.3389/fvets.2020.576204

INTRODUCTION

Unlike other species such as sheep and cattle, which ovulate spontaneously as part of their estrous cycle, female camelids are induced ovulators requiring an external stimulus in the presence of a follicle with a diameter ≥ 7 mm to elicit the ovulatory process (1, 2). The luteinizing hormone (LH) concentration increases by 20 min after copulation, peaks within 2–3 h, remains high during 5 h, and reaches basal concentration by 12 h post-mating (3). An ovulation-inducing factor identified

as beta nerve growth factor (4, 5) is present in the seminal plasma of males, which induces the LH secretion and ovulation around 30 h post-mating (6). Besides, the administration of exogenous hormones that elicit LH release such as gonadotrophin-releasing hormone (GnRH) or hormones with LH activity such as human chorionic gonadotrophin (hCG) induces ovulation within an interval of ∼29 h [GnRH: (7)] or 26 h [hCG: (1)].

In spontaneously ovulating species, increasing plasma concentration of estradiol-17β, in the absence of plasma progesterone concentration from a corpus luteum (CL), induces the preovulatory surge of LH and, consequently, ovulation (8, 9). Moreover, treatment of heifers with estradiol-17β, without exogenous progesterone or progestogen, is followed by a surge release of LH (10, 11).

In llamas, the LH secretion and the ovulatory response depend on follicular size at the time of mating. Females with follicles ≥7 mm in diameter ovulate while those with smaller ovarian follicles (<6 mm) release less LH and do not ovulate in response to copulation suggesting that this pattern could be due to a reduced estrogen priming of the hypothalamus–pituitary axis (2). Plasma estradiol-17β concentration follows a wavelike pattern with basal concentration recorded, when small follicles are present in the ovaries (12). Although the increase in plasma estrogen concentrations does not elicit the LH preovulatory peak in llamas, it has been suggested that estradiol modulates the LH secretion by the pituitary in response to the intramuscular injection of purified ovulation-inducing factor in this species (13).

All these information show some evidence that estradiol modulates LH secretion in camelids; however, to our knowledge there are no reports evaluating the effect of exogenous administration of estrogens on the ovulatory process in these species. Thus, this study aimed to investigate the effect of three different doses of estradiol-17β on ovulation and subsequent luteal development and function in llamas. The hypothesis was that estradiol-17β is capable of triggering the ovulatory process in llamas and that the response depends on the dose used.

MATERIALS AND METHODS

Field studies were performed in compliance with animal welfare regulations set by the Faculty of Veterinary Sciences, UNCPBA, where activities were conducted. Animals belong to the Faculty of Veterinary Sciences, UNCPBA, and facilities are located in Tandil, Argentina, at 37°S, 60°W. Twenty-three nonpregnant, non-lactating female llamas, ranging between 5 and 12 years of age and with an average body weight of 100 ± 15 kg, were included in the study. All animals were clinically and reproductively healthy with a mean body condition score of 3 (body condition score from 1 = thin to 5 = obese) (14). Llamas were kept in pens isolated from males and fed pasture hay and water *ad libitum*.

Experimental Design

Female llamas were examined daily by transrectal ultrasonography (Mindray, DP 6600 Vet, with 5.0/7.5 variable traducer probe) to assess ovarian status. When a growing follicle with a diameter ≥8 mm, considered ovulatory in this species (2), was observed, animals were randomly assigned to one of five treatments: control group (1.6 ml saline solution, im; $n = 3$); GnRH group (8.4 μg acetate of Buserelin, Receptal®, Intervet, Argentina, iv; $n = 6$) and E1, E2, and E3 groups that received 0.6 ($n = 4$), 1 ($n = 4$), or 1.6 mg ($n = 6$), respectively, of an intramuscular injection of estradiol-17β (17β-estradiol, Rio de Janeiro, Argentina) into the hind leg. Detection of ovulation was based on ultrasonographic visualization of disappearance of the largest follicle and subsequent presence of a newly formed CL and progesterone concentration exceeding 1 ng ml^{-1}.

Thereafter, ultrasonographic examination was performed daily for 12 days to measure the CL diameter, which was estimated by averaging two measurements of the CL diameter at right angles to each other.

Blood samples were collected daily from the day that a growing follicle ≥8 mm was detected (Day 0) and during 12 days in ovulating animals. Blood samples were collected by jugular venipuncture in tubes with heparin (Heparin Sodium, SOBRIUS®, Fada Pharma, Buenos Aires, Argentina) and centrifuged immediately after collection. Plasma was stored at −20°C until hormone assays were performed.

Hormone Determinations

Progesterone was measured using a RIA kit (IM 1188, Beckman Coulter, Immunotech, Czech Republic) previously validated for use with llama blood plasma (15). The intra-assay coefficient of variation was below 5% for concentrations between 0.1 and 40 ng ml^{-1}. The inter-assay coefficients of variation for three quality-control samples were 2.9% (0.5 ng ml^{-1}), 2.2% (2.0 ng ml^{-1}), and 5.8% (10 ng ml^{-1}). The sensitivity of the assay was 0.10 ng ml^{-1}.

Statistical Analysis

Follicle diameters at treatment and at Day 2 were compared among groups by one-way ANOVA followed by Tukey's test.

Normal distribution of the variables was tested in all cases using the Shapiro–Wilk test. For non-normally distributed variables (first day that a CL was detected, day of maximum CL diameter, first day plasma progesterone concentration was above 1 ng ml^{-1}, and day that plasma progesterone concentration returned to values below 1 ng ml^{-1}) the Mann–Whitney test was used to compare GnRH and E3 groups. For the remaining characteristics (maximum CL diameter, maximum progesterone concentration and day of maximum progesterone concentration), a Student *t*-test was applied.

The diameter of the CL and plasma progesterone concentration on the different days and between GnRH and E3 groups were analyzed by repeated measures one-way ANOVA or one-way ANOVA, respectively, followed by Tukey's test.

The percentage of animals that ovulated after the different treatments was compared using Fisher's exact test.

All statistical analyses were conducted using the Infostat Professional 2017 software package. Data are presented as mean ± S.E.M. Differences were considered to be significant when $P < 0.05$, and a tendency was considered when $P < 0.1$.

RESULTS

Mean diameter of the largest follicle at the time of treatment was similar among groups ($P = 0.39$). Ovulation rate differed between groups ($P = 0.0001$). All females treated with 1.6 mg of estradiol-17β (E3 group) ovulated after treatment as all animals injected with Buserelin and only one llama treated with 1 mg of estradiol-17β (E2 group). Ovulation, assessed by the disappearance of the largest follicle, was observed 48 h post injection (Day 2) in all llamas that ovulated in response to the different treatments. Mean diameter of the largest follicle on Day 2 differed among groups ($P = 0.0001$) (**Table 1**).

A CL was first detected after treatment between Days 4 and 5 in GnRH and E3 groups and on Day 4 in the only llama that ovulated in the E2 group. Maximum CL diameter ($P = 0.73$) and the day on which maximal CL diameter was observed ($P = 0.70$) were not different among groups (**Table 2**).

Mean CL diameter was similar between both groups during the experimental period ($P = 0.90$). An effect of day was observed in the GnRH and E3 groups ($P < 0.01$). Mean CL diameter in the GnRH group was smaller on Days 11 and 12 than on Days 6, 7, 8, and 9, and mean CL diameter in the E3 group was smaller on Day 12 than on Days 7, 8, and 9 (**Figure 1**).

The first day that the plasma progesterone concentration was above 1 ng ml^{-1} tended to be different between GnRH and E3 groups, occurring earlier in the first group ($P = 0.07$). The highest plasma progesterone concentration did not differ between both groups ($P = 0.88$). However, the day that the highest plasma progesterone concentration was achieved differed and occurred between Days 6 and 8 in the GnRH group and between Days 7 and 10 in the E3 group ($P = 0.015$). The highest plasma progesterone concentration in the llama that ovulated in the E2 group was recorded on Day 8 post treatment. The day that basal plasma progesterone concentration (below 1 ng ml^{-1}) was observed tended to be different between both groups ($P = 0.09$) and was earlier in the GnRH group (**Table 2**).

Plasma progesterone concentration was similar between groups during the different days ($P = 0.68$). An effect of day was observed in both groups ($P < 0.0001$), and the highest plasma progesterone concentration was reached on Days 7, 8, and 9 post treatment (**Figure 2**).

DISCUSSION

To our knowledge, this is the first report demonstrating that an injection of estradiol-17β is effective to induce ovulation in llamas being the response dependent of the dose. Previously, the administration of estradiol-17β was used in an attempt to control ovarian follicular development in alpacas. D'Occhio et al. (32) have reported that doses of 0.5 or 2 mg of estradiol-17β induce follicular regression and a new wave emergence regardless of the stage of follicular development without reporting ovulations. In a later study, using a dose of 1 mg of estradiol-17β, the author has observed that the diameter of the largest follicle remained >6 mm during all the study, indicating that ovulation did not occur (16). The disclosure with the later study could be related to the dose used. In the present study, an effect of the dose of estradiol-17β was demonstrated, observing ovulation in 100% of the animals when the highest dose (1.6 mg) was tested. Similarly, a positive correlation between the dose of estradiol and the magnitude of the LH surge was reported in other species such as cows (17, 18) and ewes (19, 20). Although llamas are induced ovulators requiring copulation and semen deposition to trigger the ovulatory process (21), a close relationship has been observed between follicular size, plasma concentration of estradiol-17β, and LH secretion. Females with small follicles (<6 mm) secrete lower concentrations of estradiol-17β (12, 22) and respond to mating with less LH release (2). A low percentage of llamas (23) and camels (24) may ovulate spontaneously. In non-mated dromedary camels, it has been

TABLE 2 | Effect of administration of GnRH and 1.6 mg of estradiol-17β (E3 group) on luteal development in llamas (mean ± SEM).

	GnRH	E3
First day CL detected (day)	4.2 ± 0.31^{a}	4.5 ± 0.22^{a}
Maximum CL diameter (mm)	14.8 ± 1.14^{a}	14.3 ± 0.85^{a}
Day maximum CL diameter (day)	7.8 ± 0.31^{a}	8.2 ± 0.31^{a}
First day progesterone concentration > 1 ng ml^{-1} (day)	4.5 ± 0.22^{a}	5.2 ± 0.20^{b}
Maximum progesterone concentration (ng ml^{-1})	7.1 ± 1.29^{a}	6.8 ± 1.73^{a}
Day maximum progesterone concentration (day)	7.0 ± 0.37^{a}	8.8 ± 0.49^{c}
Day progesterone concentration < 1 ng ml^{-1} (day)	10.0 ± 0.52^{a}	11.4 ± 0.40^{d}

Values with different superscripts within a row are significantly different (a vs. c P = 0.015) or show a tendency to be different (a vs. b P = 0.07 and a vs. d P = 0.09).

TABLE 1 | Effect of the different treatments on ovulation in llamas (mean ± SEM).

	Control	GnRH	E1	E2	E3
Mean follicular diameter (mm) on day 0	8.2 ± 0.62^{a}	9.7 ± 0.81^{a}	9.5 ± 0.47^{a}	9.6 ± 0.36^{a}	10.0 ± 0.31^{a}
Ovulation rate after treatment (%)	0 (0/3)^{a}	100 (6/6)^{b}	0 (0/4)^{a}	25 (1/4)^{a}	100 (6/6)^{b}
Mean follicular diameter (mm) on Day 2	9.7 ± 0.68^{x}	3.0 ± 0.42^{y}	11.4 ± 1.44^{x}	8.1 ± 2.02^{x}	2.8 ± 0.43^{y}

Values with different superscripts within a row are significantly different.
a vs. b = Proportions with different superscripts are different (P = 0.0001).
x vs. y = differences in mean follicular diameter on Day 2 between groups (P = 0.0001).

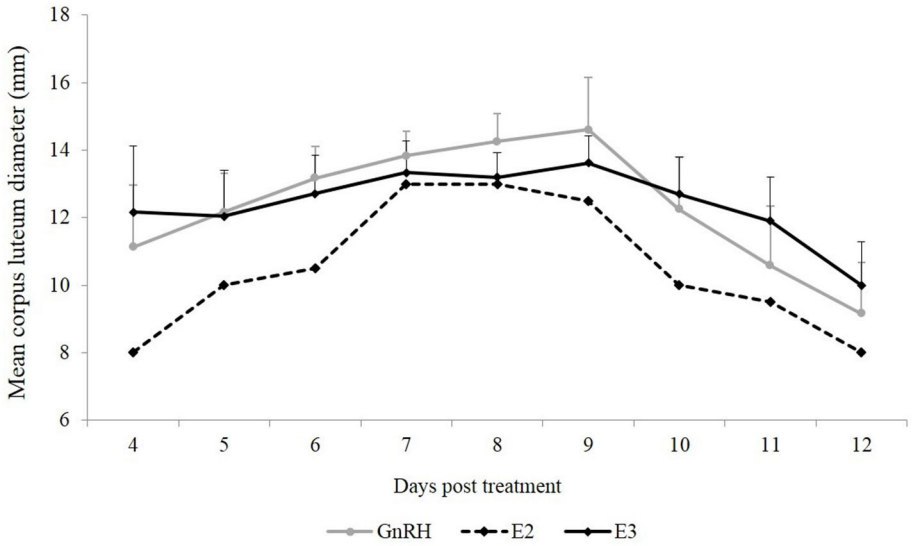


FIGURE 1 | Mean CL diameter (mm) from the first day that CL was detected until Day 12 post-treatment. Gray line: animals treated with Buserelin, analog of GnRH ($n = 6$), black line: animals treated with a dose of 1.6 mg of estradiol-17β ($n = 6$), and dotted line represents the only llama that ovulated in response to a dose of 1.0 mg of estradiol-17β.

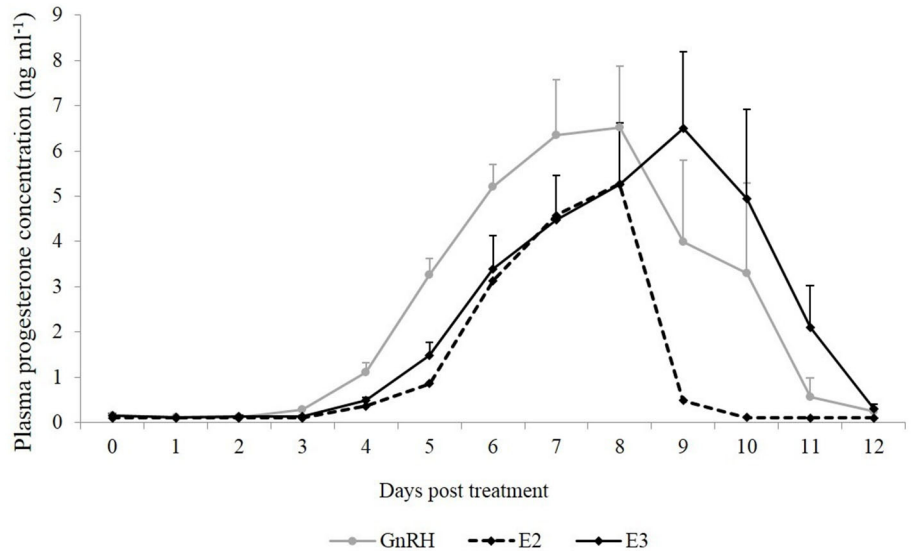


FIGURE 2 | Mean plasma progesterone concentration (ng ml^{-1}) from the day of treatment (Day 0) until Day 12 post-treatment. Gray line: animals treated with Buserelin, analog of GnRH ($n = 6$), black line: animals treated with a dose of 1.6 mg of estradiol-17β ($n = 6$), and dotted line represents the only llama that ovulated in response to a dose of 1.0 mg of estradiol-17β.

suggested that endogenous estradiol could induce GnRH release and subsequently stimulate preovulatory LH surge in a low percentage of female in specific occasions such as lactation or after a progesterone phase (24). Results hereby presented allow suggesting that a similar mechanism might be involved in llamas that ovulate spontaneously.

The pattern of plasma progesterone release after induction of ovulation with an injection of a GnRH analog in this study is similar to that previously reported in other studies in llamas. Plasma progesterone concentration starts to increase on Day 4 and reaches maximum concentration at Day 8, and a decrease is observed between Days 10 and 12 after induction of ovulation (25, 26).

The observation that females treated with 1.6 mg of estradiol-17β achieved plasma progesterone concentration above 1 ng ml^{-1} and the highest plasma progesterone concentration 1 day later than animals treated with the GnRH analog could be in relation to the time elapsed between treatment and the LH

surge and therefore the moment of ovulation. Previous studies in llamas (27, 28) and cows (29) have reported that LH peak occurs ∼2 h after injection of a GnRH analog. In addition, it has been shown in llamas that ovulation occurs around 29 h after treatment (7). Although there are no reports demonstrating the time elapsed between estradiol injection and LH surge in camelids, it could be speculated that the LH peak might occur between 12 and 18 h post injection, as previously reported in other species [cows: (30, 31); ewes: (8)]. Even though the time elapsed between treatment and ovulation was not evaluated in the present study, it could be suggested that llamas treated with estradiol-17β would have a retarded LH peak and consequently ovulate some hours later than those treated with a GnRH analog injection. Thereafter, it would determine a later development and functionality of the CL.

In summary, the results of the present study demonstrate that an injection of estradiol-17β is able to induce ovulation in llamas and that the response depends on the dose used. Most of the animals required the highest tested dose (1.6 mg) to induce the ovulatory process. Although the CL diameter in females induced to ovulate with estradiol was similar to that in llamas induced to ovulate with a GnRH analog, the rise in plasma progesterone concentration above 1 ng ml^{-1} and the peak progesterone concentration were attained 1 day later in the estradiol treated females. This information enables a better understanding of the ovulatory process in llamas, and the injection of estradiol-17β could be considered as a tool to develop new strategies to control ovarian activity in this species.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Faculty of Veterinary Sciences, UNCPBA.

AUTHOR CONTRIBUTIONS

CB Bianchi conducted the fieldwork, collected the data, performed the hormonal analysis, and interpreted the data and prepared the manuscript. MB and FV contributed with the fieldwork and collaborated with the preparation of the manuscript. MG contributed to analyzing the data, interpreting the results, and preparing the manuscript. MA contributed to the designing of the experiment, collecting data, interpreting the results, and critical reviewing of the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This research was supported by grants from Agencia Nacional de Promoción Científica y Tecnológica (PICT No. 0145) and from CONICET (PIP No. 0650).

REFERENCES

1. San Martin M, Copaira M, Zuniga J, Rodriguez R, Bustinza G, Acosta L. Aspects of reproduction in the alpaca. *J Reprod Fertil*. (1968) 16:395–9. doi: 10.1530/jrf.0.0160395
2. Bravo PW, Stabenfeldt GH, Lasley BL, Fowler ME. The effect of ovarian follicle size on pituitary and ovarian responses to copulation in domestic South American camelids. *Biol Reprod*. (1991) 45:553–9. doi: 10.1095/biolreprod45.4.553
3. Aba MA, Quiroga MA, Auza N, Forsberg M, Kindah H. Control of ovarian activity in llamas (*Lama glama*) with medroxyprogesterone acetate. *Reprod Dom Anim*. (1999) 34:471–6. doi: 10.1111/j.1439-0531.1999.tb01406.x
4. Kershaw-Young CM, Druart X, Vaughan J, Maxwell MC. β-Nerve growth factor is a major component of alpaca seminal plasma and induces ovulation in female alpacas. *Reprod Fertil Dev*. (2012) 24:1093–7. doi: 10.1071/RD12039
5. Ratto MH, Leduc YA, Valderrama XP, Van Straten KE, Delbaere LT, Pierson RA, et al. The nerve of ovulation-inducing factor in semen. *Proc Natl Acad Sci USA*. (2012) 109:15042–7. doi: 10.1073/pnas.1206273109
6. Adams GP, Ratto MH, Huanca W, Singh J. Ovulation - inducting factor in the seminal plasma of llamas and alpacas. *Biol Reprod*. (2005) 73:452–7. doi: 10.1095/biolreprod.105.040097
7. Ratto M, Huanca W, Singh J, Adams GP. Comparison of the effect of natural mating, LH and GnRH on interval to ovulation and luteal function in llamas. *Anim Reprod Sci*. (2006) 91:299–306. doi: 10.1016/j.anireprosci.2005.03.015
8. Bolt DJ, Kelley HE, Hawk WH. Release of LH by estradiol in cycling ewes. *Biol Reprod*. (1971) 4:35–40. doi: 10.1093/biolreprod/4.1.35
9. Haresign W, Foxcroft GR, Lamming GE. Control of ovulation in farm animals. *J Reprod Fertil*. (1983) 69:383–95. doi: 10.1530/jrf.0.0690383
10. Kesner JS, Padmanabhan V, Convey EM. Estradiol induces and progesterone inhibits the preovulatory surges of luteinizing hormone and follicle-stimulating hormone in heifers. *Biol Reprod*. (1982) 26:571–8. doi: 10.1095/biolreprod26.4.571
11. Bo GA, Adams GP, Pierson RA, Tribulo HE, Caccia M, Mapletoft RJ. Follicular wave dynamics after estradiol-17β treatment of heifers with or without a progestogen implant. *Theriogenology*. (1994) 41:1555–69. doi: 10.1016/0093-691X(94)90821-Y
12. Cavilla MV, Bianchi CP, Maistruarena C, Aba MA. Ultrasonographic and endocrine characterization of follicular waves in llamas with a special reference to the overlapping phenomenon during successive waves. *Reprod Dom Anim*. (2013) 48:923–30. doi: 10.1111/rda.12187
13. Silva ME, Recabarren MP, Recabarren SE, Adams GP, Ratto MH. Ovarian estradiol modulates the stimulatory effect of ovulation-inducing factor (OIF) on pituitary LH secretion in llamas. *Theriogenology*. (2012) 77:1873–82. doi: 10.1016/j.theriogenology.2012.01.004
14. Van Saun RJ. Nutritional requirements and assessing nutritional status in camelids. *Vet Clin North Am Food Anim Pract*. (2009) 25:265–79. doi: 10.1016/j.cvfa.2009.03.003
15. Bianchi CP, Benavente MA, Simonetti M, Aba MA. Synchronization of time of ovarian follicular development in llamas (*Lama glama*) using a protocol based on GnRH and $PGF_{2\alpha}$. *Anim Reprod Sci*. (2018) 192:200–5. doi: 10.1016/j.anireprosci.2018.03.011
16. Vaughan JL. *Control of Ovarian Follicular Growth in the Alpaca (Lama pacos)* (Ph.D. thesis) (2001). Central Queensland University, Rockhampton, QLD, Australia.
17. Lammoglia MA, Short RE, Bellows SE, Bellows RA, MacNeild MD, Hafs HD. Induced and synchronized estrus in cattle: dose titration of estradiol benzoate in peripubertal heifers and postpartum cows after treatment with an intravaginal progesterone-releasing insert and prostaglandin $F_{2\alpha}$. *J Anim Sci*. (1998) 76:1662–70. doi: 10.2527/1998.7661662x

18. Reames PS, Hatler TB, Hayes SH, Ray DL, Silvia WJ. Differential regulation of estrous behavior and luteinizing hormone secretion by estradiol-17β in ovariectomized dairy cows. *Theriogenology*. (2011) 75:233–40. doi: 10.1016/j.theriogenology.2010.08.009
19. Radford HM, Wheatley IS, Wallace ALC. The effects of oestradiol benzoate and progesterone on secretion of luteinizing hormone in the ovariectomized ewe. *J Endocr*. (1969) 44:135–6. doi: 10.1677/joe.0.0440135
20. Joseph IBJK, Currie WD, Ravindra JP, Cook SJ, Rawlings NC. Oestradiol and the surge release of gonadotrophins in the ewe. *Anim Reprod Sci*. (1994) 34:217–30. doi: 10.1016/0378-4320(94)90018-3
21. Ratto MH, Berland M, Silva ME, Adams GP. New insights of the role of β-NGF in the ovulation mechanism of induced ovulating species. *Reproduction*. (2019) 157:199–207. doi: 10.1530/REP-18-0305
22. Chaves MG, Aba M, Agüero A, Egey J, Berestin V, Rutter B. Ovarian follicular wave pattern and the effect of exogenous progesterone on follicular activity in non-mated llamas. *Anim Reprod Sci*. (2002) 69:37–46. doi: 10.1016/S0378-4320(01)00173-7
23. Bravo PW, Sumar J. Laparoscopic examination of the ovarian activity in Alpacas. *Anim Reprod Sci*. (1989) 21:271–81. doi: 10.1016/0378-4320(89)90034-1
24. Nagy P, Juhasz J, Wernery U. Incidence of spontaneous ovulation and development of the corpus luteum in non-mated dromedary camels (*Camelus dromedaries*). *Theriogenology*. (2005) 64:292–304. doi: 10.1016/j.theriogenology.2004.11.020
25. Bianchi CP, Cavilla MV, Jorgensen E, Benavente MA, Aba MA. Development of corpus luteum susceptibility to an analog of prostaglandin $F_{2\alpha}$, throughout the luteal phase in llamas (*Lama glama*). *Anim Reprod Sci*. (2012) 131:199–203. doi: 10.1016/j.anireprosci.2012.03.007
26. Bianchi CP, Meikle A, Alvarez MA, Benavente MA, Cavilla MV, Rodriguez E, et al. Oestrogen, progesterone and oxytocin receptors and COX-2 expression in endometrial biopsy samples from the induction of ovulation to luteolysis in llamas (*Lama glama*). *Reprod Dom Anim*. (2013) 48:681–90. doi: 10.1111/rda.12146
27. Aba MA, Forsberg M. Heterologous radioimmunoassay for llama and alpaca luteinizing hormone with a monoclonal antibody, an equine standard and a human tracer. *Acta Vet Scand*. (1995) 36:367–75.
28. Silva ME, Colazo MG, Ratto MH. GnRH dose reduction decreases pituitary LH release and ovulatory response but does not affect corpus luteum (CL) development and function in llamas. *Theriogenology*. (2012) 77:1802–10. doi: 10.1016/j.theriogenology.2011.12.024
29. Colazo MG, Kastelic JP, Davis H, Rutledge MD, Martinez MF, Small JA, et al. Effects of plasma progesterone concentrations on LH release and ovulation in beef cattle given GnRH. *Dom Anim Endocr*. (2008) 34:109–17. doi: 10.1016/j.domaniend.2006.11.004
30. Bo GA, Bergfelt DR, Brogliatti GM, Pierson RA, Adams GP, Mapletoft RJ. Local versus systemic effects of exogenous estradiol-17β on ovarian follicular dynamics in heifers with progestogen implants. *Anim Reprod Sci*. (2000) 59:141–57. doi: 10.1016/S0378-4320(00)00140-8
31. Martinez MF, Kastelic JP, Colazo MG, Mapletoft RJ. Effects of estradiol on gonadotrophin release, estrus and ovulation in CIDR-treated beef cattle. *Dom Anim Endocr*. (2007) 33:77–90. doi: 10.1016/j.domaniend.2006.04.009
32. D'Occhio MJ, Novoa C, Vera WG, Avalos R. Ovarian follicle regression and emergence of a new follicular wave after injection of 17β-estradiol in alpacas. *Austr Soc Reprod Biol*. (1997) 29:102.

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 23 October 2020
doi: 10.3389/fvets.2020.586265

β-NGF Stimulates Steroidogenic Enzyme and *VEGFA* Gene Expression, and Progesterone Secretion via ERK 1/2 Pathway in Primary Culture of Llama Granulosa Cells

Ximena Valderrama[1], *Cesar Ulloa-Leal*[2], *Mauricio Erciario Silva*[3], *Jose Goicochea*[4], *Silvana Apichela*[5], *Martin Argañaraz*[5], *Luciana Sari*[5], *Luis Paiva*[2], *Vicente Francisco Ratto*[2] *and Marcelo Hector Ratto*[2]*

[1] *Instituto de Investigaciones Agropecuarias (INIA), Osorno, Chile,* [2] *Institute of Animal Science, Faculty of Veterinary Sciences, Universidad Austral de Chile, Valdivia, Chile,* [3] *Department of Veterinary Sciences and Public Health, Faculty of Natural Resources, Universidad Catolica de Temuco, Temuco, Chile,* [4] *Department of Surgery and Reproductive Biotechnology, Faculty of Veterinary Medicine and Zootechnics, Universidad Nacional Hermilio Valdizán, Huánuco, Peru,* [5] *Instituto Superior de Investigaciones Biológicas (INSIBIO), CONICET-UNT, Facultad de Bioquímica, Instituto de Biología "Dr. Francisco D. Barbieri," Química y Farmacia, UNT, San Miguel de Tucumán, Argentina*

OPEN ACCESS

Edited by:
Regiane R. Santos, Schothorst Feed Research, Netherlands

Reviewed by:
Paulo Bayard Dias Goncalves, Federal University of Pampa, Brazil
Fabio S. Lima, University of California, Davis, United States

***Correspondence:**
Marcelo Hector Ratto
marceloratto@uach.cl

Specialty section:
This article was submitted to Animal Reproduction - Theriogenology, a section of the journal Frontiers in Veterinary Science

Received: *22 July 2020*
Accepted: *07 September 2020*
Published: *23 October 2020*

Citation:
Valderrama X, Ulloa-Leal C, Silva ME, Goicochea J, Apichela S, Argañaraz M, Sari L, Paiva L, Ratto VF and Ratto MH (2020) β-NGF Stimulates Steroidogenic Enzyme and VEGFA Gene Expression, and Progesterone Secretion via ERK 1/2 Pathway in Primary Culture of Llama Granulosa Cells. Front. Vet. Sci. 7:586265. doi: 10.3389/fvets.2020.586265

The beta-nerve growth factor (β-NGF) from llama seminal plasma exerts ovulatory and luteotrophic effects following intramuscular or intrauterine infusion in llamas and alpacas. In this study, we investigate the *in vitro* effect of llama β-NGF on the expression of genes involved in angiogenesis and progesterone synthesis as well as progesterone release in preovulatory llama granulosa cells; we also determine whether these changes are mediated via the ERK1/2 signaling pathway. From adult female llamas, we collected granulosa cells from preovulatory follicles by transvaginal ultrasound-guided follicle aspiration; these cells were pooled and incubated. After 80% confluence, the cultured granulosa cells were treated with β-NGF, β-NGF plus the MAPK inhibitor U0126, or luteinizing hormone, and the abundance of angiogenic and steroidogenic enzyme mRNA transcripts were quantified after 10 and 20 h by RT-qPCR. We also quantified the progesterone concentration in the media after 48 h by radioimmunoassay. We found that application of β-NGF increases the abundance of mRNA transcripts of the vascular endothelial growth factor (*VEGFA*) and the steroidogenic enzymes cytochrome P450 side-chain cleavage (P450scc/*CYP11A1*), steroidogenic acute regulatory protein (*STAR*), and 3β-hydroxysteroid dehydrogenase (*HSD3B1*) at 10 and 20 h of treatment. Application of the MAPK inhibitor U0126 resulted in downregulation of the genes encoding these enzymes. β-NGF also enhanced progesterone synthesis, which was prevented by the prior application of the MAPK inhibitor U0126. Finally, western blot analysis confirmed that β-NGF activates the ERK1/2 signaling pathway. In conclusion, our results indicate that β-NGF exerts direct luteotropic effects on llama ovarian tissue via the ERK 1/2 pathway.

Keywords: ***HSD3B1***, ***CYP11A1***, ***CYP19A1***, **MAPK, U0126, corpus luteum, follicle,** ***STAR***

INTRODUCTION

It is now well-established that the beta-nerve growth factor (β-NGF) present in the seminal plasma of llamas and alpacas is the essential signal inducing the luteinizing hormone (LH) surge and ovulation in these species (1). Purified llama β-NGF also has a significant luteotrophic effect after intramuscular or intrauterine infusion in llamas and alpacas (2, 3). It is reported that plasma progesterone (since day 8 from ovulation) and corpus luteum (CL) diameter at day 16 are greater in llamas given two doses of 1 mg of purified β-NGF than those given a single dose (4). The increase of the vascular area of the CL is positively correlated to progesterone production in llamas treated with β-NGF (4, 5). Interestingly, administration of β-NGF is also shown to improve luteal function in cows (6).

In llamas, the preovulatory LH surge induced by purified llama β-NGF is more sustained than that the observed after GnRH administration (7, 8), suggesting that the luteotrophic effect of β-NGF could be due to this prolonged LH secretion profile. In this sense, systemic administration of β-NGF increased CL vascularization and upregulated the expression of cytochrome P450 side-chain cleavage (P450scc encoded by *CYP11A1*) and steroidogenic acute regulatory protein (*STAR*) mRNA transcripts enhancing plasma progesterone concentrations during the early luteal phase in llamas (9). Also, systemic administration of purified llama β-NGF induced a rapid shift from estradiol to progesterone synthesis in the preovulatory follicle in llamas as evidenced by the *in vivo* increase of the progesterone/estradiol ratio in the follicular fluid and upregulation of genes related to progesterone production (10).

The presence of NGF and its high-affinity receptor trkA in granulosa, theca, and luteal cells of mammalian species (11–13) suggests that the luteotrophic effect of β-NGF may be exerted not only by the prolonged LH secretion pattern, but also directly by acting at the ovarian level. In a recent study, the addition of purified llama β-NGF upregulated the expression of *STAR* and vascular endothelial growth factor (*VEGFA*) transcripts in primary culture of llama granulosa cells (10).

Previous studies investigating the effect of NGF/trkA on steroidogenesis in the ovary have been conducted in granulosa or theca cells from cows (14) and humans (15), species classified as spontaneous ovulators, and so might not be the same than those of llamas. After copulation, female llamas have been shown to increase their plasmatic content of β-NGF concomitantly occurring with the LH discharge from the posterior pituitary (16), which hampers the discrimination of β-NGF and LH effects on follicle cells *in vivo*. The use of an *in vitro* system may serve to elucidate the potential local effects of these hormones on luteal function, and thus, the effects of β-NGF are not confounded by those of the endogenous LH release.

In this study, we investigate the effect of β-NGF on steroidogenic enzymes and *VEGFA* gene expression as well as progesterone secretion and test whether these effects are mediated via ERK1/2 signaling pathway *in vitro* using a primary culture of granulosa cells collected from llama preovulatory follicles.

MATERIALS AND METHODS

Animals

Llamas were kept at the llama research farm of the Institute of Animal Science, Universidad Austral de Chile, Valdivia, Chile (39°38'S−73°5'W and 19 m above sea level) and were maintained in pens and had access to natural pasture supplemented with hay and water *ad libitum*.

Experimental procedures were reviewed and approved by the University Bioethical Committee and were performed in accordance with the Chilean Animal Protection Act (2009) and the university animal care protocols.

Semen Collection and Protein Purification

Semen was collected from five mature male llamas, twice per week for 5 months before the start of the experiments. An artificial sheep vagina was used as previously described (2). Each ejaculate was diluted 1:1 (vol/vol) with phosphate-buffered saline (PBS; Gibco, Grand Island, NY, USA) and centrifuged for 30 min at 1,500 × g at room temperature. A pool of sperm-free seminal plasma was stored at −20°C. Purification of β-NGF was performed in a two-step procedure as previously described (17, 18). In brief, seminal plasma was loaded into a type 1 macro-prep ceramic hydroxylapatite column (1 × 10 cm, 40 μm; Bio-Rad Laboratories, Hercules, CA, USA) previously equilibrated with 10 mM sodium phosphate, pH 6.8, and a flow rate of 0.5 mL/min. An eluted fraction showing a major protein on SDS-PAGE was concentrated in PBS (pH 7.4) using a 5 kDa cutoff membrane filter device (Vivaspin; Sartorius, Göttingen, Germany) and subsequently loaded onto a gel filtration column (SEC, hi Prep 26/60 Sephacryl S-100; Amersham Laboratories, Piscataway, NJ, USA). The purification procedure was carried out at room temperature at a flow rate of 0.5 ml/min using fast protein liquid chromatography (Amersham Laboratories, Piscataway, NJ, USA). Elution was performed isocratically using PBS at pH 7.4. The bioactive fraction after gel filtration was identified using an *in vivo* llama ovulation bioassay (17).

Granulosa Cell Collection

Non-pregnant, non-lactating female llamas ($n = 48$) ≥ 6 years of age and weighing 120–150 kg were used from April to June. Llamas were submitted to transvaginal ultrasound-guided follicle ablation of all ovarian follicles ≥ 5 mm using a 19-gauge needle attached to a 5 MHz convex-array transducer to synchronize follicular wave emergence as described previously (19). The llamas were examined daily by transrectal ultrasonography using a 7.5-MHz linear-array transducer (Aloka SSD-500; Aloka Co., Ltd., Tokyo, Japan) to detect the emergence of a new preovulatory follicle. When a growing follicle ≥ 8 mm in diameter was detected (17, 18, 20), llamas were submitted to transvaginal ultrasound-guided follicle aspiration using a 5.0-MHz convex-array ultrasound transducer coupled to a 19-gauge needle as described previously (19, 21, 22) to collect granulosa cells by flushing the preovulatory follicle.

Primary Culture of llama Granulosa Cells

Primary culture of llama granulosa cells was performed as previously described (10). Chemicals and reagents were

purchased from Sigma–Aldrich Co., St. Louis, MO, USA, unless otherwise stated. In brief, follicular fluid collected from an individual female was centrifuged at 400 × g for 10 min. The cell pellet was then resuspended in 2 ml of Ham's F-12/DMEM and centrifuged again at 1,200 × g for 45 min in a 3 ml 50% Percoll column to separate granulosa cells from erythrocytes and interstitial cells. Purified granulosa cells were collected from the top of the Percoll column and washed twice by centrifugation at 400 x g for 6 min. Because a low number of granulosa cells were collected from individual animals, it was necessary to pool three animals after Percoll purification to get one biological sample. Granulosa cells were plated into 24-well culture plates (at 1×10^5 cells/well) in Ham's F-12/DMEM supplemented with 10% fetal bovine serum and antibiotics (penicillin/streptomycin and gentamicin) and incubated in an atmosphere of 95% air, 5% of CO_2 at 38°C and high humidity for 48 h. After 80% of confluence, the medium was replaced with serum-free Ham's F-12/DMEM and cells were treated with (i) control PBS, (ii) DMSO plus 10 μM of the MAPK inhibitor U0126 (Cell Signaling Technologies, Beverly, MA, USA), (iii) 30 ng/ml of LH (Lutropin-V; Vétoquinol Canada Inc., Lavaltrie, QC, Canada), (iv) 50 ng/ml of purified llama β-NGF, and (v) purified llama β-NGF plus 10 μM the MAPK inhibitor U0126 (added 30 min prior to β-NGF and remained for the whole period of treatment).

Five biological samples were used in both gene expression and progesterone secretion experiments ($n = 15$ llamas in each experiment); for each time and treatment, each biological sample was plated in 4 wells (i.e., experimental replicates) and treated as indicated above. For gene expression, cells were treated for 10 or 20 h; this time course was based on a previous report (10), in which significant changes in mRNA expression of *STAR* and *VEGFA* in primary culture of granulosa cells treated with β-NGF were detected.

RNA Isolation and Real-Time PCR (Q-PCR) Analysis

Total RNA was extracted from granulosa cells using Trizol (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's recommendations. The purity of the samples was analyzed using the Nanodrop 1000 (Thermo Fisher Scientific, Inc., Wilmington, DE, USA). One microgram of total RNA was converted to complementary DNA (cDNA) using the kit AffinityScript Q-PCR cDNA Synthesis (Agilent Technologies, Inc., Santa Clara, CA, USA) and Oligo-dT as per the manufacturer's instructions.

Brilliant III Ultra-Fast SYBR® Q-PCR master mix (Agilent Technologies, Inc., Santa Clara, CA, USA) was used to detect and quantitate the transcripts. Samples were run in triplicate and amplified in a QuantStudio 3 RT-PCR (Applied Biosystems, Foster City, CA, USA) thermocycler using the following thermal cycle conditions: one cycle at 95°C for 3 min, 40 cycles of 95°C for 5 s, 60°C for 30 s, and 72°C for 1 min. The coefficient of variation between samples ranged from 0.03 to 0.1 depending on the treatment. Data were analyzed using the thermocycler-associated software. For each sample, cycle threshold values for the assayed transcripts were normalized for total input cDNA (10 ng) concentrations using cycle threshold values for transcripts for the "normalizer" housekeeping gene, large ribosomal protein (RPLP0; (23)). The normalized values in each experiment were compared to a "calibrator" sample to determine the relative increase in the amount of the transcript. Primers for PCR were designed using Primer Express Software (PE Biosystems, Foster City, CA, USA). Llama primers for steroidogenic enzymes, *VEGFA*, and *RPLP0* transcripts and their expected size of amplified products and sequences have already been tested and previously described (9, 10). The accession numbers of gen data are shown in **Supplementary Table 1**. Primer sets for transcript amplification were used at a final concentration of 250 nM each. Data was analyzed using the thermocycler-associated software. Additionally, following amplification, the melting curves for the products were generated to ensure that the product represents a homogenous species.

Progesterone Secretion From Primary Culture of llama Granulosa Cells

Progesterone concentration was determined from the primary culture of llama granulosa cells as previously described (10). Briefly, the procedure for follicular synchronization and aspiration, granulosa cell collection, and treatments were similar to the described above. However, for progesterone secretion, granulosa cells were cultured for 48 h after confluence. After the incubation period, the media was collected and stored at −20°C until it was assayed for progesterone. Progesterone concentration was determined by a solid-phase radioimmunoassay kit (RIA-CT KIP1458; DIASource ImmunoAssays SA Louvain-la-Neuve, Belgium) as previously reported (10). The intra-assay coefficient of variation was 0.26–3.57%, the minimum detectable limit 0.05 ng/ml, coefficient of variation of internal standard < 3.87%, the limit of quantification observed for the assay was 0.45 ng/ml.

MAPK/ERK Activity in Primary Granulosa Cells Culture

From 6 biological samples ($n = 18$ llamas total), 4 experimental replicates per sample were used to determine the phosphorylation state of ERK1/2 after β-NGF treatment. In brief, llama granulosa cells were cultured as described above and treated with (i) control PBS, 50 ng/ml of β-NGF for (ii) 0, (iii) 5, (iv) 10, and (v) 15 min, β-NGF plus 10 μm of MAPK inhibitor U0126 for (vi) 5, (vii) 10, and (viii) 15 min. After the incubation period, cells (1×10^6) were lysed in 75 μl of lysis buffer containing 150 mM NaCl, 50 mM Tris-HCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1 mm sodium orthovanadate, 10 mg/ml leupeptin, 1.8 mg/ml aprotinin, 2 mM sodium fluoride, 2 mM sodium pyrophosphate, and 1 M dithiothreitol. Ten micrograms of protein from each sample were size-fractionated by 12% SDS-PAGE and then transferred to nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA, USA). Nonspecific binding was blocked by incubating the membranes in PBS (pH 7.4) containing 2% non-fat dried milk and 0.2% Tween-20. Membranes were then incubated overnight at 4°C with anti-phospho-MAPK p44/42 (ERK1/2) or anti-p44/42 (ERK1/2) rabbit polyclonal antibodies

(1:1000; Cell Signaling Technologies, Beverly, MA, USA), followed by incubation at room temperature for 2 h with a goat anti-rabbit antibody conjugated with horseradish peroxidase (1:10,000; Cell Signaling Technologies, Beverly, MA, USA). The same membranes used to detect phosphorylated ERKs were stripped and blotted against total ERKs. Proteins were detected using an enhanced chemiluminescent detection system (PerkinElmer Life Sciences, Wellesley, MA, USA). The films were scanned and analyzed with a gel doc automated digitizing system (G:BOX Chemi XRQ; Syngene, Cambridge, UK).

Statistical Analysis

Data for gene, ERK protein expression, and progesterone concentration were analyzed by the PROC MIXED procedure, including the effect of treatment, time, replicate, and their interaction. The Dunnett test was used to compare gene expression of all treatments with the control groups. If significant differences were detected, means were compared among groups using Tukey's test. All data are reported as mean ± SEM. Analyses were performed using the Statistical Analysis System software package SAS Learning Edition, version 4.1 (SAS Institute, Inc., Cary, NC, USA).

RESULTS

The effect of β-NGF or LH treatment on mRNA expression of steroidogenic enzymes and *VEGFA* in llama granulosa cells are shown in **Figures 1**, **2**. There was a significant ($P \leq 0.01$) increase of 3 beta-hydroxysteroid dehydrogenase (*HSD3B1*) and *VEGFA* mRNA abundance in cells treated with β-NGF or LH after 10 h (**Figures 1C,E**). After 20 h, the mRNA abundance of *STAR* and *CYP11A1* (P450scc) steroidogenic enzymes also significantly increased; the *HSD3B1* and *VEGFA* mRNA transcripts remained upregulated (**Figures 2B–E**). As expected, neither β-NGF nor LH affected the mRNA expression of cytochrome P450 aromatase (P450arom encoded by *CYP19A1*) that is involved in estradiol synthesis (**Figures 1A**, **2A**).

The addition of the MAPK inhibitor U0126 prior to the β-NGF treatment downregulated ($P \leq 0.01$) the mRNA abundance of all steroidogenic enzymes and *VEGFA* transcripts after 10 h (**Figures 1A–E**). Most of the genes remained downregulated after 20 h, except *CYP19A1* (P450arom), which was not significantly different from control (**Figures 2B–E**). Cells treated with the U0126 inhibitor alone did not significantly change the mRNA expression of steroidogenic enzymes and *VEGFA* at any time.

Progesterone secretion from llama granulosa cells was significantly ($P \leq 0.01$) increased 48 h after β-NGF or LH treatment; the addition of the MAPK inhibitor U0126 prior to the β-NGF treatment prevented the increase of progesterone secretion as shown in **Figure 3**.

Western blotting of llama granulosa cells treated with 50 ng/ml of β-NGF showed a significant ($P \leq 0.01$), time-dependent increase of ERK2 phosphorylation after 5, 10, and 15 min of treatment; this increase exhibited a reduced intensity for ERK1. The addition of the inhibitor U0126 prior to β-NGF administration significantly ($P \leq 0.01$) decreased the state of ERK1/2 phosphorylation (**Figures 4A,B**).

DISCUSSION

Neurotrophins, including β-NGF, are classically known for their role in neural growth and survival (24), but the relatively recent isolation and purification of β-NGF from the seminal plasma of llamas (17, 18) and also other species (25) has led to revisiting their roles in reproductive biology, emerging new ones (18, 26). Previous studies have linked NGF to ovarian development and innervation (27–29), but in the last years, evidence (1) indicates that β-NGF is involved in CL formation.

In this study, we investigated whether purified llama β-NGF exerts luteotropic effects on a primary culture of llama granulosa cells collected from preovulatory follicles. We show that llama granulosa cells treated with β-NGF upregulates steroidogenic enzymes and angiogenic transcripts after 10 and 20 h of treatment, and that this response is mediated by the ERK1/2 signaling pathway. Also, the β-NGF-induced increase of progesterone concentration is prevented by the MAPK inhibitor U0126, confirming that the biological actions of β-NGF are mediated, at least in part, by this pathway.

The first evidence of NGF receptors in the ovary was given by Dissen et al. (29), who detect low-affinity receptors in theca and also, to a lesser extent in granulosa cells of antral and preantral follicles of peripubertal rats. Subsequent studies confirm the expression of the high-affinity trkA NGF receptor in granulosa and theca cells of several species, including cows (14), rats (30), and humans (15). Binding of trkA receptors by its ligand β-NGF is known to elicit activation of the ERK1/2 signaling pathway (31, 32), including in granulosa cells (33). In line with this, our results show that the sole application of purified llama β-NGF to primary cultured llama granulosa cells results in activation of the ERK1/2 signaling pathway.

Expression of different steroidogenic enzymes is crucial for the ability of cells to synthesize steroids from cholesterol and its precursors. In the present study, we detect upregulation of *HSD3B1* mRNA transcript after 10 h of both β-NGF and LH treatments; and after 20 h, all the transcripts of the steroidogenic enzymes involved in progesterone secretion are also upregulated. Few studies address the effect of β-NGF on these enzymes; we have previously reported that β-NGF enhanced *STAR, CYP11A1*, and *HSD3B1* mRNA transcripts in llama granulosa cells *in vivo* (10), but these followed a distinct time expression pattern. Recently, Stewart et al. (6) report that the expression of steroidogenic enzymes in heifer luteal cells seems to be relatively similar to that reported here, resulting in increased circulating progesterone, albeit the expression of *HSD3B1*—which converts pregnenolone to progesterone—was unaffected.

In spontaneous ovulators, it can be expected that granulosa cells collected by follicle aspiration from a preovulatory follicle may exhibit some degree of luteinization due to the effect of the increasing LH release occurring at the end of the follicular phase. Conversely, in llamas, LH is released in response to the copulatory stimulus as this species is an induced ovulator. In line with this, Silva et al. (9) report that, in llamas treated with control PBS but not β-NGF or GnRH, plasma progesterone concentrations remain basal for up to 10 days. Although, in the present study, we did not evaluate the ratio of progesterone:

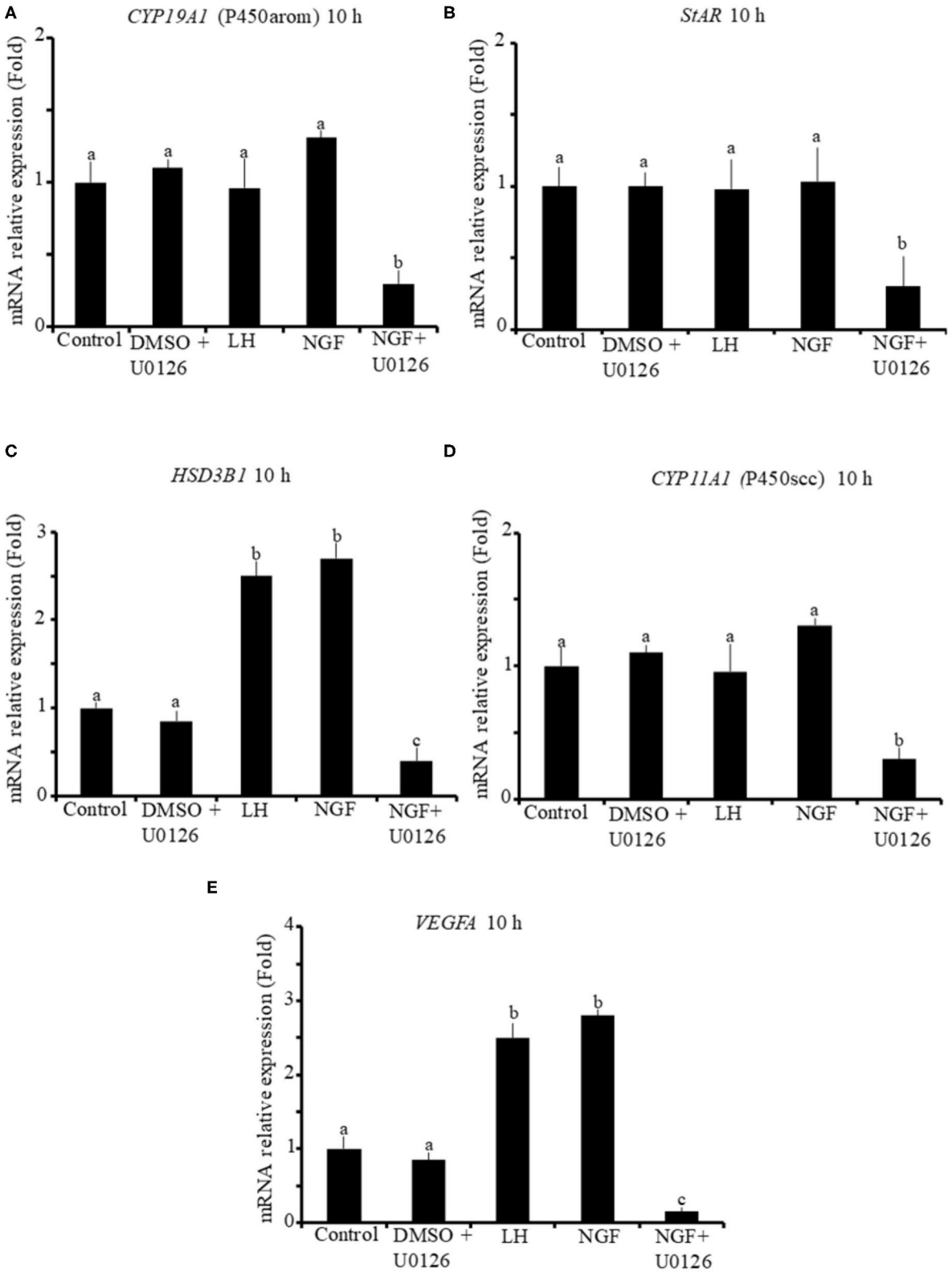


FIGURE 1 | Relative mRNA abundance of **(A)** *CYP19A1* (P450arom), **(B)** *STAR*, **(C)** *HSD3B1*, **(D)** *CYP11A1* (P450scc) steroidogenic enzymes, and **(E)** *VEGFA* angiogenic factor in primary llama granulosa cell culture treated for 10 h. Mean + SEM; $n = 5$ biological samples; within each biological sample, 4 experimental replicates were performed; a, b, c superscripts indicate significant differences ($P \leq 0.01$) between control and other groups.

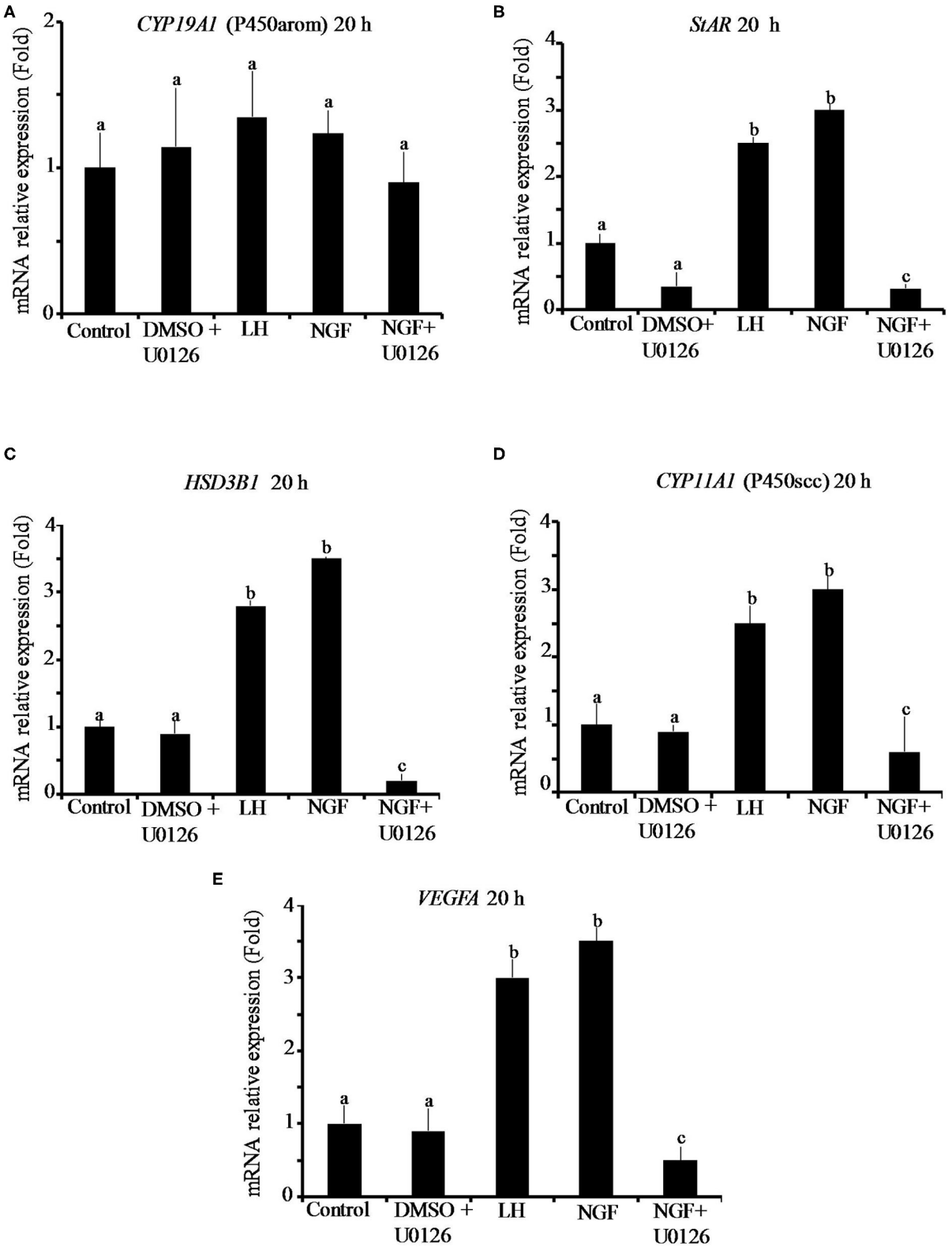


FIGURE 2 | Relative mRNA abundance of **(A)** *CYP19A1* (P450arom), **(B)** *STAR*, **(C)** *HSD3B1*, **(D)** *CYP11A1* (P450scc) steroidogenic enzymes, and **(E)** *VEGFA* angiogenic factor in primary llama granulosa cell culture treated for 20 h. Mean + SEM; $n = 5$ biological samples; within each biological sample, 4 experimental replicates were performed; a, b, c superscripts indicate significant differences ($P \leq 0.01$) between control and other groups.

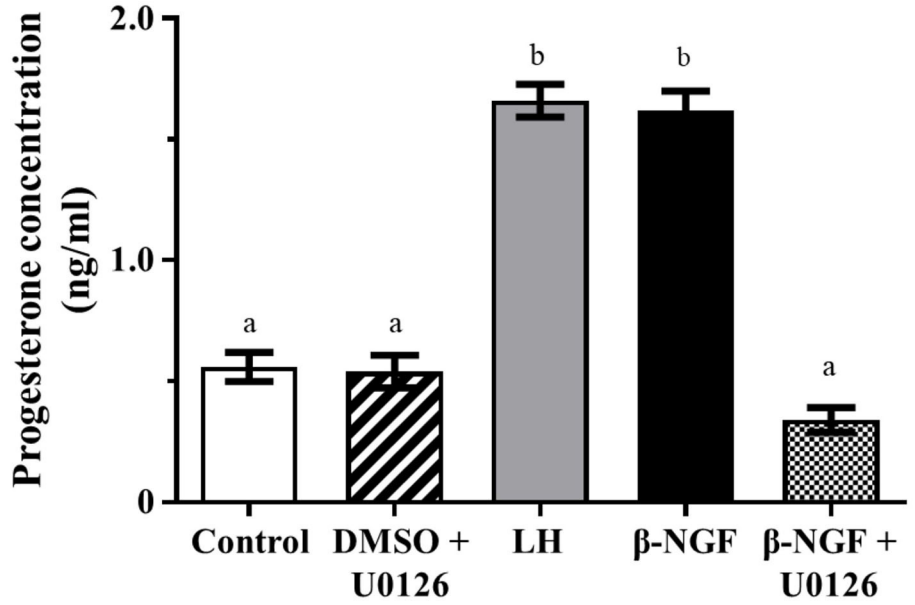


FIGURE 3 | Progesterone secretion from primary culture of llama granulosa cells after 48 h. Mean ± SEM; $n = 6$ biological samples; within each biological sample, 4 experimental replicates were performed; a, b superscripts indicate significant differences ($P \leq 0.01$) between control and other groups.

estradiol, we previously reported (10) a marked ratio increase from 0.2 to 0.8 in control and β-NGF-treated animals, respectively, and this shift to progesterone production occurred after 20 but not 10 h following β-NGF treatment. Finally, the persistent detection of *CYP19A1* (P450arom catalyzing estradiol) reported in the present study also supports that the cells cultured here preserve their granulosa phenotype over the time analyzed, and thus, the effect of β-NGF on llama granulosa cells should be carefully interpreted as prosteroidogenic rather than luteinizing itself.

Time-dependent gene expression patterns of steroidogenic enzymes following LH have been detected in cultured bovine granulosa cells, resulting in enhanced expression of *CYP11A1* (P450scc) and *HSD3B1* transcripts; paradoxically, *in vivo* expression of these genes is reduced in granulosa cells collected after the LH surge when compared to those collected before (34, 35). The parallel gene expression profiles induced by β-NGF and LH reported here may be related to phosphorylation of ERK1/2, which also occurs following activation of LH receptors in preovulatory granulosa cells (36).

Interestingly, the application of the inhibitor U0126 alone did not affect gene expression, but its use combined with β-NGF downregulated virtually all steroidogenic and *VEGFA* transcripts in the llama granulosa cells. A plausible explanation could be that, once the EKR1/2 pathway was blocked, β-NGF exerts a paradox effect by activating (or causing predominance of) other transduction pathways that downregulate the constitutive expression of the genes analyzed here. For example, in some cell types, β-NGF-activating trkA receptors stimulate cell growth and survival, whereas the absence of trkA stimulates apoptosis via the low-affinity neurotrophin receptor, p75NTR (37). Expression of p75NTR is found in granulosa cells of humans (38) and squirrels (39); whether this is expressed in llama granulosa cells is unknown.

Consistent with our findings on intracellular pathways and gene expression, we also find that β-NGF application results in an ERK1/2-dependent progesterone synthesis in primary culture of llama granulosa cells. A previous study (40) reports that *in vitro* NGF microdialysis perfusion of cow luteal tissue but not in non-proliferative luteal cell culture, results in increased progesterone (and also local oxytocin) release from ovaries at early- and mid-luteal stage. This discrepancy between experimental conditions is likely to be related to cell culture settings as thecal cells plated in low but not high density also failed to secrete the steroids in response to NGF (14). Conversely to the evidence from llama and cows, in cultured human granulosa cells collected from preovulatory follicles, NGF application is shown to increase estradiol, whereas it decreases progesterone secretion (15). Perhaps there are species-specific variations on steroid output response to β-NGF in granulosa cells.

The CL is considered one of the most vascularized body structures; it receives the greatest rate of blood flow per unit of tissue compared to any organ of the body (41), and so angiogenesis plays an important role during the CL formation. The LH surge is considered a key signal to influence the expression of the VEGF that induces angiogenesis throughout the proliferation of preexistent endothelial cells (42). In human granulosa cells obtained from *in vitro* fertilization patients, NGF is shown to promote ovarian angiogenesis by enhancing the secretion of VEGF through the activation

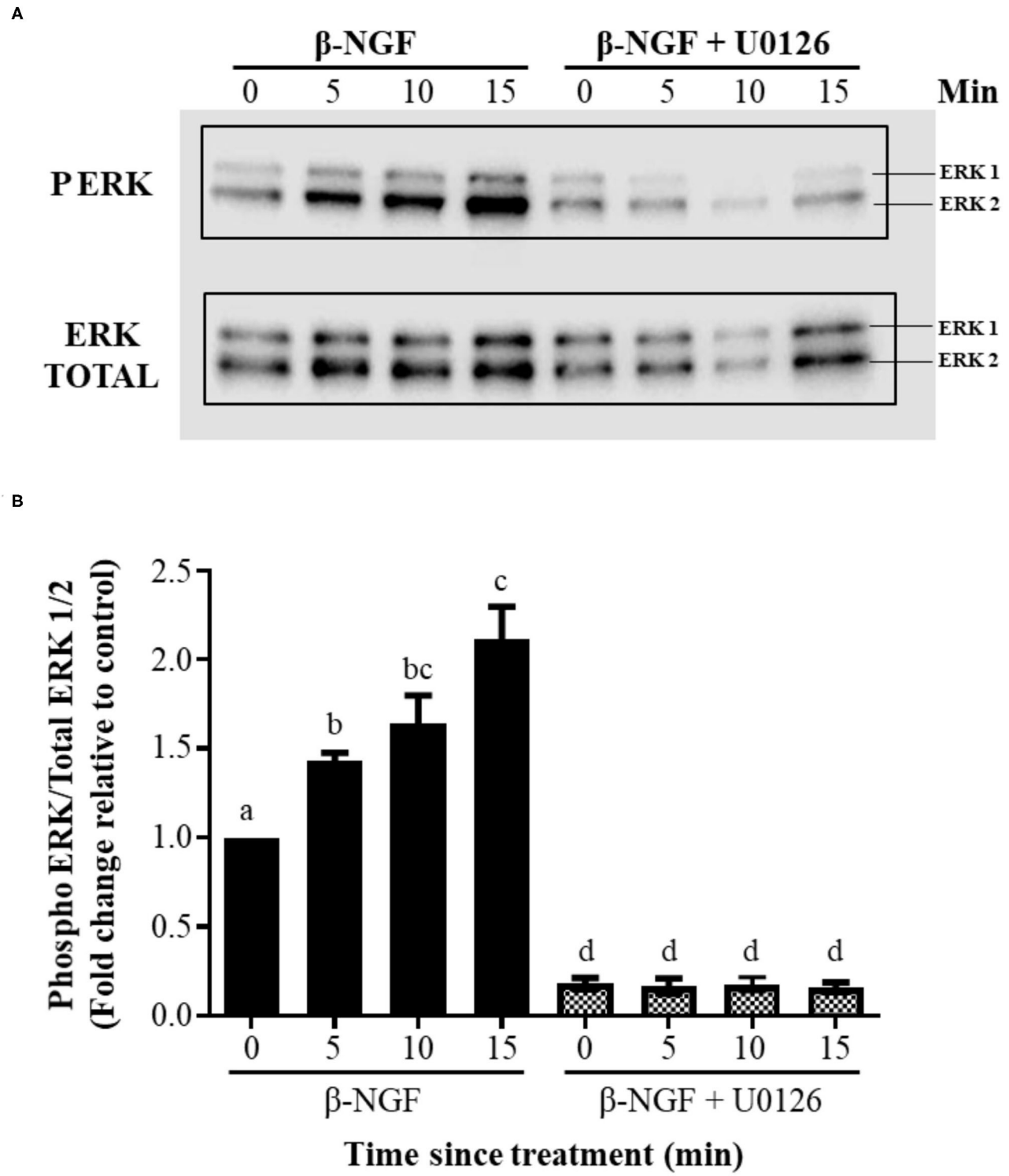


FIGURE 4 | β-NGF induces phosphorylation of ERK1/2 in llama granulosa cells *in vitro*. **(A)** Western blot showing that NGF induces a rapid, time-dependent phosphorylation of ERK2, and to lesser extent, expression of ERK1 by the same time intervals. **(B)** Densitometric analysis of the blot shown in **(A)**, expressed as the ratio of phosphorylated ERK1/2 of total ERK. Values are mean + SEM; $n = 6$ biological replicates. a, b, c, d superscripts indicate significant differences ($P \leq 0.01$) between control and the other groups.

of the ERK1/2 pathway (43). Similarly, we found that, in llama granulosa cells, *VEGFA* mRNA expression rapidly increases after 10 h to remain relatively stable after 20 h of β-NGF and LH treatments, and this increase was dependent on the ERK1/2 pathway. Enhancement of *VEFG* mRNA expression is also known to occur by different signaling pathways in other cell types (44); whether β-NGF also activates other pathways in llama granulosa cells remains to be determined.

Previous studies show that, in llamas, CL develops after ovulation induced by seminal plasma (2) or purified β-NGF (7, 8, 18) and tended to be larger, regressed later, and produced twice as much progesterone than those resulting from GnRH treatments, supporting the notion that this effect might be related to the sustained LH release profile induced by β-NGF (7, 8, 17, 45). However, decreasing pharmacological doses of the GnRH analog, gonadorelin acetate, have been shown to affect the magnitude of the LH release in a dose-dependent fashion, but the consequent reduction in LH discharge had no effect on the CL diameter and plasma progesterone concentrations in llamas (46), indicating that the enhancement of luteal function induced by β-NGF occurs locally at the ovarian level rather than upstream on the classical LH mechanism.

In this *in vitro* study, both β-NGF and LH treatments equally increased genes involved in CL angiogenesis and steroid synthesis that resulted in a similar progesterone secretion, making it difficult to distinguish the contribution to the luteotropic effect of each hormone separately, and so it could be that, when acting β-NGF and LH together, there are synergic effects at the follicular level, similarly as shown to occur with LH and insulin administration in porcine granulosa cells *in vitro* (47, 48). In line with this notion, llamas in which ovulations were induced by β-NGF displayed enhanced expression of steroidogenic enzymes (9) and plasma progesterone concentrations (49) than those of ovulations stimulated by GnRH.

In summary, here we show and identify the intracellular pathway by which β-NGF exerts a direct luteotropic effect on ovarian tissue; however, further studies are required to determine whether synergic mechanisms exist that explain the β-NGF-related enhancement of luteal function and progesterone secretion reported *in vivo*.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by University Bioethical Committee (Universidad Austral de Chile) in accordance with the Chilean Animal Protection Act (2009) and the University animal care protocols, and the Animal Bioethical Committee of CONICYT (Chilean National Council of Science and Technology).

AUTHOR CONTRIBUTIONS

XV and LP were responsible for the development of the primary culture of llama granulosa cells and the execution of RT-QPCR and western blots experiments. CU-L, MS, JG, VR, and MR were responsible for granulosa cell collection in llamas using ultrasound-guided follicular aspiration and interpretation of gene expression and western blot data. XV, MR, SA, LS, and MA were responsible for the experimental design of the study and interpretation of gene expression and western blot data. MR and LP were major contributors in writing and editing of the manuscript. All authors participated actively in the analyses, interpretation of the data, read, and approved the final manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This study was supported by the Agencia Nacional de Investigacion y Desarrollo (ANID) of Chile, grants FONDECYT Postdoctorado 3200439 (LP), and FONDECYT Regular 1160934 (MR).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2020.586265/full#supplementary-material

REFERENCES

1. Silva M, Paiva L, Ratto MH. Ovulation mechanism in South American Camelids: the active role of β-NGF as the chemical signal eliciting ovulation in llamas and alpacas. *Theriogenology*. (2020) 150:280–7. doi: 10.1016/j.theriogenology.2020.01.078
2. Adams GP, Ratto MH, Huanca W, Singh J. Ovulation-inducing factor in the seminal plasma of alpacas and llamas. *Biol Reprod*. (2005) 73:452–7. doi: 10.1095/biolreprod.105.040097
3. Ratto MH, Huanca W, Singh J, Adams GP. Comparison of the effect of ovulation-inducing factor (OIF) in seminal plasma of llamas, alpacas, and bulls. *Theriogenology*. (2006) 66:1102–6. doi: 10.1016/j.theriogenology.2006.02.050
4. Fernandez A, Ulloa-Leal C, Silva M, Norambuena C, Adams GP, Guerra M, et al. The effect of repeated administrations of llama ovulation-inducing factor (OIF/NGF) during the peri-ovulatory period on corpus luteum development and function in llamas. *Anim Reprod Sci*. (2014) 149:345–52. doi: 10.1016/j.anireprosci.2014.08.001
5. Ulloa-Leal C, Bogle OA, Adams GP, Ratto MH. Luteotrophic effect of ovulation-inducing factor/nerve growth factor present in the seminal plasma of llamas. *Theriogenology*. (2014) 81:1101–7. doi: 10.1016/j.theriogenology.2014.01.038
6. Stewart JL, Stella S, Cunha LL, Dias NW, Canisso IF, Mercadante VRG, et al. Administration of nerve growth factor-β to heifers with a preovulatory follicle enhanced luteal formation and function and promoted LH release. *Theriogenology*. (2020) 148:37–47. doi: 10.1016/j.theriogenology.2020.02.040

7. Silva M, Smulders JP, Guerra M, Valderrama XP, Letelier C, Adams GP, et al. Cetrorelix suppresses the preovulatory LH surge and ovulation induced by ovulation-inducing factor (OIF) present in llama seminal plasma. *Reprod Biol Endocrinol.* (2011) 9:74. doi: 10.1186/1477-7827-9-74
8. Silva M, Nino A, Guerra M, Letelier C, Valderrama XP, Adams GP, et al. Is an ovulation-inducing factor (OIF) present in the seminal plasma of rabbits? *Anim Reprod Sci*. (2011) 127:213–21. doi: 10.1016/j.anireprosci.2011.08.004
9. Silva M, Ulloa-Leal C, Valderrama XP, Bogle OA, Adams GP, Ratto MH. Nerve growth factor from seminal plasma origin (spβ-NGF) increases CL vascularization and level of mRNA expression of steroidogenic enzymes during the early stage of Corpus Luteum development in llamas. *Theriogenology*. (2017) 103:69–75. doi: 10.1016/j.theriogenology.2017.07.041
10. Valderrama XP, Silva M, Goicochea J, Ratto MH. The effect of seminal plasma β-NGF on follicular fluid hormone concentration and gene expression of steroidogenic enzymes in llama granulosa cells. *Reprod Biol Endocrinol.* (2019) 17:60. doi: 10.1186/s12958-019-0504-9
11. Maranesi M, Petrucci L, Leonardi L, Piro F, García-Rebollar P, Millán P, et al. New insights on a NGF-mediated pathway to induce ovulation in rabbits (*Oryctolagus cuniculus*). *Biol Reprod*. (2018) 98:634–43. doi: 10.1093/biolre/ioy041
12. Carrasco R, Singh J, Adams GP. The dynamics of trkA expression in the bovine ovary are associated with a luteotrophic effect of ovulation-inducing factor/nerve growth factor (OIF/NGF). *Reprod Biol Endocrinol*. (2016) 14:47–57. doi: 10.1186/s12958-016-0182-9
13. Dissen GA, Romero C, Paredes A, Ojeda S. Neurotrophic control of ovarian development. *Microsc Res Tech*. (2002) 59:509–15. doi: 10.1002/jemt.10227
14. Dissen GA, Parrot JA, Skinner MK, Hill DF, Costa ME, Ojeda SR. Direct effects of nerve growth factor on thecal cells from antral ovarian follicles. *Endocrinology*. (2000) 141:4736–50. doi: 10.1210/endo.141.12.7850
15. Salas C, Julio-Pieper M, Valladares M, Pommer R, Vega M, Mastronardi C, et al. Nerve growth factor-dependent activation of trkA receptors in the human ovary results in synthesis of follicle-stimulating hormone receptors and estrogen secretion. *J Clin Endocrino Metab*. (2006) 91:2396–403. doi: 10.1210/jc.2005-1925
16. Berland MA, Ulloa-Leal C, Barría M, Wright H, Dissen GA, Silva ME, et al. Seminal plasma induces ovulation in llamas in the absence of a copulatory stimulus: role of nerve growth factor as an ovulation-inducing factor. *Endocrinology*. (2017) 157:3224–32. doi: 10.1210/en.2016-1310
17. Ratto MH, Delbaere LTJ, Leduc YA, Pierson RA, Adams GP. Biochemical isolation and purification of ovulation-inducing factor (OIF) in seminal plasma of llamas. *Repro Biol Endocrinol*. (2011) 9:24. doi: 10.1186/1477-7827-9-24
18. Ratto MH, Leduc Y, Valderrama XP, van Straaten K, Delbaere L, Pierson R, et al. The nerve of ovulation inducing factor. *Proc Natl Acad Sci USA*. (2012) 109:15042–7. doi: 10.1073/pnas.1206273109
19. Ratto MH, Singh J, Huanca H, Adams GP. Ovarian follicular wave synchronization and fixed-time natural mating in llamas. *Theriogenology*. (2003) 60:1645–56. doi: 10.1016/S0093-691X(03)00176-6
20. Urra F, Ratto MH, Silva M. Evaluation of mating, intrauterine deposition of raw seminal plasma or seminal plasma purified β-NGF on endometrial vascularization in llamas. *Theriogenology*. (2018) 125:18–23. doi: 10.1016/j.theriogenology.2018.10.007
21. Ratto MH, Singh J, Huanca H, Adams GP. *In vivo* and *in vitro* maturation of llama oocyte. *Theriogenology*. (2004) 63:2445–57. doi: 10.1016/j.theriogenology.2004.09.053
22. Berland MA, von Baer A, Ruiz J, Parraguez VH, Morales P, Adams GP, et al. *In vitro* fertilization and development of cumulus oocytes complexes collected by ultrasound-guided follicle aspiration in superstimulated llamas. *Theriogenology*. (2011) 75:1482–8. doi: 10.1016/j.theriogenology.2010.11.047
23. Luo W, Gumen A, Haughiam JM, Wiltbank MC. The role of Luteinizing hormone in regulating gene expression during selection of the dominant follicle in cattle. *Biol Reprod*. (2011) 84:369–78. doi: 10.1095/biolreprod.110.085274
24. Huang EJ, Reichardt LF. Neurotrophins: roles in neuronal development and function. *Annu Rev Neurosci*. (2001) 24:677–736. doi: 10.1146/annurev.neuro.24.1.677
25. Bogle O, Carrasco R, Ratto MH, Singh J, Adams GP. Source and localization of ovulation- inducing factor/nerve growth factor in male reproductive tissues among mammalian species. *Biol Reprod*. (2018) 99:1194–204. doi: 10.1093/biolre/ioy149
26. Pinet-Charvet C, Fleurot R, Derouin-Tochon F, de Graaf S, Druart X, Tsikis G, et al. Beta-nerve growth factor stimulates spontaneous electrical activity of *in vitro* embryonic mouse GnRH neurons through a P75 mediated-mechanism. *Sci Rep*. (2020) 10:10654. doi: 10.1038/s41598-020-67665-4
27. Lara HE, Hill DF, Katz KH, Ojeda SR. The gene encoding nerve growth factor is expressed in the immature rat ovary: effect of denervation and hormonal treatment. *Endocrinology*. (1990) 126:357–63. doi: 10.1210/endo-126-1-357
28. Lara HE, McDonald JK, Ojeda SR. Involvement of nerve growth factor in female sexual development. *Endocrinology*. (1990) 126:364–75. doi: 10.1210/endo-126-1-364
29. Dissen GA, Hill DF, Costa ME, Ma YJ, Ojeda SR. Nerve growth factor receptors in the peripubertal rat ovary. *Mol Endocrinol*. (1991) 5:1642–50. doi: 10.1210/mend-5-11-1642
30. Dissen GA, Hill DF, Costa ME, Les Dees CW, Lara HE, Ojeda SR. A role for trkA nerve growth factor receptors in mammalian ovulation. *Endocrinology*. (1996) 137:198–209. doi: 10.1210/endo.137.1.8536613
31. Huang EJ, Reichardt LF. Trk receptors: roles in neuronal signal transduction. *Annu Rev Biochem.* (2003) 72:609–42. doi: 10.1146/annurev.biochem.72.121801.161629
32. Van Kanegan MJ, Strack S. The protein phosphatase 2A regulatory subunits B'beta and B'delta mediate sustained TrkA neurotrophin receptor autophosphorylation and neuronal differentiation. *Mol Cell Biol*. (2009) 29:662–74. doi: 10.1128/MCB.01242-08
33. Moore RK, Otsuka F, Shimasaki S. Role of ERK1/2 in the differential synthesis of progesterone and estradiol by granulosa cells. *Biochem Biophys Res Commun*. (2001) 289:796–800. doi: 10.1006/bbrc.2001.6052
34. Voss AK, Fortune JE. Levels of messenger ribonucleic acid for cholesterol side-chain cleavage cytochrome P-450 and 3 beta-hydroxysteroid dehydrogenase in bovine preovulatory follicles decrease after the luteinizing hormone surge. *Endocrinology*. (1993) 132:888–94. doi: 10.1210/endo.132.2.8425501
35. Voss AK, Fortune JE. Levels of messenger ribonucleic acid for cytochrome P450 17 alpha-hydroxylase and P450 aromatase in preovulatory bovine follicles decrease after the luteinizing hormone surge. *Endocrinology*. (1993) 132:2239–45. doi: 10.1210/endo.132.5.8477668
36. Salvador LM, Maizels E, Hales DB, Miyamoto E, Yamamoto H, Hunzicker-Dunn M. Acute signaling by the LH receptor is independent of protein kinase C activation. *Endocrinology*. (2002) 143:2986–94. doi: 10.1210/endo.143.8.8976
37. Casaccia-Bonnefil P, Kong H, Chao MV. Neurotrophins: the biological paradox of survival factors eliciting apoptosis. *Cell Death Differ*. (1998) 5:357–64. doi: 10.1038/sj.cdd.4400377
38. Buyuk E, Santoro N, Cohen HW, Charron MJ, Jindal S. Reduced neurotrophin receptor tropomyosin-related kinase A expression in human granulosa cells: a novel marker of diminishing ovarian reserve. *Fertil Steril*. (2011) 96:474–8. doi: 10.1016/j.fertnstert.2011.05.017
39. Li B, Sheng X, Bao L, Huang S, Li Q, Liu Y, et al. Seasonal changes in expression of nerve growth factor and its receptors TrkA and p75 in the ovary of wild ground squirrel (*Citellus dauricus* Brandt). *J Ovarian Res*. (2014) 7:3. doi: 10.1186/1757-2215-7-3
40. Miyamoto A, Okuda K, Schweigert FJ, Schams D. Effects of basic fibroblast growth factor, transforming growth factor- and nerve growth factor on the secretory function of the bovine corpus luteum *in vitro*. *J Endocrinol.* (1992) 135:103–14. doi: 10.1677/joe.0.1350103
41. Wiltbank MC, Dysko RC, Gallagher KP, Keyes PL. Relationship between blood flow and steroidogenesis in the rabbit corpus luteum. *J Reprod Fertil.* (1988) 84:513–20. doi: 10.1530/jrf.0.0840513
42. Dvorak HF, Nagy JA, Feng D, Brown LF, Dvorak AM. Vascular permeability factor/vascular endothelial growth factor and the significance of microvascular hyperpermeability in angiogenesis. In: Claesson-Welsh L, editor. *Vascular Growth Factors and Angiogenesis. Current Topics in Microbiology and Immunology*. Berlin: Springer (1999). p. 97–132.
43. Julio-Pieper M, Lozada P, Tapia V, Vega M, Miranda C, Vantman D, et al. Nerve growth factor induces vascular endothelial growth factor expression in granulosa cells via a trkA receptor/mitogen-activated protein Kinase-extracelullarly regulated kinase 2-dependent pathway. *J Clin Endocrinol Metab.* (2009) 94:3065–71. doi: 10.1210/jc.2009-0542

44. Pagès G, Pouysségur J. Transcriptional regulation of the vascular endothelial growth factor gene–a concert of activating factors. *Cardiovasc Res.* (2005) 65:564–73. doi: 10.1016/j.cardiores.2004.09.032
45. Tanco VM, Ratto MH, Lazzarotto M, Adams GP. Dose-response of female llamas to ovulation-inducing factor from seminal plasma. *Biol Reprod.* (2011) 85:452–6. doi: 10.1095/biolreprod.111.091876
46. Silva ME, Colazo MG, Ratto MH. GnRH dose reduction decreases pituitary LH release and ovulatory response but does not affect corpus luteum (CL) development and function in llamas. *Theriogenology*. (2012) 77:1802–10. doi: 10.1016/j.theriogenology.2011.12.024
47. Sekar N, Garmey JC, Veldhuis JD. Mechanisms underlying the steroidogenic synergy of insulin and luteinizing hormone in porcine granulosa cells: joint amplification of pivotal sterol-regulatory genes encoding the low-density lipoprotein (LDL) receptor, steroidogenic acute regulatory (stAR) protein and cytochrome P450 side-chain cleavage (P450scc) enzyme. *Mol Cell Endocrinol*. (2000) 159:25–35. doi: 10.1016/S0303-7207(99)00203-8
48. Sekar N, Lavoie HA, Veldhuis JD. Concerted regulation of steroidogenic acute regulatory gene expression by luteinizing hormone and insulin (or insulin-like growth factor I) in primary cultures of porcine granulosa-luteal cells. *Endocrinology*. (2000) 141:3983–92. doi: 10.1210/endo.141.11.7763
49. Silva M, Ulloa-Leal C, Norambuena C, Fernández A, Adams GP, Ratto MH. Ovulation-inducing factor (OIF/NGF) from seminal plasma origin enhances Corpus Luteum function in llamas regardless the preovulatory follicle diameter. *Anim Reprod Sci*. (2014) 148:221–7. doi: 10.1016/j.anireprosci.2014.05.012

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 28 October 2020
doi: 10.3389/fvets.2020.587596

Effect of Cooling and Freezing on Llama (*Lama glama*) Sperm Ultrastructure

Renato Zampini[1,2], Ximena A. Castro-González[1], Luciana M. Sari[1], Alfredo Martin[3,4], Ana V. Diaz[1], Martin E. Argañaraz[1,2] and Silvana A. Apichela[1,5]**

[1] Instituto Superior de Investigaciones Biológicas (INSIBIO), Consejo Nacional de Investigaciones Científicas y Técnicas-Universidad Nacional de Tucumán (CONICET-UNT), Instituto de Biología "Dr. Francisco D. Barbieri", Facultad de Bioquímica, Química y Farmacia, Universidad Nacional de Tucumán (UNT), San Miguel de Tucumán, Argentina, [2] Cátedra de Biología Celular y Molecular, Facultad de Bioquímica, Química y Farmacia, Universidad Nacional de Tucumán (UNT), San Miguel de Tucumán, Argentina, [3] Cátedra de Técnicas Quirúrgicas, Facultad de Agronomía y Zootecnia, Universidad Nacional de Tucumán (UNT), San Miguel de Tucumán, Argentina, [4] Instituto de Investigación Animal del Chaco Semiárido (IIACS), Instituto Nacional de Tecnología Agropecuaria (INTA), Leales, San Miguel de Tucumán, Argentina, [5] Cátedra de Zootecnia General I, Facultad de Agronomía y Zootecnia, Universidad Nacional de Tucumán (UNT), San Miguel de Tucumán, Argentina

Semen cryopreservation in South American camelids has a low efficiency. Post-thaw viability of sperm is low, and poor results are obtained when artificial insemination is performed with cryopreserved semen, impeding advances both in accelerated genetic progress and selection. This study aimed to describe the effect of a conventional method of camelid semen cryopreservation on the llama sperm ultrastructure during cooling and freezing, using transmission and scanning electron microscopy (TEM, SEM). Sperm motility, vigor, viability, and DNA integrity during those steps were also examined. Ejaculates from five fertile adult llama males were obtained by electroejaculation. For cooling, semen samples were washed with Hepes-balanced salt solution (HBSS), diluted in Tris-citric acid-fructose egg yolk extender (TCF-EY), and then cooled until 5°C for 24 h. For freezing, sperm samples were washed with HBSS, diluted in TCF-EY and cooled until 5°C for 2.5 h. Samples were equilibrated with TCF-EY, supplemented with 6% glycerol at 5°C for 20 min, and then stored in liquid nitrogen for a month before thawing. TEM and SEM analyses were carried out on sperm samples prior to cryopreservation, after cooling down until 5°C for 2.5 and 24 h, and after the freeze-thaw process. Ultrastructural injury was noticed during cooling, even though sperm motility, vigor, viability, and DNA integrity were not significantly affected. Analysis revealed plasma membrane and acrosome damage, loss of mitochondria, and axoneme and periaxonemal structure disorganization after 2.5 h of cooling. During freezing, a significant decrease in sperm motility and viability was observed after thawing. TEM and SEM revealed prominent signs of post-thawing damage. The plasma membrane was lost or exhibited various degrees of swelling, undulation, and perforations. Besides, the sperm presented vacuoles in the nucleus and broken acrosomes. Mitochondria in the midpiece showed vacuolization and structural disorganization. In conclusion, SEM and TEM revealed that cryopreservation induced ultrastructural damages in llama sperm that initiated during cooling and intensified during freezing. These details provide valuable data for further studies to minimize cryodamage in camelid sperm.

Keywords: cryodamage, cryopreservation, *Lama glama*, ultrastructure, South American camelids, spermatozoa

OPEN ACCESS

Edited by:
Regiane R. Santos, Schothorst Feed Research, Netherlands

Reviewed by:
Ivan Cunha Bustamante-Filho, Universidade Do Vale Do Taquari - Univates, Brazil
Abouzar Najafi, University of Tabriz, Iran

****Correspondence:***
Renato Zampini
renatozampini@gmail.com
Silvana A. Apichela
silvanaapichela@gmail.com

Specialty section:
This article was submitted to Animal Reproduction - Theriogenology, a section of the journal Frontiers in Veterinary Science

Received: *26 July 2020*
Accepted: *24 September 2020*
Published: *28 October 2020*

Citation:
Zampini R, Castro-González XA, Sari LM, Martin A, Diaz AV, Argañaraz ME and Apichela SA (2020) Effect of Cooling and Freezing on Llama (Lama glama) Sperm Ultrastructure. Front. Vet. Sci. 7:587596. doi: 10.3389/fvets.2020.587596

INTRODUCTION

Semen cryopreservation is a widely used technique to preserve and supply sperm for breeding and maintenance of genetic diversity in wildlife. During a standard cryopreservation protocol, different processing steps are involved (dilution of semen at 37°C with the extender and cooling until 5°C, addition of a cryoprotectant and equilibration, and freezing in liquid nitrogen at −196°C), and it is not entirely clear how each step affects the sperm cell (1). However, it has been shown that cryopreservation induces deleterious alterations in sperm structure and function (2–5). These involve thermal stress due to the change in temperature during cooling, freezing and thawing, as well as osmotic stress caused by the addition of high concentrations of cryoprotective agents and crystallization (6). As a result, a reduction in overall sperm fertility has been reported in different domestic livestock when performing artificial insemination (AI) (7, 8).

In South American camelids, poor results have been obtained after AI with cryopreserved semen (9–14). Llama and alpaca pregnancy rates with frozen-thawed semen ranged from 0 to 26% (9–12), while maximum pregnancy (33%) was obtained with cooled semen at 5°C (14).

Ultrastructural damage after cryopreservation has been reported in bull (1), goat (15), dromedary (16), ram (17), dog (18), and human (19), many of which cannot be detected by conventional assessment. Ultrastructural analysis requires high magnification, which is not possible with a light microscope due to the low resolution. Electron microscopy is an extremely useful tool for this purpose. Specifically, scanning electron microscopy (SEM) offers a three-dimensional image of surface structures and transmission electron microscopy (TEM) provides a high magnification image of cellular components, including the cytoskeleton, membrane systems, organelles, as well as specialized structures in differentiated cells.

To date, no studies have examined sperm ultrastructure alterations in llamas caused by cooling or freezing. Deeper knowledge about these procedures could be useful to improve the most critical steps during cryopreservation. Therefore, the present study aimed to evaluate the effects of cryopreservation on the ultrastructural characteristics of llama sperm during cooling until 5°C and freezing. Standard assessment of sperm motility, vigor, viability and DNA integrity was also performed.

MATERIALS AND METHODS

Animals

Five llama males between 4 and 5 years old from the CEEC (Centro Experimental de Estudios en Camélidos Sudamericanos), Faculty of Agronomy and Zootechnics of the National University of Tucumán (26°50′11.4"S 65°16′58.3"W, and 440 m altitude, Tucumán, Argentina) were used. The animals were kept on natural pasture and strategically supplemented with bales of alfalfa, and water was provided *ad libitum*. Semen was obtained during the winter-spring of 2019.

Semen Collection and Handling

Semen collections were carried out using electroejaculation (EE) under general anesthesia with 0.2 mg/kg of xylazine IV (Xilazina, Richmond, Argentina) and 1.5 mg/kg of ketamine IV (Ketamina, Holliday, Argentina). The same males were subjected to EE with an interval of 15 days between successive semen collections. All procedures were in line with the UNT 002/18 Protocol approved by the Committee for the Care and Use of Laboratory Animals (CICUAL) from the Universidad Nacional de Tucumán, Argentina.

A stimulator similar to an Electrojac V (Sistel, Argentina) with a rectal probe with three linear electrodes was used for EE. The probe was lubricated, gently inserted into the rectum and orientated so that the electrodes were positioned ventrally to the prostate. The device was used in automatic mode, applying stimulus cycles of 2 s with 2 s intervals between stimuli. Voltage was increased one volt every five cycles (starting with 2 V), until erection occurred (∼8 V). Then, voltage was increased with 1 V increments every 10 cycles until ejaculation. According to the sensitivity of each animal to electro-stimuli, the minimum voltage required to obtain ejaculation was applied (maximum 13 V), without exceeding 10 min of electro-stimulation.

Semen was obtained in 50 ml Falcon tubes and immediately placed in a 37°C water bath. At this time, an aliquot of 12 μl was used to evaluate sperm viability, motility, and vigor in raw semen. In order to remove seminal plasma, each ejaculate was diluted 4-fold with Hepes balanced salt solution (HBSS: 25 mM Hepes, 130 mM NaCl, 5 mM KCl, 0.36 mM NaH_2PO_4, 0.49 mM $MgCl_2$, and 2.4 mM $CaCl_2$, pH 7.4, 290 mOsm/kg) and subsequently centrifuged at 800 × g for 8 min at room temperature. Particularly highly viscous semen was first diluted and then gently pipetted to break down the gel before centrifuging. The sperm pellet was suspended in 200–300 μl of HBSS, and its concentration was calculated using a Makler counting chamber. At this point, some samples were used for cooling (C0: fresh sperm before cooling procedure) and others for freezing (F0: fresh sperm before freezing procedure) protocols.

Sperm Cryopreservation

Sperm samples were cryopreserved by cooling down until 5°C for 24 h or freezing in liquid nitrogen at −196°C for a month. The experimental design is shown in **Figure 1**.

Cooling Protocol

Sperm suspensions ($n = 5$, $r = 2$) were diluted with Tris citric acid fructose-egg yolk extender (TCF-EY: 250 mM Tris, 80 mM citric acid, 60 mM fructose, 20% egg yolk, 0.5% Equex, 80,000 IU Penicillin G sodium, and 0.1% Streptomycin sulfate) to obtain a final concentration of 30–40 × 10^6 spermatozoa/ml. Samples were subsequently placed in a 37°C water bath and then put in a refrigerator. The temperature was monitored until reaching 5°C in ± 2.5 h (cooling rate 0.2°C/min). Samples were then maintained at 5°C for 24 h. After this period, the cooled sperm cells were warmed up to 37°C in a water bath (C24) to carry out sperm evaluations.

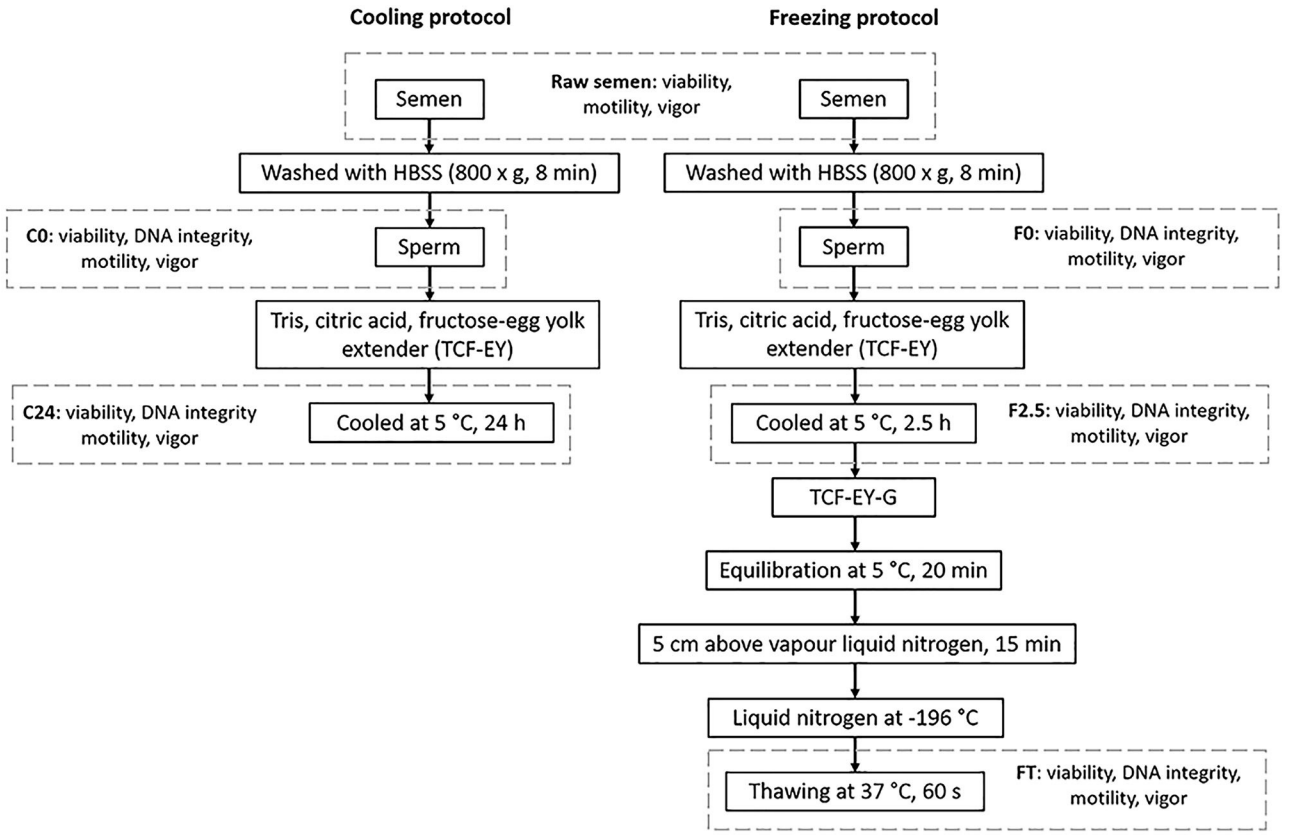


FIGURE 1 | Experimental design of cryopreservation procedures for llama sperm.

Freezing Protocol

Sperm suspensions ($n = 5$, $r = 3$) were diluted with TCF-EY until a final concentration of 120–160 $\times$ 10^6 spermatozoa/ml, and subsequently cooled down until 5°C for $\pm$ 2.5 h (cooling rate 0.2°C/min) (F2.5). Then, sperm cells were diluted 1:1 with freezing diluent that consisted of TCF-EY supplemented with 12% (v/v) glycerol as cryoprotectant (TCF-EY-G) (final glycerol concentration was 6%) followed by equilibration for 20 min at 5°C. After the equilibration period, the samples were loaded in 0.5 ml straws (30–40 $\times$ 10^6 spermatozoa per straw), which were placed 5 cm above liquid nitrogen vapor for 15 min in a freezing unit for straws (Minitube, Madison, WI, USA). Straws were then plunged into liquid nitrogen at −196°C for storage. After a month the samples were thawed in a 37°C water bath for 60 s (FT: freeze-thawing) and evaluated as follows.

Sperm Features Assessment

The following sperm traits were evaluated: viability, motility, vigor and DNA integrity. The traits were assessed at different time points: C0 and C24 for cooling; and F0, F2.5, and FT for freezing. Additionally, viability, motility, and vigor were also assessed in raw semen.

The percentage of live spermatozoa was determined by the eosin-nigrosin staining. Briefly, 5 μl of sperm suspension was placed on a slide and mixed with the same volume of eosin-nigrosin solution. After 30–40 s, a thin smear was prepared and observed under a light microscope at 400X magnification. At least 200 spermatozoa from two different slides were counted per sample (viable sperm remained colorless, while non-viable sperm stained pink).

Assessments of motility and vigor were made by placing a 7 μl aliquot of spermatozoa on a slide under heating stage and coved with a warmed coverslip (18 $\times$ 18 mm), using a bright-field microscope (Numak, model Zenith DO-1L, Buenos Aires, Argentina) at 400X. The patterns observed were: oscillatory motility (OM) and progressive motility (PM). In addition, total sperm motility (TM = OM + PM) was determined. Sperm vigor was evaluated by using a score from 0 to 5 following **Table 1** criteria, considering the amount of sperm with movement, the presence of progressive movement, and the beat frequency of the sperm.

To assess sperm DNA integrity, acridine orange assay was carried out on sperm samples. Briefly, the sperm were diluted with HBSS and centrifuged at 800 $\times$ g for 8 min to remove the extender, suspended in 100 μl of HBSS (except for C0 and F0 because they have already been washed with HBSS). Then, thin smears were prepared from the sperm suspensions, fixed for 3 h in Carnoy's solution (methanol/acetic acid, 3:1) and

TABLE 1 | Sperm vigor classification.

Score	Description
5	Sperm have very fast oscillatory or progressive movement. 60% of cells are motile
4	Sperm have vigorous oscillatory movement, with fast beat frequency. About 40–50% of cells are motile
3	Sperm have mainly oscillatory movement, with slow beat frequency, and low progressive movement. Less than 40% of cells are motile
2	Sperm have weak oscillatory movement. Progressive motility is not observed
1	Total motility is poor. Very few sperm (about 10%), have weak movements
0	Sperm have no movement

stained with acridine orange solution (0.19% in phosphate citrate buffer, pH = 2.5) for 5 min. The slides were gently washed with distilled water for 5 min and air-dried. The stained smears were then observed under a fluorescence microscope (wavelengths of 450–490 nm, Olympus, Tokyo, Japan) at 400X magnification. Sperm with normal DNA content present a green fluorescence, whereas sperm with abnormal DNA content emit fluorescence in a spectrum varying from yellow-green to red. At least 100 spermatozoa per slide were counted.

Ultrastructural Assessment

Sperm ultrastructure analysis was performed by scanning and transmission electron microscopy at the Centro Integral de Microscopía Electrónica (CIME), CONICET, Tucumán-Argentina.

Scanning Electron Microscopy (SEM)

SEM analysis was carried out on sperm samples obtained before cryopreservation (fresh sperm), after cooling at 5°C for 2.5 and 24 h, and after freeze-thawing. Sperm samples (concentration≥ 80×10^6 spermatozoa/ml, $n = 2$ per each group) were washed with 1 ml of HBSS and centrifuged twice at 800 × g for 5 min. The supernatant was removed and the pellets formed by spermatozoa were fixed in Karnovsky's reagent (paraformaldehyde 2.7% and glutaraldehyde 1.7% in sodium phosphate buffer, pH 7.2) for 24 h at 4°C. Then, 100 μl of sperm suspension was placed on a glass previously covered with 2% agar for 1 h. After that, the samples were dehydrated in increasing ethanol concentrations (30, 50, 70, 90, and 100%) for 10 min each, and subjected to two acetone baths for 10 min. The samples were critical point dried with liquid carbon dioxide (Denton Vacuum DCP-1 Critical Point Dryer, NJ, USA), mounted on aluminum stubs and metalized with gold (JEOL ion sputter JFC-1100, Tokyo, Japan) for later observation using a scanning electron microscope (Zeiss SUPRA 55VP, Oberkochen, Germany). Different fields were randomly chosen, and 100 sperm per sample were examined.

Transmission Electron Microscopy (TEM)

TEM analysis was carried out on fresh sperm, after cooling at 5°C for 2.5 and 24 h, and after freeze-thawing. Similar to SEM technique, the sperm samples (concentration≥ 80×10^6 spermatozoa/ml, $n = 2$ per each group) were washed with 1 ml of HBSS and centrifuged twice at 800 × g for 5 min. The sperm pellets were fixed in Karnovsky's reagent for 24 h at 4°C. Then, sperm were centrifuged and the pellets were embedded in 1.2% agar solution. Afterward, the samples were washed three times with sodium phosphate buffer, post-fixed in 2% osmium tetroxide in the same buffer at 4°C overnight, and treated with an aqueous solution of 2% uranyl acetate for 30 min. Samples were serially dehydrated in ethanol, passed through acetone and embedded in Spurr resin overnight at 60°C. Ultrafine sections (60–70 nm) were examined on copper grids using a Zeiss Libra 120 electron microscope (Carl Zeiss, Oberkochen, Germany). Micrographs were examined focusing on the different sperm structures (plasma membrane, acrosome, nucleus, mitochondria in the midpiece, and axoneme and periaxonemal in the principal piece), and observations were made paying particular attention to whether the structures were preserved or presented some type of alteration (100 sperm heads and tails per sample including longitudinal and cross-sections were examined) to describe the sperm quality features in the different groups.

TABLE 2 | Percentage of llama sperm viability, and DNA integrity during cooling procedure.

	Viability (%)	DNA integrity (%)
C0	67.4 ± 3.8[a]	86.2 ± 4.5[a]
C24	72.7 ± 3.7[a]	82.4 ± 8.6[a]

Values are mean ± standard error of the mean.
[a] *The same letter within columns indicates no significant difference ($p > 0.05$).*

Statistical Analysis

All data were expressed as the mean ± standard error of the mean, and analyzed by statistical packages Infostat Version 2011p and R 3.1. Linear mixed-effects models were used to evaluate the effect of cryopreservation on sperm motility, viability, and DNA integrity, with animals as a random effect, and sampling time as fixed effects. Mean comparisons were performed using Fisher's LSD test. The same effects on vigor were evaluated using the Kruskal–Wallis test. $p < 0.05$ was considered statistically significant.

RESULTS

Effect of Cryopreservation on Sperm Traits

Raw semen presented 64.6 ± 3.2% live sperm and 47.0 ± 4.3% total motility; most of the sperm showed oscillatory movement (41.0 ± 4.6%), but very little progressive movement was observed (6.0 ± 2.6%). Mean sperm vigor was 2.7 ± 0.3 vigor score.

Concerning sperm motility (total, progressive, and oscillatory), vigor, viability, and DNA integrity, no differences were found between C0 and C24 sperm samples during cooling (**Tables 2**, **3**).

The freezing procedure resulted in a significant reduction in spermatozoa viability after thawing (FT) compared with F0 and F2.5 values ($p < 0.05$), but no differences were detected regarding DNA integrity (**Table 4**). A marked decrease in oscillatory and

TABLE 3 | Percentage of llama sperm motility (TM, total motility; PM, progressive motility, and OM, oscillatory movement) and vigor (scale 0–5) during cooling procedure.

	TM (%)	PM (%)	OM (%)	Vigor (vigor score)
C0	51.5 ± 6.2[a]	11.0 ± 6.6[a]	40.3 ± 7.4[a]	4.0 ± 0.4[a]
C24	58.3 ± 5.7[a]	24.2 ± 6.0[a]	34.0 ± 6.8[a]	3.6 ± 0.4[a]

Values are mean ± standard error of the mean.
[a]The same letter within columns indicates no significant difference ($p > 0.05$).

TABLE 4 | Percentage of llama sperm viability, and DNA integrity during freezing procedure.

	Viability (%)	DNA integrity (%)
F0	66.9 ± 2.4[a]	88.7 ± 4.5[a]
F2.5	66.2 ± 2.4[a]	88.0 ± 4.5[a]
FT	36.7 ± 2.4[b]	90.4 ± 4.4[a]

Values are mean ± standard error of the mean.
[a,b]Within columns, different letters between rows indicate significant differences for each of the sperm characteristic ($p < 0.05$).

TABLE 5 | Percentage of llama sperm motility (TM, total motility, PM, progressive motility, and OM, oscillatory movement) and vigor (scale 0–5) during freezing procedure.

	TM (%)	PM (%)	OM (%)	Vigor (vigor score)
F0	41.0 ± 4.1[a]	17.3 ± 3.6[ab]	23.7 ± 3.6[a]	3.2 ± 0.3[a]
F2.5	47.0 ± 4.1[a]	23.3 ± 3.6[b]	23.7 ± 3.6[a]	3.3 ± 0.3[a]
FT	16.3 ± 4.1[b]	9.3 ± 3.6[a]	7.0 ± 3.7[b]	2.5 ± 0.3[a]

Values are mean ± standard error of the mean.
[a,b]Within columns, different letters between rows indicate significant differences ($p < 0.05$).

total motility was observed after thawing (FT) when compared to F0 and F2.5 sperm samples. Particularly, progressive motility of F2.5 samples was higher than FT ones (**Table 5**). Sperm vigor was not affected during the freezing protocol (**Table 5**).

Fresh Sperm Ultrastructure

Prior to cryopreservation, sperm cells showed the typical morphology of llama spermatozoa (**Figure 2A**). Three well-differentiated regions could be detected in the sperm head: the anterior acrosomal region, the equatorial segment or posterior acrosomal region, and the post-acrosomal region (**Figures 2B,C**). While the anterior acrosomal region could be distinguished from the posterior acrosomal region by a straight line, the post-acrosomal region was virtually separated by a curved line. Lack of acrosomes was observed in some cells as a result of a premature acrosome reaction, revealed by the disruption of the plasma membrane in the anterior acrosomal region, whereas the post-acrosomal segment and its plasma membrane were preserved (**Figure 2C**).

TEM micrographs of fresh sperm showed intact plasma membranes with homogeneous condensed chromatin

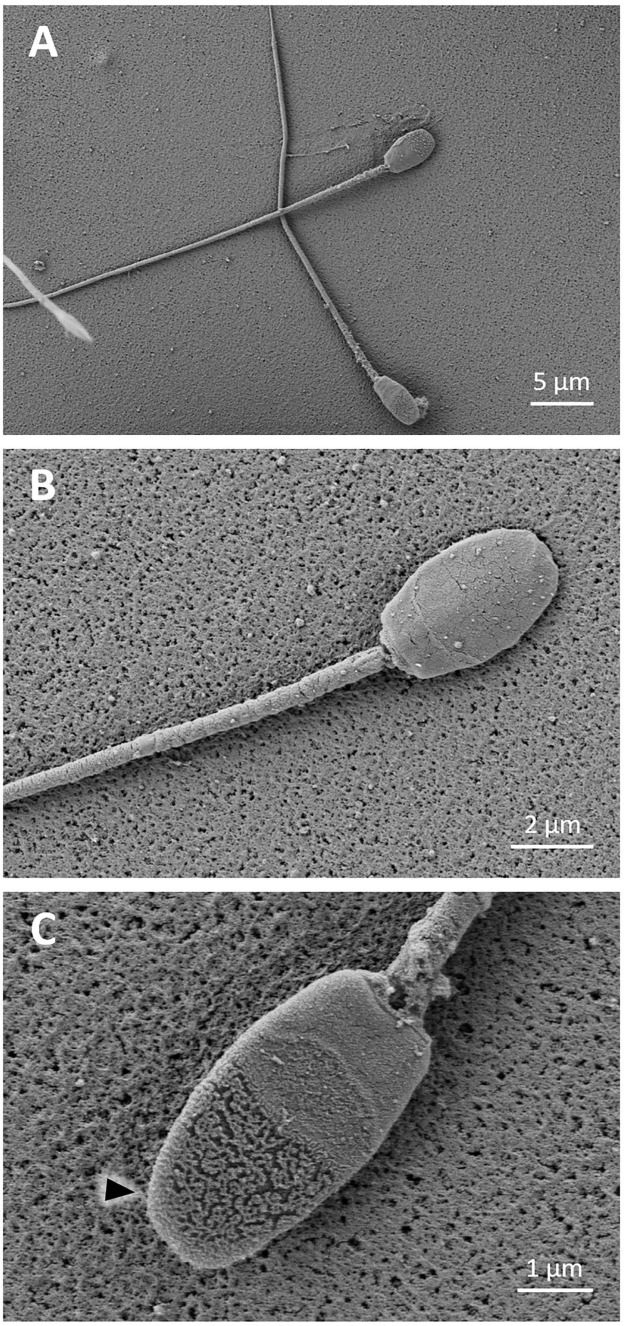


FIGURE 2 | SEM images of fresh llama sperm. **(A)** Typical llama sperm morphology. **(B)** Sperm showing a slightly rough or cracked surface; the anterior acrosomal region, the equatorial segment, and the post-acrosomal region can be distinguished in the sperm head. **(C)** Sperm showing a typical acrosome reaction (triangle).

(**Figure 3A**); only a tiny number of cells showed slightly undulated membranes in the sperm head. Acrosomal membranes and their content were preserved in most cells (**Figure 3A**), with only a few reacted sperm cells (**Figure 3B**). Well-preserved midpieces with the characteristic mitochondrial sheath surrounded by the plasma membrane were observed

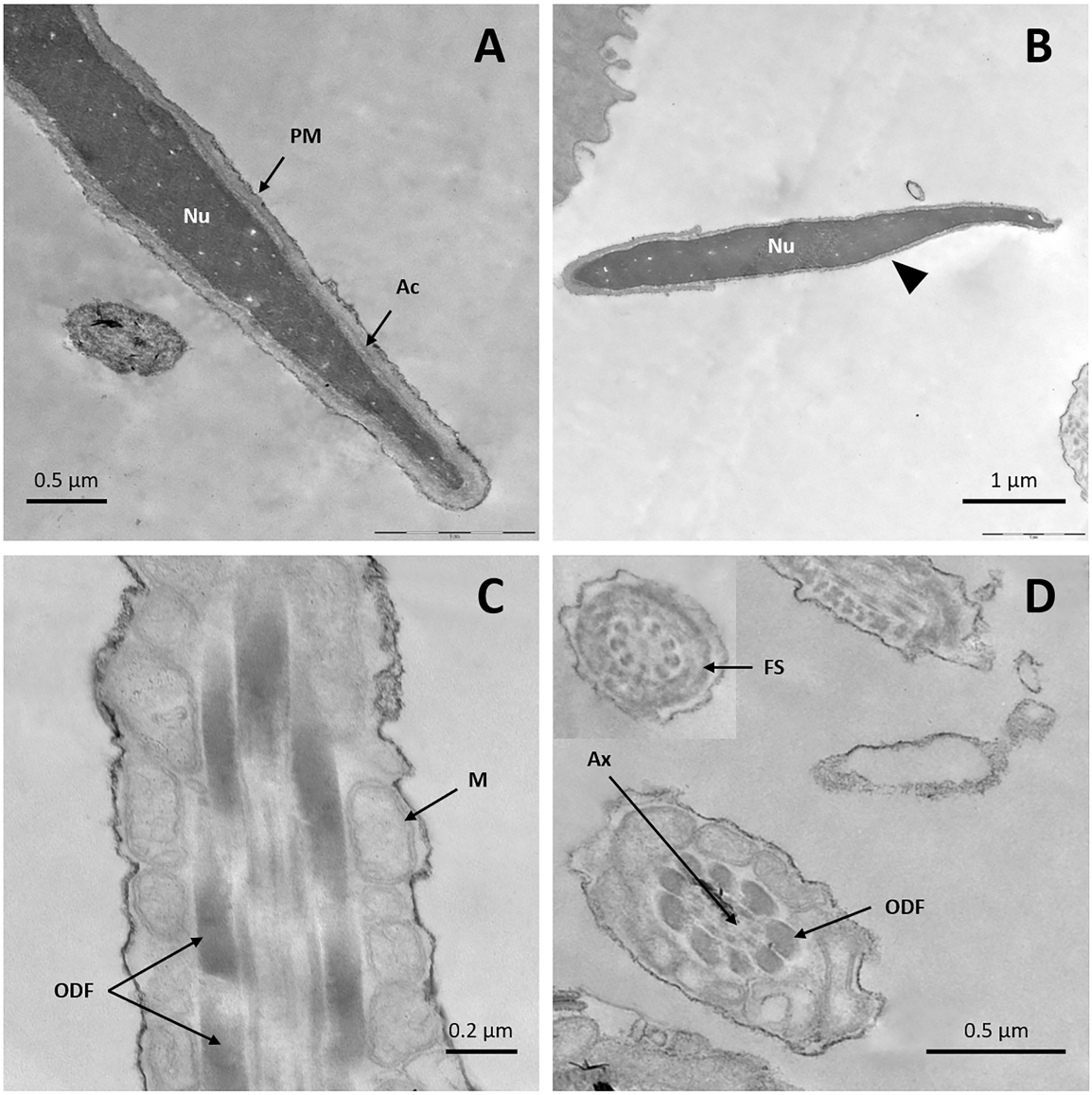


FIGURE 3 | TEM images of fresh llama sperm. **(A)** Longitudinal section of the spermatozoon head showing an intact nucleus, acrosome, and plasma membrane. **(B)** Longitudinal section of the sperm head showing an intact nucleus and reacted acrosome (triangle). **(C)** Longitudinal section of sperm midpiece showing an intact plasma membrane and mitochondrial sheath. **(D)** Cross-section of the sperm tail showing the axoneme, the outer dense fibers and the fibrous sheath. Ac, acrosome; Ax, axoneme; FS, fibrous sheath; M, mitochondria; Nu, nucleus; ODF, outer dense fibers; PM, plasma membrane.

(**Figure 3C**). In the principal and mid pieces of the tail, presence of an intact axoneme with conventional structure was identified (**Figure 3D**), as well as presence of the typical nine outer dense fibers with drop formats around the axoneme (**Figure 3D**), and an external fibrous sheath (inset **Figure 3D**).

Effect of Cooling on the Sperm Ultrastructure

Sperm showed signs of damage after 24 h of cooling. Some sperm cells showed bent tails (**Figures 4A,B**), with a rough or cracked surface, and other cells even showed loss of the plasma membrane (**Figure 4C**). Sperm cells with reacted acrosomes were also observed (**Figure 4B**).

TEM images showed many sperm cells with a swollen or irregular undulated plasmalemma in the head region, and a lack of acrosomal content (**Figures 5A,B**). In the midpiece, loss of the plasma membrane and mitochondria was detected (**Figures 5C,D**). Some alteration in the axoneme and periaxonemal structures such as changes in the number and arrangement of the microtubule doublets, or abnormal size and position of the outer dense fibers was observed (**Figure 5C**).

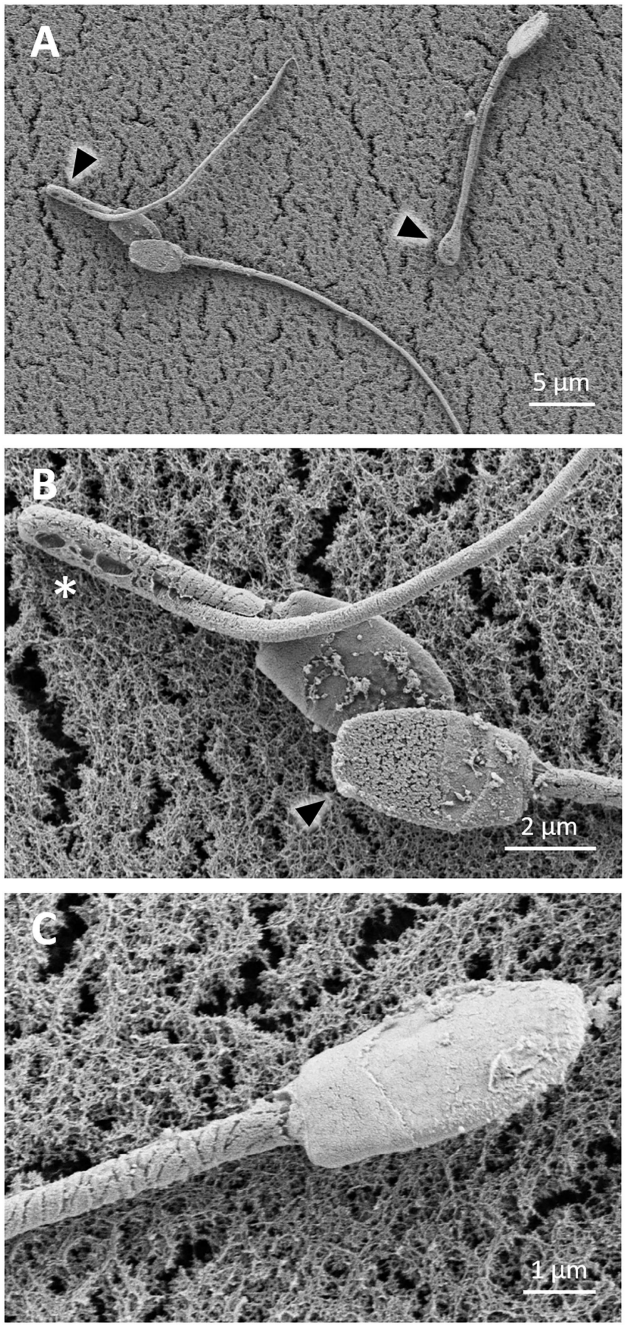


FIGURE 4 | SEM images of llama sperm cooled for 24 h. **(A)** Sperm showing bent tails (triangle). **(B)** Sperm showing rough surface, bent tail (asterisk) and acrosome reaction (triangle). **(C)** Sperm showing cracked surface at the head level and loss of the plasma membrane in the midpiece.

Effect of Freezing on the Sperm Ultrastructure

During the freezing procedure, sperm suffered cryodamage. After 2.5 h of cooling until 5°C, some detached heads and coiled tails were observed (**Figure 6A**). Sperm showed an irregular surface (**Figure 6B**), and broken membranes and loss of mitochondria were detected in sperm midpieces (**Figure 6C**). Similarly, after thawing, sperm showed detached heads as well as bent and coiled tails (**Figures 7A,B**). In the sperm head, the plasma membrane showed perforations (**Figure 7B**). In the midpieces, loss of the membrane and mitochondria could be distinguished (**Figure 7C**).

After sperm cells were cooled until 5°C, but prior to freezing with liquid nitrogen, TEM images revealed detachment of the plasma membrane in many sperm heads (**Figure 8A**). Sperm without acrosome and with vesicles of fused plasma and outer acrosomal membranes was also observed, features that indicate the acrosome reaction (**Figures 8B,C**). In addition, some sperm cells displayed vacuoles in the nucleus (**Figures 8B,C**). In the midpiece, broken plasma membranes, mitochondrial vacuolization and even loss of mitochondria were noticed (**Figures 8C,D**). In the tail, some sperm cells presented an abnormal microtubule pattern (**Figure 8C**).

After freezing/thawing, great damage was detected with TEM. Examination of sperm cells revealed the presence of vacuoles in the sperm nucleus (**Figure 9A**). Acrosomal membranes appeared broken and there was a lack of acrosomal content (**Figures 9B,C**). Besides, certain cells exhibited the acrosome reaction with vesicles of fused plasma and outer acrosomal membranes (**Figure 9E**). The plasma membrane, particularly at the head level, was remarkably affected: spermatozoa were swollen, irregularly undulated, and demonstrated a broken plasma membrane (**Figures 9B–D**). Similar membrane alterations were detected in transverse sections of the midpiece and principal piece (**Figures 9F–H**). Examination of the sperm midpiece showed mitochondrial damaged: a distorted cristae structure and vacuolization (**Figures 9F,G**). The axoneme structure and outer dense fibers were conserved along the tail, but the fibrous sheath was damaged (**Figure 9H**).

DISCUSSION

Application of electron microscopy techniques to evaluate spermatozoa allowed detection of distressed cell structures after cryopreservation. In order to improve preservation procedures, llama sperm samples were first subjected to a cooling or freezing protocol and then evaluated using high-resolution microscopy techniques (SEM and TEM), to reveal any morphological injuries associated with cryopreservation methods by comparison with fresh sperm cells.

When llama spermatozoa were cooled until 5°C, sperm traits (viability, DNA integrity, motility, and vigor) were preserved after 24 of cooling. The presence of EY in the extender seems to be essential to maintain these sperm traits, as reported previously in llamas (13, 20). Even though cooling is not the most aggressive technique, SEM and TEM images revealed ultrastructural alterations affecting sperm quality after 24 h at 5°C. This might partly explain the poor results obtained when cryopreserved llama sperm is used in AI procedures (9–14). The ultrastructural changes observed would indicate that cooling protocols for llama semen need to be reviewed, evaluating

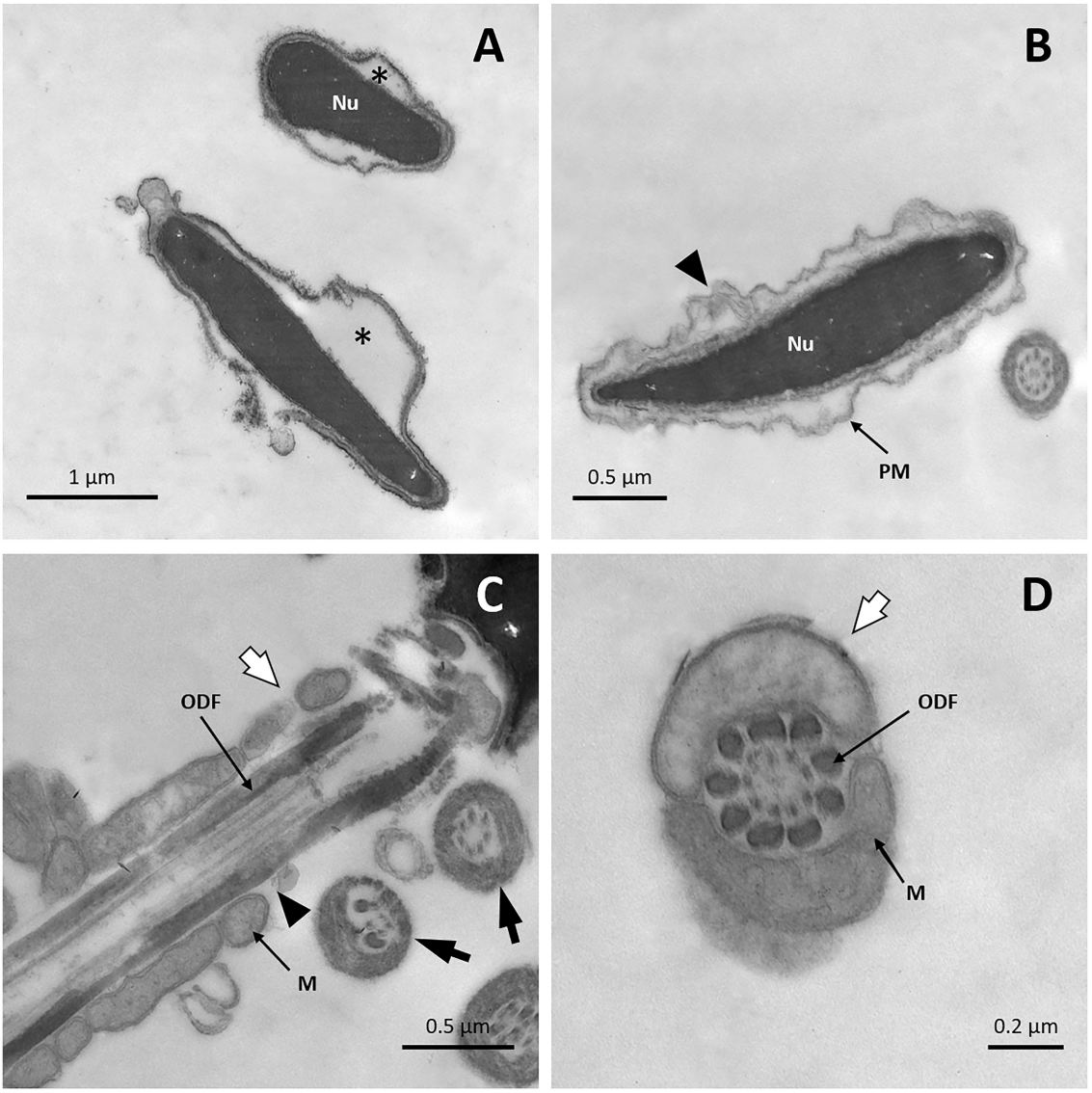


FIGURE 5 | TEM images of llama sperm after 24 h of cooling. **(A)** Spermatozoa showing plasma membrane and acrosome detachment (asterisk). **(B)** Section of the spermatozoon head showing an irregularly undulated plasma membrane (triangle). **(C)** Longitudinal section of a sperm midpiece showing loss of the plasma membrane (white arrow) and mitochondria (triangle). Cross-sections of sperm tails with disorganized outer dense fibers and microtubules (black arrow). **(D)** Cross-section of the midpiece of a sperm cell showing an entirely loss of the plasma membrane (white arrow). M, mitochondria; Nu, nucleus; ODF, outer dense fibers; PM, plasma membrane.

different cooling rates, and diluents from an ultrastructural point of view.

Specifically, after 24 h of cooling, sperm showed signs of damage like cracked or loose membranes, reacted acrosomes, and bent and coiled tails. TEM observations also confirmed acrosomal damage, loss of mitochondria, and disorganization of the axoneme and periaxonemal structures. Several studies have demonstrated increased levels of reactive oxygen species (ROS) in semen preserved at 4°C (21–24). When the balance between ROS production and detoxification by antioxidants is disrupted, an excess of ROS creates oxidative stress. Consequences of oxidative damage are numerous, ranging from membrane damage and lipid peroxidation, inhibition of respiration, leakage of intracellular enzymes, axonemal protein damage, and mitochondrial membrane damage (25), some of which are in coincidence with our observations. The swollen and detached plasmalemma from the sperm head observed with TEM might also be associated with osmotic stress suffered during the cooling procedure, as has been seen in other species (26, 27).

During freezing, sperm traits were evaluated at the beginning of the process, after cooling until 5°C for 2.5 h, and after thawing. Similar to the cooling procedure, during which sperm

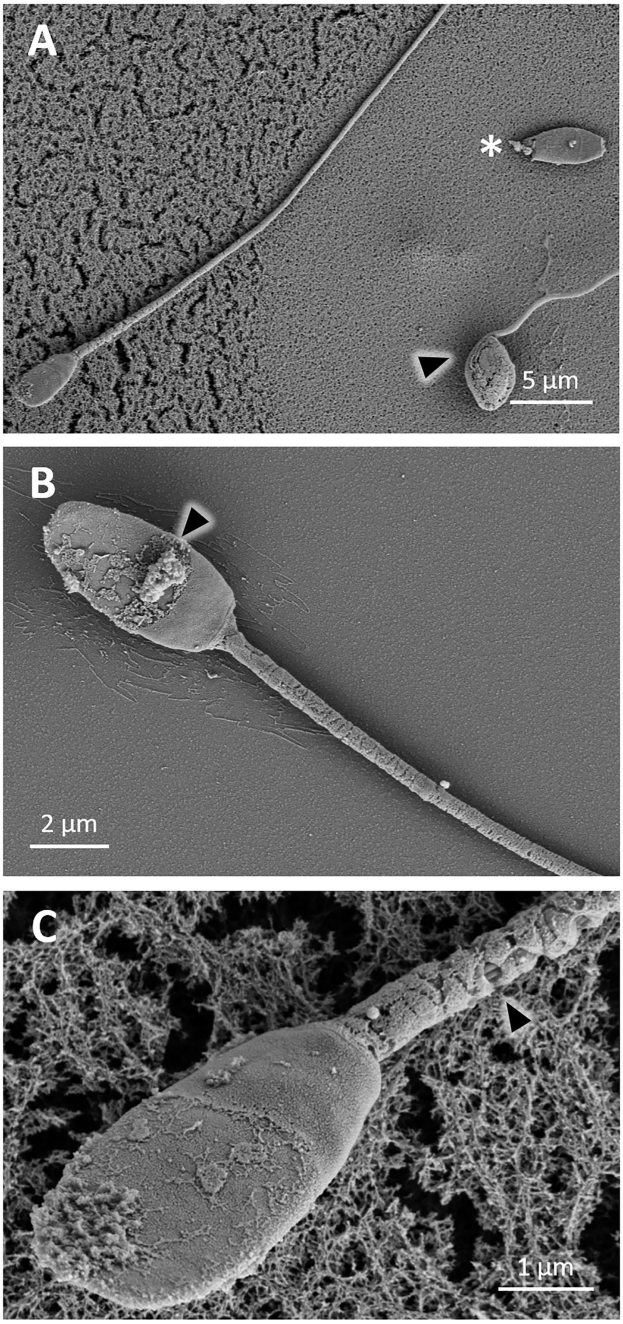


FIGURE 6 | SEM images of llama sperm after cooling for 2.5 h. **(A)** Sperm cells showing detached head (asterisk) and coiled tail (triangle). **(B)** Sperm cell showing a rough surface (triangle). **(C)** Sperm cell showing loss of the plasma membrane and mitochondria (triangle) in the midpiece.

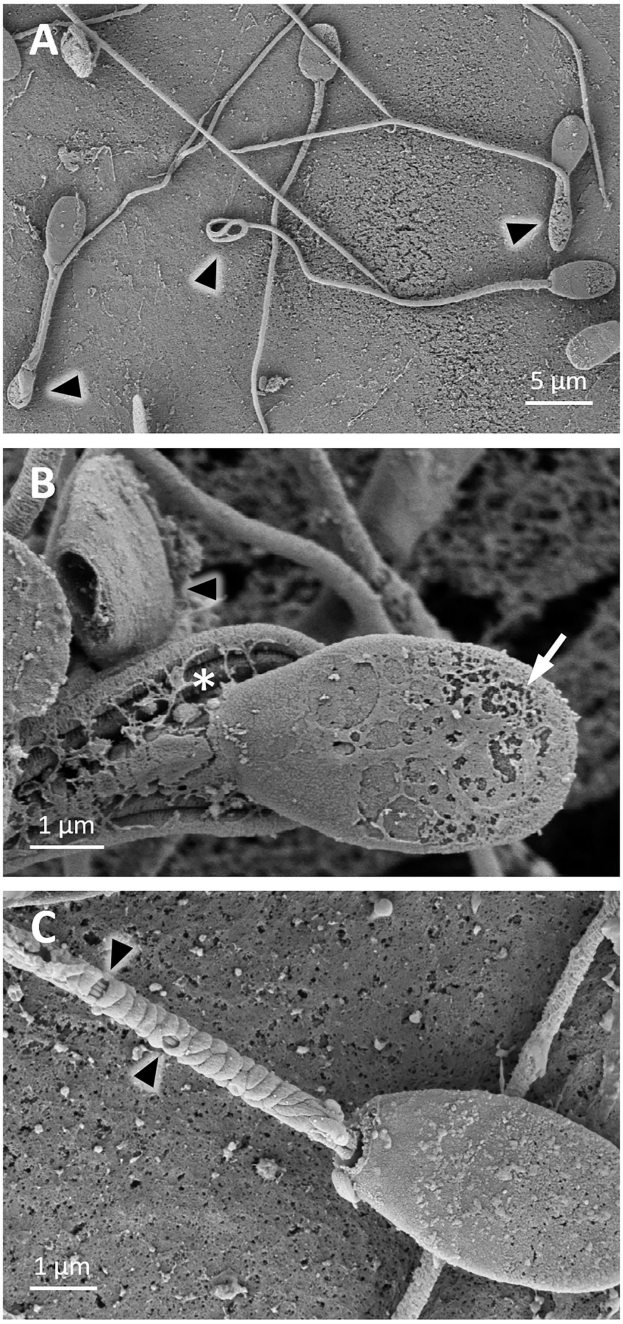


FIGURE 7 | SEM images of llama sperm after thawing. **(A)** Sperm cells with bent and coiled tails (triangle). **(B)** Sperm cell with plasma membrane perforations (arrow) and coiled tail (asterisk), and a detached head (triangle). **(C)** Sperm cell showing loss of the plasma membrane and mitochondria (triangles) in the midpiece.

cells were chilled for 24, after 2.5 h of cooling no differences were detected regarding sperm viability, DNA integrity, motility, and vigor compared with F0. As described for other species, the step from 5°C to liquid nitrogen seems to be the most critical one during freezing (4, 28), being sperm cells exposed to cold shock, ice crystal formation, and cellular dehydration, which severely compromise sperm quality (29). Indeed, post-thawing sperm viability decreased from 66.9 ± 2.4% in F0 samples to 36.7 ±2.4% in frozen/thawed samples (∼55% of sperm viability was retained with the protocol used in the

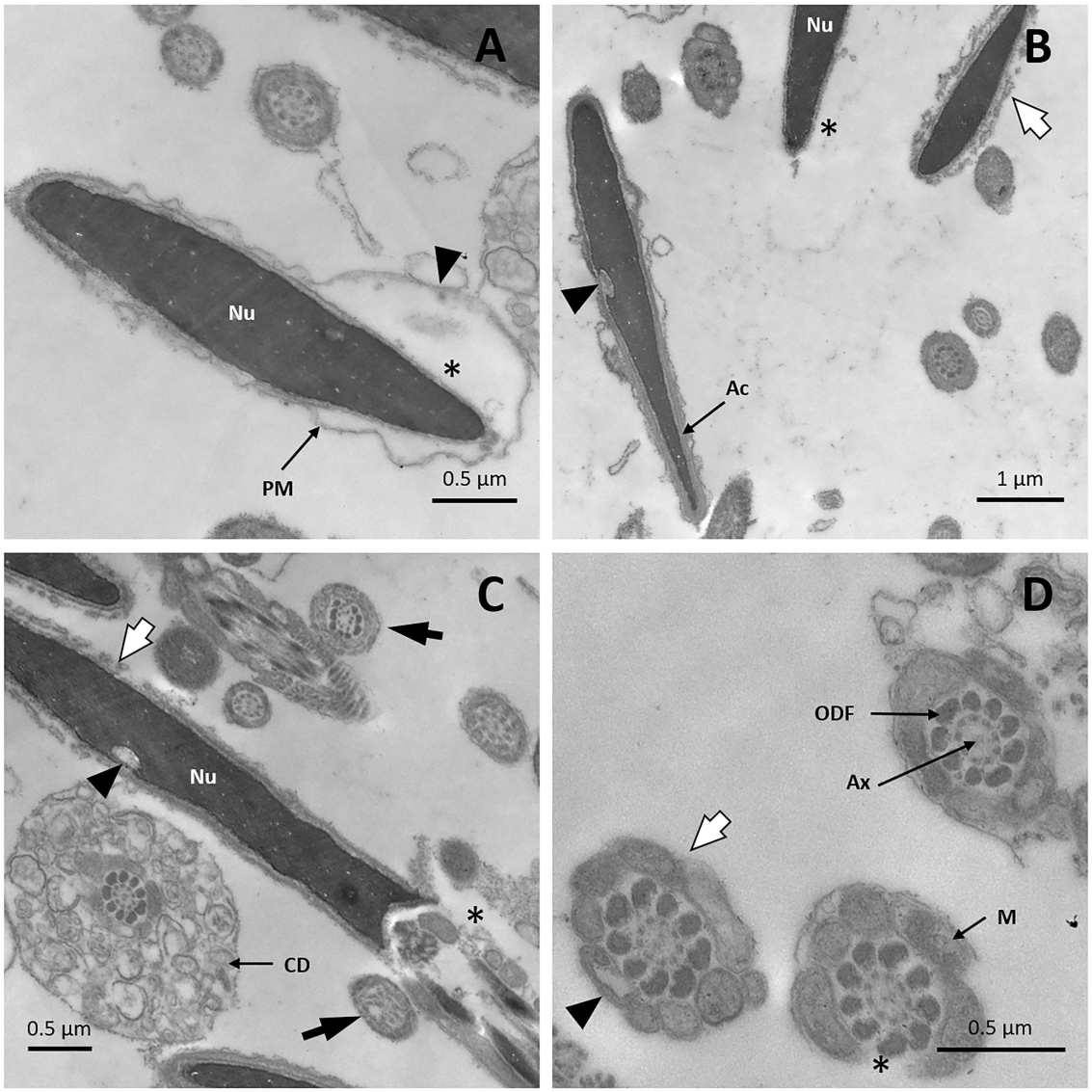


FIGURE 8 | TEM images of llama sperm after 2.5 h of cooling. **(A)** Spermatozoon showing plasma membrane detachment (triangle) and a lack of acrosomal content (asterisk). **(B)** Sperm cells showing invagination in the nucleus (triangle) and acrosome reaction: it can be noticed that the outer acrosomal membrane and the plasma membrane form bubbles (white arrow), and that the acrosome is completely absent (asterisk). **(C)** Spermatozoon showing invagination in the nucleus (triangle), acrosome-reaction (white arrow), and loss of the plasma membrane in the midpiece (asterisk). Axonemal microtubule disorganization is also detected (black arrow). **(D)** Cross-section of the midpiece of a sperm cell showing the entire loss of the plasma membrane (white arrow). Missing (asterisk) and vacuolated (triangle) mitochondria are also observed. Ax, axoneme; CD, cytoplasmic droplet; M, mitochondria; Nu, nucleus; ODF, outer dense fibers; PM, plasma membrane.

present study). This is in agreement with previous reports on South American camelids, which described a similar decrease in sperm viability after thawing, even though different freezing protocols were employed (12, 30–32). With regard to sperm motility, a significant decrease in the percentage of total motility was observed after thawing when compared to F0 and F2.5. This decrease would be related to the decline in post-thaw viability. However, as reported in other studies, the impairment of mitochondrial activity (23, 33, 34), and axonemal protein damage (5) observed during freezing–thawing might also explain this result. On the other hand, DNA integrity would not be affected by the freezing procedure, and sperm cells also would be able to conserve their vigor with the protocol used in this study.

Although SEM and TEM images revealed similar alterations like those observed with the cooling protocol, sperm cryodamage seemed to be more severe after thawing. The plasma membrane surrounding the sperm head and tail were partially lost or exhibited various degrees of swelling, undulations, and perforations. Similar membrane injuries were detected after freezing-thawing in others species, e.g., bull (1), goat (15),

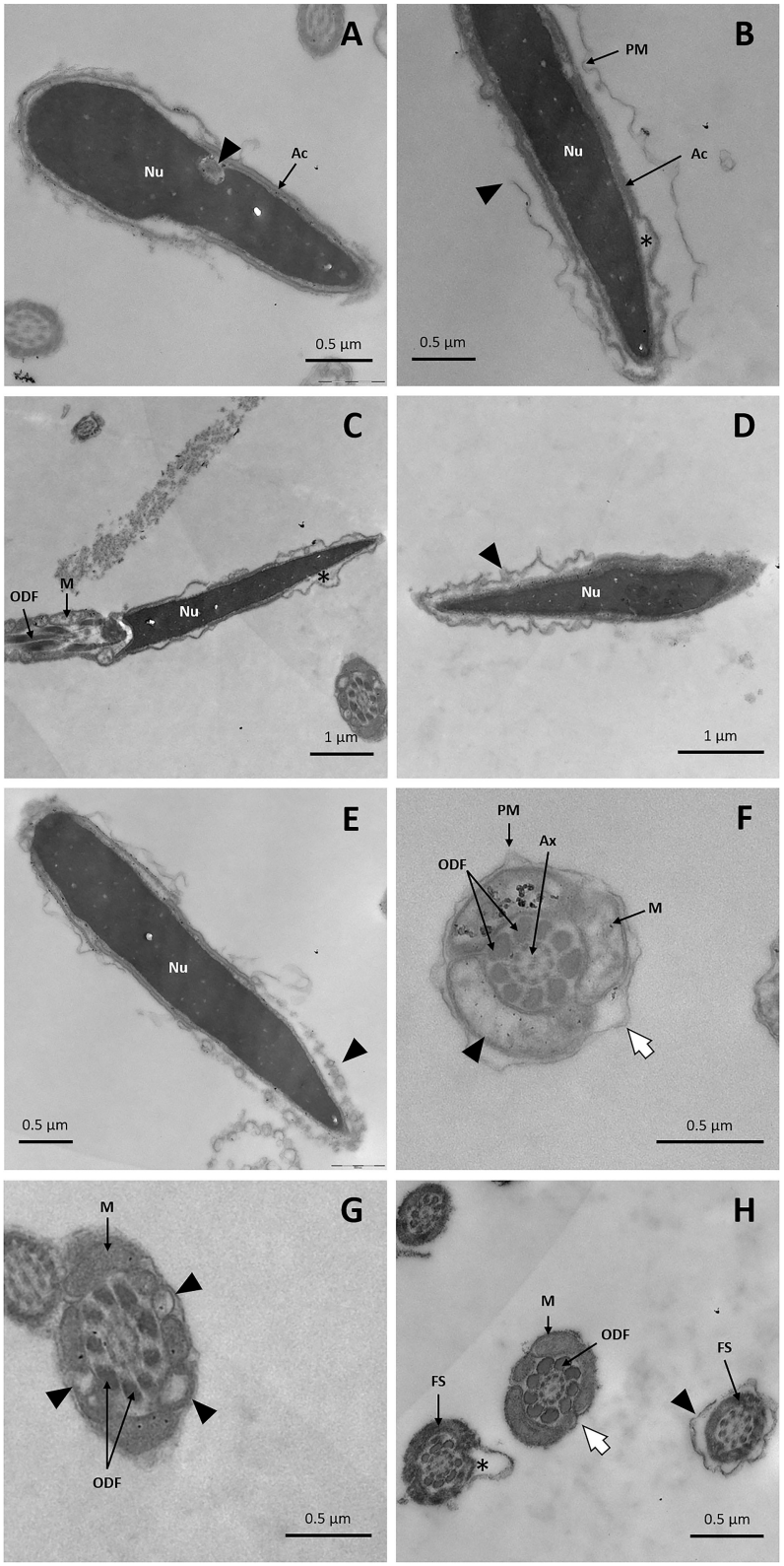


FIGURE 9 | TEM images of llama sperm after thawing. **(A)** Presence of invagination (triangle) in the sperm nucleus. **(B)** Longitudinal section of the spermatozoon head showing an intact nucleus with broken plasma membrane (triangle) and a lack of acrosomal content (asterisk). **(C)** Longitudinal section of the sperm head and midpiece showing an irregularly undulated plasma membrane and the lack of acrosomal content (asterisk). **(D)** Sperm with swollen plasma membrane.

(Continued)

FIGURE 9 | (E) Acrosome-reacted spermatozoon: it can be noticed that the outer acrosomal membrane and the plasma membrane form bubbles (triangle). **(F)** Cross-section of the midpiece of a sperm tail showing plasma membrane detachment (white arrow) and mitochondria with distorted cristae (triangle). **(G)** Cross-section of a sperm midpiece showing mitochondria with vacuolization (triangles). **(H)** Cross-sections of sperm tails showing detachment of the plasma membrane in the principal piece (triangle), the entire loss of the plasma membrane in the midpiece (white arrow) and fibrous sheath disorganization (asterisk). Ac, acrosome; Ax, axoneme; FS, fibrous sheath; M, mitochondria; Nu, nucleus; ODF, outer dense fibers; PM, plasma membrane.

dromedary (16), ram (17), and dog (18), demonstrating that the plasma membrane is one of the most sensitive parts of the sperm cell. Indeed, during freezing and thawing, the temperature, and osmotic variations induce tremendous alterations in cell water volume, which confer considerable mechanical stress on the cell membranes (7). Particularly, the swollen plasma membrane observed in all these species might be associated with osmotic shock suffered during freezing-thawing. It is clear that the plasma membrane exerts a fundamental role in the cell coating and cellular homeostasis, which means that membrane damage compromises the sperm function by altering the selective permeability of sperm membranes, and disrupting the ability of sperm to interact with cells of the oviduct and the oocyte (4, 7). In line with the above-mentioned issues, bull and buffalo sperm have shown a remarkable loss of capacity to regulate the intracellular concentrations of ions after cryopreservation (35, 36). Moreover, reports in several mammals have demonstrated that fewer cryopreserved sperm attached to the oviductal epithelial cells compared to fresh semen (37–39). Reduced sperm binding is likely a consequence of membrane injury, possibly by structural damage to the sperm receptors or by incomplete receptor aggregation (7).

After thawing, acrosomal integrity was seriously compromised. This became evident because of the absence of acrosomal content and acrosomal membrane damage observed with TEM. The acrosomal membrane is another sensitive part of the sperm cell to damage during cryopreservation (3). The loss of the acrosome has been associated with the mechanical stress suffered by the sperm during freezing or cryo-capacitation events that could lead to a premature acrosome reaction (7). In mammals, the acrosome reaction should occur in the vicinity of the mature cumulus-oocyte complex (40), and hence a premature acrosome reaction might result in reduced fertilization rates.

In the current study, presence of intranuclear vacuoles was observed after thawing, as has been detected in different species during semen cryopreservation (1, 15, 17). Even though vacuoles in the human sperm head were not associated with altered sperm traits or DNA damage (41), reports on different species have indicated that nuclear vacuoles are associated with abnormal chromatin packaging or chromatin damage (1, 42), and exert a negative effect on embryo development (43).

The mitochondrial structure was also affected by the freezing-thawing process. TEM and SEM images revealed vacuolization, cristae distortion and even loss of mitochondria in the midpiece of frozen sperm. Mitochondrial damage during freezing and thawing would lead to a decrease in spermatozoa mitochondrial membrane potential (MMP). Indeed cryopreservation (cooling and freezing) procedures significantly reduced the sperm MMP (23, 44, 45). Since high sperm MMP is required for mitochondrial ATP synthesis, the ability of the sperm cell to produce ATP could be compromised and therefore influence the sperm motility. Another sperm structure distorted by the freezing process was the fibrous sheath, which is a cytoskeletal structure surrounding the axoneme and outer dense fibers in the principal piece region of the sperm flagellum. The fibrous sheath acts as a scaffold for proteins, and is believed to influence the degree of flexibility, the plane of flagellar motion, and the shape of the flagellar beat (46). Other studies indicate that some cytoskeletal proteins of the tail decrease in abundance (e.g., ODF2, ROPN1, actin) or change their distribution during freezing–thawing in bull, ram, and buffalo sperm (47–50). These findings along with our observations might be associated with the loss of sperm motility during freezing-thawing.

Overall, the ultrastructural damage of llama spermatozoa caused by cryopreservation protocols appears to be irreversible, directly affecting sperm survival.

In conclusion, this is the first time that the ultrastructure of llama sperm and ultrastructural damage caused by cooling and freezing procedures have been characterized. Evaluations with SEM and TEM allowed us to detect several sperm ultrastructural injuries that would not be noticed by routine seminal assessments, and reveal alterations that start during the cooling procedure and intensify with freezing. In this context, further studies should be focused on a reduction in damage of the sperm plasma membrane, acrosome and mitochondria during cooling and freezing in order to improve cryopreservation protocols of llama semen.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

Protocol UNT 002/18 approved by the Committee for the Use and Care of Laboratory Animals (CICUAL) from Universidad Nacional de Tucumán.

AUTHOR CONTRIBUTIONS

RZ and SAA conceived and designed the study and interpreted the data. RZ, XAC-G, LMS, AM, AVD, MEA, and SAA collected samples and performed the experiments. RZ and LMS performed the statistical analysis. RZ wrote the draft manuscript. MEA and SAA revised and discussed the manuscript. All authors read and approved the manuscript for publication.

FUNDING

This study was supported by the ANPCyT (grant number BID-PICT 2016-2277).

ACKNOWLEDGMENTS

The authors would like to thank Magister Osvaldo Arce for his support with statistical analyses.

REFERENCES

1. Khalil WA, El-Harairy MA, Zeidan AE, Hassan MA, Mohey-Elsaeed O. Evaluation of bull spermatozoa during and after cryopreservation: structural and ultrastructural insights. *Int J Vet Sci Med.* (2018) 6:S49–56. doi: 10.1016/j.ijvsm.2017.11.001
2. Watson PF. The causes of reduced fertility with cryopreserved semen. *Anim Reprod Sci.* (2000) 60:481–92. doi: 10.1016/S0378-4320(00)00099-3
3. Pesch S, Bergmann M. Structure of mammalian spermatozoa in respect to viability, fertility and cryopreservation. *Micron.* (2006) 37:597–612. doi: 10.1016/j.micron.2006.02.006
4. Ezzati M, Shanehbandi D, Hamdi K, Rahbar S, Pashaiasl M. Influence of cryopreservation on structure and function of mammalian spermatozoa: an overview. *Cell Tissue Bank.* (2020) 21:1–15. doi: 10.1007/s10561-019-09797-0
5. Peris-Frau P, Soler AJ, Iniesta-Cuerda M, Martín-Maestro A, Sánchez-Ajofrín I, Medina-Chávez DA, et al. Sperm cryodamage in ruminants: understanding the molecular changes induced by the cryopreservation process to optimize sperm quality. *Int J Mol Sci.* (2020) 21:2781. doi: 10.3390/ijms21082781
6. Chantler E, Abraham-Peskir JV. Significance of midpiece vesicles and functional integrity of the membranes of human spermatozoa after osmotic stress. *Andrologia.* (2004) 36:87–93. doi: 10.1111/j.1439-0272.2004.00609.x
7. Bailey JL, Bilodeau JF, Cormier N. Semen cryopreservation in domestic animals: a damaging and capacitating phenomenon. *J Androl.* (2000) 21:1–7. doi: 10.1002/j.1939-4640.2000.tb03268.x
8. Curry MR. Cryopreservation of semen from domestic livestock. *Rev Reprod.* (2000) 5:46–52. doi: 10.1530/revreprod/5.1.46
9. Bravo PW, Skidmore JA, Zhao XX. Reproductive aspects and storage of semen in *Camelidae*. *Anim Reprod Sci.* (2000) 62:173–93. doi: 10.1016/S0378-4320(00)00158-5
10. Aller JF, Rebuffi GE, Cancino AK, Alberio RH. Influencia de la criopreservación sobre la motilidad, viabilidad y fertilidad de espermatozoides de llama (*Lama glama*). *Arch Zootec.* (2003) 52:15–23. Available online at: https://www.redalyc.org/articulo.oa?id=49519702
11. Vaughan J, Galloway D, Hopkins D. Artificial insemination in alpacas (*Lama pacos*). Kingston, ACT: RIRDC Rural Industries Research and Development Corporation. (2003).
12. Stuart CC, Vaughan JL, Kershaw CM, De Graaf SP, Bathgate R. Effect of diluent type, cryoprotectant concentration, storage method and freeze/thaw rates on the post-thaw quality and fertility of cryopreserved alpaca spermatozoa. *Sci Rep.* (2019) 9:12826. doi: 10.1038/s41598-019-49203-z
13. Giuliano SM, Chaves MG, Trasorras VL, Gambarotta, M, Neild D, et al. Development of an artificial insemination protocol in llamas using cooled semen. *Anim Reprod Sci.* (2012) 131:204–10. doi: 10.1016/j.anireprosci.2012.03.010
14. García W, Alarcón V, Bravo PW. Inseminación artificial de alpacas con semen refrigerado y con inclusión de dos tipos de yema de huevo. *Rev Investig Vet Perú.* (2017) 28:337–44. doi: 10.15381/rivep.v28i2.13080
15. Shi L, Ren Y, Zhou H, Hou G, Xun W, Yue W, et al. Effect of rapid freezing–thawing techniques on the sperm parameters and ultrastructure of Chinese Taihang black goat spermatozoa. *Micron.* (2014) 57:6–12. doi: 10.1016/j.micron.2013.09.004
16. Shahin MA, Khalil WA, Saadeldin IM, Swelum AAA, El-Harairy MA. Comparison between the effects of adding vitamins, trace elements, and nanoparticles to shotor extender on the cryopreservation of dromedary camel epididymal spermatozoa. *Animals.* (2020) 10:78. doi: 10.3390/ani10010078
17. Arando A, Delgado JV, Arrebola FA, León JM, Alcalá CJ, Pérez-Marín CC. Vitrification induces critical subcellular damages in ram spermatozoa. *Cryobiology.* (2019) 87:52–9. doi: 10.1016/j.cryobiol.2019.02.005
18. Cerdeira J, Sánchez-Calabuig MJ, Pérez-Gutiérrez JF, Hijon M, Castaño C, Santiago-Moreno J. Cryopreservation effects on canine sperm morphometric variables and ultrastructure: comparison between vitrification and conventional freezing. *Cryobiology.* (2020) 95:164–70. doi: 10.1016/j.cryobiol.2020.03.007
19. Ozkavukcu S, Erdemli E, Isik A, Oztuna D, Karahuseyinoglu S. Effects of cryopreservation on sperm parameters and ultrastructural morphology of human spermatozoa. *J Assist Reprod Genet.* (2008) 25:403–11. doi: 10.1007/s10815-008-9232-3
20. Carretero MI, Giuliano SM, Arraztoa CC, Santa Cruz RC, Fumuso FG, Neild DM. Comparison of two cooling protocols for llama semen: with and without collagenase and seminal plasma in the medium. *Andrologia.* (2017) 49:e12691. doi: 10.1111/and.12691
21. Wang AW, Zhang H, Ikemoto I, Anderson DJ, Lioughlin KR. Reactive oxygen species generation by seminal cells during cryopreservation. *Urology.* (1997) 49:921–5. doi: 10.1016/S0090-4295(97)00070-8
22. Vishwanath R, Shannon P. Storage of bovine semen in liquid and frozen state. *Anim Reprod Sci.* (2000) 62:23–53. doi: 10.1016/S0378-4320(00)00153-6
23. Kadirvel G, Kumar S, Kumaresan A. Lipid peroxidation, mitochondrial membrane potential and DNA integrity of spermatozoa in relation to intracellular reactive oxygen species in liquid and frozen-thawed buffalo semen. *Anim Reprod Sci.* (2009) 114:125–34. doi: 10.1016/j.anireprosci.2008.10.002
24. Evangelista-Vargas S, Santiani A. Detection of intracellular reactive oxygen species (superoxide anion and hydrogen peroxide) and lipid peroxidation during cryopreservation of alpaca spermatozoa. *Reprod Domest Anim.* (2017) 52:819–24. doi: 10.1111/rda.12984
25. Aitken RJ, Gordon E, Harkiss D, Twigg JP, Milne P, Jennings Z, et al. Relative impact of oxidative stress on the functional competence and genomic integrity of human spermatozoa. *Biol. Reprod.* (1998) 59:1037–46. doi: 10.1095/biolreprod59.5.1037
26. González-Fernández L, Morrell JM, Peña FJ, Macías-García B. Osmotic shock induces structural damage on equine spermatozoa plasmalemma and mitochondria. *Theriogenology.* (2012) 78:415–22. doi: 10.1016/j.theriogenology.2012.02.021
27. Sieme H, Oldenhof H, Wolkers WF. Sperm membrane behaviour during cooling and cryopreservation. *Reprod Domest Anim.* (2015) 50:20–6. doi: 10.1111/rda.12594
28. Neild DM, Gadella BM, Chaves MG, Miragaya MH, Colenbrander B, Agüero A. Membrane changes during different stages of a freeze–thaw protocol for equine semen cryopreservation. *Theriogenology.* (2003) 59:1693–705. doi: 10.1016/S0093-691X(02)01231-1
29. Hammerstedt RH, Graham JK, Nolan JP. Cryopreservation of mammalian sperm: what we ask them to survive. *J Androl.* (1990) 11:73–88.
30. Santiani A, Huanca W, Sapana R, Huanca T, Sepúlveda N, Sánchez R. Effects on the quality of frozen-thawed alpaca (*Lama pacos*) semen using two different cryoprotectants and extenders. *Asian J Androl.* (2005) 7:303–9. doi: 10.1111/j.1745-7262.2005.00021.x
31. Santiani A, Evangelista S, Valdivia M, Risopatrón J, Sánchez R. Effect of the addition of two superoxide dismutase analogues (Tempo and Tempol) to alpaca semen extender for cryopreservation. *Theriogenology.* (2013) 79:842–6. doi: 10.1016/j.theriogenology.2012.12.012
32. Carretero MI, Neild DM, Ferrante A, Caldevilla M, Arraztoa CC, Fumuso FG, et al. Effect of cryoprotectant and equilibration temperature on cryopreservation of *Lama Glama* spermatozoa. *Andrologia.* (2015) 47:685–93. doi: 10.1111/and.12319
33. Yoon SJ, Kwon WS, Rahman MS, Lee JS, Pang MG. A novel approach to identifying physical markers of cryo-damage in bull spermatozoa. *PLoS ONE.* (2015) 10:e0126232. doi: 10.1371/journal.pone.0126232
34. Kumar D, Kumar P, Singh P, Yadav SP, Yadav PS. Assessment of sperm damages during different stages of cryopreservation in water buffalo by fluorescent probes. *Cytotechnology.* (2016) 68:451–8. doi: 10.1007/s10616-014-9798-9

35. Bailey JL, Buhr MM. The impact of cryopreservation on Ca2+ regulation by bovine spermatozoa. *Can J Anim Sci.* (1994) 74:45–51. doi: 10.4141/cjas94-007
36. Kadirvel G, Kumar S, Kumaresan A, Kathiravan P. Capacitation status of fresh and frozen-thawed buffalo spermatozoa in relation to cholesterol level, membrane fluidity and intracellular calcium. *Anim Reprod Sci.* (2009) 116:244–53. doi: 10.1016/j.anireprosci.2009.02.003
37. Dobrinski I, Thomas PG, Ball PA. Cryopreservation reduced the ability of equine spermatozoa to attach to oviductal epithelial cells and zonae pellucidae *in vitro*. *J Androl.* (1995) 16:536–42.
38. Goldman EE, Ellington JE, Foote RH. Reaction of fresh and frozen bull spermatozoa incubated with fresh and frozen bovine oviduct epithelial cells. *Reprod Nutr Dev.* (1998) 38:281–8. doi: 10.1051/rnd:19980308
39. Burgess CM, Clutterbuck AL, England GCW. The effect of cryopreservation on the capacitation status and epithelial cell attachment capability of dog spermatozoa. *Vet J.* (2012) 192:398–402. doi: 10.1016/j.tvjl.2011.08.026
40. Yanagimachi R. Mammalian sperm acrosome reaction: where does it begin before fertilization? *Biol Reprod.* (2011) 85:4–5. doi: 10.1095/biolreprod.111.092601
41. Fortunato A, Boni R, Leo R, Nacchia G, Liguori F, Casale S, et al. Vacuoles in sperm head are not associated with head morphology, DNA damage and reproductive success. *Reprod Biomed Online.* (2016) 32:154–61. doi: 10.1016/j.rbmo.2015.10.009
42. Franco Jr JG, Mauri AL, Petersen CG, Massaro FC, Silva LFI, Felipe V, et al. Large nuclear vacuoles are indicative of abnormal chromatin packaging in human spermatozoa. *Int J Androl.* (2012) 35:46–51. doi: 10.1111/j.1365-2605.2011.01154.x
43. Vanderzwalmen P, Hiemer A, Rubner P, Bach M, Neyer A, Stecher A, et al. Blastocyst development after sperm selection at high magnification is associated with size and number of nuclear vacuoles. *Reprod Biomed Online.* (2008) 17:617–27. doi: 10.1016/S1472-6483(10)60308-2
44. Martin G, Sabido O, Durand P, Levy R. Cryopreservation induces an apoptosis-like mechanism in bull sperm. *Biol Reprod.* (2004) 71:28–37. doi: 10.1095/biolreprod.103.024281
45. Guthrie HD, Welch GR. Determination of intracellular reactive oxygen species and high mitochondrial membrane potential in viable boar sperm using fluorescence activated flow cytometry. *J Anim Sci.* (2006) 84:2089–100. doi: 10.2527/jas.2005-766
46. Eddy EM, Toshimori K, O'Brien DA. Fibrous sheath of mammalian spermatozoa. *Microsc Res Techniq.* (2003) 61:103–15. doi: 10.1002/jemt.10320
47. Felipe-Pérez YE, Valencia J, de Lourdes Juárez-Mosqueda M, Pescador N, Roa-Espitia AL, Hernández-González EO. Cytoskeletal proteins F-actin and β-dystrobrevin are altered by the cryopreservation process in bull sperm. *Cryobiology.* (2012) 64:103–9. doi: 10.1016/j.cryobiol.2011.12.004
48. He Y, Wang K, Zhao X, Zhang Y, Ma Y, Hu J. Differential proteome association study of freeze-thaw damage in ram sperm. *Cryobiology.* (2016) 72:60–8. doi: 10.1016/j.cryobiol.2015.11.003
49. Naresh S. Effect of cooling (4 °C) and cryopreservation on cytoskeleton actin and protein tyrosine phosphorylation in buffalo spermatozoa. *Cryobiology.* (2016) 72:7–13. doi: 10.1016/j.cryobiol.2015.12.004
50. Yoon SJ, Rahman MS, Kwon WS, Ryu DY, Park YJ, Pang MG. Proteomic identification of cryostress in epididymal spermatozoa. *J Anim Sci Biotechnol.* (2016) 7:67. doi: 10.1186/s40104-016-0128-2

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 30 October 2020
doi: 10.3389/fvets.2020.555261

Plasma IGF1 and 17β-Estradiol Concentrations During the Follicular Wave in Llamas

María F. Gallelli[1,2], Carolina Bianchi[1,3], Enzo Zampini[1,2], Marcelo Aba[3], M. Gambarotta[4] and Marcelo Miragaya[2]*

[1] Consejo Nacional de Investigaciones Científicas y Técnicas, Buenos Aires, Argentina, [2] Facultad de Ciencias Veterinarias, Instituto de Investigación y Tecnología en Reproducción Animal, Universidad de Buenos Aires, Buenos Aires, Argentina, [3] Laboratorio de Endocrinología, Facultad de Veterinaria, Universidad Nacional del Centro de la Provincia de Buenos Aires, Tandil, Argentina, [4] Departamento de Bioestadística, Facultad de Ciencias Veterinarias, Universidad de Buenos Aires, Buenos Aires, Argentina

The aim of this study was to characterize the temporal association between follicular waves and circulating concentrations of 17β-estradiol (E2) and IGF1 in llamas. Follicular waves could be clearly divided in three phases: growth, plateau and regression; with a mean duration of 18.8 ± 0.32 days. All follicular waves showed overlapping, so that as one dominant follicle was regressing, another one was growing. E2 plasma concentration showed a wavelike pattern, similar to that followed by the dominant follicle; reaching its maximum concentration at the end of the growth phase and decreasing at the end of the plateau phase. IGF1 also showed variations during the follicular wave. It tended to increase during the growth phase and decreased toward Days 14 and 16. IGF1 reached its maximum concentration before E2 did (5 ± 0.8 vs. 7.2 ± 0.5 days after wave emergence) and before the maximum follicular diameter was attained (10.2 ± 0.46 days after wave emergence). Both hormones started to rise again in coincidence with the development of a new follicular wave. The observed profiles allow to suggest that IGF1 could have a role on folliculogenesis and ovarian steroideogenesis in llamas, as reported for other species.

Keywords: estradiol, folliculogenesis, follicular wave, IGF1, llama

OPEN ACCESS

Edited by:
Marina Rufino Salinas Fortes, The University of Queensland, Australia

Reviewed by:
Bouchra El Amiri, Institut National de la Recherche Agronomique de Settat, Morocco
Silvana Andrea Apichela, Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina

****Correspondence:***
María F. Gallelli
mgallelli@fvet.uba.ar

Specialty section:
This article was submitted to Animal Reproduction - Theriogenology, a section of the journal Frontiers in Veterinary Science

Received: *24 April 2020*
Accepted: *05 October 2020*
Published: *30 October 2020*

Citation:
Gallelli MF, Bianchi C, Zampini E, Aba M, Gambarotta M and Miragaya M (2020) Plasma IGF1 and 17β-Estradiol Concentrations During the Follicular Wave in Llamas. Front. Vet. Sci. 7:555261. doi: 10.3389/fvets.2020.555261

INTRODUCTION

Llamas are induced ovulators requiring a stimulus in presence of a mature follicle to trigger the ovulatory process (1, 2). In absence of this stimulus, ovarian activity occurs in waves of follicular growth and regression, in which one follicle becomes dominant, grows to maturity and finally regresses (3, 4). Consequently, follicular waves may be divided into three phases: growth, mature and regression; with a total duration of 17–22 days, according to different authors (3, 5–7). Regression and growth of successive dominant follicles usually overlap, so that as one dominant follicle is regressing, another one is growing to dominance (7). A positive correlation between follicle size and plasma estradiol has been reported in llamas, with maximum concentrations coinciding with maximum follicle size (3, 4, 7). Thus, the follicular wave pattern and the hormonal profiles are responsible for long periods of behavioral estrous. On the contrary, plasma progesterone levels remain low during the follicular wave, since ovulation does not occur (3).

Although in the last years hormonal regulation of folicullogenesis in camelids has been studied, the mechanisms underlying the process are not yet fully elucidated (4, 8). Conversely in other species, it has been reported that folicullogenesis is regulated not only by gonadotrophins, but also by local ovarian hormones (like activin or inhibin), and other factors such as Insulin like growth factor 1 (IGF1) (9, 10). This hormone stimulates proliferation and differentiation of granulosa cells, and it would play a role in follicle selection (9, 11–13). In fact, in many domestic animals, it has been reported that IGF1 concentrations in follicular fluid are related to follicle size (11, 14). In addition, IGF1 stimulates ovarian steroideogenesis and vascularization (12, 15, 16) acting throughout its own receptor (IGF1R) which is expressed in ovarian follicles in different species [cattle: (12); ewe: (17); dog: (16)]. Recently, IGF1R was identified in ovarian follicles of alpacas (18) and llamas (unpublished data); being its expression greater in granulosa than in theca interna cells of tertiary follicles, especially in absence of a corpus luteum. This information suggests a possible effect of IGF1 on camelids ovaries.

The objective of this study was to characterize the temporal association between follicular waves and circulating concentrations of 17β-estradiol (E2) and IGF1 in llamas.

MATERIALS AND METHODS

Animals

This study was approved by the ethics committee of the Faculty of Veterinary Sciences of Buenos Aires University (CICUAL N° 2016/27). Animals belong to the Faculty of Veterinary Sciences of Buenos Aires University (Argentina) and the experimental procedures were performed from January to August at this institution (34°36′ S, 58°22′ W, at sea level). Ten (n= 10) non-pregnant, non-lactating, sexually mature, clinically healthy llamas, ranging between 5 and 10 years of age and with an average body weight of 120 ± 20 kg were included in the study. All females were kept isolated from males. They were kept at grass and were fed hay bale or pellets and water *ad libitum*. They were all in good nutritional status with a mean body condition score of 3 (body condition score from 1 = thin to 5 = obese) (19).

Follicular Activity Characterization

Animals were examined daily by rectal palpation and transrectal ultrasonography to assess ovarian status (Berger® LC 2010 plus attached to a 5.0 MHz linear-array electronic transducer, Buenos Aires, Argentina). The examination procedure was similar to that described by Bravo et al. (1). A follicular wave was defined as the simultaneous growth of a group of follicles; and a dominant follicle was defined as one that grew to at least 7 mm and whose diameter exceeded that of all other follicles in the cohort (5). The day of wave emergence (Day 0) was defined retrospectively, as the day on which the dominant follicle was first detected, at a diameter of 3–4 mm (5). The follicular wave was divided in three phases: growth, plateau and regression. The growth phase was defined as the period comprehended between the day on which the follicle emerged until it maintained its growth around the maximum diameter. The plateau phase, that followed the growth phase, was characterized by variations in follicle's diameter ≤ 0.5 mm. The regression phase was characterized by two consecutive decreasing measurements of the follicle and finished when it reached 3–4 mm at the end of the wave (5, 7). In each occasion, the diameter of each follicle was measured three times, and the averaged diameters were considered the diameter of that follicle.

Blood Sampling

For the evaluation of plasma E2 and IGF1 concentrations, blood samples were collected by jugular venipuncture every other day. Blood samples were collected in tubes with heparin and centrifuged immediately. Plasma was stored at −20°C until IGF1 and E2 assays were performed.

Hormone Determinations

Plasma E2 concentration was determined using a commercial RIA kit (Estradiol double antibody, KE2D, Siemens Medical Solutions Diagnostics, CA, USA) reported for use in bovine plasma (20) and validated for use with llama plasma after minor modifications (2). The sensitivity of the assay was 1.5 pg ml^{-1} and the intra-assay and the inter-assay coefficient was below 11 and 8% for concentrations between 1.5 and 48 pg ml^{-1}, respectively. All samples were measured in duplicates.

IGF1 was determined by RIA as reported in cattle (21). Briefly, samples and standards were subjected to the acid-ethanol cryoprecipitation method previously described by Breier et al. (22). Concentrations of IGF-I were determined using an antibody (UB2-495) (rabbit anti-hIGF-I) provided by L. Underwood and J. J. Van Wyk and distributed by the Hormone Distribution Program of the NIDDK. Serially diluted llama plasma samples containing high IGF1 concentrations produced curves parallel to the standard curve. Assay sensitivity was 15.6 ng/ml. Intra- and interassay coefficients of variation were below 7.2 and 12.8%, respectively. All samples were measured in duplicates.

Statistical Analysis

Hormone concentrations along the follicular wave were analyzed by repeated measures one-way ANOVA, followed by the Bonferroni test (Graph Pad 5, USA). Llamas were considered as a blocking factor. Pearson's correlation was calculated to study the relationship between IGF1 and E2. Values are expressed as mean ± SEM. Differences were considered significant when P-values were < 0.05 and a tendency was considered when $P < 0.1$.

RESULTS

Follicular Wave Characteristics

In all the studied animals, the follicular waves could be clearly divided in the mentioned phases (growth, plateau, and regression) (**Figure 1**). Mean follicular wave duration was 18.8 ± 0.32 days. The mean maximum follicular diameter was 9.9 ± 0.1 mm and it was attained at 10.2 ± 0.46 days after emergence. In all cases, the dominant follicle showed ovulatory diameter (≤ 7 mm) at day 7 after wave emergence.

The follicular growth phase averaged 9 ± 0.3 days, the plateau phase 4.2 ± 0.3 days, and the regression phase 6.7 ±0.26 days.

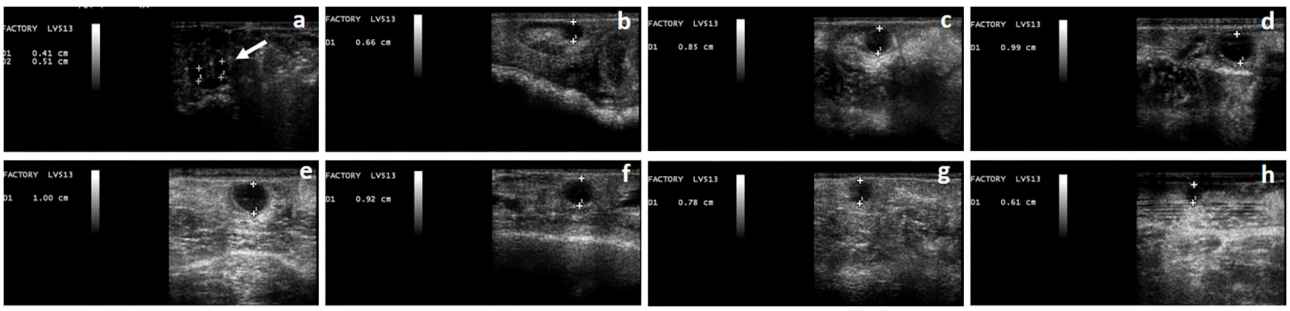


FIGURE 1 | Phases of the follicular wave in a llama. The growth phase **(a–d)**, plateau phase **(d,e)**, regression phase **(f–h)**, can be observed. At the beginning of the growth phase, the simultaneous growth of a group of follicles can be observed **(a)**, one of which will become dominant (indicated by the arrow). Follicle's diameter is expressed in cm.

The mean growth rate was 0.66 ± 0.03 mm/day while the mean regression rate was 0.71 ± 0.04 mm/day.

Mean diameter of the dominant follicle differed along the follicular wave ($P < 0.001$). Significant differences were detected from Day 0 until Day 6 ($P < 0.001$). Mean follicular diameter did not differ significantly later between Days 6 vs. 8, 10, 12, and 14 ($P > 0.05$). However, mean follicular diameter was greater on Day 6 than Days 16 and 18 ($P < 0.001$).

All follicular waves showed overlapping, so that as one follicle was regressing, another follicle was growing. Ovulatory follicles developed 80% times in the left ovary and 20%, in the right ovary. The sequential development of ovulatory follicles alternated between ovaries in 55% of cases. No ultrasonographical evidences of ovulation were recorded during the study.

Plasma E2 and IGF1 Concentrations During the Follicular Wave

Estradiol

Plasma estradiol concentration showed significant differences during the follicular wave ($P = 0.0007$). This hormone showed a wavelike pattern similar to that described by the dominant follicle. Plasma E2 concentration increased during the growth phase of the follicular wave, reached its maximum concentration at the end of this phase and then started to decrease at the end of the plateau phase. The E2 mean wave length was of 12.9 ± 0.7 days, being shorter than the mean duration of the follicular wave. Thus, E2 started to decline before the structural regression of the follicle. As all follicular waves showed overlapping, plasma E2 concentration started to increase during the regression phase of the previous wave in association with the growth phase of the successive follicular wave (**Figure 2**).

The mean maximum E2 concentration reached was 38.2 ± 4.5 pmol/l (10.34 ± 1.2 pg/ml) and it was attained at 7.2 ± 0.5 days after wave emergence. Mean E2 concentration was significantly greater on Days 6 vs. 0, 12, 14, and 16 ($P < 0.05$) and showed a tendency to be greater on Days 6 vs. 2 ($P < 0.1$). It also was significantly greater on Days 8 vs. 0, 2, 12, 14, and 16 ($P < 0.05$) (**Figure 2**). The mean diameter of the dominant follicle at the day of mean maximum E2 concentration was 8.7 ± 0.3 mm.

IGF1

Plasma IGF1 concentration showed differences during the follicular wave ($P= 0.0004$). Mean IGF1 plasma concentration tended to increase from Day 0 toward Days 4 and 6 ($P < 0.1$). Then it started to decrease, being significantly lower on Days 14 and 16 vs. 4 and 6 ($P < 0.05$) (**Figure 3**).

During the regression phase and simultaneously with the growth phase of the successive follicular wave, plasma IGF1 concentration started to increase (**Figure 3**).

The mean maximum IGF1 concentration was 460 ± 52 ng/ml and it was attained at 5 ± 0.8 days after wave emergence. Therefore, the mean maximum IGF1 concentration was attained during the growth phase, before the day when the mean maximum follicular diameter was reached (10.2 ± 0.46 days after wave emergence) and before the day of maximum plasma E2 concentration (7.2 ± 0.5 days). The mean diameter of the dominant follicle at the day of mean maximum IGF1 concentration was 7 ± 0.7 mm and mean plasma E2 concentration was 7.5 ±0.6. The mean lowest IGF1 concentration was 250 ± 39 ng/ml and it was attained at 11± 1.3 days after wave emergence, previous to the structural regression of the dominant follicle (regression phase) (**Figure 3**).

Correlation Analysis

There was correlation between IGF1 and E2 plasma concentration ($r^2 = 0.31$, $P = 0.008$).

DISCUSSION

In the present study, a temporal association between follicular waves and circulating concentrations of E2 and IGF1 in llamas has been demonstrated.

In agreement with previous studies, the development of the dominant follicle showed a wavelike pattern that could be divided into three phases (growth, plateau, and regression) (3, 7). The mean length of the follicular waves and their phases was shorter than that previously reported by some authors (3, 7) but similar to that described by others (5, 6). However, it must be considered that follicular wave length is highly variable, lasting from 17 to 25 days, and closely related with the maximum diameter reached

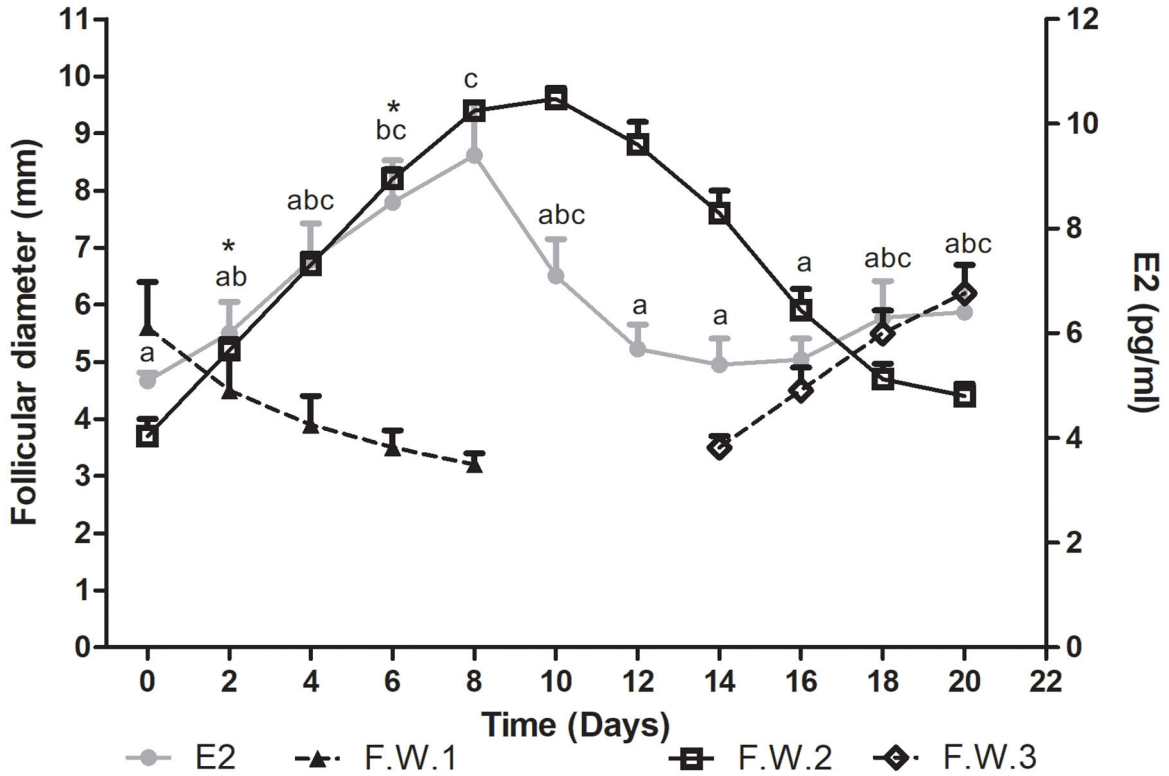


FIGURE 2 | Mean follicular diameter (mm) and plasma E2 concentration (pg/ml) during the follicular wave of llamas. Follicular wave 2 (F.W.2) emerged at Day 0 and the dominant follicle went through its different phases (growth, plateau, and regression) while the dominant follicle of the follicular wave 1 (F.W.1) (previous follicular wave) was regressing. E2 plasma concentration showed a wavelike pattern similar to the one described by the dominant follicle of F.W.2. At the end of the study, as a new follicular wave initiated (F.W.3), E2 plasma concentration started to rise again. Values are expressed as mean ± SEM. Different letters indicate E2 concentrations significantly different ($P < 0.05$). * Indicates that E2 concentration showed a tendency to be greater at Days 6 vs. 2 ($P < 0.1$).

by the dominant follicle (7). The phenomenon of overlapping of follicular waves has been observed in all cases. Likewise, in a study performed in llamas it has been reported that 100% of follicular waves were superimposed on the preceding ones (7). On the contrary, Bravo et al. (1) referred absence of overlapping in some follicular waves, in coincidence with basal E2 concentration. The underlying cause of the differences between all the mentioned studies is not clear, but it might be related to environmental or genetic factors that could have an impact on follicular activity.

Plasma E2 concentration showed a wavelike pattern, resembling that described by the dominant follicle, as previously reported in llamas (1, 3, 7). This hormone concentration increased during the growth phase, reaching its maximum at the end of this phase, and then decreased when the plateau phase was finishing. Also, E2 mean wave length was shorter than the mean duration of the follicular wave. Therefore, the structural regression of the dominant follicle was preceded by the decline in E2 concentration, so that the dominant follicle function was affected before ultrasonagraphic changes were observed. These results are consistent with those described by Cavilla et al. (7), who observed an increase of E2 concentration in coincidence with follicular wave emergence and a decrease during the plateau phase. Likewise, in cows and sheep, E2 reached its maximum concentration at the end of the growth phase and started to decrease during the static phase (23, 24). Thus, it has been proposed that the decline in E2 concentration during this phase could have a role in the development of the subsequent follicular wave (24). In the present study, during the regression phase, a new follicular wave started to develop, leading to a new increase in plasma E2 concentration.

Plasma IGF1 concentration showed variations during the follicular wave; being the first time that this factor was evaluated in llamas. IGF1 concentration tended to increase during the growth phase and then decreased toward Days 14 and 16. As the new follicular wave emerged, IGF1 started to rise again. To the authors knowledge, there are no reports regarding the effect of IGF1 on ovarian function in llamas. However, in other species it has been proposed that this factor would promote folliculogenesis, stimulating granulosa cells proliferation and follicular steroideogenesis [sheep: (25); pig: (26); cattle: (27, 28); goat: (29); horse: (13)]. Considering the observed IGF1 profile, it would be possible that in llamas this factor might be involved in the stimulation of follicular development, as in other species. Besides, variations in IGF1 plasma concentration during the follicular phase of the estrous cycle have been observed in domestic species [sheep: (25, 30); goat: (29); cattle: (28)]. These

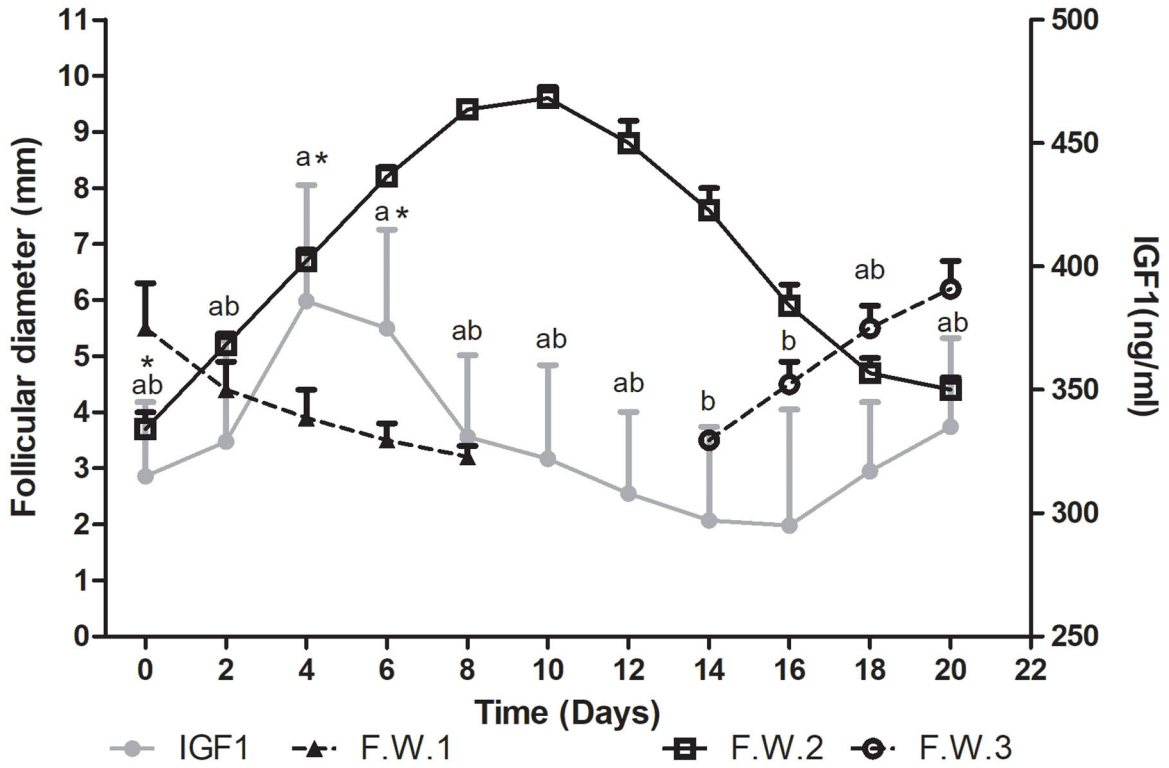


FIGURE 3 | Mean follicular diameter (mm) and mean plasma IGF1 concentration (ng/ml) during the follicular wave of llamas. Follicular wave 2 (F.W.2) emerged at Day 0 and the dominant follicle went through its different phases (growth, plateau, and regression) while the dominant follicle of the follicular wave 1 (F.W.1) (previous follicular wave) was regressing. IGF1 plasma concentration reached its greatest concentration during the growth phase. At the end of the study, as a new follicular wave initiated (F.W.3), IGF1 plasma concentration started to rise again. Values are expressed as mean ± SEM. Different letters indicate IGF1 concentrations significantly different ($P < 0.05$). * Indicates that IGF1 concentration showed a tendency to be greater at Days 4 and 6 vs. 0 ($P < 0.1$).

authors reported that plasma IGF1 concentration increased in association to estrous, in coincidence with maximum plasma E2 concentration. Moreover, *in vitro* studies have shown that IGF1 stimulates E2 production by granulosa cells in a dose dependent manner (31–33). Conversely, it has also been proposed that E2 would stimulate IGF1 production (25, 28). In the present study, a positive correlation between IGF1 and E2 has been observed. Opposite to what has been reported in other species, mean IGF1 greatest concentration was not attained simultaneously to E2 peak, reaching its maximum concentration before E2 did (5 ± 0.8 vs. 7.2 ± 0.5 days after wave emergence). These results might suggest that in llamas IGF1 would stimulate follicular E2 production, although an effect of E2 on IGF1 should not be discarded. Although correlation between E2 and IGF1 was observed, the association between these variables was not strong. This might be explained by the fact that ovarian function is regulated by multiple factors, including gonadotropins (34). In other species, it has been proposed that IGF1 might regulate follicular activity directly and/or by increasing follicle's sensitivity to gonadotropins action (11, 12, 27). The exact mechanisms by which IGF1 is involved in ovarian function regulation in camelids remains to be studied.

In conclusion, in llamas, E2 showed a wavelike pattern while IGF1 tended to increase toward Days 4 and 6 and then decreased; reaching its greatest values before E2 and the diameter of the dominant follicle did. This suggests that IGF1 might have a role in ovarian folliculogenesis and steroideogenesis. Further research is needed to elucidate the mechanism of action of IGF1 on ovarian tissue in this species.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Comité Institucional de Cuidado y Uso de Animales de Experimentación, Facultad de Ciencias Veterinarias, Universidad de Buenos Aires.

AUTHOR CONTRIBUTIONS

MGal designed and performed the study and wrote the manuscript. CB collaborated in the study design, performed E2 determinations, and revised the manuscript. EZ collaborated in field performance of the study. MGam performed statistical

analysis and revised the manuscript. MA revised and edited the manuscript. MM collaborated in the study design and revised and edited the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This research was supported by grants from Agencia Nacional de Promoción Científica y Tecnológica (PICT No. 2016-0145).

REFERENCES

1. Bravo PW, Fowler ME, Stabenfeldt GH, Lasley BL. Ovarian follicular dynamics in the llama. *Biol Reprod*. (1990) 43:579–85. doi: 10.1095/biolreprod43.4.579
2. Aba MA, Forsberg M, Kindhal H, Sumar J, Edqvist L-E. Endocrine changes after mating in pregnant and non-pregnant llamas and alpacas. *Acta Vet Scand*. (1995) 36:489–98.
3. Chaves MG, Aba M, Aguero A, Egey J, Berestin V, Rutter B. Ovarian follicular wave pattern and the effect of exogenous progesterone on follicular activity in non-mated llamas. *Anim Reprod Sci*. (2002) 69:37–46. doi: 10.1016/S0378-4320(01)00173-7
4. Vaughan J. Ovarian function in South American camelids (alpacas, llamas, vicunas, guanacos). *Anim Reprod Sci*. (2011) 124:237–43. doi: 10.1016/j.anireprosci.2010.08.031
5. Adams GP, Griffin PG, Ginther OJ. Effect of lactational and reproductive status on ovarian follicular waves in llamas (Lama glama). *J Reprod Fertil*. (1990) 90:535–45. doi: 10.1530/jrf.0.0900535
6. Aller JF, Alberio RH. Dinámica folicular en llamas en la época otoño-invernal. *Rev Arg Prod Anim*. (1996) 16:319–23.
7. Cavilla MP, Bianchi CP, Maistruarena C, Aba M. Ultrasonographic and endocrine characterization of follicular waves in llamas with a special reference to the overlapping phenomenon during successive waves. *Reprod Dom Anim*. (2013) 48:923–30. doi: 10.1111/rda.12187
8. Trasorras VL, Carretero MI, Neild DM, Chaves MG, Giuliano SM, Miragaya MH. Production, Preservation, and Transfer of South American Camelid embryos. *Front Vet Sci*. (2017) 4:190. doi: 10.3389/fvets.2017.00190
9. Fortune JE, Rivera GM, Yang M-Y. Follicular development: the role of the follicular microenvironment in selection of the dominant follicle. *Anim Reprod Sci*. (2004) 82:109–26. doi: 10.1016/j.anireprosci.2004.04.031
10. Beg MA, Ginther OJ. Follicle selection in cattle and horses: role of intrafollicular factors. *Reproduction*. (2006) 132:365–77. doi: 10.1530/rep.1.01233
11. Monget P, Fabre S, Mulsant P, Lecerf F, Elsen J, Mazerbourg S, et al. Regulation of ovarian folliculogenesis by IGF and BMP system in domestic animals. *Domest Anim Endocrinol*. (2002) 23:139–54. doi: 10.1016/S0739-7240(02)00152-2
12. Schams D, Berisha B, Kosmann M, Amselgruber WM. Expression and localization of IGF family members in bovine antral follicles during final growth and in luteal tissue during different stages of estrous cycle and pregnancy. *Domest Anim Endocrinol*. (2002) 22:51–72. doi: 10.1016/S0739-7240(01)00116-3
13. Ginther OJ, Gastal EL, Gastal MO, Beg MA. Intrafollicular effect of IGF1 on development of follicle dominance in mares. *Anim Rep Sci*. (2008) 105:417–23. doi: 10.1016/j.anireprosci.2007.11.007
14. Shimizu T. Molecular and cellular mechanisms for the regulation of ovarian follicular function in cows. *J Reprod Dev*. (2016) 62:323–9. doi: 10.1262/jrd.2016-044
15. Spicer LJ, Chamberlain CS, Maciel SM. Influence of gonadotropins on insulin- and insulin-like growth factor-I (IGF-I)-induced steroid production by bovine granulosa cells. *Domest Anim Endocrinol*. (2002) 22:237–54. doi: 10.1016/S0739-7240(02)00125-X
16. Balogh O, Müller L, Boos A, Kowalewski MP, Reichler IM. Expression of insulin like growth factor 1 and its receptor in preovulatory follicles and in the corpus luteum in the bitch. *Gen Comp Endocrinol*. (2018) 269:68–74. doi: 10.1016/j.ygcen.2018.08.016
17. Sosa C, Carriquiry M, Chalar C, Crespi D, Sanguinetti C, Cavestany D, et al. Endometrial expression of leptin receptor and members of the growth hormone-Insulin-like growth factor system throughout the estrous cycle in heifers. *Anim Reprod Sci*. (2010) 122:208–14. doi: 10.1016/j.anireprosci.2010.08.011
18. Gallelli MF, Bianchi C, Lombardo D, Rey FM, Rodriguez F, Castillo VA, et al. Leptin and IGF1 receptors in alpaca (Vicugna pacos) ovaries. *Anim Reprod Sci*. (2019) 200:96–104. doi: 10.1016/j.anireprosci.2018.12.001
19. Van Saun RJ. Nutritional requirements and assessing nutritional status in camelids. *Vet Clin Anim Food*. (2009) 25:265–79. doi: 10.1016/j.cvfa.2009.03.003
20. Sirois J, Fortune JE. Lengthening the bovine estrous cycle with low levels of exogenous progesterone: a model for studying ovarian follicular dominance. *Endocrinology*. (1990) 127:916–25.
21. Lacau-Mengido LM, Mejía ME, Díaz-Torga GS, Gonzalez Iglesias A, Formía N, Libertun C, et al. Endocrine studies in ivermectin-treated heifers from birth to puberty. *J Anim Sci*. (2000) 78:817–24. doi: 10.2527/2000.784817x
22. Breier BH, Gallaher BW, Gluckman PD. Radioimmunoassay for insulin-like growth factor-I: Solutions to some potential problems and pitfalls. *J Endocrinol*. (1991) 28:347–57. doi: 10.1677/joe.0.1280347
23. Souza CJH, Campbell BK, Baird DT. Follicular waves and concentrations of steroids and inhibin A in ovarian venous blood during the luteal phase of the oestrous cycle in ewes with an ovarian autotransplant. *J Endocrinol*. (1998) 156:563–72. doi: 10.1677/joe.0.1560563
24. Noseir WMB. Ovarian follicular activity and hormonal profile during estrous cycle in cows: the development of 2 versus 3 waves. *Reprod Biol Endocrin*. (2003) 1:50. doi: 10.1186/1477-7827-1-50
25. Spicer LJ, Hanrahan JP, Zavy MT, Enright WJ. Relationship between ovulation rate and concentrations of insulin-like growth factor-1 in plasma during the oestrous cycle in various genotypes of sheep. *J Reprod Fertil*. (1993) 97:403–9. doi: 10.1530/jrf.0.0970403
26. Kolodziejczyk J, Gertler A, Leibovich H, Rzasa J, Gregoraszczuk EL. Synergistic action of growth hormone and insulin-like growth factor I (IGF-I) on proliferation and estradiol secretion in porcine granulosa and theca cells cultured alone or in coculture. *Theriogenology*. (2003) 60:559–70. doi: 10.1016/S0093-691X(03)00032-3
27. Lucy MC. Regulation of ovarian follicular growth by somatotropin and insulin-like growth factors in cattle. *J Dairy Sci*. (2000) 83:1635–47. doi: 10.3168/jds.S0022-0302(00)75032-6
28. Kawashima C, Katsuya K, Hayashi K, Amaya Montoya C, Kaneko E, Matsunaga N, et al. Changes in plasma metabolic hormone concentrations during the ovarian cycles of Japanese black and Holstein cattle. *J Reprod Dev*. (2007) 53:247–54. doi: 10.1262/jrd.18104
29. Hashizume T, Ohtsuki K, Matsumoto N. Plasma insulin-like growth factor-I concentrations increase during the estrous phase in goats. *Domest Anim Endocrinol*. (2000) 18:253–63. doi: 10.1016/S0739-7240(99)00082-X
30. Spicer LJ, Zavy MT. Concentrations of insulin-like growth factor-I in serum of sheep with different ovulation rates: changes during the estrous cycle. *Theriogenology*. (1992) 37:395–405. doi: 10.1016/0093-691X(92)90198-Z
31. Spicer LJ, Alpizar E, Echternkamp SE. Effects of insulin, insulin-like growth factor I, and gonadotropins on bovine granulosa cell proliferation, progesterone production, estradiol production, and(or) insulin-like growth factor I production *in vitro*. *J Anim Sci*. (1993) 71:1232–41. doi: 10.2527/1993.7151232x

32. Yoshimura Y, Ando M, Nagamatsu S, Iwashita M, Adachi T, Sueoka K, et al. Effects of Insulin-Like Growth Factor-I on follicle growth, oocyte maturation, and ovarian steroidogenesis and plasminogen activator activity in the rabbit. *Biol Reprod*. (1996) 55:152–60. doi: 10.1095/biolreprod55.1.152
33. Gutiérrez CG, Campbell BK, Webb R. Development of a long-term bovine granulosa cell culture system: induction and maintenance of estradiol production, response to follicle-stimulating hormone, and morphological characteristics. *Biol Reprod*. (1997) 56:608–16. doi: 10.1095/biolreprod56.3.608
34. Silva JRV, Figueiredo JR, van den Hurk R. Involvement of growth hormone (GH) and insulin-like growth factor (IGF) system in ovarian folliculogenesis. *Theriogenology*. (2009) 71:1193–208. doi: 10.1016/j.theriogenology.2008.12.015

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer SA declared a shared affiliation with several of the authors MGal and CB to the handling editor at the time of review.

frontiers in Veterinary Science

MINI REVIEW
published: 06 November 2020
doi: 10.3389/fvets.2020.586858

Semen Handling in South American Camelids: State of the Art

Jane M. Morrell and Maria Celina Abraham*

Clinical Sciences, Swedish University of Agricultural Sciences, Uppsala, Sweden

Reproductive biotechnologies such as artificial insemination could be very useful for South American camelids, allowing widespread use of semen from breeding males with desirable genetics. However, artificial insemination is not widely employed in these species and is considered to have low overall efficiency. This is due in part to incomplete knowledge about the physiology of conception in these species, and also to challenges presented by semen collection and handling. Several recent reviews have centered on female camelid reproduction; therefore, in this review, the focus is on semen handling. Various semen collection methods are presented. Different methods of reducing seminal viscosity are compared, such as needling, enzyme treatment, and colloid centrifugation. Use of enzymes remains controversial because of widely differing results among research groups. Colloid centrifugation, particularly single layer centrifugation, has proved to be successful in facilitating development of sperm handling techniques in dromedary camels, and has also been used with llama semen. Therefore, protocols for colloid centrifugation of alpaca semen could be developed in the future.

Keywords: viscous seminal plasma, ovulation inducing factor, dribble ejaculators, single layer centrifugation, epididymal spermatozoa, sperm preservation

OPEN ACCESS

Edited by:
Marcelo H. Ratto,
Austral University of Chile, Chile

Reviewed by:
Warren Eliot Johnson,
Smithsonian Institution, United States
Mauricio Silva,
Temuco Catholic University, Chile

****Correspondence:***
Jane M. Morrell
jane.morrell@slu.se

Specialty section:
This article was submitted to Animal Reproduction - Theriogenology, a section of the journal Frontiers in Veterinary Science

Received: *27 July 2020*
Accepted: *12 October 2020*
Published: *06 November 2020*

Citation:
Morrell JM and Abraham MC (2020) Semen Handling in South American Camelids: State of the Art. Front. Vet. Sci. 7:586858. doi: 10.3389/fvets.2020.586858

INTRODUCTION

Although artificial insemination (AI) is common in other domestic livestock, the use of this reproductive biotechnology in camelids is limited, but especially so for South American camelids (SACs) (1). In any species, many production traits can be improved by careful selective breeding stock with desirable genetics (2). Fiber quality in SACs is no exception: it can be improved by selective breeding using animals with high quality fiber (3). In theory, AI could be very useful for improving fiber quality in SACs by allowing more widespread breeding from genetically elite individuals, but protocols for this reproductive biotechnology have not been optimized in these species.

Knowledge about reproductive physiology in SACs is currently incomplete (4), thus hindering the development of protocols for AI. Thus, factors such as the optimum timing of AI relative to ovulation, the number of spermatozoa required, and the site of semen deposition need to be defined for the different species. In addition, techniques for semen preservation require optimization (5). Moreover, SACs are induced ovulators, with an ovulation-inducing factor (recently identified as nerve growth factor) in seminal plasma being the main ovulation-inducing agent (6). Therefore, if AI is used, it may be necessary to induce ovulation, either by injecting hormones, mating to a vasectomized male, or inseminating seminal plasma at the same time as the spermatozoa. The optimal timing of ovulation induction relative to semen deposition is not known.

The viscous character of camelid semen is one of the main limiting factors in the development of AI (7). Other important factors limiting reproduction in alpacas are their low overall fertility, which is due both to low sperm production and a high incidence of pseudo pregnancy or early embryo mortality (8), and the physiological capacity of females that usually have no more than four offspring throughout their life (9).

As an alternative to AI, embryo transfer has been attempted in SACs (10). Successful within-species embryo transfer was achieved in both llamas (11–14) and alpacas (15, 16). Cross-species embryo transfer between SACs was also accomplished with *in vivo* generated embryos (12, 17, 18). Embryos have been produced *in vitro* from llamas (19), and recently also from alpacas (20). However, it is not clear whether offspring resulted from transfer of such *in vitro* derived embryos.

A recent review on SAC (5) summarized the available literature on timing of AI, sperm numbers, deposition site, and how ovulation was induced, together with the outcome. The purpose of the present review, therefore, is to examine the problems associated with semen handling in SACs and look at possible solutions that have been successful or partially successful in a related species, the dromedary camel.

SEMEN COLLECTION

All reproductive biotechnologies require a source of good quality semen. Semen collection in SACs is challenging, not least because mating takes place in sternal recumbency and is of long duration (21). Semen can be collected by different methods, including an artificial vagina, vaginal sponges, and electro-ejaculation, as reviewed recently (22). Creating a urethral fistula provides access to small quantities of spermatozoa without seminal plasma but is not practical as a regular supply of spermatozoa for AI. There may be ethical and welfare aspects related to some of these methods. Thus, for example, anesthesia is required, or is mandatory in some countries, for electroejaculation, since it can cause extreme muscular contractions. Surgical alterations to provide a source of spermatozoa without seminal plasma would not be considered ethical in many countries, and may present welfare issues. The advantages and disadvantages of the different methods are summarized in **Table 1**.

TABLE 1 | Advantages and disadvantages of various semen collection methods in alpacas [modified from (22)].

Technique	Advantages	Disadvantages
Artificial vagina	Relatively clean "physiological" samples; No contact with female reproductive tract secretions.	Males must be trained; cannot be used for isolated semen collections in untrained individuals.
Electroejaculation	Any mature individual.	Anesthesia required, restricting number of collections from each individual; ejaculate may not be complete or may be contaminated with urine*.
Post-mating aspiration	No training of males needed.	Semen contaminated with secretions from female tract, bacteria etc. Not universally acceptable as source of semen for AI. Restraint of females may cause stress.
Epididymal spermatozoa (castration; post mortem)	By-product from slaughterhouses or from normal husbandry technique.	Only one sample from each male unless hemicastration performed.
Urethral fistula	Provides spermatozoa without viscous seminal plasma.	Small numbers of spermatozoa collected. Fistula must be kept open*.
Others (intravaginal sacs or condom, vaginal sponges, aspiration after mating)	No training of males needed.	Insertion of intravaginal devices not easy and stressful if animals not accustomed to handling. Devices may hamper intromission; Devices or aspiration can cause injury. Contamination with vaginal secretions, bacteria.

**may not be considered acceptable in some countries on welfare grounds.*

An artificial vagina is the preferred method for collecting samples in alpacas and llamas (23). The samples collected are not usually contaminated with urine, which can be a problem with electroejaculation (21), and contains the contributions from the accessory sex glands in the physiological proportions. Once the male has mounted a female or a phantom, the erect penis is introduced into the artificial vagina and ejaculation occurs into a sterilized collection vessel. In the dromedary camel, using a phantom instead of a live female enabled a sample to be collected without contamination (24). In a study comparing semen collection by artificial vagina and electroejaculation in llamas, the proportion of successful semen collections was reported to be greater when using an electro-ejaculator than an artificial vagina (25). Semen volume was greater and both sperm motility and membrane integrity were higher in samples collected by electroejaculation than by artificial vagina. However, the method may not be suitable for repeated use on the same males in the field and requires anesthesia because the intense muscular contractions produced can be painful (4).

Aspiration of semen from the reproductive tract of a mated female, or extraction from vaginal sponges or condoms, results in samples that may be contaminated by other secretions and cells (21), resulting in poor sperm survival. Sperm recovery may be stressful for females that are not accustomed to being handled (22) and the method is not practical for most alpaca husbandry systems. Although this method is a source of ejaculated spermatozoa, aspiration from the vagina for transfer into other females is not desirable for biosecurity [(26), cited in (22)].

SEMEN CHARACTERISTICS

As previously mentioned, camelid semen is highly viscous (7), forming a gel immediately after ejaculation. Although the gel is rich in glycosaminoglycans (GAG), this component is not considered to cause the viscosity, since treatment with enzymes specific for GAGs does not reduce the viscosity appreciably

whereas treatment with proteases does (27). The viscosity is, instead, attributed to the high mucin content as detailed in Kershaw-Young and Maxwell (27). More recent studies on the protein components of camelid seminal plasma have been mostly directed toward characterizing the ovulation-inducing factor in the seminal plasma, which is beyond the scope of this review [e.g., (28)].

The spermatozoa are retained within this gelatinous mass, making it difficult to separate the spermatozoa from the seminal plasma. Thus, simple techniques, such as evaluating sperm concentration or sperm motility by conventional means, are challenging. The viscous seminal plasma might be an adaptation to retain the spermatozoa in the female's reproductive tract until ovulation, which occurs ∼30 h after copulation in the alpaca (29). Sperm motility in this gelatinous mass is oscillatory rather than progressive (30).

Camelids ejaculate in fractions throughout the whole copulation ("dribble ejaculation"); sometimes only gelatinous seminal plasma is ejaculated without any spermatozoa (31). The volume of the ejaculate varies, e.g., from 0.4 to 4.3 mL, and the average seminal plasma volume was 1.5 ± 0.1 mL (32). The semen is usually opalescent to milky white, depending on sperm concentration (33), which ranges from 62 to 750 × 10^6 spermatozoa/mL, with an average motility of 68–85% (21).

TABLE 2 | Summary of various studies on enzyme treatment of semen from South American camelids.

Enzyme	Species	Effect	Source
Trypsin	Alpaca	Sperm motility ↓	(35)
Trypsin, collagenase, hyaluronidase, and fibrinolysin	Alpaca and llama	Not possible to obtain progressive motility	(36)
Trypsin	Alpaca and llama	Detached heads	(37)
Collagenase at 0.5, 1.0, 2.0, and 4.0 mg/mL	Alpaca	Toxic to sperm	(38)
1 mg/ml collagenase	Llama	Spermatozoa are not adversely affected	(19, 39)
Hyaluronidase, chondroitinase ABC, and keratinase) and proteases (papain and proteinase K)	Alpaca	Papain was most promising in reducing thread formation	(40)
Papain	Alpaca	Does not reduce sperm motility, viability, DNA integrity or acrosome integrity	(41)
200 or 600 units/mL catalase	Alpaca	Spermatozoa are not adversely affected	(5)

↓, *decreased.*

SEMEN HANDLING

The high viscosity of the seminal plasma creates difficulties in sperm assessment and handling. The spermatozoa are trapped within the gel and show an oscillatory motility pattern rather than a progressive pattern (32). It is almost impossible to make smears from this material, and the gel appears to prevent dyes from penetrating the sperm membrane for evaluation of membrane integrity. The presence of seminal plasma may also hinder penetration of cryoprotectants (27). Certainly, alpaca spermatozoa freeze poorly using currently available protocols, and no offspring have been produced following AI with frozen semen (5). However, there are reports of successful freezing of llama spermatozoa (19), with offspring born after AI.

Several methods to reduce seminal plasma viscosity and release trapped spermatozoa have been attempted. These include needling, pipetting, sperm washing, addition of enzymes, and colloid centrifugation. Needling and pipetting, i.e., repeated aspiration of semen through a needle or a pipette, respectively, may help liquefaction but sperm quality tends to be reduced, possibly due to physical damage or by release of reactive oxygen species that may subsequently affect sperm membranes and chromatin. Prior extension of the semen sample with a buffered semen extender, followed by gentle pipetting during incubation, was shown to liquefy semen from dromedary camels (34). Removal of the freed spermatozoa from the seminal plasma is needed as the gel tends to reappear with time, trapping the spermatozoa once more. Colloid centrifugation (see sperm selection) was more effective for removing spermatozoa from liquefied seminal plasma than centrifugation without a colloid, since it allowed the spermatozoa to be completely separated from the seminal plasma (34).

The gel fraction of the seminal plasma is thought to be due to the presence of proteins such as mucins. In an attempt to reduce the viscosity of the semen, researchers have tested various proteases with varying degrees of success (summarized in **Table 2**). However, the use of enzymes is controversial since they may damage spermatozoa (10). One explanation for the differing results presented by various researchers could be the considerable variation in viscosity among camelid ejaculates. The degree of viscosity affects the concentration of enzyme needed, or the time required for it to act. However, exposing spermatozoa to any enzymes could be expected to have an adverse effect on their membranes. Thus, the affected spermatozoa might still be able to function in IVF shortly after enzyme treatment, or if the spermatozoa are rapidly removed from the media containing the enzymes, whereas they are unable to function if preserved for subsequent use in AI.

Colloid centrifugation is a so-called biomimetic sperm preparation technique, whereby the sperm selection that occurs in the female reproductive tract is mimicked *in vitro*. Briefly, in the female reproductive tract, motile spermatozoa migrate away from seminal plasma; non-motile spermatozoa are removed by back-flow (42). Spermatozoa that are free of seminal plasma interact with the uterine and oviductal epithelial cells, and are thought to be retained in the crypts of the uterotubal junction where they initiate the changes that occur during capacitation. They are released when ovulation occurs, and locate the oocyte for fertilization.

Several biomimetic techniques are available that simulate this *in vivo* sperm selection (43). These include migration techniques, e.g., "swim-up," filtration, magnetic activated cell sorting, and colloid centrifugation. Colloids can be used as a density gradient, i.e., with two or more layers of colloid of different densities, or with only one layer of colloid (Single Layer Centrifugation, SLC). This technique has been used for sperm selection in many different species [reviewed by (43)]. Since spermatozoa are separated from seminal plasma as well as selecting the robust spermatozoa (44), the method could be beneficial in extracting camelid spermatozoa from seminal plasma. Of the different selection techniques, colloid centrifugation looks to be quite promising, at least for llama spermatozoa (10, 19), and is now used regularly when preparing dromedary camel semen for reproductive biotechnologies (45). Use of a low density gradient made from a colloid designed for human spermatozoa was also reported for preparing alpaca spermatozoa (5). It should be noted that in the case of the low density colloid, the purpose of the colloid is merely to separate the spermatozoa from the seminal plasma rather than to select robust spermatozoa from the rest of the ejaculate (46). Higher density colloids are used for selection of robust spermatozoa that are more likely to be capable of achieving fertilization.

A comparison of swim-up and colloid centrifugation of llama spermatozoa (after treatment of the ejaculate with collagenase) concluded that colloid centrifugation was the method of choice for preparing spermatozoa (39). In studies on dromedary camel semen that had been subjected to gentle pipetting, i.e., without enzyme treatment, Malo et al. (34) showed that sperm quality parameters and *in vitro* fertilization ability of spermatozoa were improved by SLC compared with simple sperm washing. The colloid separated live motile spermatozoa from seminal plasma, dead cells, debris, and extender. The same researchers were able to cryopreserve dromedary camel sperm samples (47–49) and obtain offspring after AI with the thawed samples. Since colloid centrifugation seems to represent a reliable, repeatable, and relatively simple way of extracting camelid spermatozoa without damage, it could provide the way forward when working with alpaca semen.

Semen Extenders

A variety of different extenders have been used for camelid semen, as reviewed recently (5). Evaluating which extenders function best for each species is problematic because it is not known which methods for evaluating sperm quality are reliable as indicators of fertility in SACs. Once a method for AI in these species has been optimized, it may be possible to relate sperm quality in different extenders to fertility, thus facilitating development of optimized extenders for these species.

Pregnancy Rates

The success of any one semen handling method compared to another is usually measured in terms of pregnancy rate and births. Although some pregnancies and live births have been achieved following AI in SACs, the success rate is low (5). In an alpaca study, 1 out of 42 inseminated females gave birth (4). A 21.7% success rate was reported for a study on llamas (50). Such low success rates imply that the methods used are still sub-optimal. A comparison of the different methodologies is provided by (5). It would be interesting to see pregnancy rates from the use of sperm samples prepared by colloid centrifugation without the use of enzymes, since pregnancy rates are higher in other species following colloid selection, e.g., stallion (51).

CURRENT CHALLENGES AND POSSIBLE SOLUTIONS

The protocols currently available for semen collection, extracting spermatozoa from the ejaculate, and sperm preservation are not effective for alpaca spermatozoa.

As presented here, there are indications that colloid centrifugation presents a practical solution for viscosity reduction in llamas and dromedary camel semen, either following brief enzyme treatment or after pipetting in the presence of buffer. Therefore, optimizing these protocols for alpaca semen is strongly recommended. In the meantime, initial studies on developing preservation or cryopreservation protocols could be carried out with epididymal spermatozoa, which can be obtained either as a byproduct of castration or from slaughterhouse material. Although they represent a useful source of spermatozoa for describing the characteristics of alpaca spermatozoa (52), or for testing extenders and freezing protocols [(52), personal communication] they have limitations for more general use in AI. For obvious reasons, regular sperm collections from the same male are not possible and therefore biological replicates of experiments are not feasible, and it can be difficult to harvest the spermatozoa without blood or cellular contamination. Furthermore, it is not known whether extenders and preservation protocols derived using epididymal spermatozoa are relevant for working with ejaculated spermatozoa. However, this material could be a useful starting point in the development of sperm preservation protocols.

FURTHER CONSIDERATIONS

Developing sperm handling procedures is only a first step in developing AI. If AI is to become a reality in SACs, timing of ovulation relative to AI, sperm numbers deposited, and the site of semen deposition have to be established. The timing of ovulation induction relative to insemination should be investigated, e.g., with the help of ultrasound to pinpoint ovulation. Obtaining a consistent supply of ejaculated spermatozoa that can be manipulated is essential to carrying out studies that are reliable and repeatable. One point is clear; there is still plenty of opportunity for research in these interesting species.

AUTHOR CONTRIBUTIONS

JM and MA researched the literature. JM drafted the review. MA checked it. Both authors contributed to the article and approved the submitted version.

FUNDING

JM was funded by the Veterinary Faculty, Swedish University of Agricultural Sciences. The library at SLU provided a discount on OPen Access fees.

REFERENCES

1. Santiani A, Huanca W, Sapana R, Huanca T, Sepulveda N, Sanchez R. Effects on the quality of frozen-thawed alpaca (lama pacos) semen using two different cryoprotectants and extenders. *Asian J Androl.* (2005) 7:303–9. doi: 10.1111/j.1745-7262.2005.00021.x
2. León-Velarde CU, Guerrero J. *Improving Quantity and Quality of Alpaca Fiber*. Available online at: https://www.researchgate.net/publication/254416124 (downloaded July 3, 2020).
3. Kristjanson P, Anirudh K, Radeny M, Kuan J. Pathways into and out of poverty and the role of livestock in the Peruvian Andes. In: *2006 Annual Meeting*. International Association of Agricultural Economists (2006).
4. Adams GP, Ratto MH, Collins CW, Bergfelt DR. Artificial insemination in South American camelids and wild equids. *Theriogenology.* (2009) 71:166–75. doi: 10.1016/j.theriogenology.2008.09.005
5. Morton KM, Vaughan JL, Maxwell WMC. *Continued Development of Artificial Insemination Technology in Alpacas*. Rural Industries Research and Development Corporation Publication Number 08/057 (2018).
6. Adams GP, Ratto MH, Huanca W, Jaswant S. Ovulation-inducing factor in the seminal plasma of alpacas and llamas. *Biol Reprod.* (2005) 73:452–7. doi: 10.1095/biolreprod.105.040097
7. Tibary A, Vaughan J. Reproductive physiology and infertility in male South American camelids: a review and clinical observations. *Small Ruminant Res.* (2006) 61:283–98. doi: 10.1016/j.smallrumres.2005.07.018
8. San Martin M, Copaira M, Zuniga J, Rodriguez R, Bustinza G, Acosta L. Aspects of reproduction in the alpaca. *J Reprod Fertil.* (1968) 16:395–9. doi: 10.1530/jrf.0.0160395
9. Huanca W, Adams GP. Semen collection and artificial insemination in llamas and alpacas. In: *Youngquist R, Threlfall W, editors. Current Therapy in Large Animal Theriogenology, 2 edn.* St Louis, MO: Saunders - Elsevier Inc. (2007). p. 869–73.
10. Trasorras VL, Carretero MI, Neild DM, Chaves MG, Giuliano SM, Miragay MH. Production, preservation, and transfer of South American camelid embryos. *Front Vet Sci.* (2017) 4:190–203. doi: 10.3389/fvets.2017.00190
11. Wiepz DW, Chapman RJ. Non-surgical embryo transfer and live birth in a llama. *Theriogenology.* (1985) 24:254–7. doi: 10.1016/0093-691X(85)90189-X
12. Sumar J. Embryo transfer in domestic South American camelids. *Anim Reprod Sci.* (2013) 136:170–7. doi: 10.1016/j.anireprosci.2012.10.029
13. Huanca W, Condori R, Chileno M, Garcia P, Cainzo J, Becerra G. Effect of time of incubation on nuclear maturation and cleavage post *in vitro* fertilization of alpaca oocytes (in Spanish). *Rev Inv Vet Peru.* (2014) 25:468–76. doi: 10.15381/rivep.v25i4.10782
14. Trasorras V, Giuliano S, Miragaya M. *In vitro* production of embryos in South American camelids. *Anim Reprod Sci.* (2013) 136:187–93. doi: 10.1016/j.anireprosci.2012.10.009
15. Vaughan J, Mihm M, Wittek T. Factors influencing embryo transfer success in alpacas - a retrospective study. *Anim Reprod Sci.* (2013) 136:194–204. doi: 10.1016/j.anireprosci.2012.10.010
16. Tibary A, Pearson LK, Campbell A. Embryo transfer in camelids. *Spermova.* (2015) 5:234–525. doi: 10.18548/aspe/0002.44
17. Pacheco JI, Tollig S, Winblad Von Walter A, Pezo D, Velez V. Repeated embryo collection and interspecies transfer in alpacas and llamas during non-breeding season (in Spanish). *Spermova.* (2015) 5:6–9. doi: 10.18548/aspe/0002.1
18. Palomino JM, Jones L, Vanhanen T, Mastromonaco GF, Busato R, Adams GP. Alpaca embryo transfer on a private Canadian farm. *Can vet J.* (2018) 5:631–4.
19. Trasorras VL, Chaves MG, Carretero MI, Miragaya MH. *Ovarian Superovulation in South American Camelids.* ICAR (2012).
20. Gamarra G, Huaman E, Klón S, Carpio M, Alvarado E, Asparrin M, et al. First *in vitro* embryo production in alpacas (*Lama pacos*). *Reprod Fertil Dev.* (2020) 21:177–8. doi: 10.1071/RDv21n1Ab157
21. Bravo PW, Skidmore JA, Zhao XX. Reproductive aspects and storage of semen in Camelidae. *Anim Reprod Sci.* (2000) 62:173–93. doi: 10.1016/S0378-4320(00)00158-5
22. Abraham MC, de Verdier K, Båge R, Morrell JM. Semen collection methods in alpacas. *Vet Rec.* (2017) 180:613–4. doi: 10.1136/vr.104074
23. Bravo PW, Alarcon V, Baca L, Cuba Y, Ordoñez C, Salinas J, et al. Semen preservation and artificial insemination in domesticated South American camelids. *Anim Reprod Sci.* (2013) 136:157–63. doi: 10.1016/j.anireprosci.2012.10.005
24. Ziapour S, Niasari-Naslaji A, Mirtavousi M, Keshavarz M, Kalantari A, Adel H. Semen collection using phantom in dromedary camel. *Anim Reprod Sci.* (2014) 151:15–21. doi: 10.1016/j.anireprosci.2014.09.016
25. Giuliano S, Director A, Gambarotta M, Trasorras V, Miragaya M. Collection method, season and individual variation on seminal characteristics in the llama (*Lama glama*). *Anim Reprod Sci.* (2008) 104:359–69. doi: 10.1016/j.anireprosci.2007.02.016
26. Ampuero Casquino E, Ordoñez Rodríguez C, Huanca Mamani H, Ruíz Béjar J., Sumar Kalinoswki J, Cucho Dolmos H, et al. *Reproductive Biotechnologies and Genetic Improvement in Alpacas*. Universidad Nacional de San Antonio Abad del Cusco, Peru (2014) (in Spanish).
27. Kershaw-Young CM, Maxwell WM. Seminal plasma in camelids and comparisons with other species. *Reprod Domest Anim.* (2012) 47:369–75. doi: 10.1111/j.1439-0531.2012.02100.x
28. El-Allali K, El-Bousmaki N, Ainani H, Simmoneaux V. Effect of the camelid's seminal plasma ovulation-inducing factor/ß-NGF. A kisspeptin target hypothesis. *Front Vet Sci.* (2017) 4:99. doi: 10.3389/fvets.2017.00099
29. Tibary A, Memon MA. Reproductive physiology in the female South American camelidae. *J Camel Pract Res.* (1999) 6:217–33.
30. Bravo PW, Mellado W, Ampuero E, Alarcon V. Effect of daily breeding on fertility of male alpaca. *Andrology.* (1997) 9:143–53.
31. Lichtenwalner AB, Woods GL, Weber JA. Seminal collection, seminal characteristics and pattern of ejaculation in llamas. *Theriogenology.* (1996) 46:293–305. doi: 10.1016/0093-691X(96)00186-0
32. Garnica J, Achata R, Bravo PW. Physical and biochemical characteristics of alpaca semen. *Anim Reprod Sci.* (1993) 32:85–90. doi: 10.1016/0378-4320(93)90059-Z
33. Urquieta B, Flores P, Munoz C, Bustos-Obregon E, Garcia-Huidobro J. Alpaca semen characteristics under free and directed mounts during a mating period. *Anim Reprod Sci.* (2005) 90:329–39. doi: 10.1016/j.anireprosci.2005.01.019
34. Malo C, Crichton EG, Morrell JM, Pukazhenthi BS, Skidmore JA. Single layer centrifugation of fresh dromedary camel semen improves sperm quality and *in vitro* fertilization capacity compared with simple sperm washing. *Reprod Domes Anim.* (2017) 52:1097–103. doi: 10.1111/rda.13036
35. Bravo PW, Pacheco C, Quispe G, Vilcapaza L, Ordoñez C. Degelification of alpaca semen and the effect of dilution rates on artificial insemination outcome. *Arch Androl.* (1999) 43:239–46. doi: 10.1080/014850199262562
36. Bravo PV, Callo P, Garnica J. The effect of enzymes on semen viscosity in Llamas and Alpacas. *Small Ruminant Res.* (2000) 38:91–5. doi: 10.1016/S0921-4488(00)00142-5
37. Poblete P, von Baer A, von Baer L, Del Campo. M. Evaluación de la morfología espermática del semen de camélidos sudamericanos tratados con tripsina. In: *Memorias III Congreso Mundial Sobre Camélidos*. Potosí (2003). p. 765–8.

38. Maxwell C, Evans G, Morton KM. The development of collection, processing and storage technologies for alpaca semen. In: *Extender Abstracts WBC/ICAR Satellite Meeting on Camelid Reproduction.* Budapest (2008). p. 19–25.
39. Santa Cruz R, Giuliano SM, Gambarotta MC, Morrell JM, Abraham MC, Miragaya MH, et al. Comparison of different methods of sperm selection of llama raw semen. *Anim Reprod Sci.* (2016) 173:8–12. doi: 10.1016/j.anireprosci.2016.08.001
40. Kershaw-Young CM, Stuart C, Evans G, Maxwell WMC. The effect of glycosaminoglycan enzymes and proteases on the viscosity of alpaca seminal plasma and sperm function. *Anim Reprod Sci.* (2013) 138:261–7. doi: 10.1016/j.anireprosci.2013.02.005
41. Kershaw CM, Evans G, Rodney R, Maxwell WMC. Papain and its inhibitor E-64 reduce camelid semen viscosity without impairing sperm function and improve post-thaw motility rates. *Reprod Fertil Dev*. (2016) 29:1107–14. doi: 10.1071/RD15261
42. Morrell JM, Rodriguez-Martinez H. Biomimetic techniques for improving sperm quality in animal breeding: a review. *Open Androl J.* (2009) 1:1–9. doi: 10.2174/1876827X00901010001
43. Morrell JM, Rodriguez-Martinez H. Colloid centrifugation of semen: applications in assisted reproduction. *Am J Anal Chem.* (2016) 7:597–610. doi: 10.4236/ajac.2016.78055
44. Kruse R, Dutta PCB, Morrell JM. Colloid centrifugation removes seminal plasma and cholesterol from boar spermatozoa. *Reprod Fertil Dev.* (2011) 23:858–65. doi: 10.1071/RD10260
45. Skidmore JA, Billah M, Binns M, Short RV, Allen WR. Hybridizing old and new world camelids: *Camelus dromedarius* × *Lama guanicoe*. *Proc Biol Sci.* (1999) 266:649–56. doi: 10.1098/rspb.1999.0685
46. Morrell JM, Núnez-Gonzalez A, Crespo-Felez I, Martínez-Martínez S, Martínez Alborcia M-J, Fernandez-Alegre E, et al. Removal of bacteria from boar semen using a low-density colloid. *Theriogenology.* (2019) 126:272–8. doi: 10.1016/j.theriogenology.2018.12.028
47. Malo C, Crichton EG, Morrell JM, Pukazhenthi BS, Johannisson A, Splan R, et al. Colloid centrifugation of fresh semen improves post-thaw quality of cryopreserved dromedary camel spermatozoa. *Anim Reprod Sci.* (2018) 192:28–34. doi: 10.1016/j.anireprosci.2018.02.005
48. Malo C, Elwing B, Soederstroem L, Lundeheim N, Morrell JM, Skidmore JA. Effect of different freezing rates and thawing temperatures on cryosurvival of dromedary camel spermatozoa. *Theriogenology. (2*019a) 125:43–8. doi: 10.1016/j.theriogenology.2018.07.037
49. Malo C, Grundin J, Morrell JM, Skidmore JA. Individual male dependent improvement in post-thaw dromedary camel sperm quality after addition of catalase. *Anim Reprod Sci. (2*019b) 209:106168. doi: 10.1016/j.anireprosci.2019.106168
50. Aller JF, Rebuffi GE, Cancino AK, Alberio RH. Fetal mortality diagnosis by ultrasound in the vicuña *(Vicugna vicugna). Reprodu Fertil Dev.* (2003) 15:125–8. doi: 10.1071/rd02097
51. Morrell JM, Richter J, Martinsson G, Stuhtmann G, Hoogewijs M, Roels K, et al. Pregnancy rates are higher after artificial insemination with cooled stallion spermatozoa selected by Single Layer Centrifugation than with control semen doses. *Theriogenology.* (2014) 82:1102–5. doi: 10.1016/j.theriogenology.2014.07.028
52. Santiani A, Ugarelli A, Evangelista-Vargas S. Characterization of functional variables in epididymal alpaca (*Vicugna pacos*) sperm using imaging flow cytometry. *Anim Reprod Sci.* (2016) 173:47–55. doi: 10.1016/j.anireprosci.2016.08.010

Conflict of Interest: JM is the inventor and one of the patent holders of the colloids mentioned in this article.

The remaining author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 12 November 2020
doi: 10.3389/fvets.2020.595889

Development of a GnRH-PGF$_{2\alpha}$ Based Synchronization and Superstimulation Protocol for Fixed-Time Mating in Llama Embryo Donors

Enzo German Zampini[1,2]*, *Maria Fernanda Veiga*[1], *Fernanda Gabriela Fumuso*[1,2], *Luciana Cabido*[3], *Deborah Margarita Neild*[1], *Maria Graciela Chaves*[1], *Marcelo Horacio Miragaya*[1] and *Virginia Luz Trasorras*[1,2]

[1] Universidad de Buenos Aires, Facultad de Ciencias Veterinarias, Instituto de Investigación y Tecnología en Reproducción Animal, Cátedra de Teriogenología, Buenos Aires, Argentina, [2] Consejo Nacional de Investigaciones Científicas y Técnicas, Buenos Aires, Argentina, [3] Dirección Provincial de Desarrollo Ganadero, Gobierno de la Provincia de Jujuy, Jujuy, Argentina

OPEN ACCESS

Edited by:
Nick Wheelhouse,
Edinburgh Napier University,
United Kingdom

Reviewed by:
Bouchra El Amiri,
Institut National de la Recherche Agronomique de Settat, Morocco
Narayanan Krishnaswamy,
Indian Veterinary Research Institute (IVRI), India

***Correspondence:**
Enzo German Zampini
egzampini@fvet.uba.ar

Specialty section:
This article was submitted to Animal Reproduction - Theriogenology, a section of the journal Frontiers in Veterinary Science

Received: 17 August 2020
Accepted: 23 October 2020
Published: 12 November 2020

Citation:
Zampini EG, Veiga MF, Fumuso FG, Cabido L, Neild DM, Chaves MG, Miragaya MH and Trasorras VL (2020) Development of a GnRH-PGF$_{2\alpha}$ Based Synchronization and Superstimulation Protocol for Fixed-Time Mating in Llama Embryo Donors. Front. Vet. Sci. 7:595889. doi: 10.3389/fvets.2020.595889

The aim of the present study was to evaluate the application of a GnRH-PGF$_{2\alpha}$ based synchronization and superstimulation protocol for fixed-time natural mating in llama embryo donors. All females ($n = 8$) received 8 μg IM of GnRH analog (GnRHa; buserelin) on day 0, regardless of follicular status. After eight days, another GnRHa dose was administered followed by 250 μg IM PGF$_{2\alpha}$ (cloprostenol). A dose of 1000 IU IM of equine chorionic gonadotrophin (eCG) was applied on day 12 and a new dose of PGF$_{2\alpha}$ was administered on day 13. All embryo donors were mated with a male of proven fertility followed by a GnRHa dose on day 18. 24 h later, mating was repeated with a different male. Transcervical uterine flushing for embryo recovery was carried out on all females on day 26. Recipient females received one dose of GnRHa (day 0) two days after the first mating of embryo donor females. A 75% (6/8) of embryo donors responded to the superstimulation treatment with a range of 2 to 5 corpus luteums (CLs) on embryo recovery day. A total of 24 CLs were registered, with a mean of 4 ± 0.9 CLs per female. Embryo recovery rate was 66.7% (16/24), with a range of 0 to 4 embryos and a mean of 2.7 ± 1.5 embryos per female. Regarding quality of the recovered embryos, 56.2% were grade I, 6.2% were grade II and 37.5% were grade V (untransferable; arrested morulae). Grade I and II embryos ($n = 10$) were transcervically transferred into recipient females ($n = 10$) six days after inducing their ovulation. At 24 days after embryo transfer (ET), a 50% pregnancy rate was registered. In conclusion, a group of llama embryo donors can be synchronized and superstimulated using a fixed-time mating protocol based on GnRHa, PGF$_{2\alpha}$, and eCG without the necessity of using ultrasonography in the field.

Keywords: camelids, follicular wave, ovarian dynamic, embryo transfer, ovarian superstimulation

INTRODUCTION

Breeding llamas (*Lama glama*) is an economically relevant animal husbandry activity for a large sector of the high-altitude populations of South America, as the by-products of meat, fiber and manure are all put to use. Llamas are also employed for transporting cargo and represent an important cultural symbol for the region. Total llama population is estimated to be 5 million animals (1) in South America, concentrated mainly in Bolivia, Peru, and Argentina. Over the last few years, an increase in the consumption of llama meat has occurred because its palatability and low cholesterol content respond to current market demands, thus suggesting a favorable scenario for the production of these species. One of the existing problems llama producers face is the phenotypical expression of malformations (prognathism, conformation defects, heterochromatism, etc.), as a consequence of intense consanguinity in the herds due to internal replacement and mating of closely related animals. On the other hand, although this species presents a high capacity for adaptation to different adverse environmental conditions, conception and embryo mortality rates are highly variable under natural conditions (2). The mean annual fertility (birthing rate) in alpacas (*Vicugna pacos*) and llamas in South America can be as low as 45% (3). This low reproductive performance has different causes, for example, a late puberty (4), short reproductive seasonality in their natural habitat (5), long gestations (335 to 360 days) and birthing only one offspring at a time (6–8). Conventional reproductive management currently undertaken by most llama producers, consists in observing the sexual behavior of the females when confronted with a male and indicating natural mating when receptivity is detected, this being a labor-intense activity which demands a considerable amount of time (9). However, it is known that conception rates are lower (50–75%) when the females are mated based on their sexual behavior rather than when mating is indicated in the presence of a dominant growing follicle detected by ultrasonography (10, 11). Therefore, the establishment of a protocol that allows the presence of one or more dominant follicles at a fixed time, would not only make it possible to achieve better pregnancy rates, but would also facilitate the practices of herd management (12). In addition, applying ovarian superstimulation treatments combined with embryo transfer (ET) in genetically valuable animals, would make it possible to obtain a greater number of offspring per female per year, thus maximizing their reproductive efficiency (13, 14). In this way, the generation interval would be shortened, and genetic progress would accelerate (15).

The objective of this study was to evaluate the efficiency of a simple, fixed-time synchronization-ovarian superstimulation protocol in llamas, for use in an embryo transfer program, that does not depend on ultrasonography and is based on the administration of two doses of a GnRH analog, prostaglandin and equine chorionic gonadotrophin.

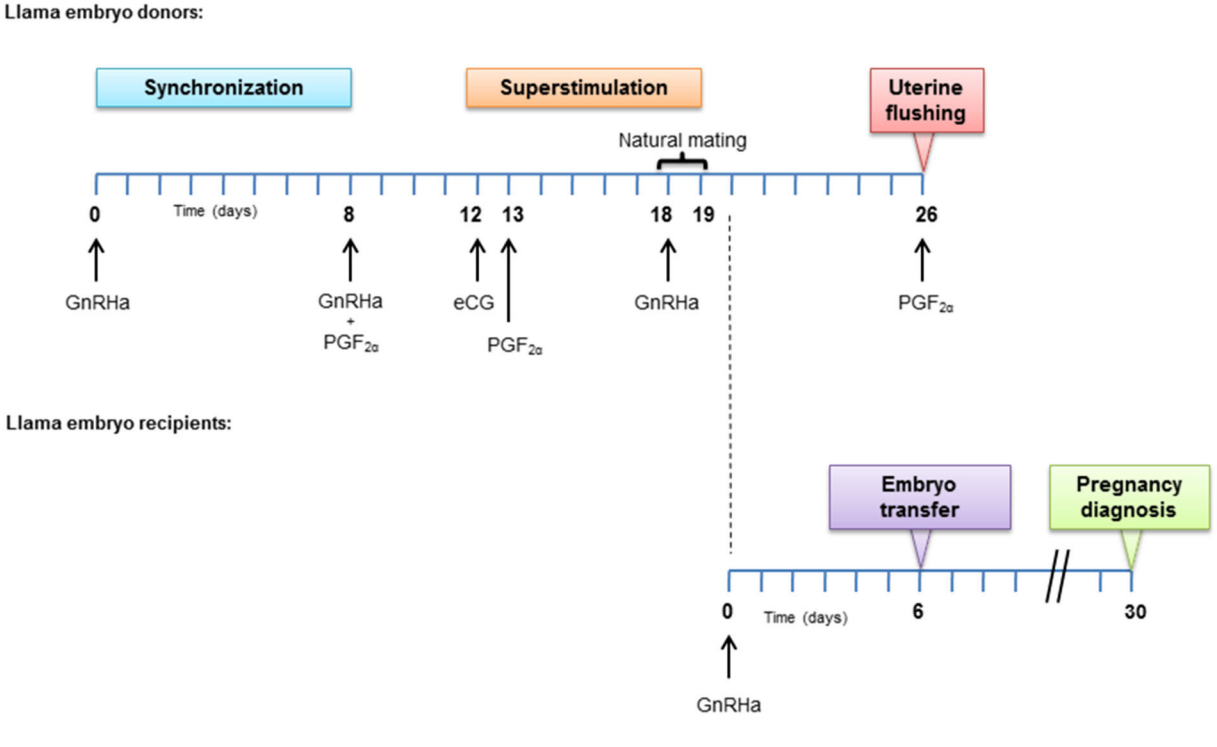


FIGURE 1 | Experimental design. Hormone treatment applied in embryo donors and recipient females.

MATERIALS AND METHODS

Environmental Conditions

This study was carried out during the month of May in the La Intermedia establishment in the town of Abra Pampa, located in the Department of Cochinoca of the Province of Jujuy, Argentina. The area is an Andean plateau situated 3,480 meters above sea level (22° 38' S−65° 69' W). This area corresponds to the Ecoregion of the dry Puna, with rainfall that varies between 100 and 400 mm per year, concentrated mainly between November and March (250 to 350 mm), a time of year when maximum temperatures reach 25°C. Winter is dry, with maximum temperatures in the region of 20°C, being warm during the day and fresh at night.

Animals

A total of 48 non-gestating and non-lactating females, between 2 and 8 years old, with a body condition score of 3 (body condition score from 1 = thin to 5 = obese) were used in this study. They were kept separate from the group of males and were fed with natural pastures and had free access to water.

Experimental Design

Treatment in Female Donors

The complete experimental design is described in **Figure 1**. On day 0, all donor females ($n = 8$) received 8 μg IM of GnRH analog (GnRHa; buserelin acetate; Receptal®, Intervet, Buenos Aires, Argentina) for inducing endogenous LH release. Eight days later, another GnRHa dose was administered followed by 250 μg IM of $PGF_{2\alpha}$ (cloprostenol; Ciclase DL®, Syntex S.A., Buenos Aires, Argentina). On day 12 an IM dose of 1,000 IU of equine chorionic gonadotrophin (eCG; Novormon®, Syntex, Argentina) was injected (16). On day 13, another dose of $PGF_{2\alpha}$ was administered and 5 days later (day 18) natural mating with a male *Lama glama* of proven fertility was indicated for each female ($n = 6$). After natural mating, all females received a dose of GnRHa and 24 h later, natural mating was repeated with a different male, with the objective of minimizing the male effect.

Embryo Recovery and Evaluation

Uterine flushing was carried out non-surgically for embryo recovery, 8 days after the first mating (17). The maneuvers were carried out with the female either standing or in sternal recumbency. The animal was restrained in stocks, the tail was wrapped, and the rectum was emptied of feces. The perineum was then scrubbed using a hypoallergenic detergent, rinsed carefully with clean water and then dried. Restless females received 0.2 mg/kg IV xylazine (Xilazina® 10%, PRO-SER S.A., Buenos Aires, Argentina) before flushing. A Foley catheter (12 or 16 Fr, according to female size), with a stylet inserted into the catheter to keep it from bending during recto-vaginal manipulation, was used. Uterine flushing was done by placing the catheter cuff cranial to the internal cervical os and insufflating it with 5 or 10 ml of air (according to catheter gauge). The whole uterus was flushed 4 to 5 times using Ringer-Lactate solution, previously warmed (30-35°C), with a total volume of 500 ml. The recovered medium was filtered through a 70 μm EmCon™ filter for embryos. After the flushing, donor females received a single IM dose of $PGF_{2\alpha}$ to induce luteolysis. The fluid in the flushing filter was placed in warmed reticulated Petri dishes and embryos were identified using a stereomicroscope. Embryos were classified according to their morphology following the criteria set by Tibary and Anouassi (18), using a grade scale from 1 to 5. Only morphologically normal (grade 1 and 2) blastocyst stage embryos were transferred to recipient females.

Management of the Recipient Females

All recipient females ($n = 40$) received 8 μg IM of GnRHa two days after the first natural service of the embryo donors (recipients' day 0). Transcervical ET was carried out on day 6 after GnRHa administration (17). The preparation of recipient females was as for donor females. A lubricated gloved hand was inserted in the rectum to hold the cervix while an assistant separated the vulva labia and an ET pipette, covered with a sterile sheath (IMV®, France) and carrying the 0.25 ml straw (IMV®, France) containing a single embryo, was inserted into the vagina. Cervical threading was performed aided by transrectal manipulation and the embryo was deposited in the uterine horn ipsilateral to the CL (17).

Pregnancy Diagnosis

Pregnancy diagnosis was carried out by transrectal ultrasonography, observing an embryonic vesicle 24 days after ET (corresponding to pregnancies of 32 days).

RESULTS

Donor females were evaluated by transrectal ultrasonography before carrying out the uterine flushing on day 26. Of the 8 females used, 75% (6/8) responded to the ovarian superstimulation treatment with a range of 2 to 5 CLs. A total of 24 CLs were registered, with a mean of 4 ± 0.9 CLs per female, and an occurrence of 58.3% in the left ovary (LO; 2.3 ± 0.5) and 41.7% in the right ovary (RO; 1.7 ± 1). A 25% (2/8) of the females presented a large anovulatory luteinized follicle.

A total of 16 embryos were recovered from the 6 donor females that were flushed (range: 0 to 4 embryos per animal), with a mean of 2.7 ± 1.5 embryos recovered per female (**Figure 2**). Embryo recovery rate (number of embryos recovered / number of CLs observed) was 66.7% (16/24). Of the recovered embryos, 56.2% (9/16) were grade I, 6.2% (1/16) grade II and the remaining 37.5% (6/16) were grade V (non-transferrable; arrested morulae).

Only grade I and II hatched blastocysts ($n = 10$) were transferred to the uterus of recipient females. An 80% (8/10) of the recipient llamas presented a CL in the left ovary and a 20% (2/10) in the right ovary.

The pregnancy rate was 50% (5/10) twenty four days after ET (3 pregnancies with a CL in the LO and 2 pregnancies with a CL in the RO) and pregnancies were again confirmed 80 days post ET.

DISCUSSION

The protocol evaluated in the present study was efficient in superstimulating embryo donor llamas without the need for

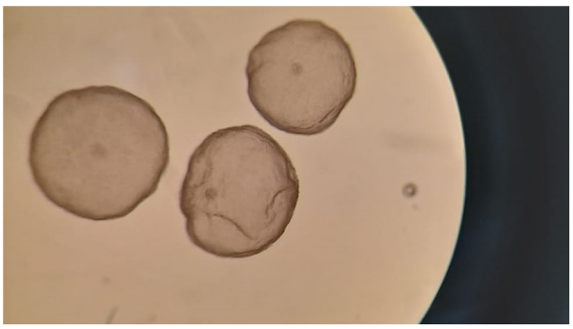


FIGURE 2 | Hatched blastocysts (grade I) obtained 8 days after natural mating of an ovarian-superstimulated female llama.

ultrasonography. Ovarian response to the induction of multiple follicle growth in llamas is extremely variable, ranging between 0 and 17 ovulations or CLs and a recovery of 0 to 4 transferrable embryos per animal (13). One of the causes of this high variability in response is the stage of ovarian dynamics at the beginning of the superstimulatory treatment as the presence of a dominant follicle induces atresia in the rest of the follicles of that same wave (19). According to Miragaya et al. (20), beginning ovarian superstimulatory treatment in the presence of a follicle >5 mm in diameter induces the growth of only that follicle. To accomplish this latter objective, a natural luteal phase can be generated by inducing ovulation of the dominant follicle, with the resulting formation of a functional CL. In this manner, the inhibitory effect on follicular activity of progesterone produced by the CL is achieved (21). There are some protocols to induce multiple follicular growth in llamas based on the application of a single dose (1000 IU) of eCG, at different days after administering GnRH (22–24) or LH (25, 26) to induce ovulation of the dominant growing follicle previously monitored by ultrasound. In the current study, 75% of the llamas responded positively to the superstimulation protocol with a mean 4 CLs per female. These results were superior to those obtained by Bourke et al. (23), who achieved a 47% response to treatment, with an average of 2.4 ± 0.55 CLs per animal. In this last study, multiple follicular development was stimulated with eCG seven days after inducing ovulation with GnRH. It is possible that another dominant follicle could have already developed at the time of applying the ovarian superstimulatory treatment, because a new follicular wave emerges on average 2 to 3 days after inducing ovulation (9, 27). In addition, according to Ferrer et al. (28), follicle diameter is maintained below 7 mm until 6 days after induction. It is probable that the presence of this dominant follicle has an inhibitory effect on the development of the new follicles, thus reducing the response to ovarian superstimulatory treatment. In the Bourke et al. study (23), 1.4 ± 0.45 embryos were obtained per female, a lower result than the 2.7 ± 1.5 embryos recovered in the present study. These authors used 19 females, 14 of which received a single natural mating, and the remaining 5 received two matings separated by 24 h, obtaining an embryo recovery rate of 22 and 72%, respectively. They attributed the low results to the implementation of a single natural mating for each superstimulated female. Other studies have used the same ovarian superstimulation treatment, two days after administering GnRH in the presence of a dominant follicle, registering in the case of Vásquez (24), a mean of 6.5 ± 3.1 CLs/female and recovering 4 ± 2.6 embryos/female, while Aller et al. (22) observed 5.5 ± 3.1 CLs/female and obtained 2.1 ± 2.2 embryos/female. The embryo recovery rate achieved by Vásquez (24) was similar to that obtained in our study (65.3 vs. 66.7% respectively), both of which were superior to the rate of 40.7% obtained by Aller et al. (22). Based on inducing ovulation with LH, Huanca et al. (26) registered a higher mean of CLs (10.1 ± 2.9 CLs/animal) at the time of embryo recovery after administering eCG two days after LH. Evangelista et al. (25) used this same protocol, but the ovarian superstimulatory treatment was applied three days after LH, obtaining 9.3 ± 3.4 CLs/female. In these two studies, embryo recovery rate was 48.3% (26) and 37.7% (25), and this last low percentage could be attributed to the single natural mating used for each superstimulated female donor. Implementing double natural mating with different males on embryo donors that have been subjected to an ovarian superstimulation treatment, not only improves embryo recovery rates (23), it also allows genetic diversity to be increased, as has been recently proven in alpacas (29). In this way, not only is genetic variability increased, but phenotypical expression of congenital malformations could be avoided.

In an ET program, not only is the number of embryos recovered important, but it is also essential to consider the embryo quality. In our study, 62.5% of the embryos recovered were good quality and in condition to be transferred. Perhaps, the 37.5% that were arrested at the morula stage could be attributed to superstimulation treatments producing an increase in the number of recruited follicles and accelerating their growth and development, hence possibly leading to the ovulation of oocytes with immature nuclei or cytoplasm, thus affecting their future fertilization and/or embryo development (30). Furthermore, according to Aller et al. (22), there is a high positive correlation (0.91) between the number of follicles present on the day of natural mating and the estrogen plasma concentration in superstimulated llamas, and, based on studies in cattle (31, 32), these estrogen plasma levels could generate a reduction in the secretory activity of the oviductal epithelial cells and alter normal fertilization and/or early embryo development. Although to date no studies confirm this same effect in camelids, 5 of the arrested morulae obtained came from females that presented an ovarian cyst on the day of uterine flushing. According to Huanca et al. (26), ovarian cysts could affect results in superstimulated llamas in one of two ways: if hormonally active, they could alter correct embryo development and/or transport along the oviduct or, they could physically impede the normal ovulatory mechanism for the rest of the follicles.

In all the above-mentioned superstimulation protocols, trained personnel and transrectal ultrasonography are required, as prior synchronization of follicular dynamics was not carried out in the embryo donors. Thus, these methods are not very practical for applying in the field. In our study, we were

able to establish a simple protocol that controls follicular growth in a group of females and guarantees the presence of two or more dominant follicles at a fixed-time, without the need of previous reproductive evaluation. Although it would be important to increase the number of females treated, implementing this protocol could improve pregnancy rates, facilitate management and allow natural mating and/or artificial insemination to be programmed simultaneously in larger groups of animals. In addition to being used for ET programs, it could also be applied for obtaining embryos for cryopreservation and even to harvest oocytes in an ovum pick-up program.

CONCLUSION

It is possible to implement a synchronization-superstimulation fixed-time protocol in llamas using two doses of a GnRH analog, with two doses of prostaglandin F_2 alfa prior to eCG, without the need for ultrasonography. This would make field work much easier, gaining independence from the need for personnel trained in imaging diagnostics.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Comité Institucional de Cuidado y uso de Animales de Laboratorio (CICUAL).

AUTHOR CONTRIBUTIONS

VT and EZ contributed to the conception and design of the study. EZ, MV, FF, LC, and VT provided help with field work. EZ and VT wrote the manuscript. MC, DN, and MM made critical revisions to the paper and DN contributed to language editing. All authors read, edited and approved the final manuscript.

FUNDING

This research was supported by grants from the University of Buenos Aires (UBACyT 2018) and the Agencia Nacional de Promoción Científica y Tecnológica (PICT 2016-0145).

ACKNOWLEDGMENTS

The authors thank Syntex Laboratories for donating the Novormon® for this study, Javier Giubergia, director of Dirección Provincial de Desarrollo Ganadero of Jujuy province and all their technical staff.

REFERENCES

1. *FAOstat*. (2018). Available online at: http://www.fao.org/faostat/en/#data/QA.
2. Sumar JB. Reproduction in llamas and alpacas. *Anim Reprod Sci.* (1996) 42:405–15. doi: 10.1016/0378-4320(96)01538-2
3. Rodriguez JS, Pearson LK, Tibary A. *Infertility Subfertility in the Female Camelid*. Christopher C, Anderson DE, Van Saun TA, Robert J, Johnson V, LaRue W, editors. Linda Duncan (2014).
4. Leyva V, Sumar JB. Evaluación del peso corporal al empadre sobre la capacidad reproductiva de hembras alpaca de un año de edad. *Proc Int Conv*. (1981) 1:7–18.
5. Pollard JC, Littlejohn RP, Moore GH. Seasonal and other factors affecting the sexual behaviour of alpacas. *Anim Reprod Sci.* (1995) 37:349–56. doi: 10.1016/0378-4320(94)01339-N
6. Johnson LW. *Llama Reproduction*. Veternairy Clinic of North America. Food Animal Practice (1989). p. 37–54.
7. Leon JB, Smith BB, Timm KI, Lecren G. Endocrine changes during pregnancy, parturition and the early post-partum period in the llama (Lama glama). *J Reprod Fertility*. (1990) 88:503–11. doi: 10.1530/jrf.0.0880503
8. Sumar JB. Removal of the ovaries or ablation of the corpus luteum and its effect on the maintenance of gestation in the alpaca and llama. *Acta Vet Scandin*. (1988) 83:133–41.
9. Ratto MH, Singh J, Huanca W, Adams GP. Ovarian follicular wave synchronization and pregnancy rate after fixed-time natural mating in llamas. *Theriogenology*. (2003) 60:1645–56. doi: 10.1016/S0093-691X(03)00176-6
10. Campbell AJ, Pearson LK, Spencer TE, Tibary A. Double ovulation and occurrence of twinning in alpacas (Vicugna pacos). *Theriogenology*. (2015) 84:421–4. doi: 10.1016/j.theriogenology.2015.03.027
11. Tibary A, Anouassi A, Sghiri A, Khatir H. Current knowledge and future challenges in camelid reproduction. *Soc Reprod Ferti Suppl.* (2007) 64:297–313. doi: 10.5661/rdr-vi-297
12. Gallelli MF, Bianchi C, Trasorras V, Zampini E, Aba M, Miragaya M. Synchronization of time of development of ovarian follicular waves in South American Camelids. *Anim Reprod Sci.* (2019) 6:208. doi: 10.1016/j.anireprosci.2019.06.017
13. Ratto MH, Silva ME, Huanca W, Huanca T, Adams GP. Induction of superovulation in South American camelids. *Anim Reprod Sci.* (2013) 136:164–9. doi: 10.1016/j.anireprosci.2012.10.006
14. Trasorras VL, Chaves MG, Aguero A. *Comparación de Tres Tratamientos Para Sincronizar La Dinámica Ovárica en la llama (Lama glama) Con Estrógeno y Progesterona*. Curitiba: IV Congreso Latinoamericano de Especialistas En Pequenos Rumiantes y Camélidos Sudamericanos (2005). p. 18–20.
15. Trasorras VL, Carretero MI, Neild DM, Chaves MG, Giuliano SM, Miragaya MH. Production, preservation, and transfer of South American camelid embryos. *Front Vet Sci.* (2017) 4:190. doi: 10.3389/fvets.2017.00190
16. Trasorras VL, Chaves MG, Miragaya MH, Pinto M, Rutter B, Flores M, et al. Effect of eCG superstimulation and buserelin on cumulus-oocyte complexes recovery and maturation in Llamas (Lama glama). *Reprod Dom Anim.* (2009) 44:359–64. doi: 10.1111/j.1439-0531.2007.00972.x
17. Trasorras VL, Chaves MG, Neild DM, Gambarotta M, Aba MA, Agüero A. Embryo transfer technique: Factors affecting the viability of the corpus luteum in llamas. *Anim Reprod Sci.* (2010) 121:279–85. doi: 10.1016/j.anireprosci.2010.06.004
18. Tibary A, Anouassi A. Theriogenology in Camelidae. Anatomy, physiology, pathology and artificial breeding. In: *Reproductive Physiology in Female Camelidae*. Actes Éditions, Institut Agronomique et Vetérinaire Hassan II (Maroc), printed by Abu Dhabi Printing and Publishing Company (1997).
19. Adams GP, Sumar J, Ginther OJ. Effects of lactational and reproductive status on ovarian follicular waves in llamas (Lama glama). *J Reprod Fertility*. (1990) 90:535–45. doi: 10.1530/jrf.0.0900535
20. Miragaya MH, Chaves MG, Agüero A. Reproductive biotechnology in South American camelids. *Small Rumin Res.* (2006) 61:299–310. doi: 10.1016/j.smallrumres.2005.07.017

21. Aba MA, Forsberg M, Kindahl H, Sumar J, Edqvist LE. Endocrine changes after mating in pregnant and non-pregnant llamas and alpacas. *Acta Vet Scand.* (1995) 36:489–98.
22. Aller JF, Abalos MC, Acuña F, Cancino AK. Plasma steroid profiles and ovarian response in llamas treated with eCG for superovulation combined with exogenous progesterone during early luteal phase. *An Reprod Sci.* (2019) 2019:208. doi: 10.1016/j.anireprosci.2019.106108
23. Bourke DA, Kyle CE, Mcevoy TG, Young P, Adam CL. Superovulatory responses to eCG in llamas (Lama glama). *Theriogenology.* (1995) 44:255–68. doi: 10.1016/0093-691X(95)00175-8
24. Vásquez ME. *Evaluación de dos Métodos de Criopreservación de embriones Sobre las Tasas de Sobrevivencia In vitro y Preñez en llamas*. Master Thesis. Lima, Peru. Faculty of veterinary medicine. National University of San Marcos (2008). Available online at: https://cybertesis.unmsm.edu.pe/handle/20.500.12672/1248.
25. Evangelista S, Cordero A, Santiani A, Vásquez M, Cárdenas O, Huanca W. Equine chorionic gonadotrophin (eCG) stimulation during the luteal and non-luteal phases on ovarian response and embryo quality in llamas. *Rev In Vet Perú.* (2009) 20:33–40. http://www.scielo.org.pe/pdf/rivep/v20n1/a06v20n1.pdf.
26. Huanca W, Cordero A, Huanca T, Cardenas O, Adams GP, Ratto MH. Ovarian response and embryo production in llamas treated with equine chorionic gonadotropin alone or with a progestin-releasing vaginal sponge at the time of follicular wave emergence. *Theriogenology.* (2009) 72:803–8. doi: 10.1016/j.theriogenology.2009.05.019
27. Bianchi CP, Benavente MA, Simonetti M, Aba MA. Synchronization of time of ovarian follicular development in llamas (Lama glama) using a protocol based on GnRH and PGF2α. *Anim Reprod Sci.* (2018) 192:200–5. doi: 10.1016/j.anireprosci.2018.03.011
28. Ferrer MS, Agüero A, Chaves MG, Russo AF, Rutter B. Sincronización de la onda follicular mediante el uso de buserelina en la llama (Lama glama). *InVet.* (2002) 4:7–11. Available online at: http://www.sidalc.net/cgi-bin/wxis.exe/?IsisScript=agrinpa.xis&method=post&formato=2&cantidad=1&expresion=mfn=023194 (accessed November 21, 2020).
29. Forshey BS, Moraes CR, Lakritz J, Pinto CR. F., Coffman E, Schanbacher BJ, et al. Embryo production by superovulation and dual siring in alpacas (Vicugna pacos). *Small Rumin Res.* (2018) 162:63–8. doi: 10.1016/j.smallrumres.2018.03.006
30. Sirard MA, Blondin P. Oocyte quality embryo production in cattle. *Int J Anim Sci.* (1998) 78:102. doi: 10.4141/A98-102
31. Callesen H, Greve T, Hyttel P. Preovulatory endocrinology and oocyte maturation in superovulated cattle. *Theriogenology.* (1986) 25:71–86. doi: 10.1016/0093-691X(86)90184-6
32. van de Leemput EE, Vos PLAM, Zeinstra EC, Bevers MM, van der Weijden GC, Dielemana SJ. Improved *in vitro* embryo development using *in vivo* matured oocytes from heifers superovulated with a controlled preovulatory LH surge. *Theriogenology.* (1999) 52:335–49. doi: 10.1016/S0093-691X(99)00133-8

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 12 November 2020
doi: 10.3389/fvets.2020.595856

Basal Levels and hCG Responses of Serum Testosterone and Estrogen in Male Alpacas

Aymen El Zawam[1], *Ahmed Tibary*[2]* *and Cristian Patino*[2]

[1] Department of Surgery and Theriogenology, College of Veterinary Medicine, University of Tripoli, Tripoli, Libya, [2] Comparative Theriogenology, Department of Veterinary Clinical Science, Center for Reproductive Biology, College of Veterinary Medicine, Washington State University, Pullman, WA, United States

Steroid response to human Chorionic Gonadotropin (hCG) administration has been used in various species to study testicular function and for diagnostic purposes. In this study, two experiments were conducted to determine serum testosterone concentration response to administration of hCG and its correlation with testicular weight. In the second experiment the relationship between age, testosterone and estrogen response to hCG, and testicular histometry was in pre-pubertal and post-pubertal male alpacas. For experiment 1, males in two age groups (2 to 3 years, $n = 9$) and (4 to 7 years; $n = 15$) received 3,000 IU hCG IV, 36 to 48 h before castration. Serum testosterone concentration was determined before (T0), 1 h (T1), 2 h (T2), 8 h (T8), and 24 h (T24) after administration of hCG. Basal concentrations of serum testosterone was significantly different ($P < 0.01$) between age groups. Serum testosterone concentrations increased over time and doubled 2 h after treatment. The highest change (250 to 300% increase from T0) was observed at 8 h (3.5 ± 0.3 ng/ml). A significant correlation ($P < 0.01$; $r = 0.64$) was found between serum testosterone concentration and total testicular weight. For experiment 2, 60 males ranging in age from 6 to 60 months were used. Serum testosterone and estrogen was determined in samples taken just prior to and 2 h after administration of 3,000 IU hCG. Basal serum testosterone concentrations were very low ($\leq$0.1 ng/mL) until 9 months of age then increased steadily with age. There was a significant variation amongst males within the same age group. Serum testosterone concentration increased by 2- to 4-fold 2 h after hCG injection ($P \leq 0.05$). Males in the 13 to 14 months of age group had the highest rise. Estrogen concentration increased in response to hCG administration and was detected only in males with high testosterone. We conclude that administration of 3,000 IU of hCG IV can be used reliably to detect testicular tissue and study its steroidogenic activity. The response is correlated with testicular weight and Leydig cell number. Testicular growth and sensitivity to LH stimulation increases between the ages of 13 and 14 months. The aromatizing ability of Leydig cells increased significantly in post-pubertal male alpacas.

Keywords: camelid, testis, reproduction, steroidogenesis, Leydig cell

OPEN ACCESS

Edited by:
Marcelo H. Ratto,
Austral University of Chile, Chile

Reviewed by:
Sergio Recabarren,
University of Concepcion, Chile
Martha E. Valdivia,
National University of San Marcos, Peru

***Correspondence:**
Ahmed Tibary
tibary@wsu.edu

Specialty section:
This article was submitted to Animal Reproduction - Theriogenology, a section of the journal Frontiers in Veterinary Science

Received: *17 August 2020*
Accepted: *17 September 2020*
Published: *12 November 2020*

Citation:
El Zawam A, Tibary A and Patino C (2020) Basal Levels and hCG Responses of Serum Testosterone and Estrogen in Male Alpacas. Front. Vet. Sci. 7:595856. doi: 10.3389/fvets.2020.595856

INTRODUCTION

Research on sexual development in male alpacas is scarce. The age at puberty is variable, and factors regulating sexual development and maturation in this species are often a subject of controversy (1, 2). Male camelids are born with descended testes that are relatively small in comparison to other domestic livestock, soft and difficult to palpate (3). Early studies showed that testicular growth is slow and reaches a plateau at 30 months of age. There is a wide variation in testicular size at all ages suggesting that other factors such as nutrition and genetics are also important (4, 5). Penile detachment from the prepuce has been traditionally used as a sign of puberty because it is directly under testosterone influence (3, 6, 7). Young males display sexual interest and mounting at 1 year of age but at this age only 8% of males had lost the peno-preputial attachment and could achieve intromission. Loss of preputial adhesions is achieved in 70 and 100% of the males at 2 and 3 years of age, respectively (3). In one study, serum testosterone concentrations in alpacas were 202, 254, 708, and 1,510 pg/mL at 9, 10, 11, and 12 months of age, respectively (2).

Measurement of testosterone and estrogens from a single blood sample is not a reliable indicator of testis function because their secretion varies during the day (8). The human chorionic gonadotropin (hCG) stimulation test is a reliable dynamic test for the evaluation of testicular steroidogenesis (9–12). This approach provides a diagnostic index of steroidogenic capacity of the testes. Administration of hCG was shown to induce a rapid increase of serum testosterone concentration in several species including bulls (13, 14), cats (15, 16), dogs (17), humans (11, 18), rams (19–21), and stallions (22–24).

The testicular steroidogenic response to hCG administration has been characterized in several species and used for diagnostic purposes to differentiate between cryptorchidism and male-like behavior in absence of testicular tissue, primarily in stallions (23, 25), dogs (16), and cats (15, 16). Response to hCG stimulation allows differential diagnosis between cryptorchidism and monorchism (24). Steroidogenic response to hCG stimulation has also been investigated as a method for evaluation of testicular function disorders in older animals (26). In one study, young stallions (8–10 years old) show a more pronounced response than older stallions (19–25 years). However, these observations remain limited in numbers (27). hCG stimulation also results in an increase in serum estrogens concentration and provides information on aromatizing activity within the testis (28–31).

In camelids, studies on steroidogenic testicular response to hCG stimulation test are limited. In llamas, administration of 750 IU of hCG resulted in a 3-fold increase in serum testosterone concentration within 1 h of treatment (32). In one cryptorchid llama, plasma testosterone concentrations in samples taken before and 18 h after administration of hCG (5,000 IU, IM) were 20,802 and 39,697 nmol/L, respectively (33). Studies on the effect of hCG stimulation in adult alpacas show the same effect as in llamas (2). To our knowledge, there are no studies comparing pre-pubertal and post-pubertal male response to hCG.

We report here two experiments designed to investigate the endocrinological response to hCG stimulation in male alpacas to gain better insight on sexual development in this species. The primary objective of the first experiment was to further characterize endocrine response over time to hCG stimulation and its correlation to testicular weight. The objective of the second experiment was to investigate the serum testosterone and estrogen concentration in response to a single dose of hCG in pre- and post-pubertal alpacas and its relation to testicular histology.

MATERIALS AND METHODS

Two experiments were conducted on intact male alpaca (*Vicugna pacos*). The objective of the first experiment was to characterize the testosterone response to a single injection of human Chorionic gonadotropin. The second experiment was designed to study the steroidogenic response of pre- and post-pubertal alpaca males to a single injection of hCG.

Animals and Animal Care

For experiment 1, twenty-four (24) intact Huacaya males in two age groups (2 to 3 years, $n = 9$) and (4 to 7 years; $n = 15$) were used. For experiment 2, sixty (60) Huacaya males aged from 6 months to 5 years were used and divided into seven age groups: 6 to 8 months ($n = 13$); 9 to 11 months ($n = 6$); 12 to 14 months ($n = 10$); 15 to 17 months ($n = 13$); 18–20 months ($n = 6$); 22 to 30 months ($n = 5$); and 36 to 60 months ($n = 7$). All males were normal with respect to testicular palpation and ultrasonography. The scrotal contents (testicles, epididymides, and testicular envelopes) of all males were examined by ultrasonography to rule out any abnormalities using a 7.5 MHz linear transducer (Alkoa 500, Inc. Tokyo, Japan) (1). The animals were housed at Washington State University Veterinary Teaching Hospital during the study. They were fed free choice Orchard grass hay and had free access to water. All procedures were approved by Washington State University Animal Care and Use Committee.

hCG Stimulation and Blood Sampling

For experiment 1, the animals were restrained in a chute for IV catheter placement. Each animal was clipped over the jugular vein and the area aseptically prepped with betadine scrub. A local block with 1 ml of 2% lidocaine was placed subcutaneously. A #15 blade was used to make a stab incision into the skin to allow easy placement of the IV catheter. A 16-gauge IV catheter was placed into the jugular vein. Each male received an intravenous injection of 3,000 IU hCG (Chorulon®, Intervet, Holland) 36 to 48 h before scheduled castration. Blood was drawn from the IV jugular catheter using the three-syringe technique. A pre-hCG blood sample (T0) was taken to measure initial levels of testosterone. Subsequent samples were drawn at 1 (T1), 2 (T2), 8 (T8), and 24 (T24) h following hCG administration. In experiment 2, all animals received 3,000 IU of hCG intravenously (10,000 IU/10 mL, Chorulon, Intervet International B.V. Boxmeer-Holland). Blood samples were collected just prior and 2 h following hCG administration. Blood was withdrawn by jugular venipuncture into vacutainer

tubes without anticoagulant (MONOJECT, Tyco Healthcare LP, Mansfield, MA, USA). Samples were left to clot at 4°C overnight then centrifuged at 3,000 × g for 20 min at 4°C. Sera from the samples were transferred into polypropylene vials and stored at −20°C until assayed for testosterone and total estrogen concentration.

Castration and Testicular Processing

Males in both experiments underwent standard surgical castration under general anesthesia. The animals were anesthetized by intramuscular administration of a combination of Ketamine (4 mg/kg, Ketaset® 100 mg/mL, Fort Dodge Animal Health, Fort Dodge, Iowa), Butorphanol (0.04 mg/kg IM, Torbugesic® 10 mg/mL, Fort Dodge Animal Health, Fort Dodge, Iowa), and Xylazine (0.4 mg/kg; AnaSed® 100 mg/mL, 0.4 mg/kg IM, Lloyd, Shenandoah, Iowa). The alpaca was placed in lateral recumbency on a mat. The scrotum was clipped and prepared aseptically with betadine scrub. A 2.5 cm incision was made on the craniodorsal aspect of the down testicle extending over the ventral testicle to the tail of the epididymis. The testicle was exteriorized and a transfixation suture placed around the vascular cone of the testicular cord. The testicle was removed by transection of the spermatic cord ventrally to the transfixing suture. The procedure was repeated on the other side. The scrotal skin incision was left to heal by second intention. The animal was monitored until recovery from anesthesia was complete. The testicles were dissected free of the epididymis and their weight determined with a milligram precision using an electronic scale.

In experiment 2, cubic pieces of testicular parenchyma tissue were collected from each animal after castration and fixed in 4% paraformaldehyde overnight, then stored in 70% ethanol. The tissues were later dehydrated in alcohol, embedded in paraffin, sectioned at 8 μm and placed on glass slides. Slides were deparaffinized in histoclear® (AGTC Bioproducts LLC, Wilmington, MA 01887 USA), rehydrated in serial ethanol concentrations, and stained with hematoxylin and eosin to evaluate germ and somatic cells using light microscope.

Histological Evaluation

The diameters of 10 round seminiferous tubules were measured at 10X magnification using an eyepiece micrometer and averaged. All slides were evaluated by a single operator in a random order. Representative cross-sections of testes from each age group were processed for detection of adult Leydig cells. Leydig cells were evaluated based on morphology parameters (shape, size, number, and maturation status). The morphology of stained sections was evaluated using light microscopy at 100X magnification, and digital images were captured. Adult Leydig cells were counted in randomly selected fields through the entire sections without overlap. Mature Leydig cells were identified in the interstitial tissue of prepubertal males (**Figure 1**) and adult males (**Figure 2**) by their large rounded nucleus with an obvious nucleolus, little or no cytoplasmic lipid droplets, and a relatively thicker peripheral rim of heterochromatin in their nuclei compared to other interstitial cells.

Histochemical detection of apoptosis-associated DNA fragmentation in testicular tissue was performed by the TUNEL assay (DeadEnd™ Colorimetric TUNEL System). The TUNEL System end-labels the fragmented DNA of apoptotic cells using a modified TUNEL assay. Biotinylated nucleotide is incorporated at the 3'-OH DNA ends using the Terminal Deoxynucleotidyl Transferase Recombinant (rTdT) enzyme. Horseradish peroxidase-labeled streptavidin (Streptavidin HRP) is then bound to these biotinylated nucleotides, which are detected using the peroxidase substrate, hydrogen peroxide and the stable chromogen, diaminobenzidine (DAB). Using this procedure, apoptotic nuclei are stained dark brown (Promega, Part# TB199, www.promega.com Madison, WI. USA).

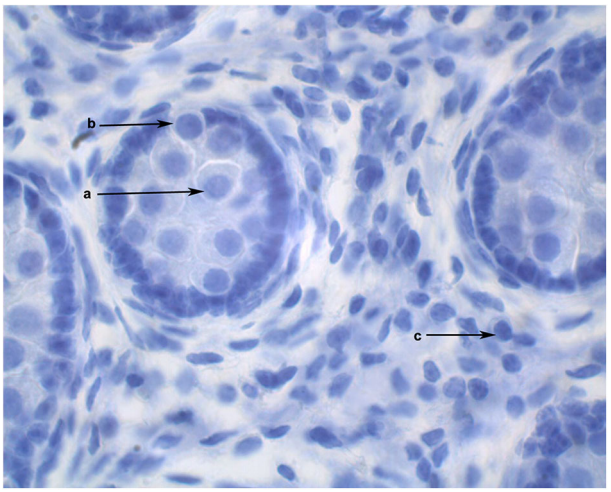


FIGURE 1 | Cross section of alpaca testis at 6 months of age showing gonocytes in the center of the seminiferous cords (a) and gonocytes that have begun to migrate to the basement membrane (b). Mature Leydig cells are present in the interstitial space (c).

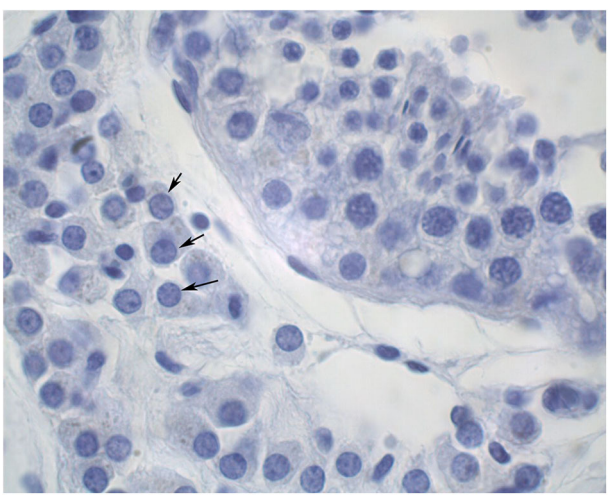


FIGURE 2 | Cross section of an adult alpaca testis showing mature Leydig cells (arrows) in the interstitial space.

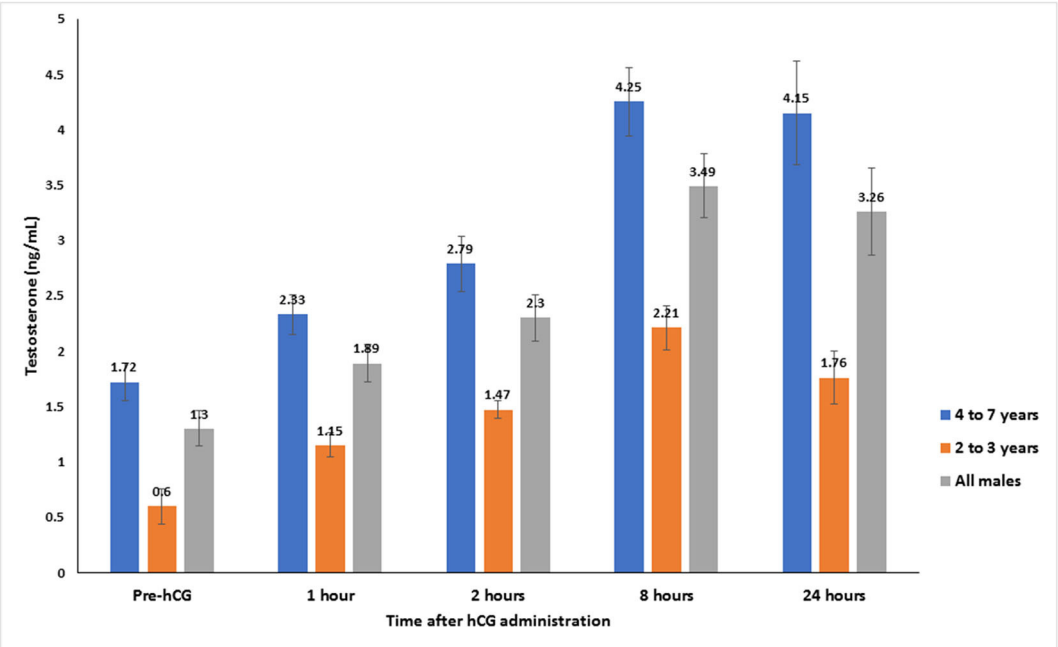


FIGURE 3 | Serum testosterone concentration (mean ± SEM, ng/mL) before and after hCG injection (3,000 IU, IV) in alpacas.

The TUNEL assay was carried out on paraffin-embedded testis sections. In brief, testis tissue sections were pretreated by deparaffinized and rehydrated in xylene and decreasing concentrations of ethanol followed by washing in 0.85% NaCl for 5 min. Sections were washed twice in PBS for 5 min and after each step in this assay. The tissues were permeabilized with 20 μg/mL proteinase K incubation for 25 min at room temperature. Sections on the slides were covered with 100 μl of equilibration buffer for 10 min followed by addition of 100 μl of rTdT TUNEL reaction mixture to the sections for 60 min at 37°C in a humidified chamber. The enzyme reaction was stopped by dipping slides into 20XSCC (liquid buffers Sodium Chloride and Sodium Citrate) for 15 min. Sections on slides were then incubated with 100 μl of HRP-conjugated streptavidin (diluted 1:500 in PBS) for 30 min at room temperature, followed by 100 μl of DAB until a light brown background appeared. The sections were counterstained with hematoxylin and evaluated under a light microscope. To count TUNEL-positive cells in the tissues, five different microscopic fields were scanned at magnification of 40X.

Hormones Assays

The concentration of testosterone and estrogen was determined in serum samples collected pre and post-hCG treatment using a commercial double-antibody RIA kit (Coat-A-Count® total testosterone; Siemens Healthcare Diagnostics Inc. Los Angeles, CA 90045 USA; testosterone Catalog # TKT1, estrogen Catalog # KE2D1). The calibrators contain 8, 16, 96, 414, 792, and 1,685 nanograms of testosterone per deciliter (ng/dl) in processed human serum. Whereas, the estrogen calibrators contain 0, 5,10,20,50,150, and 50 picograms of estrogen per milliliter (pg/mL) in processed human serum. The sensitivity of the assay for testosterone and estrogen was 0.1 ng/mL (intra-assay CV, inter-assay CV were 2.4 and 8.5%, respectively) and 1 pg/mL (intra-assay CV, inter-assay CV were 4 and 5%, respectively), respectively.

Statistical Analysis

All data are presented as mean ± SEM. In experiment 1, response to hCG stimulation was evaluated by repeated measure ANOVA for all males and by age group. Correlation between testosterone concentrations and total testicular weight was established using a linear regression model. Data from experiment 2 were analyzed using ANOVA (Tukey test). All analyses were performed using statistical software (Statistix 10, Analytical Software, Tallahassee, FL, USA). Significance of results is reported if $p < 0.05$.

RESULTS

Experiment 1

Serum testosterone concentrations before and after administration of hCG are presented in **Figure 3**. Serum testosterone concentration pre-hCG administration (T0) showed large individual variation (range: 0.3–3.2 ng/mL). The mean serum testosterone concentration at T0 for all males was 1.3 ± 0.2 ng/mL. There was a significant difference ($p < 0.001$) in the mean basal serum testosterone concentrations between the two age groups (1.7 ± 0.2 ng/mL for males 4–7 years of age vs. 0.6 ± 0.2 ng/mL for males 2–3 years of age) (**Table 1**). Serum testosterone concentration increased over time following hCG administration. Both age groups showed a similar trend in serum testosterone response to hCG administration. Overall,

TABLE 1 | Serum testosterone concentration (mean ± SEM, ng/mL) in male alpacas before and after injection of hCG (3,000 IU, IV).

Age	N	Pre-hCG	1 h	2 h	8 h	24 h
2–3 years	9	0.6 ± 0.16^{a1}	1.15 ± 0.11^{b1}	$1.47 \pm 0.08^{c,e1}$	2.21 ± 0.2^{d1}	1.76 ± 0.24^{e1}
3.5–4 years	15	1.71 ± 0.17^{a2}	2.33 ± 0.18^{b2}	2.79 ± 0.25^{b2}	$4.25 \pm 0.31^{c,d2}$	4.15 ± 0.47^{d2}
All males	24	$1.3 \pm 0.16^{a1,2}$	$1.89 \pm 0.17^{b1,2}$	2.3 ± 0.21^{c2}	$3.49 \pm 0.29^{d1,2}$	3.26 ± 0.39^{d2}

[a,b,c,d,e]Values with different superscripts in the same row are statistically different ($p < 0.05$).
[1,2,3]Values with different superscripts within the same column are statistically different ($p < 0.05$).

TABLE 2 | Change in serum testosterone concentration (pg/mL) over time (expressed as ratio between basal testosterone concentration at T0 and other sampling times).

Age	*N*	Ratio T1/T0	Ratio T2/T0	Ratio T8/T0	Ratio T24/T0
2–3 years	9	$1.45 \pm 0.13^{a,1}$	$1.82 \pm 0.13^{b,1}$	$2.74 \pm 0.28^{c,1}$	$2.71 \pm 0.38^{d,1}$
3.5–4 year	15	$2.43 \pm 0.3^{a,2}$	$3.29 \pm 0.48^{a,2}$	$4.7 \pm 0.68^{b,1}$	$4.03 \pm 0.82^{b,1}$
All males	24	$1.82 \pm 0.17^{a,1,2}$	$2.37 \pm 0.27^{b,1,2}$	$3.48 \pm 0.36^{c,1,2}$	$3.21 \pm 0.4^{c,1}$

Blood sample timing, T0 = before hCG treatment, T1 = 1 h after hCG, T2 = 2 h after hCG, T8 = 8 h after hCG, T24 = 24 h after hCG.
[a,b,c]Values with different subscripts within the same row are statistically different ($p < 0.05$).
[1,2]Values with different subscripts within the same column are statistically different ($p < 0.05$).

TABLE 3 | Testicular weight in alpacas (Mean ± SEM, grams) of different age groups.

Age group	N	Left testicle	Right testicle	Paired testicular weight
2–3 years of age	9	$12.10 \pm 0.79^{a,1}$	$12.02 \pm 0.49^{a,1}$	24.12 ± 1.26^{1}
>3 years of age	15	$20.36 \pm 0.94^{b,2}$	$19.88 \pm 0.99^{b,2}$	40.24 ± 1.91^{2}
All males	24	17.27 ± 1.06^{c}	16.93 ± 1.02^{c}	34.19 ± 2.06

[a,b,c]Values with different subscripts within the same row are statistically different ($p < 0.05$).
[1,2]Values with different subscripts within the same column are statistically different ($p < 0.05$).

serum testosterone concentration doubled by 2 h and peaked at 8 h post-hCG administration (**Table 2**). Older males had a significantly higher ($p < 0.05$) magnitude of serum testosterone changes than younger males at 1 and 2 h after hCG injection. Serum testosterone concentrations were not significantly different ($p > 0.05$) at 8 and 24 h post-hCG administration.

Mean total testicular weight (± SEM) was significantly ($P < 0.001$) different between the two age groups (**Table 3**). Mean left and right testicular weight were not significantly different within each group ($p > 0.05$). Paired testicular weight was significantly correlated with serum testosterone level at all sampling times (**Table 4**). However, when the data was analyzed by age groups, a significant correlation was found only between serum testosterone concentration at T0 and paired testicular weight in young (2–3 year-old) males.

Experiment 2

Serum testosterone concentrations before and after administration of hCG for different age groups are presented in **Figure 4**. Basal serum testosterone concentrations were below the detection level of the assay (0.1 ng/mL) for alpacas 6 to 11 months of age. Serum testosterone concentration increased after 11 months with large individual variations among individual animals within the same age group. A significant increase in serum testosterone concentration ($p < 0.05$) was observed 2 h after administration of hCG. The magnitude of the response to hCG varied according to age of alpacas (**Table 5**). It is interesting to note that the lowest magnitude of serum testosterone concentration change occurred in males between 15 and 16 months of age.

The proportion of animals with detectable estrogen levels (>1 pg/mL) after administration of hCG is presented in **Table 6**. The youngest animal with detectable estrogen was 14 month-old. There was a significant correlation between serum estrogen levels and serum testosterone concentration (**Table 7**).

The histomorphometry analysis of testicular samples obtained in this study are summarized in **Table 8**. Seminiferous tubule diameter increased significantly ($p < 0.05$) from 13 months onwards. There was no difference in the size of the seminiferous tubules between the alpacas in the 22 to 30 months age group and those older than 36 months. Testosterone concentration in pre- and post-hCG treatment samples were correlated to tubule diameter (**Table 7**). The proportion of mature Leydig cells as well as the number of Leydig cell in each section increased with advancing age as well as with tubule diameter. There was no DNA fragmentation detected by TUNEL analysis to identify apoptotic cells in all age groups. However, some positively stained germ cells were present within seminiferous tubules among the different ages examined (**Figure 5**). There was no significant difference in apoptotic cells amongst age groups.

DISCUSSION

Data on testicular steroidogenesis in camelids is very limited. In alpacas, serum testosterone concentration is reported to be low until 9 months of age then increases rapidly starting at 11 months of age and reaches adult levels by 2 years of age (32, 34). In camels, the pattern of testosterone section is strongly affected by season and diurnal rhythms particularly in rutting camels (35, 36). Seasonal effect of basal testosterone levels has also been described in the vicuña (*Vicugna vicugna*) (37) and the alpaca (3). In the vicuña, plasma testosterone concentration was higher in February (summer, peak breeding season) than in August (winter) and testis, seminiferous tubule and Leydig cell diameter were larger in February than in August. Spermatogenic activity was lower in Aug. than in Feb (37). In alpacas, Sumar in 1991 reported significantly higher serum testosterone concentration

TABLE 4 | Correlations matrix between paired testicular weight (Paired TW, grams) and serum testosterone concentration before (0 h) and after hCG administration.

	T 0 h	1 h	2 h	8 h	24 h
T 0 h	–				
1 h	0.83 ($p < 0.001$)	–			
2 h	0.46 ($p = 0.02$)	0.59 ($p = 0.002$)	–		
8 h	0.65 ($p < 0.001$)	0.81 ($p < 0.001$)	0.92 ($p < 0.001$)	–	
24 h	0.46 ($p = 0.02$)	0.65 ($p < 0.001$)	0.91 ($p < 0.001$)	0.91 ($p < 0.001$)	–
Paired TW	0.46 ($p = 0.02$)	0.44 ($p = 0.03$)	0.72 ($p < 0.001$)	0.64 ($p < 0.001$)	0.69 ($p < 0.001$)

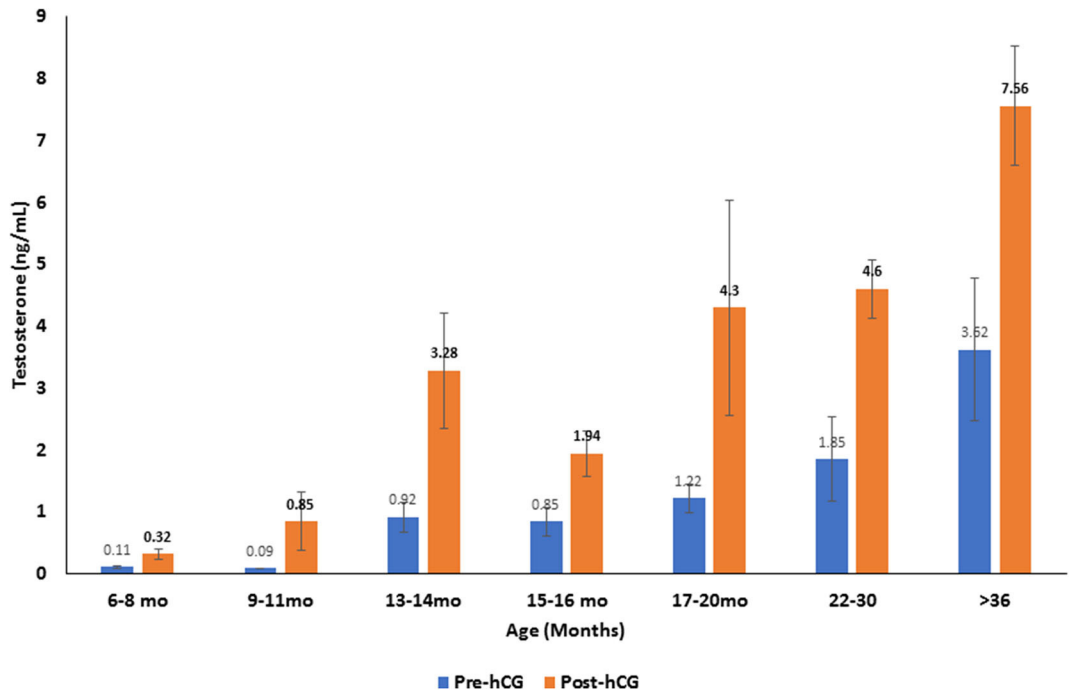


FIGURE 4 | Serum testosterone concentration (mean ±SEM, ng/mL) before and 2 h after hCG administration (3,000 IU, IV) in alpacas of different age groups.

in spring (2 to 3 times higher than in other seasons) (38). However, evaluation of circulating testosterone levels on a single sample is difficult because of its fluctuation during the day. This fluctuation of serum testosterone concentration has been attributed to a diurnal rhythm of LH section in several animal species, most notably in bulls (39–41), dogs (42), and stallions (43, 44). In dogs, serum plasma testosterone concentration can show a 26 to 62% variation within the same 24 h period (42). In rams, a true diurnal rhythm could not be established (19, 45). Other factors such as sexual and other social stimulation may also affect serum testosterone concentration. A large variation in basal serum testosterone concentration was also observed in both experiments reported here. This large variability of serum testosterone concentration between and within individuals of the same age makes interpretation difficult. The hCG stimulation test could help standardize comparison of steroidogenic activity in males. Serum testosterone response to hCG stimulation test was reported previously in alpacas. An increase in serum testosterone concentration occurred within 30 min after treatment (32). This response appeared to be short lasting compared to our results, and testosterone concentrations returned to pre-injection levels by 2 h after injection. However, the dose used (750 IU) was lower than in the present experiment. In the present study, the mean serum testosterone concentration almost doubled at 1 h post-hCG administration and peaked (300% above T0) at 8 h post-hCG administration. Studies in other species have shown that administration of hCG to intact and cryptorchid males results in an immediate (30 min to 1 h) increase in serum testosterone concentration followed by a decline, then a second, delayed, and more intense rise (14, 20, 23, 39). Administration of hCG to bulls (6,000 IU, IV) produced an increase in testosterone level within 30 min with a peak at 3 h and a second higher peak (2- to 3-fold basal level) at 2 days with levels remaining elevated for 3 to 4 days (39). In stallions, administration of 10,000 IU hCG to intact or cryptorchid animals produced peaks in serum testosterone concentration at 60 min and 24 h with the 24 h concentration reaching 2- to 10-fold the level observed at 60 min (23). In other studies, the peak response was found to occur as late as

TABLE 5 | Serum testosterone concentration (Mean ± SEM, ng/mL) and magnitude of response (expressed as a ratio between serum testosterone 2 h (T2) after treatment and basal testosterone T0) to hCG administration.

Age	N	Testosterone T0	Testosterone T2	T2/T0 ratio
6–8	13	$0.11 \pm 0.02^{a,1}$	$0.32 \pm 0.09^{a,2}$	2.71 ± 0.43^{a}
9–11	6	$0.09 \pm 0^{a,1}$	$0.85 \pm 0.47^{a,1}$	9.41 ± 5.41^{a}
13–14	10	$0.92 \pm 0.24^{b,1}$	$3.28 \pm 0.93^{b,2}$	5.8 ± 1.55^{a}
15–16	13	$0.85 \pm 0.23^{b,1}$	$1.94 \pm 0.37^{b,2}$	1.12 ± 1.23^{a}
17–20	6	$1.22 \pm 0.24^{b,1}$	$4.30 \pm 1.74^{b,2}$	4.72 ± 2.89^{a}
22–30	5	$1.85 \pm 0.68^{b,1}$	$4.60 \pm 0.48^{c,2}$	4.40 ± 1.51^{a}
>36	7	$3.62 \pm 1.15^{b,1}$	$7.56 \pm 0.96^{d,2}$	3.05 ± 0.8^{a}

^{a,b,c}Values with different subscripts within the same column are statistically different ($p < 0.05$).
^{1,2}Values with different subscripts within the same column are statistically different ($p < 0.05$).

TABLE 6 | Serum estrogen concentration (Mean ± SEM, pg/mL) and magnitude of response (expressed as a ratio between serum estrogen 2 h (T2) after treatment and basal estrogen T0) to hCG administration in different age group alpacas.

Age group	*N*	% with detectable estrogen level	Estrogen at T0	Estrogen at 2 h	Ratio E2/E0
6–8	13	0	0	0	–
9–11	6	0	0	0	–
13–14	10	10	0.1	0.49	4.86
15–16	13	23.1	0.25 ± 0.13^{a}	0.3 ± 0.16^{a}	1.12 ± 1.23^{a}
17–20	6	33.3	1.3 ± 0.83^{a}	3.62 ± 0.35^{a}	2.97 ± 1.23^{a}
22–30	5	100	$1.45 \pm 0.25^{a,1}$	$2.6 \pm 0.34^{a,2}$	1.99 ± 0.40^{a}
>36	7	100	$3.32 \pm 0.58^{b,1}$	$5.59 \pm 0.44^{b,2}$	1.91 ± 0.27^{a}

^{a,b,c}Values with different subscripts within the same column are statistically different ($p < 0.05$).
^{1,2}Values with different subscripts within the same column are statistically different ($p < 0.05$).

TABLE 7 | Correlations matrix between serum testosterone and estrogen concentration before (0 h) and 2 h after hCG administration and seminiferous tubule diameter, total Leydig cell number and proportion of mature Leydig cells in histological sections.

	Test. T0	Test. T2	Estr. T0	Estr. T2	STD	TLCN
Test. T0	–					
Test. T2	0.66	–				
Estr. T0	0.82		–			
Estr. T2	0.67	0.87		–		
STD	0.60	0.68	0.57	0.60	–	
TLCN	0.52	0.78	0.63	0.80	0.56	–
PMLC	0.51	0.56	0.50	0.55	0.58	0.67

All correlations are highly significant ($p < 0.001$).
Test. T0: serum testosterone concentration before hCG injection, Test. T2: serum testosterone concentration 2 h apres hCg administration.
Estr. T0: serum estrogen concentration before hCG injection, Estr. T2: serum testosterone concentration 2 h after hCg administration.
STD, seminiferous tubule diameter.
TLCH, Total Leydig cell number.
PMLC, Percent mature Leydig cells.

TABLE 8 | Mean ± SEM of Seminiferous tubule diameter (STD), Total Leydig cell number (TLCN), and Percent mature Leydig cells (PMLC), per microscope field in alpacas of different age group.

Age	STD (μm)	TLCN	PMLC (%)
6–8	71.56 ± 6.21^{a}	17.29 ± 7.52^{a}	35 ± 12.4^{a}
9–11	73.33 ± 8.82^{a}	33.4 ± 9.30^{a}	27.3 ± 7.56^{a}
13–14	151.48 ± 15.19^{b}	43.72 ± 15.94^{a}	54.79 ± 12.03^{a}
15–16	127.85 ± 12.75^{b}	39.3 ± 7.38^{a}	50.85 ± 10.08^{a}
17–20	158.67 ± 14.6^{b}	54.23 ± 15.38^{a}	60.5 ± 17.2^{a}
22–30	221.2 ± 24.32^{c}	$89.44 \pm 23.62^{a,b}$	80 ± 9.66^{a}
>36	196.43 ± 11.38^{c}	121.83 ± 17.9^{b}	87.33 ± 1.58^{a}

^{a,b,c}Values with different subscripts within the same column are statistically different ($p < 0.05$).

2 to 3 days after hCG administration with levels returning to baseline 8 to 10 days after injection (46). In the alpacas of the present study, serum testosterone concentration seemed to be on a decreasing trend at 24 h post hCG injection. Therefore, at least a first peak in response may have occurred between 8 and 24 h after hCG administration. It would be interesting to determine the long-term effect on testosterone concentration beyond 24 h. As expected, the response to hCG stimulation test was highly correlated to age and paired testicular weight. There was a significant effect of age (i.e., testicular weight) of on the magnitude of serum testosterone concentration change with older males registering the highest increase. This effect is most likely related to a higher number of Leydig cells and better steroidogenic activity in older males. The highest correlation between paired testicular weight and serum testosterone response to hCG stimulation occurred after 2 h.

In the second experiment, basal serum testosterone concentration increased with advancing age. The basal concentration of the testosterone in prepubertal ages of this study was similar to that reported previously (0.06 to 0.09 ng/mL) (32). The large variation in serum testosterone concentration and Leydig cell number amongst males within each age group suggests that there may be genetic factors involved in sexual development which are worthy of investigation. The magnitude of serum testosterone concentration in response to hCG injection was different for different age groups. The largest increase was seen in the 13 to 14 month age group. This supports earlier observation that this is the stage of onset of puberty in some animals (3, 34, 38). An interesting observation in the present study is a drop in the response to hCG stimulation in the 15 to 16 month age group before increasing again after 17 months of age. The bimodal response to hCG has been described in rabbits by Hall and Young (47). The first response may include two mechanisms: hCG may stimulate the release of testosterone present in the Leydig cells or hCG may stimulate 20 α-hydroxylase activity, thereby increasing the conversion of cholesterol to 20 α-hydroxycholesterol and finally to testosterone. The second response of hCG is thought to be the result of a general increase in microsomal production of enzymes involved in the synthesis of testosterone.

Strong evidence exists concerning the implication of estrogen in male reproductive function. Estrogens have an essential role in regulating the hypothalamus–pituitary–testis axis and thus

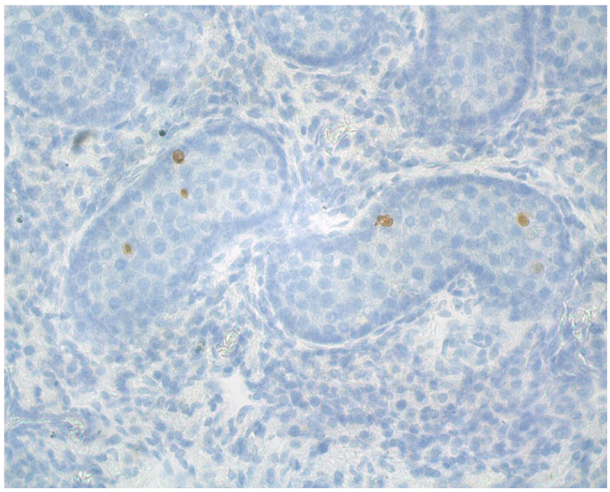


FIGURE 5 | Representative alpaca (8 months of age) testis cross-section used for analysis of apoptosis (TUNEL staining for apoptotic cells).

indirectly regulate luteinizing hormone (LH) and testosterone balance through a feedback loop (48). Activity of estrogen on spermatogenesis involves germ cell proliferation, differentiation and the final maturation of spermatids as well as germ cell survival (49). Estrogen is produced in sizable quantities in the testis and it is also present in very high concentrations in semen of several species and seems to be associated with male fertility although the exact mechanism remains unknown (29, 30). In bulls, serum estradiol concentration in prepubertal animals was found to be positively correlated to testicular growth (50). In stallions, estrogens and not testosterone were reported to be significantly lower in infertile stallions (51).

In adult stallions and bulls, estrogen concentration increases after administration of hCG or GnRH (28, 30). To our knowledge, the present study is the first that demonstrates the aromatizing ability of the testicular tissue and serum estrogen changes in response to hCG administration in alpacas. Estrogen was below detectable levels before 14 months of age. It is interesting to note that the only male with detectable estrogen at 14 months of age had four times more Leydig cells than the male with the second highest Leydig cells count in the same group. Based on our histological evaluation, differentiation and maturation of adult Leydig cells in alpacas is initiated before 6 months of age. These stages of differentiation and maturation are responsible for increased testosterone production. There was no evidence of apoptosis in samples analyzed by the TUNEL assay from different groups. This suggests that the changes in steroid production observed are due mainly to changes in steroidogenic ability of Leydig cells.

Our observation on size of seminiferous tubules in adult males (196.43 $\pm$11.38 μm) are in agreement with previously reported (174 to 240 μm) studies on alpacas (52). The first dramatic increase in the diameter occurred after 12 months of age and was accompanied with a strong response to hCG and increased Leydig cell number. The second increase in tubule diameter was seen after 21 months of age along with an increase in Leydig cells number and serum testosterone This may be explained as an accommodation of the tubules for the release and flow of spermatozoa along the tubules.

CONCLUSION

The present experiment confirmed the high variability of basal serum testosterone concentration in serum in male alpacas even within the same age group. Response to hCG stimulation test using 3,000 IU IV is correlated to testicular weight. For diagnostic purposes, two samples—one taken before hCG administration and one taken 2 or 8 h after hCG administration -should be sufficient to evaluate the presence and function of testicular tissue. Further studies are needed to evaluate the effect of hCG stimulation beyond 24 h. The intensity of steroidogenic response to hCG is age dependent. A sharp increase in testosterone following hCG treatment is observed in males between 12 and 14 months of age and after 21 months of age, confirming that the ages between 12 and 20 months are critical in sexual development in male alpacas. In addition, significant changes in serum estrogen concentration were detected in animals that exceeded 3 ng/mL serum testosterone concentration. This suggests a high aromatizing ability in the testes of alpacas. The response was correlated to number and differentiation of Leydig cells. Further studies are needed to clarify the role of estrogens in male alpaca testis development.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee, Washington State University.

AUTHOR'S NOTE

The content of this manuscript has been published in part as part of the thesis (53).

AUTHOR CONTRIBUTIONS

AE and AT contributed to experimental design, data collection, data analysis, and manuscript preparation. CP contributed to data analysis and manuscript preparation. All authors contributed to the article and approved the submitted version.

FUNDING

This research was funded by donations from alpacas breeders in WA, ID, MT, OR, CA.

ACKNOWLEDGMENTS

The authors wish to thank Ms. Shirley Sandoval and Mr. David DeAvila for their technical help.

REFERENCES

1. Tibary A, Vaughan J. Reproductive physiology and infertility in male South American camelids: a review and clinical observations. *Small Ruminant Res.* (2006) 61:283–98. doi: 10.1016/j.smallrumres.2005.07.018
2. Losno W, Coyotupa J. *Testoterona Serica en Alpacas Macho Prepuberes*. Lima: Res Proyectos de Investigacion Univ Nacional Mayor de San Marcos (1979). p. 116.
3. Sumar J. Reproductive physiology in South American camelids. In: Land RB, Robinson DW, editors. *Genetics of Reproduction in Sheep*. London: Butterworths (1985). p. 81–95.
4. Galloway D. The development of the testicles in alpacas in Australia. In: *The Australian Alpaca Industry Conference*. Canberra, ACT (2000). p. 21–3.
5. Abraham MC, Puhakka J, Ruete A, Al-Essawe EM, de Verdier K, Morrell JM, et al. Testicular length as an indicator of the onset of sperm production in alpacas under Swedish conditions. *Acta Vet Scand.* (2016) 58:10. doi: 10.1186/s13028-016-0191-x
6. Chuna P, Leyva V, Franco J. Adherencias pene-prepuciales y niveles de testosterone circulantes en alpacas. *Rev Inv Vet Peru.* (1999) 10:11–6. doi: 10.15381/rivep.v10i2.6684
7. San Miguel C, Leyva V, Garcia W. Admnistracion de testosterona en alpaca con adherencias peni-preputial. *Rev Inv Vet Peru.* (2002) 13:28–36. doi: 10.15381/rivep.v13i2.7327
8. Cox J. Behaviour of the false rig: causes and treatments. *Vet Rec.* (1986) 118:353–6. doi: 10.1136/vr.118.13.353
9. Grant DB, Lauwrence BM, Atherden SM. HCG stimulation test in children with abnormal sexual development. *Arch Dis Child.* (1976) 51:596. doi: 10.1136/adc.51.8.596
10. Kogan SJ. Cryptorchidism. In: Kelalis PP, King LR Belman AB, editors. *Clinical Pediatric Urology*. Philadelphia, PA: W. B. Saunders (1992). p. 1063.
11. Kolon TF, Miller O. Comparison of single versus multiple dose regimens for the human chorionic gonadotropin stimulatory test. *J Urol.* (2001) 166:1451–4. doi: 10.1016/S0022-5347(05)65807-7
12. Levitt SB, Kogan SJ, Schneider KM, Becker JM, Sobel EH, Mortimer RH, et al. Endocrine tests in phenotypic children with bilateral impalpable testes can reliably predict "congenital" anorchism. *Urology*. (1978) 11:11–7. doi: 10.1016/0090-4295(78)90192-9
13. Sundby A, Velle W. Plasma concentration of testosterone in young bulls in relation to age, rate of weight gain and stimulation with human chorionic gonadotrophin. *J Endocrinol.* (1980) 86:465–9. doi: 10.1677/joe.0.0860465
14. Sundby A, Velle W. Relationship between growth rate in bulls and human chorionic gonadotropin-induced plasma testosterone concentrations. *J Anim Sci.* (1983) 56:52–7. doi: 10.2527/jas1983.56152x
15. Memon MA, Ganjam VK, Pavletic MM, Schelling SH. Use of human chorionic gonadotropin stimulation test to detect a retained testis in a cat. *J Am Vet Med Assoc.* (1992) 201:1602.
16. Memon MA, Tibary A. Canine and feline cryptorchidism. In: Caoncannon PW, England G, Verstegen J, editors. *Recent Advances in Small Animal Reproduction.* Ithaca, NY: International Veterinary Information Services (WWW.IVIS.org) (2001).
17. Kawakami E, Hori T, Tsutsui T. Changes in plasma LH and testosterone levels and semen quality after a single injection of hCG in two dogs with spermatogenic dysfunction. *J Vet Med Sci.* (1998) 60:765–7. doi: 10.1292/jvms.60.765
18. Christiansen P, Andersson AM, Skakkebaek NE, Juul A. Serum inhibin B, FSH, LH and testosterone levels before and after human chorionic gonadotropin stimulation in prepubertal boys with cryptorchidism. *Eur J Endocrinol.* (2002) 147:95–101. doi: 10.1530/eje.0.1470095
19. Falvo RE, Buhl AE, Reimers TJ, Foxcroft GR, Dunn MH, Dziuk PJ. Diurnal fluctuations of testosterone and LH in the ram: effect of HCG and gonadotrophin-releasing hormone. *J Reprod Fertil.* (1975) 42:503–10. doi: 10.1530/jrf.0.0420503
20. Garnier F, Saez JM. Response of plasma testosterone to human chorionic gonadotropin stimulation in the ram. *Biol Reprod.* (1980) 22:832–6. doi: 10.1095/biolreprod22.4.832
21. Bartke A, Voglmayr JK. Effects of gonadotropins on androgen levels in rete testis fluid of the ram. *Biol Reprod.* (1977) 16:274–80. doi: 10.1095/biolreprod16.2.274
22. Cox JE. Testosterone concentrations in normal and cryptorchid horses - response to human chorionic-gonadotropin. *Anim Reprod Sci.* (1989) 18:43–50. doi: 10.1016/0378-4320(89)90006-7
23. Arighi M, Bosu WTK. Comparison of hormonal methods for diagnosis of cryptorchidism in horses. *J Equine Vet Sci.* (1989) 9:20–6. doi: 10.1016/S0737-0806(89)80112-1
24. Petrizzi L, Varasano V, Robbe D, Valbonetti L. Monorchidism in an appaloosa stallion. *Vet Rec.* (2004) 155:424–5. doi: 10.1136/vr.155.14.424
25. Cox JE, Redhead PH, Dawson FE. Comparison of the measurement of plasma testosterone and plasma estrogens for the diagnosis of cryptorchidism in the horse. *Equine Vet J.* (1986) 18:179–82. doi: 10.1111/j.2042-3306.1986.tb03589.x
26. Clement F, Plongere G, Magistrini M, Palmer E. Assessment of the sexual function in the stallion. *Point Vet.* (1998) 29:61–6.
27. Okuda K, Ishimoto C, Ohishi H, Maekawa N, Sato K. Age-related responses of serum testosterone to human chorionic gonadotropin in the stallion. *Japan J Anim Reprod.* (1991) 37:21–5. doi: 10.1262/jrd1977.37.21
28. Bollwein H, Schulze J, Miyamoto A, Sieme H. Testicular blood flow and plasma concentrations of testosterone and total estrogen in the stallion after the administration of human chorionic gonadotropin. *J Reprod Dev.* (2008) 54:335–9. doi: 10.1262/jrd.20014
29. Devkota B, Koseki T, Matsui M, Sasaki M, Kaneko E, Miyamoto A, et al. Relationships among age, body weight, scrotal circumference, semen quality and peripheral testosterone and estradiol concentrations in pubertal and postpubertal Holstein bulls. *J Vet Med Sci.* (2008) 70:119–21. doi: 10.1292/jvms.70.119
30. Devkota B, Takahashi KI, Matsuzaki S, Matsui M, Miyamoto A, Yamagishi N, et al. Basal levels and GnRH-induced responses of peripheral testosterone and estrogen in Holstein bulls with poor semen quality. *J Reprod Dev.* (2011) 57:373–8. doi: 10.1262/jrd.10-136T
31. Silberzahn P, Pouret E, Zwain I. Androgen and oestrogen response to a single injection of hCG in cryptorchid horses. *Equine Vet J.* (1989) 21:126–9. doi: 10.1111/j.2042-3306.1989.tb02116.x
32. Bravo P, Johnson LW. Reproductive physiology of the male camelid. *Vet Clin Nth Am Food Anim Pract.* (1994) 10:259–64. doi: 10.1016/S0749-0720(15)30560-0
33. Perkins NR, Frazer GS, Hull BL. Endocrine diagnosis of cryptorchidism in a llama. *Aust Vet J.* (1996) 74:275–7. doi: 10.1111/j.1751-0813.1996.tb13773.x
34. Bravo WP. Chapter 15 - reproductive anatomy and physiology in the male. In: Cebra C, Anderson DE, Tibary A, van Saun RJ, Johnson LW, editors. *Llama and Alpaca Care*. St. Louis, MO: W.B. Saunders (2014). p. 150–61.
35. Al-Qarawi AA, Abdel-Rahman HA, El-Belely MS, El-Mougy SA. Age-related changes in plasma testosterone concentrations and genital organs content of bulk and trace elements in the male dromedary camel. *Anim Reprod Sci.* (2000) 62:297–307. doi: 10.1016/S0378-4320(00)00146-9
36. Agarwal SP, Rai AK, Khanna ND. Seasonal variation in the concentration of steroid hormones in seminal plasma of camel. *Indian Vet J.* (1997) 74:82–3.
37. Urquieta B, Cepeda R, Cáceres JE, Raggi LA, Rojas JR. Seasonal variation in some reproductive parameters of male vicuña in the High Andes in northern Chile. *J Arid Environ.* (1994) 26:79–87. doi: 10.1006/jare.1994.1012
38. Sumar J. Fisiología de la reproducción del macho y manejo reproductivo. In: Fernandez-Bac S, editor. *Avances y Perspectivas del Conocimiento de los Camélidos Sud Americanos*. Santiago: FAO (1991). p. 111–48.
39. Sundby A, Tollman R, Velle W. Long-term effect of HCG on plasma testosterone in bulls. *J Reprod Fertil.* (1975) 45:249–54. doi: 10.1530/jrf.0.0450249
40. Katongole V, Naftolin F, Short R. Relationship between blood levels of lutenizing hormone and testosterone in bulls, and the effects of sexual stimulation. *J Endocrinol.* (1971) 50:457–66. doi: 10.1677/joe.0.0500457
41. Sanwal PC, Sindby A, Edqvist LE. Diurnal variation of periperal plasma levels of testosterone in bulls measured by a rapid radioimmunossay procedure. *Acta Vet Scand.* (1974) 15:90–9.
42. Coster RD, Beckers JF, Wouters-Ballman P, Ectors F. Diurnal variations in plasma testosterone and LH in the dog. *Ann Med Vet.* (1979) 123:423–8.
43. Bono G, Minori D, Gaiani R, Mattioli M. Concentrations of estrone sulfate, androgens and Lh in the peripheral plasma of mating stallions. *J Reprod Fertil.* (1982) 66:17–22. doi: 10.1530/jrf.0.0660017

44. Kirkpatrick JF, Vail R, Devous S, Schwend S, Baker CB, Wiesner L. Diurnal variation of plasma testosterone in wild stallions. *Biol Reprod.* (1976) 15:98–101. doi: 10.1095/biolreprod15.1.98
45. Schanbacher BD, D'Occhio MJ, Kinder JE. Initiation of spermatogenesis and testicular growth in oestradiol-17 beta -implanted bull calves with pulsatile infusion of luteinizing hormone releasing hormone. *J Endocrinol.* (1982) 93:183–92. doi: 10.1677/joe.0.0930183
46. Silberzahn P, Zwain I, Guerin P, Benoit E, Jouany JM, Bonnaire Y. Testosterone response to human chorionic gonadotropin injection in the stallion. *Equine Vet J.* (1988) 20:61–3. doi: 10.1111/j.2042-3306.1988.tb01456.x
47. Hall P, Young D. Site of action of trophic hormones upon the biosynthetic pathways to steroid hormones. *Endocrinology.* (1968) 82:559–68. doi: 10.1210/endo-82-3-559
48. O'Donnell L, Robertson K, Jones M, Simpson RS. Estrogen and spermatogenesis. *Endocr Rev.* (2001) 22:289–318. doi: 10.1210/edrv.22.3.0431
49. Carreau S, Hess R. Oestrogens and spermatogenesis. *Phil Trans R Soc B.* (2010) 365:1517–35. doi: 10.1098/rstb.2009.0235
50. Moura A, Erickson B. Age-related changes in peripheral hormone concentrations and their relationships with testis size and number of Sertoli and germ cells in yearling beef bulls. *J Reprod Fertil.* (1997) 111:183–90. doi: 10.1530/jrf.0.1110183
51. Roser JF. Regulation of testicular function in the stallion: an intricate network of endocrine, paracrine and autocrine systems. *Anim Reprod Sci.* (2008) 107:179–96. doi: 10.1016/j.anireprosci.2008.05.004
52. Montalvo C, Cevallos E, Copaira M. Estudio microscopico del parenquima testicular de la alpaca durante las estaciones del ano. In: *Porc XI Cong Nac De Viencias Veterinarias.* Lima (1979). p. 37.
53. El Zawam, A. *Establishment of spermatogenesis following testicular tissue ectopic xenografting in alpaca* [Ph.D. thesis]. Washington State University, Pullman, WA, United States (2013).

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 17 November 2020
doi: 10.3389/fvets.2020.597960

Uterine and Corpus Luteum Blood Flow Evaluation Prior to Uterine Flushing in Llama Embryo Donors

Enzo G. Zampini[1,2], María F. Gallelli[1,2], María G. Chaves[1], Deborah M. Neild[1], Mariana Gambarotta[3], Marcelo H. Miragaya[1] and Virginia L. Trasorras[1,2]*

[1] Universidad de Buenos Aires (UBA), Facultad de Ciencias Veterinarias (FCV), Instituto de Investigación y Tecnología en Reproducción Animal, Cátedra de Teriogenología, Buenos Aires, Argentina, [2] Consejo Nacional de Investigaciones Científicas y Técnicas, Buenos Aires, Argentina, [3] Universidad de Buenos Aires (UBA), Facultad de Ciencias Veterinarias (FCV), Cátedra de Bioestadística, Buenos Aires, Argentina

The aim of this study was to assess the uterine blood flow (UBF) and corpus luteum blood flow (CLBF) in llamas 8 days post-mating, using color-Doppler ultrasonography (CDU), to determine the possible relationship between vascularization and the presence of an embryo. Adult females ($n = 25$) were used to monitor ovarian dynamics by palpation and transrectal ultrasonography until detection of a ≥6 mm growing follicle. Females were randomly assigned to one of two groups: Group I ($n = 19$), were mated and ovulation was induced by a single dose of buserelin (GnRH analog) that same day (Day 0); and Group II ($n = 6$), only ovulation was induced (control). On Day 8, UBF and CLBF were evaluated transrectally in both groups. The color-flow images obtained were analyzed with Image J1.52a software to determine the vascularization area and the percentage of corpus luteum with blood flow emission (CLBF%) together with the percentage for each uterine horn (UBF%). Statistical analysis was performed using an ANOVA test. In Group I, uterine flushing was performed to obtain the embryos, thus dividing the females into Group I+ ($n = 10$), when an embryo was recovered and Group I- ($n = 9$), when no embryo was recovered. Embryo recovery rate was 52.63% (10/19). In Group I+, UBF% was significantly higher compared to Group I- and Group II ($P < 0.05$). UBF appears to be a good predictor for embryo presence, with an area under the curve (AUC) of 0.9 and an optimal cut-off value of 9.37% (with a sensitivity of 90% and specificity of 88.9%). The CLBF% did not differ between groups ($P > 0.05$). In conclusion, it is possible to detect a local increase of UBF in the presence of an embryo on day 8 post-mating in llamas. This could be useful to achieve an early pregnancy diagnosis or to decide whether to carry out the uterine flushing in a llama embryo transfer program.

Keywords: camelids, embryo transfer, Doppler ultrasonography, pregnancy, biotechnologies

OPEN ACCESS

Edited by:
Khalid El Allali, Agronomic and Veterinary Institute Hassan II, Morocco

Reviewed by:
Dale Kelley, Oklahoma State University, United States
Tom Stout, Utrecht University, Netherlands

****Correspondence:***
Enzo G. Zampini
egzampini@fvet.uba.ar

Specialty section:
This article was submitted to Animal Reproduction - Theriogenology, a section of the journal Frontiers in Veterinary Science

Received: *23 August 2020*
Accepted: *12 October 2020*
Published: *17 November 2020*

Citation:
Zampini EG, Gallelli MF, Chaves MG, Neild DM, Gambarotta M, Miragaya MH and Trasorras VL (2020) Uterine and Corpus Luteum Blood Flow Evaluation Prior to Uterine Flushing in Llama Embryo Donors. Front. Vet. Sci. 7:597960. doi: 10.3389/fvets.2020.597960

INTRODUCTION

Llamas are monotocous and have a prolonged gestation period [335–360 days; (1)], hence early pregnancy diagnosis would allow a greater efficiency to obtain one offspring per female per year when conception fails and gives better productive indices. To date, the methods used to diagnose gestation in South American camelids (SAC) are limited.

One of the methods that is widespread among SAC producers is to observe the sexual behavior of the females when confronted with a male, from 11 to 13 days after natural mating (2, 3). Although this is an inexpensive technique as it doesn't require sophisticated tools or equipment, behavior interpretation is subjective and prone to error. There are even dominant females that, in absence of high progesterone levels, still reject the male without being pregnant. In addition, this method does not provide information on the number of embryos or fetuses present, their state of development or health. Another diagnostic tool is measuring plasma progesterone levels 11–13 days post-mating (4, 5) as it reflects the presence of a functional corpus luteum (CL), necessary to maintain pregnancy in SAC (6). However, validated progesterone dosage tests for SAC are scarce and difficult to access and the time lag between blood collection and availability of the results is also problematical. Furthermore, reproductive disorders that prolong the luteal phase, such as spontaneous ovulations and luteinized cystic follicles, can produce a 15% of false positive results (5). But, similarly to the first method, this technique doesn't provide information on the number, development, and health of the embryo or fetus either. Pregnancy evaluation by transrectal palpation is possible 35 days after mating in llamas, with greater accuracy 45–50 days after mating (2, 3, 7). This is a simple, low-cost maneuver that although provides fetal viability data, the estimation of gestational age, and therefore prediction of probable date of parturition, are not precise (8). So far, the most effective, precise and early method for diagnosing gestation in SAC is transrectal ultrasonography in Brightness mode (B-mode), which allows visualization of the embryo vesicle 12–14 days after mating, although is more precise as of 16–23 days (9). Unlike the above methods, it not only gives information regarding the number, development and viability of the embryo/s or fetus/es, it also allows one to approximate gestational age (10), even when the date of natural mating is unknown, and hence estimate the probable date of parturition.

In SAC, the embryo reaches the uterine lumen as a hatched blastocyst, between 6 and 6.5 days after ovulation (11, 12), and produces increasing quantities of estradiol-17β during days 7–15 of gestation (13). According to Ford (14), the estradiol produced by embryos of domestic species is responsible for the local increase in uterine blood flow (UBF) registered during early pregnancy. However, it is not known if there is a connection between the presence of the embryo and uterine blood flow in llamas in the early stages of gestation. Color Doppler ultrasonography (CDU) not only allows evaluation of the bidimensional structure of the different organs but also offers the possibility of observing their vascular system (15) as it overlays the color signals of blood flow over the B-mode images (16). To calculate the degree of vascularization of an organ, the percentage of tissue with color signals can be estimated or the images that are captured can be processed and analyzed by a computer using software that allows an objective calculation of the number of colored pixels over the B-mode area (17). The aim of this study was to assess the uterine blood flow and corpus luteum blood flow in llamas, 8 days post-mating using color Doppler ultrasonography to determine the possible relationship between vascularization and the presence of an embryo.

MATERIALS AND METHODS

Animals

Non-pregnant, non-lactating female llamas ($n = 25$) ranging between 4 and 12 years of age and with an average body weight of 120 ± 22 kg were used in this study. All animals were in a good nutritional status (body condition), both healthy and reproductively active at the time of the trial. Females were kept separate from the males and fed with hay and water *ad-libitum*. The study was conducted between March 2018 and December 2019 at the Faculty of Veterinary Sciences of the University of Buenos Aires, Buenos Aires, Argentina, situated 34° 36' S and 58° 26′ W at sea level. This study was approved by the Committee for the Use and Care of Laboratory Animals (CICUAL, 2017/67) of the Faculty of Veterinary Sciences of the University of Buenos Aires.

Experimental Design

Ovarian dynamics were monitored by transrectal palpation and ultrasonography (Berger LC 2010 plus with a 5 MHz linear-array electronic transducer) until the presence of a growing follicle ≥6 mm was detected. At that moment, all females received a single IV dose of 8 μg of buserelin (GnRH analog; Receptal®, Intervet, Buenos Aires, Argentina) (day 0) to induce endogenous LH release and ovulation. After buserelin injection, llamas were randomly divided in two groups: Group I ($n = 19$), embryo donor females were mated with a male with proven fertility and uterine flushings were done for embryo recovery on day 8; Group II ($n = 6$), females without natural mating (control group). Ovulation was confirmed using transrectal ultrasonography based on the disappearance of the dominant follicle on day 2, and control of the ovarian dynamics was carried out every other day (B-mode ultrasonography) until day 8.

Uterine and Corpus Luteum Vascularization

All females were examined by transrectal CDU (MyLab™ 30Gold VET ESAOTE, attached to a 5 MHz linear-array electronic transducer) in order to assess the UBF and corpus luteum blood flow (CLBF) on day 8. The settings (B-mode frequency: 5 MHz with a depth of 8 cm and a gain of 52%; CFM pulse repetition frequency: 1.4 KHz and a gain of 70%) were standardized and remained constant for all examinations. Briefly, the transducer was placed over the middle segment of each uterine horn (UH) as described in llamas (18, 19), to display signals for blood flow in the vessels of all the endometrium, myometrium, and perimetrium, and over the CL. A 3 s video-clip of the vascularization of each structure was registered and downloaded. The video-clips were examined frame by frame (Adobe Premiere Pro CS6®) to select images that showed the maximum vascular signal, three of each UH and three over the maximum cross-sectional area of the CL. A total of 225 images (nine images per female) were saved in tagged image file format (TIFF) and analyzed by an operator without knowledge of the identity of each animal or the result of uterine flushing, using ImageJ 1.52i software (National Institute of Health, USA). The degree of vascularization was estimated by measuring the

colored area (cm^2) of the vascular flow signals (Doppler mode) over the left UH, right UH and the CL area (cm^2) (B-mode). Thus, percent area of vascularization was calculated by the following equation: percent of blood flow area (BF%) = (vascular area/total organ's area) × 100. The average of the three images was considered as the final value for each uterine horn (UBF%) and for the CL (CLBF%) of each animal. In females with two CLs, we calculated the CLBF% of both and considered the average. Uterine flushing was performed in Group I after Doppler ultrasonographic evaluation.

Embryo Recovery and Evaluation

In Group I, uterine flushing was carried out non-surgically for embryo recovery, 8 days after mating (20). The maneuvers were performed with the female either standing or in sternal recumbency. The animal was restrained in stocks, the tail was wrapped and the rectum was emptied of feces. The perineum was then scrubbed using a hypoallergenic detergent, rinsed carefully with clean water and then dried. Restless females received 0.2 mg/kg IV xylazine (Xilazina® 10%, PRO-SER S.A., Buenos Aires, Argentina) before flushing. A Foley catheter (Vortex™; Agtech, Inc.; Manhattan, USA.) 12 or 16 Fr, according to female size, with a stylet inserted into the catheter to keep it from bending during recto-vaginal manipulation, was used. Uterine flushing was done by placing the catheter cuff cranial to the internal cervical os and insufflating it with 5 or 10 ml of air (depending on catheter gauge). The whole uterus was flushed 4 to 5 times using Ringer-Lactate solution (Laboratorios Rivero, Buenos Aires, Argentina), previously warmed (30–35°C), with a total volume of 500 ml. The recovered medium was filtered through a 70 μm EmCon™ filter (Agtech, Inc.) for embryos. The fluid in the flushing filter was placed in warmed reticulated Petri dishes and embryos were identified using a stereomicroscope. Embryos were measured and classified according to their morphology following the criteria set by Tibary and Anouassi (21), using a grade scale from 1 to 5. After flushing, donor females received 250 μg IM of $PGF_{2\alpha}$ (cloprostenol; Ciclase DL®, Syntex S.A., Buenos Aires, Argentina) to induce luteolysis. The number of embryos collected, and positive pregnancy diagnoses were recorded. Thus, females were divided into Group I+, when an embryo was recovered (positive uterine flushing) and Group I-, when no embryo was recovered (negative uterine flushing).

Statistical Analysis

Statistical analyses were performed using InfoStat software (version 2020; FCA, National University of Córdoba, Argentina). After verifying normal distribution of the data using a Shapiro-Wilks test, an ANOVA test was applied to evaluate uterine and CL vascularization. These data were subjected to Tukey's test to determine significance. In all females, the uterine horn with highest UBF% was chosen. Furthermore, we performed receiver operating characteristics (ROC) analyses focusing on UBF% on day 8, to identify the optimal cutoff value for predicting pregnancy. We evaluated every candidate cutoff value for the optimal cutoff value by geometric distance for 100% sensitivity and specificity. The optimal cutoff value was determined using the data point that minimized the distance (22). Prognostic value was expressed as the area under the curve (AUC) with a 95% confidence interval and a test of significance. A paired *T*-test was used to evaluate the relationship between the blood flow of each uterine horn and the location of the CL in the females in Group I+. Pearson or Spearman's correlation analysis were used to compare various parameters (Follicle and CL diameter, CLBF). The correlations between parameters were classified according to Taylor (23) as weak ($r \leq 0.35$), moderate ($r = 0.36$–0.67) or strong ($r = 0.68$–1.00). Values were expressed as mean ± SEM. Differences were considered significant when $P < 0.05$.

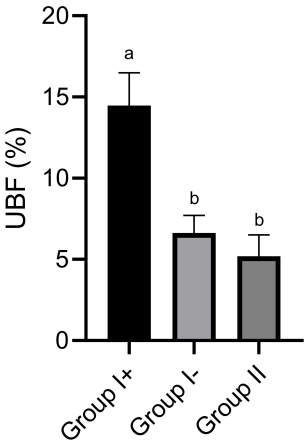


FIGURE 1 | Uterine blood flow (UBF) in the three groups: llamas with an embryo recovered (Group I+), llamas without an embryo recovered (Group I-) and control group (Group II, ovulated, non-mated females). Values are mean ± SEM. [a,b]Groups with different letters are significantly different ($P < 0.05$).

RESULTS

Embryo Recovery Rate and Quality

Embryo recovery rate was 52.63% (10/19), with a total of 11 embryos recovered from 19 flushing procedures (two embryos were obtained from one female). Hence, females in Group I were divided into Group I+: $n = 10$ and Group I-: $n = 9$. Of the 11 recovered embryos, nine were grade I, one was grade II and one was an arrested morula (grade V). Embryo size ranged from 350 to 1,500 μm.

Uterine Blood Flow in Pregnant and Non-pregnant Females

In Group I+, UBF% was significantly higher (14.5 ± 1.5%) than in Group I- (6.6 ± 1.2 %) and Group II (5.19 ± 1.94 %; $P < 0.05$). Moreover, no differences were found between Group I- and Group II ($P > 0.05$) (**Figures 1**, **2**).

Predicting the Presence of an Embryo Using a ROC Curve

To prepare the Receiver Operating Characteristic (ROC) curve, the uterine horn with highest UBF% on day 8 from each female was taken. Differentiation between pregnant and non-pregnant llamas was possible with this technique with an area under the

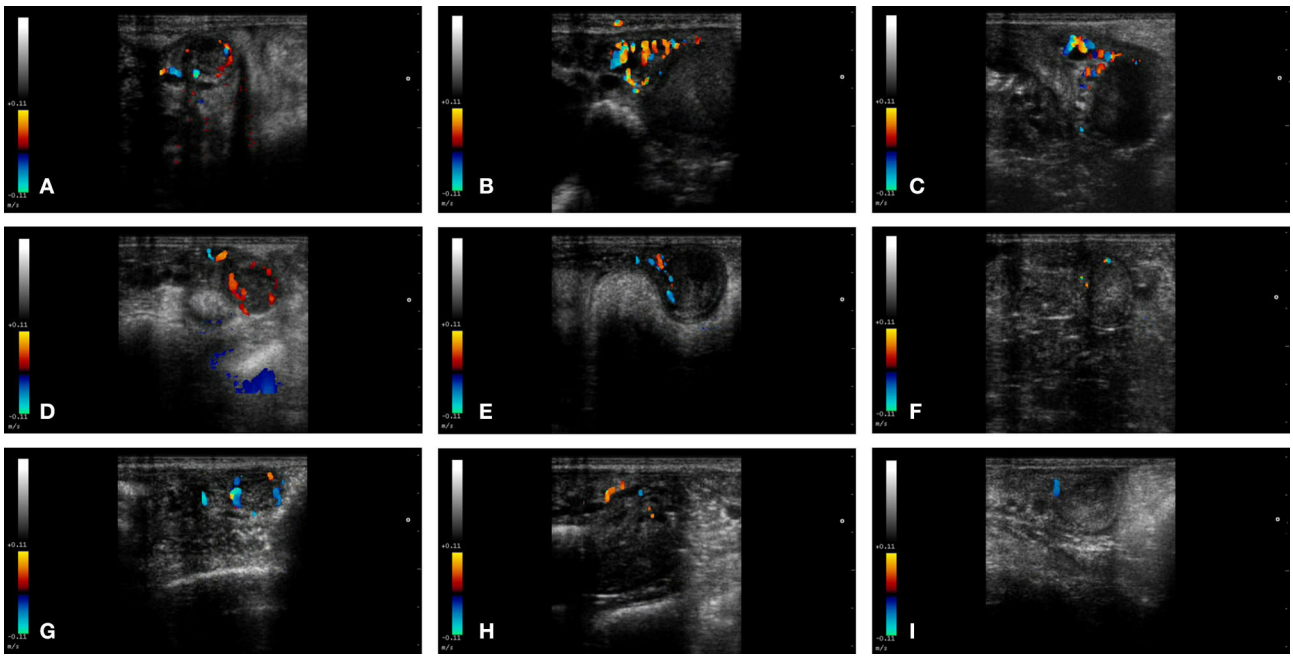


FIGURE 2 | Color-Doppler images from a female llama from each group in the study. From left to right: CL, left uterine horn and right uterine horn. Group I+ **(A–C)**, Group I- **(D–F)**, and Group II **(G–I)**.

curve (AUC) of 0.900. The optimal cut-off value was 9.37%, with a sensitivity of 90% and specificity of 88.9% (**Figure 3**).

Blood Flow of Each Uterine Horn and Its Relationship With the Location of the CL in Pregnant Females

Females in Group I+ showed no significant differences in UBF% between both uterine horns irrespective of the location of the CL ($P > 0.05$). However, those with a CL in the left ovary showed a tendency to have a higher UBF% in the left UH compared to the right UH ($P = 0.06$), whereas when the CL was in the right ovary, UBF% was similar between uterine horns ($P = 0.97$).

Corpus Luteum Diameter and Vascularization in Pregnant and Non-pregnant Females

Diameter of the CL on day 8 was similar between all groups (Group I+: 1.06 ± 0.07 cm; Group I-: 1.25 ± 0.06 cm; Group II: 1.25 ± 0.07 cm) ($P = 0.76$). Neither were significant differences detected in CL blood flow (CLBF%) between Groups I+ (23.29 ± 2.05 %), I- (22.76 ± 2.16 %) and II (22.93 ± 2.64 %) ($P = 0.98$) (**Figure 4**).

Correlation Analysis

Follicular diameter on day 0 was moderately correlated to CL diameter on day 8 (Spearman's coefficient of correlation: $s\rho = 0.61$; $P = 0.0013$) but was not correlated to CLBF% on day 8 (Pearson's coefficient of correlation: $\rho = -0.28$; $P = 0.18$); neither was CL diameter on day 8 correlated to CLBF% on the same day ($\rho = 0.08$; $P = 0.72$).

DISCUSSION

Embryo transfer is a powerful tool for increasing the number of offspring from a specific donor, but it is also becoming increasingly important for the genetic improvement of livestock herds (20, 24–26). Flushing for embryo recovery from the donor llama's uterus is normally performed on day 8 post-mating, without a previous pregnancy diagnosis. Using CDU, we observed that mated llamas with an embryo recovered (Group I+), had a significantly higher UBF% than mated females without an embryo recovered (Group I-) and llamas that ovulated but not were mated (Group II; control). This increase in UBF in pregnant females vs. non-pregnant females was also observed in embryo donor mares, where Doppler ultrasonography was used to make an early pregnancy diagnosis 8 days after artificial insemination, prior to embryo recovery. They established a cut-off value of 35.55 mm^2 to distinguish adult pregnant mares from those that were not gestating, with a 97.2% sensitivity and an 85.7% specificity (27). In inseminated buffalo, increases in UBF were registered after ovulation, with a marked difference observed between pregnant and non-pregnant females from 7 days post-ovulation; hence it was possible to diagnose pregnancy on that date (28). An adequate uterine perfusion is essential in mammals for a correct development of gestation (29), as according to Habara et al. (30), uterine blood supply could regulate endometrial receptivity. It has been suggested that

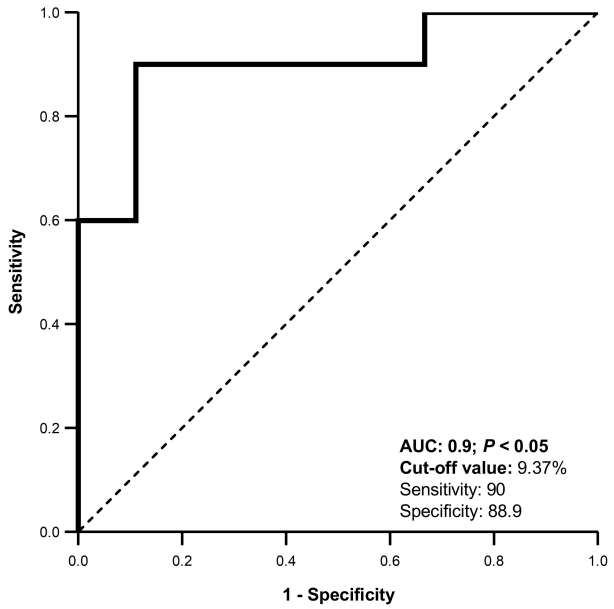


FIGURE 3 | Receiver Operating Characteristic (ROC) curve for uterine blood flow (%). AUC, Area under the curve. The curve was able to detect pregnant llamas when UBF% was >9.37%, with a sensitivity of 90% and a specificity of 88.9%.

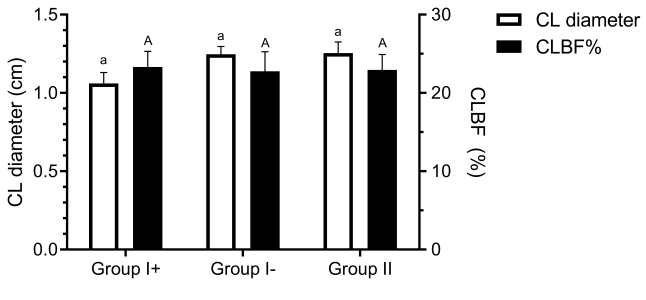


FIGURE 4 | Corpus luteum diameter (cm) and blood flow (CLBF%) on day 8 in the three groups: llamas with an embryo recovered (Group I +), llamas without an embryo recovered (Group I-) and control group (Group II, ovulated, non-mated females). Both variables were expressed as mean ± SEM. ^{a,A}indicate no significant differences between groups for both parameters ($P > 0.05$).

quantifying the increase in uterine blood flow during the luteal phase could be a good predictor for the success of embryo implantation (31). Silva et al. (17) observed that the UBF in mares increased locally at the site of the embryo during the migration phase (days 6 to 16 post-ovulation). They attributed this increase to the possible vasodilator action exerted by vasoactive agents, such as estrogens and prostaglandins, secreted by the equine embryo during that period. This increase in vascularization of the uterine horn ipsilateral to the embryo was also observed by Honnens et al. (32) in pregnant dairy cows from day 11 of gestation. Although the llama with a grade V embryo recovered (arrested morula) was classified in to Group I+ (positive uterine flushing), its UBF was 5.4%, comparable to the mean value registered in mated females without an embryo recovered (Group I-: 6.6%) and llamas that ovulated but not were mated (Group II: 5.19%). This could be due to the metabolic inactivity of the arrested morula and thus, to the lack of secretion and consequent action of its vasoactive agents.

In SAC, ovulations occur with equal frequency from both ovaries, however, most pregnancies are located in the left uterine horn [alpaca: 97.5 and 99.3% with a CL in the right and left ovaries, respectively, (33, 34); llama: 100%, (6)]. For this reason, an embryo resulting from an ovulation in the right ovary needs to migrate or emit some type of signal from the right uterine horn to the left to somehow indicate its presence to both uterine horns and thus prevent luteolysis (11, 20, 35, 36). Although the antiluteolytic signal has not yet been identified, it is believed that the estradiol-17β secreted by the blastocyst could be involved (37). The possible migration and resulting contact with both uterine horns of the embryo proceeding from an ovulation in the right ovary could explain the increase in blood flow registered in both uterine horns in the females of Group I+ with a CL in the right ovary. Powell et al. (13), suggested that llama embryo mobility through the uterus could be mediated by the estradiol-17β it secretes during the early stages of pregnancy, generating a localized increase in myometrial contractility and so allowing its propulsion toward the left uterine horn. Embryo estrogens would exercise this action by binding to the estrogen receptor β, whose expression in the myometrium and perimetrium is increased in the presence of a CL and is greater in pregnant vs. non-pregnant females (35). Whereas, if ovulation occurs in the left ovary, the embryo would not need to migrate because the right uterine horn is incapable of lysing a CL in the left ovary (11, 20, 36). Estradiol has a potent vasodilator effect (18, 38, 39) and it is possible that for this reason, the females in Group I+ with a CL in the left ovary register higher levels of blood flow in the ipsilateral uterine horn. However, increasing the number of animals evaluated could take this difference in UBF% between uterine horns to become statistically significant.

The positive correlation between the follicle diameter on day 0 and the CL diameter on day 8 that we detected was also observed in cows (40, 41), ewes (42), and mares (43–45) and could be because the size of the pre-ovulatory follicle would regulate the size of the CL in its early stages, mainly due to a spatial constraint (46). Nevertheless, the vertical diameter of the CL on day 8 was similar between pregnant females (Group I+) and non-pregnant females (Groups I- and II). According to previous studies in llamas (47, 48), after mating or inducing ovulation, the CL that is formed reaches its maximum diameter 10 days after the stimulus, both in pregnant and non-pregnant females. Only after this date does the CL commence to decrease in size in non-pregnant females while maintaining a plateau in pregnant animals and the difference between both groups becomes evident only after 14 days. Similarly, CLBF% on day 8 showed no significant differences between pregnant and non-pregnant females. Considering that the CL is the most highly vascularized temporary tissue of the body and

receives the greatest rate of blood flow per unit of tissue (49), no apparent differences between pregnant and non-pregnant females in luteal blood flow would be expected during early angiogenesis in CL development (40). Our findings coincide with a recent report by Gallelli et al. (48), who evaluated CL blood flow using CDU in llamas after natural mating and observed that the CLBF was similar in all females up to 8 days post-mating. After that, CLBF remained high in pregnant females, while in non-pregnant animals it decreased dramatically until it disappeared between 14 and 16 days after mating. The difference in CLBF between pregnant and non-pregnant llamas was only evident after day 12 (48). Similar CLBF profiles between non-pregnant females were also observed using Doppler ultrasound in llamas (50, 51), alpacas (52), and dromedaries (53, 54), after inducing ovulation. In these cases, CLBF reached maximum values between 7 and 9 days after induction of ovulation and after that, they began to descend and reached basal levels between days 12 and 14. In all cases, CLBF reduction in non-pregnant females coincided with the process of luteolysis, which occurs over this period in these species. According to different authors, CL function (progesterone production) would be determined by its vascularity more than by its size [llamas: (48); dromedaries: (53); bovines: (55–57)]. In cows, de Tarso et al. (40, 41) evaluated the diameter and blood flow of the CL and found a moderate correlation ($\rho = 0.43$ a 0.6) between both parameters. However, in our study no correlation between the diameter of the CL and its blood flow was observed on day 8, similar to what had been previously reported by Gallelli et al. (48).

According to our study, CDU would predict the outcome of transcervical uterine flushings based on the UBF% 8 days after natural mating in llamas. By constructing a ROC curvewith an AUC of 0.900 we established that, if 9.37% is used as the cut-off value, it is possible to estimate the result of a uterine flushing with a 90% sensitivity and an 88.9% specificity. This would provide valuable information when deciding whether or not to perform a uterine flushing in an embryo transfer program. In addition, females diagnosed as pregnant could be separated early to adopt management and nutrition maneuvers appropriate to this new reproductive status, first adapting their nutritional plan and minimizing stress situations in order to prevent embryo mortality (58) which is high in these species (3). On the other hand, non-pregnant females detected early could be separated to be mated again. However, due to the high percentage of early embryo losses registered in these species, it would be best to combine this early detection method with other methods implemented later on, such as B-mode ultrasonography or transrectal palpation, to confirm pregnancy diagnosis.

CONCLUSION

In conclusion, the results of the present study indicate that evaluation of uterine blood flow by color Doppler ultrasound combined with computer assisted analysis of images are reliable techniques for detection of early pregnancy prior to embryo recovery on day 8 post-mating in llamas.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Committee for the Use and Care of Laboratory Animals (CICUAL).

AUTHOR CONTRIBUTIONS

VT and EZ contributed conception, design of the study, and wrote the manuscript. EZ, VT, and MFG provided help with field work. MG performed the statistical analysis. MC, DN, and MM made critical revisions to the paper. DN contributed to language editing. All authors contributed to the article and approved the submitted version.

FUNDING

This research was supported by grants from the University of Buenos Aires (UBACyT 2014-2017 and UBACyT 2018) and the Agencia Nacional de Promoción Científica y Tecnológica (PICT 2015-2220, PICT 2016-0145, and PICT 2018-2527).

ACKNOWLEDGMENTS

The authors thank M. F. Veiga for her help in the experiments.

REFERENCES

1. Aba MA. *Hormonal Interrelationships in Reproduction of Female Llamas and Alpacas*. (Doctoral Thesis). Sweden: Swedish University of Agriculture. (1998).
2. Calderón W, Novoa C, Franco E. *Exámen de la Preñez en la alpaca*. Lima: Cuarto Boletín Extraordinario, IVITA. (1970). p. 43–48.
3. Fernandez-Baca S, Hansel W, Novoa C. Embryonic mortality in the Alpaca. *Biol Reprod*. (1970) 3:243–51. doi: 10.1093/biolreprod/3.2.243
4. Aba MA, Forsberg M, Kindahl H, Sumar J, Edqvist LE. Endocrine changes after mating in pregnant and non-pregnant llamas and alpacas. *Acta Vet Scand*. (1995) 36:489–98.
5. Adam CL, Moir CE, Shiach P. Plasma progesterone concentrations in pregnant and non-pregnant llamas (*Lama glama*). *Vet Record*. (1989) 125:618–20.
6. Sumar JB. Removal of the ovaries or ablation of the corpus luteum and its effect on the maintenance of gestation in the alpaca and llama. *Acta Vet Scand Supl*. (1988) 83:133–41.
7. Alarcon V, Sumar JB, Riera Foote WC. Comparison of three methods of pregnancy diagnosis in alpacas and llamas. *Theriogenology*. (1990) 34:1119–27. doi: 10.1016/S0093-691X(05)80011-1
8. Aller J, Alberio R, Rebuffi G. Pregnancy and age of gestation diagnosis by means rectal palpation and ultrasonography in llamas (*Lama glama*). *Archiv Zootecnia*. (1998) 47:43–50.

9. Bravo WP, Mayta MM, Ordoñez CA. Growth of the conceptus in alpacas. *Am J Vet Res.* (2000) 61:1508–11. doi: 10.2460/ajvr.2000.61.1508
10. Parraguez VH, Cortéz S, Gazitúa FJ, Ferrando G, MacNiven V, Raggi LA. Early pregnancy diagnosis in alpaca (*Lama pacos*) and llama (*Lama glama*) by ultrasound. *Anim Reprod Sci.* (1997) 47:113–21. doi: 10.1016/S0378-4320(96)01630-2
11. Picha Y, Tibary A, Memon M, Kasimanickam R, Sumar JB. Chronology of early embryonic development and embryo uterine migration in alpacas. *Theriogenology.* (2013) 79:702–8. doi: 10.1016/j.theriogenology.2012.11.027
12. Tibary A, Campbell A, Pearson L. Evolution of embryo transfer in domestic animals. *Spermova.* (2013) 3:1–9.
13. Powell SA, Smith BB, Timm KI, Menino AR. Estradiol production by preimplantation blastocysts and increased serum progesterone following estradiol treatment in llamas. *Anim Reprod Sci.* (2007) 102:66–75. doi: 10.1016/j.anireprosci.2006.10.002
14. Ford SP. Control of uterine and ovarian blood flow throughout the estrous cycle and pregnancy of ewes, sows and cows. *J Anim Sci.* (1982) 55:32–42.
15. Ginther OJ. How ultrasound technologies have expanded and revolutionized research in reproduction in large animals. *Theriogenology.* (2014) 81:112–25. doi: 10.1016/j.theriogenology.2013.09.007
16. Ginther OJ, Gastal EL, Gastal MO, Utt MD, Beg MA. Luteal blood flow and progesterone production in mares. *Anim Reprod Sci.* (2007) 99:213–20. doi: 10.1016/j.anireprosci.2006.05.018
17. Silva LA, Gastal EL, Beg MA, Ginther OJ. Changes in vascular perfusion of the endometrium in association with changes in location of the embryonic vesicle in mares. *Biol Reprod.* (2005) 72:755–61. doi: 10.1095/biolreprod.104.036384
18. Silva M, Urra F, Ratto M. Uterine endometrial vascularization during ovarian follicular growth in llamas: the effect of estradiol plasma concentration. *Theriogenology.* (2018) 106:164–9. doi: 10.1016/j.theriogenology.2017.10.027
19. Urra F, Ratto MH, Silva M. Evaluation of the effect of mating, intrauterine deposition of raw seminal plasma or seminal plasma purified β-NGF on endometrial vascularization in llamas. *Theriogenology.* (2019) 125:18–23. doi: 10.1016/j.theriogenology.2018.10.007
20. Trasorras VL, Chaves MG, Neild DM, Gambarotta M, Aba M, Agüero A. Embryo transfer technique: factors affecting the viability of the corpus luteum in llamas. *Anim Reprod Sci.* (2010) 121:279–85. doi: 10.1016/j.anireprosci.2010.06.004
21. Tibary A, Anouassi A. Theriogenology in Camelidae. Anatomy, physiology, pathology and artificial breeding. In: *Reproductive Physiology in female camelidae*. *Actes Éditions*, Rabat: Institut Agronomique et Vetérinaire Hassan II (Maroc), printed by Abu Dhabi Printing and Publishing Company. (1997).
22. Perkins NJ, Schisterman EF. The inconsistency of "optimal" cutpoints obtained using two criteria based on the receiver operating characteristic curve. *Am J Epidemiol.* (2006) 163:670–5. doi: 10.1093/aje/kwj063
23. Taylor R. Interpretation of the correlation coefficient: a basic review. *J Diagnostic Med Sonogr.* (1990) 6:35–9. doi: 10.1177/875647939000600106
24. Sumar JB. Embryo transfer in domestic South American camelids. *Anim Reprod Sci.* (2013) 136:170–7. doi: 10.1016/j.anireprosci.2012.10.029
25. Vaughan J, Mihm M, Wittek T. Factors influencing embryo transfer success in alpacas-A retrospective study. *Anim Reprod Sci.* (2013) 136:194–204. doi: 10.1016/j.anireprosci.2012.10.010
26. Trasorras VL, Carretero MI, Neild DM, Chaves MG, Giuliano SM, Miragaya MH. Production, preservation, and transfer of South American Camelid embryos. *Front Vet Sci.* (2017) 4:190. doi: 10.3389/fvets.2017.00190
27. Nieto-Olmedo P, Martín-Cano FE, Gaitskell-Phillips G, Ortiz-Rodríguez JM, Peña FJ, Ortega-Ferrusola C. Power Doppler can detect the presence of 7–8 day conceptuses prior to flushing in an equine embryo transfer program. *Theriogenology.* (2020) 145:1–9. doi: 10.1016/j.theriogenology.2020.01.015
28. Lasheen ME, Badr HM, Kandiel MMM, Abo El-Maaty AM, Samir H, Farouk M, et al. Predicting early pregnancy in Egyptian buffalo cows via measuring uterine and luteal blood flows, and serum and saliva progesterone. *Trop Anim Health Prod.* (2018) 50:137–42. doi: 10.1007/s11250-017-1413-6
29. Mansour GM, Hussein SH, Abd El Hady RM, Mohammed HF, Abd El Gawad MM, AbouGabal AI, et al. Uterine artery flow velocity waveform (FVW) type and subendometrial vascularity in recurrent pregnancy loss. *J Maternal Fetal Neonatal Med.* (2020) 33:527–32. doi: 10.1080/14767058.2018.1495190
30. Habara T, Nakatsuka M, Konishi H, Asagiri K, Noguchi S, Kudo T. Elevated blood flow resistance in uterine arteries of women with unexplained recurrent pregnancy loss. *Hum Reprod.* (2002) 17:190–4. doi: 10.1093/humrep/17.1.190
31. Rossal LP, Bellver Pradas J, Escudero E, Gaytán J, Pellicer A. Ecografía-Doopler en el estudio de la Implantación. *Rev Iberoamericana Fertilidad Reprod Humana.* (2002) 19:309–17.
32. Honnens A, Voss C, Herzog K, Niemann H, Rath D, Bollwein H. Uterine blood flow during the first 3 weeks of pregnancy in dairy cows. *Theriogenology.* (2008) 70:1048–56. doi: 10.1016/j.theriogenology.2008.06.022
33. Fernandez-Baca S, Hansel W, Saatman R, Sumar JB, Novoa C. Differential luteolytic effects of right and left uterine horns in the Alpaca. *Biol Reprod.* (1979) 20:586–95. doi: 10.1095/biolreprod20.3.586
34. Fernández-Baca S, Sumar JB, Novoa C, Leyva V. Relación entre la ubicación del cuerpo lúteo y la localización del embrión en la alpaca. *Revista Investigaciones Pecuarias.* (1973) 2:131–5.
35. Powell SA, Smith BB, Timm KI, Menino AR. Expression of estrogen receptors α and β in the corpus luteum and uterus from non-pregnant and pregnant llamas. *Mol Reprod Dev.* (2007) 74:1043–52. doi: 10.1002/mrd.20684
36. Sumar JB, Leyva V. *Relationship between location of the corpus luteum and location of the embryo in the llama (Lama glama)*. In Res. Proyectos Investigación, Univ. Nac. Mayor de San Marcos. Lima, Perú. (1979).
37. Bianchi CP, Meikle A, Benavente MA, Álvarez MA, Trasorras VL, Miragaya MH, et al. Oestrogen and progesterone receptors and COX-2 Expression In Endometrial Biopsy Samples During Maternal Recognition Of Pregnancy In Llamas (*Lama glama*). *Reprod Domestic Anim.* (2015) 50:980–8. doi: 10.1111/rda.12618
38. Mattioli M, Barboni B, Turriani M, Galeati G, Zannoni A, Castellani G, et al. Follicle activation involves vascular endothelial growth factor production and increased blood vessel extension. *Biol Reprod.* (2001) 65:1014–9. doi: 10.1095/biolreprod65.4.1014
39. Moor RM, Hay MF, Seamark RF. The sheep ovary: regulation of steroidogenic, haemodynamic and structural changes in the largest follicle and adjacent tissue before ovulation. *J Reprod Fertil.* (1975) 45:595–604. doi: 10.1530/jrf.0.0450595
40. de Tarso SGS, Apgar GA, Gastal MO, Gastal EL. Relationships between follicle and corpus luteum diameter, blood flow, and progesterone production in beef cows and heifers: preliminary results. *Anim Reprod.* (2016) 13:81–92. doi: 10.21451/1984-3143-AR797
41. de Tarso SGS, Gastal GDA, Bashir ST, Gastal MO, Apgar GA, Gastal EL. Follicle vascularity coordinates corpus luteum blood flow and progesterone production. *Reprod Fertil Dev.* (2017) 29:448–57. doi: 10.1071/RD15223
42. Murdoch WJ, Kirk EA van. Luteal dysfunction in ewes induced to ovulate early in the follicular phase. *Endocrinology.* (1998) 139:3480–4. doi: 10.1210/endo.139.8.6137
43. Ginther OJ. *Reproductive biology of the mare: basic and applied aspects, 2nd edn.* (1992). Available online at: https://agris.fao.org/agris-search/search.do?recordID=US19930099223 (accessed August 20, 2020).
44. Ishak GM, Bashir ST, Gastal MO, Gastal EL. Pre-ovulatory follicle affects corpus luteum diameter, blood flow, and progesterone production in mares. *Anim Reprod Sci.* (2017) 187:1–12. doi: 10.1016/j.anireprosci.2017.09.003
45. Silva LA, Gastal EL, Gastal MO, Beg MA, Ginther OJ. Relationship between vascularity of the preovulatory follicle and establishment of pregnancy in mares. *Anim Reprod.* (2006) 3:339−46. Available online at: https://www.animal-reproduction.org/journal/animreprod/article/5b5a6080f7783717068b47cd (accessed August 20, 2020).
46. Mann GE. Corpus luteum size and plasma progesterone concentration in cows. *Anim Reprod Sci.* (2009) 115:296–9. doi: 10.1016/j.anireprosci.2008.11.006
47. Adams GP, Sumar JB, Ginther OJ. Form and function of the corpus luteum in llamas. *Anim Reprod Sci.* (1991) 24:127–38. doi: 10.1016/0378-4320(91)90088-H
48. Gallelli MF, Bianchi C, Zampini E, Trasorras V, Gambarotta M, Miragaya M. Corpus luteum vascularization during the maternal recognition of pregnancy in llamas (*Lama glama*). *Reprod Domestic Anim.* (2020) 55:13588. doi: 10.1111/rda.13588
49. Wiltbank MC, Dysko RC, Gallagher KP, Keyes PL. Relationship between blood flow and steroidogenesis in the rabbit corpus luteum. *J Reprod Fertil.* (1988) 84:513–20. doi: 10.1530/jrf.0.0840513

50. Fernández A, Ulloa-Leal C, Silva M, Norambuena C, Adams GP, Guerra M, et al. The effect of repeated administrations of llama ovulation-inducing factor (OIF/NGF) during the peri-ovulatory period on corpus luteum development and function in llamas. *Anim Reprod Sci.* (2014) 149:345–52. doi: 10.1016/j.anireprosci.2014.08.001
51. Ulloa-Leal C, Bogle OA, Adams GP, Ratto MH. Luteotrophic effect of ovulation-inducing factor/nerve growth factor present in the seminal plasma of llamas. *Theriogenology.* (2014) 81:38. doi: 10.1016/j.theriogenology.2014.01.038
52. Norambuena MC, Hernández F, Maureira J, Rubilar C, Alfaro J, Silva G, et al. Effects of leptin administration on development, vascularization and function of *Corpus luteum* in alpacas submitted to pre-ovulatory fasting. *Anim Reprod Sci.* (2017) 182:28–34. doi: 10.1016/j.anireprosci.2017.04.006
53. Rawy MS, Derar DR, El-Sherry TM, Megahed GA. Characterisation of follicular and luteal blood flow in female dromedary camel induced to ovulate using GnRH analogue. *J Camel Pract Res.*(2012) 19:269–75.
54. Rawy MS, Derar RI, El-Sherry TM, Megahed GA. Plasma steroid hormone concentrations and blood flow of the ovarian structures of the female dromedary (*Camelus dromedarius*) during growth, dominance, spontaneous ovulation, luteinization and regression of the follicular wave. *Anim Reprod Sci.* (2014) 148:137–44. doi: 10.1016/j.anireprosci.2014.05.004
55. Baumgartner U. *Farbdopplersonographische untersuchung der Arteria uterina und des Corpus luteum beim Rind*. Munchen: Tierarztliche Fakultat Der Ludwig-Maximilians-Universitat Munchen (1998) 67–89.
56. Herzog K, Brockhan-Lüdemann M, Kaske M, Beindorff N, Paul V, Niemann H, et al. Luteal blood flow is a more appropriate indicator for luteal function during the bovine estrous cycle than luteal size. *Theriogenology.* (2010) 73:691–7. doi: 10.1016/j.theriogenology.2009.11.016
57. Herzog K, Voss C, Kastelic JP, Beindorff N, Paul V, Niemann H, et al. Luteal blood flow increases during the first 3 weeks of pregnancy in lactating dairy cows. *Theriogenology.* (2011) 75:549–54. doi: 10.1016/j.theriogenology.2010.09.024
58. Ferrer MS. Diagnosis of pregnancy and evaluation of high-risk pregnancy. In: Cebra C, Anderson DE, Tibary A, Van Saun RJ, Johnson V, and LaRue W, editors. *Llama and Alpaca Care. Medicine, Surgery, Reproduction, Nutrition, and Herd Health*. Linda Duncan (2014). p. 250–6. doi: 10.1016/B978-1-4377-2352-6.00022-5

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 30 November 2020
doi: 10.3389/fvets.2020.597954

Comparison of Extenders With the Addition of Egg Yolk for Cooling Alpaca Sperm Obtained From Deferent Ducts

Mariana Lucía Bertuzzi[1,2]*, *Edita Yola Torres*[3], *Teodosio Huanca*[4†], *Deborah Neild*[1] and *María Ignacia Carretero*[1,2]

[1] Facultad de Ciencias Veterinarias, INITRA, Cátedra de Teriogenología, Universidad de Buenos Aires, Ciudad Autónoma de Buenos Aires, Buenos Aires, Argentina, [2] Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Ciudad Autónoma de Buenos Aires, Buenos Aires, Argentina, [3] Laboratorio de Reproducción Animal, Facultad de Medicina Veterinaria y Zootecnia, Universidad Nacional del Altiplano Puno, Puno, Peru, [4] Instituto Nacional de Innovación Agraria (INIA), Centro de Investigación y Producción Quinsachata, Puno, Peru

OPEN ACCESS

Edited by:
Marcelo H. Ratto, Austral University of Chile, Chile

Reviewed by:
Abouzar Najafi, University of Tehran, Iran
Joan Enric Rodríguez-Gil, Autonomous University of Barcelona, Spain

***Correspondence:**
Mariana Lucía Bertuzzi
marianabertuzzi27@gmail.com

[†]*Deceased*

Specialty section:
This article was submitted to Animal Reproduction - Theriogenology, a section of the journal Frontiers in Veterinary Science

Received: *22 August 2020*
Accepted: *02 November 2020*
Published: *30 November 2020*

Citation:
Bertuzzi ML, Torres EY, Huanca T, Neild D and Carretero MI (2020) Comparison of Extenders With the Addition of Egg Yolk for Cooling Alpaca Sperm Obtained From Deferent Ducts. Front. Vet. Sci. 7:597954. doi: 10.3389/fvets.2020.597954

The use of non-commercial and commercial extenders for cooling alpaca sperm has already been reported, the latter showing certain advantages over the first. The Andromed® (AM) extender was created for use in ruminants and has also been tested in ejaculated and epididymal alpaca sperm. According to the manufacturer, this extender does not need the addition of egg yolk (EY); however, it is known that the addition of EY to some extenders improves the preservation of cooled sperm. The objective of this study therefore was to compare a non-commercial extender (Tris) with the addition of EY vs. the commercial extender AM with and without the addition of EY, for cooling alpaca sperm obtained from diverted deferent ducts. Fifteen pools of deferent duct sperm were formed using samples from two or three different males for each. Each sperm pool was evaluated and then divided into three aliquots that were diluted to a final concentration of 30×10^6 sperm ml-1 (0 h) with either: (1) Tris with 20% EY (T-EY), (2) AM, or (3) AM with 20% EY (AM-EY). Samples were cooled to 5°C and the following sperm parameters were evaluated after 24 and 48 h of storage: motility, viability, membrane function, acrosome integrity, morphology, and chromatin condensation. Motility was also evaluated after 72 h of storage. The samples that best preserved progressive and total sperm motility at the 24 and 48 h evaluation periods were the ones diluted with AM-EY, observing that with this extender these motility patterns decreased significantly after 72 h of storage compared to time 0 h ($p < 0.05$). A significant decrease ($p < 0.05$) in total and progressive motility was observed at 48 h for the T-EY and AM extender compared to 0 h. AM was the only extender in which the percentages of viable sperm decreased significantly ($p < 0.05$) after 48 h of conservation. For the rest of sperm parameters evaluated, no significant differences were observed between any of the extenders at any evaluation time. The Andromed® extender with the addition of 20% EY could be an alternative option for cooling alpaca sperm obtained from deferent ducts.

Keywords: extenders, cooling, egg yolk, sperm, alpaca, deferent ducts

INTRODUCTION

One of the most important reproductive biotechnologies for production is artificial insemination (AI) which, when compared to natural mating, maximizes the use of genetically superior males and achieves a rapid genetic progress. However, in South American Camelids (SACs) this biotechnology is currently limited to use with diluted raw semen, with a maximum pregnancy rate of 77% in experimental centers and no higher than 50% in private establishments (1, 2). This methodology using raw semen presents some disadvantages such as inadequate laboratory environments for conditioning the samples and the need to transport the males to the site of insemination due to the very large distances between farms. As an alternative to this, cooling semen for subsequent AI presents many advantages, such as prevention and control of venereal diseases and not requiring the presence of the breeding males on the premises, thus eliminating the costs of upkeep and providing greater security for the animals because transport is avoided. However, pregnancy rates obtained when using cooled SAC semen preserved for 24 h have been lower than those using diluted raw semen [0–33%: (2–4)]. Murillo et al. (5) obtained slightly higher pregnancy rates (40–46.7%) when using alpaca semen obtained by post-mating aspiration from the female's vagina and cooled with Andromed® and Triladyl®. However, they only preserved the samples for 6 h.

It has been postulated that the lower pregnancy rates obtained when inseminating with SAC cryopreserved semen could be linked to the rheological characteristics, such as thread formation and the high structural viscosity, of semen from these species (6–9) because these characteristics prevent an efficient homogenization of the ejaculate with the diluents (9–11). Hence, collecting sperm from the deviated deferent ducts is an interesting technique to apply in SACs as the samples thus obtained do not show thread formation, permitting a better homogenization with the extenders and as a result could have a beneficial effect on the cryopreservation of the samples.

The use of commercial extenders to cryopreserve sperm samples presents certain advantages over non-commercial ones: they require less equipment and supplies, are easy to prepare and are less variable in composition, which allows more repeatable results to be obtained. In alpacas, cooling sperm has been carried out using both commercial and non-commercial extenders (3–5, 12–14). The non-commercial extender most used for cooling alpaca sperm is Tris with the addition of different types of egg yolk (4) or membrane protective agents (13). Within commercial extenders, Triladyl® (3, 5), Biladyl® and Androhep® (12) have been assayed for cooling alpaca semen. Furthermore, the Andromed® (AM) extender, although created to be used in ruminants, has also been tested in alpaca sperm, both from ejaculates and from epididymis (5, 14). According to the manufacturers, one of the advantages of the AM extender is that it does not require the addition of EY thus avoiding the use of a product of animal origin. However, the addition of egg yolk to extenders is known to exert a protective effect on sperm, especially those of SACs, and this effect is attributed to its ability to interact with the plasma membrane lipid bilayer (15, 16) preventing the phase transition events of membrane lipids (16, 17). To our knowledge, no commercial extenders have been used to maintain cooled sperm obtained from deviated deferent ducts, neither have the effects of the addition of EY to commercial extenders been evaluated in these species.

In this context, the objective of this study was to compare a non-commercial extender (Tris) with the addition of EY vs. the commercial extender AM with and without the addition of EY, in cooled alpaca sperm obtained from diverted deferent ducts.

Abbreviations: SAC, South American Camelids; EY, Egg yolk; AM, Andromed®; AM-EY, Andromed® extender with 20% of egg yolk; T-EY, Tris extender with 20% of egg yolk; TM, Total motility; PM, Progressive motility; CM, circular motility.

MATERIALS AND METHODS

Reagents

Hoescht 33342, Propidium iodide, and the reagents for the PBS medium and for the HOS test were purchased from Sigma Chemicals (Sigma Aldrich, Buenos Aires, Argentina). Coomassie blue was purchased from Bio-Rad, California, USA.

Experimental Design

Fifteen pools of deferent duct sperm were formed using samples from two or three different males each. After collection, sperm motility, concentration, membrane integrity (viability), membrane function and acrosome integrity, morphology and chromatin condensation were evaluated in each pool and the samples were then divided into 3 aliquots. With the objective of standardizing sperm concentration, each aliquot was diluted to 30×10^6 sperm/ml with the following extenders, previously warmed to 37°C: (1) Tris with 20% EY (T-EY), (2) Andromed® (AM), and (3) AM with 20% EY (AM-EY). Once diluted, motility and viability were again evaluated, and this was considered time 0 h. After this the samples were cooled to 5°C in a controlled cooling device with a digital thermometer, that brought temperature down from 37 to 5°C, over a lapse of 7 h, and then maintained temperature at 5°C for a period of 72 h. At 24 and 48 h of conservation, an aliquot of each sample was warmed to 37°C for 15 min and the sperm parameters of motility, viability, membrane function and acrosome integrity, morphology and chromatin condensation were evaluated. Finally, at 72 h, motility was again evaluated.

Animals and Location

The study was carried out during the month of October, in the Quimsachata experimental station, owned by the National Institute of Agricultural Innovation (Instituto Nacional de Innovación Agraria; INIA), which is located in the Santa Lucia district in the Lampa province of the Puno region, Peru. This is an agroecological area of the Dry Puna and is situated 4200 meters above sea level (15°41′39″ latitude and 70°36′24″ longitude). Five adult Huacaya-breed alpaca (*Vicugna pacos*) males, between 4 and 10 years of age, were used. Their deferent ducts were surgically deviated using the technique described by Pérez and Apaza (18). Briefly, the males were sedated with acepromazine and were held in dorsal recumbency. The surgical field in the inguinal region was prepared, local anesthesia administered and

an incision 4 cm from the base of the penis was made to locate the deferent duct. After dissecting 7 cm of deferent duct, the freed extremity was redirected below the subcutaneous, fixed to the inner face of the femoral region and protected by a temporary patch. The animals were kept in pens, consisting of covered grazing areas with natural grasses, and had free access to water throughout the study.

Obtaining Sperm From the Deferent Ducts

Sperm samples were obtained following a modification of the method described by Pérez et al. (19). Briefly, the male was held in lateral recumbency and the area surrounding the fistula was disinfected. A soft massage using fingertips was applied with a caudo-craneal direction, from the tail of the epididymis, helping move the sperm toward the exit of the fistula and once a white droplet (spermatic portion) was observed, it was gently aspirated with a 10 μl micropipette using tips that had already been soaked in Tris extender. The sperm sample was then deposited in a tube with Tris previously warmed to 37°C and kept in a water bath at the same temperature. Once the sperm extraction was completed, the surrounding area was once again gently disinfected, dried and covered with solid Vaseline. The collection maneuver was repeated in two or three other males and a pool was made with the different samples. Each deferent duct from every male was collected every 2 days to avoid sperm accumulation and resulting cell death.

Evaluation of Sperm Parameters

The following sperm parameters were evaluated: motility, concentration, membrane integrity (viability), membrane function and acrosome integrity, morphology and chromatin condensation.

Sperm motility was evaluated using a phase contrast microscope (100×) and a warm stage (37°C). The patterns observed were: oscillatory motility (OM), progressive motility (PM) and circular motility (CM). In addition, total sperm motility was determined (TM = OM + PM + CM). Spermatozoa with OM are those that move in their place, without advancing whereas sperm with PM move in a straight line or describing large circles. Lastly, sperm with CM are those that move in very tight circles.

Sperm concentration was calculated using a Neubauer hemocytometer.

To evaluate membrane integrity, 12.5 μl of the sample were incubated at 37°C for 10 min in 127 μl of the staining medium, which contained 2 μl of a Hoescht 33342 solution (10 μg/ml in PBS) and 125 μl of a saline medium [described by Harrison and Vickers (20)]. After the first 10 min of incubation, 2 μl of a solution of Propidium iodide (0.5 mg/ml in isotonic saline solution) was added and the sample was incubated a further 10 min at 37°C. A minimum of 200 sperm were evaluated per sample, using an epifluorescence microscope with UV filter (400X) (Zeiss AxioScope). Sperm that fluoresced blue were classified as viable with intact membranes, and those that had a pink fluorescent nucleus were classified as non-viable, with damaged membranes.

To evaluate membrane function and acrosome integrity, the dual technique of combining the HOS test with Coomassie blue (CB) stain was carried out according to Carretero et al. (21). Briefly, first the HOS test was carried out on the sperm samples and after the 20 min of incubation, samples were fixed with 4% paraformaldehyde in PBS, incubated and centrifuged at 800 g and room temperature for 10 min. The pellet was re-suspended in 100 μl of PBS, microdroplets of the suspension were placed on slides and left to dry after which they were stained with 0.22% CB and observed using light microscopy (1000x). A total of 200 sperm were evaluated and classified into one of the following categories: (1) sperm with a functional membrane (HOS+: with tail swelling) and with an acrosome (CB+: violet acrosome staining), (2) sperm with a functional membrane (HOS+: with tail swelling) and without an acrosome (CB−: no acrosome staining), (3) sperm with a non-functional membrane (HOS−: no swelling) and with an acrosome (CB+: violet acrosome staining), and (4) sperm with a non-functional membrane (HOS−: no swelling) and no acrosome (CB−: no acrosome staining).

Sperm morphology was evaluated using phase contrast microscopy (1000x), after placing a 10 μl drop of sample on a glass slide with a coverslip. For each sample, 200 spermatozoa were evaluated, and were classified into one of the following categories: normal, abnormal head, detached head, abnormal midpiece, abnormal tail and cytoplasmic droplet. Thus, percentages of spermatozoa with normal or altered morphology were determined.

The degree of chromatin condensation was evaluated with the Toluidine Blue (TB) stain according to Carretero et al. (22). Briefly, samples were smeared onto clean slides, fixed with ethanol 96° for 2 min and stained during 5 min with 0.02% TB. Preparations were observed directly under immersion oil using a phase contrast microscope (1000x), evaluating a minimum of 200 spermatozoa per smear. Sperm were classified into three patterns: light blue (TB negative), light violet (TB intermediate) and dark blue-violet (TB positive). TB negative sperm were considered to have normal, highly condensed chromatin and TB intermediate + TB positive sperm were considered to have decondensed chromatin (between moderate and high decondensation).

Statistical Analysis

Statistical analysis was carried out using InfoStat software (Student Version) (https://www.infostat.com.ar/index.php?mod=page&id=15). In all cases normal distribution and homogeneity of variances of the data were corroborated using Shapiro-Wilk's Normality test and an ANOVA, respectively. The level of significance was set at 0.05 for all analysis.

Because sperm motility and chromatin condensation did not show a normal distribution or homogeneity of variances, these data were compared using a Kruskal–Wallis test.

To compare viability of collected sperm before dilution to the samples diluted at time 0 h, an ANOVA was applied using a factorial design with one factor with 4 levels (collected sperm before dilution, T-EY 0 h, AM 0 h and AM-EY 0 h).

To compare viability between diluted cooled samples, an ANOVA was applied using a factorial design with two factors:

extender (with 3 levels: T-EY, AM and AM-EY) and time (with 3 levels: 0, 24 and 48 h).

For analyzing the parameters of sperm morphology and of the HOS/CB test (sperm membrane function and acrosome), an ANOVA was applied using a factorial design with one factor with 7 levels (collected sperm prior to dilution, T-EY 24 h, AM 24 h, AM-EY 24 h, T-EY 48 h, AM 48 h and AM-EY 48 h).

RESULTS

Oscillatory, progressive, circular and total motility in the samples diluted at 0 h were not different to that of sperm collected from the deviated deferent ducts prior to dilution ($p > 0.05$). The samples that best preserved progressive and total sperm motility at 24 and 48 h evaluation times were the ones diluted with AM-EY ($p > 0.05$), observing that with this extender these motility patterns decreased significantly only after 72 h of storage compared to time 0 h ($p < 0.05$). Whereas a significant decrease ($p < 0.05$) in total and progressive motility was observed at 48 h for the T-EY and AM extender compared to time 0 h. It is interesting to highlight that in the samples cooled in AM, mainly oscillatory motility was observed at 48 and 72 h of conservation whereas, in the samples cooled in T-EY and AM-EY the motility pattern observed was mostly progressive, similar to the collected sample (54.5 ± 16.1%) (**Table 1**).

Percentages of viable sperm in the samples diluted at 0 h were not different to that of sperm collected from the deviated deferent ducts prior to dilution ($p > 0.05$). AM was the only extender in which the percentages of viable sperm decreased significantly ($p < 0.05$) after 48 h of conservation compared to 0 h (**Figure 1**). However, no significant differences were observed in viability between the extenders at any of the different evaluation times (**Figure 1**).

TABLE 1 | Oscillatory motility (OM), progressive motility (PM), circular motility (CM), and total motility (TM) in alpaca spermatozoa collected from deviated deferent ducts and diluted (0 h) in Tris with 20% egg yolk (Tris-EY), Andromed® (AM) and AM with 20% egg yolk (AM-EY) evaluated at 24, 48 and 72 h of storage at 5°C.

	OM (%)	PM (%)	CM (%)	TM (%)
Tris-EY 0 h	1.1 ± 2.7^{a}	52.0 ± 11.3^{a}	0.2 ± 0.7^{a}	53.3 ± 10.2^{a}
AM 0 h	1.8 ± 3.0^{a}	47.5 ± 13.5^{a}	1.4 ± 2.1^{ab}	50.7 ± 10.4^{a}
AM-EY 0 h	0.3 ± 0.8^{a}	53.4 ± 9.9^{a}	0.3 ± 0.7^{ab}	54.0 ± 9.1^{a}
Tris-EY 24 h	2.1 ± 2.8^{a}	41.8 ± 8.8^{a}	0.8 ± 1.5^{ab}	44.7 ± 6.6^{ab}
AM 24 h	17.7 ± 12.8^{b}	19.8 ± 17.2^{b}	2.1 ± 3.1^{ab}	39.6 ± 8.5^{ab}
AM-EY 24 h	1.0 ± 1.8^{a}	46.2 ± 8.7^{a}	0.8 ± 1.4^{ab}	48.0 ± 7.7^{a}
Tris-EY 48 h	3.7 ± 4.1^{a}	29.4 ± 12.5^{b}	4.4 ± 3.1^{bc}	37.5 ± 10.8^{bc}
AM 48 h	17.4 ± 10.8^{b}	9.1 ± 11.6^{b}	2.1 ± 3.2^{ab}	28.8 ± 8.5^{bc}
AM-EY 48 h	2.0 ± 3.7^{a}	40.9 ± 11.6^{ab}	3.9 ± 3.0^{ab}	46.8 ± 9.1^{ab}
Tris-EY 72 h	2.7 ± 4.1^{a}	21.7 ± 10.1^{b}	8.6 ± 6.8^{c}	33.0 ± 9.7^{c}
AM 72 h	20.0 ± 11.3^{b}	0.7 ± 1.6^{c}	1.0 ± 2.1^{ab}	21.7 ± 11.8^{c}
AM-EY 72 h	2.1 ± 2.3^{a}	35.7 ± 8.5^{b}	3.2 ± 3.3^{ab}	41.0 ± 7.7^{b}

Values are expressed as mean ± SD (n = 15). ^{a,b,c}Different letters between rows within each column, indicate significant differences (p < 0.05).

In addition, no significant differences ($p > 0.05$) were observed in the percentages of sperm with functional membranes and presence of acrosomes (HOS+/CB+) between collected sperm before dilution and cooled samples at 24 and 48 h, with the exception of cooled samples diluted with T-EY which presented a significant decrease of HOS+/CB+ sperm at 48 h compared to collected sperm before dilution (**Figure 2**).

No significant differences were observed over time in the percentage of sperm with normal morphology (**Table 2**) and condensed chromatin (**Figure 3**) nor between any of the extenders at any of the evaluation times ($p > 0.05$).

DISCUSSION

Given the current importance of the use of commercial extenders for cryopreserving the male gamete, the objective of this study was to compare a non-commercial extender to a commercial one for cooling alpaca sperm obtained from deviated deferent ducts. In this context, we observed that the AM extender with the addition of EY was the one that presented the highest percentage of sperm with total and progressive motility after 48 h and even after 72 h of conservation. The total motility observed (47% after 48 h) was higher than that reported by other authors that assayed commercial extenders for cooling alpaca sperm. For example, Morton et al. (12) obtained 36% and 16% TM at 24 and 48 h, respectively, in alpaca samples cooled in Biladyl, an extender that has EY in its preparation. Others, such as Vaughan et al. (3) and Murillo et al. (5) obtained similar results to the present study, with extenders such as Triladyl and even AM. However, in the case of Murillo et al. (5) cooling was only for 6 h and in the case of Vaughan et al. (3), the initial TM at 0 h was not mentioned and could have been superior to the one we observed in our study, thus affecting the total decrease in motility observed in their samples. In our study TM decreased a 13% after 48 h of conservation compared to the initial sample (54% TM at 0 h). The main difference between these three studies with the current one is the method of obtaining the sperm, as the other studies used either an artificial vagina or vaginal aspiration. In these methods, an ejaculate is obtained, complete with the secretions of the accessory glands that generate seminal plasma. Seminal plasma contributes the very distinctive rheological characteristics of the ejaculates of these species: thread formation and high structural viscosity, which make dilution of the samples very difficult (8, 9). In our study, because we obtained sperm from the deferent ducts, the sperm had not yet come into contact with seminal plasma, and so the samples do not show the characteristic thread formation of SAC ejaculates and therefore can be homogenized better with the extenders. In addition, sperm were probably able to interact to a greater degree with the components of the extenders, for example the egg yolk, facilitating the adherence or inclusion of molecules that protect the membranes at low temperatures during cooling. Moreover, Chahuayo and Paytán (14) carried out a similar study to ours, comparing T-EY and AM extenders to dilute epididymal spermatozoa. Thus, they also worked with sperm that had not had contact with seminal plasma. However, their motility results were lower than ours when cooling for 48 h at 4°C (TM: 21% and 3% for T-EY and AM, respectively) having started out with an 80% motility at 0 h.

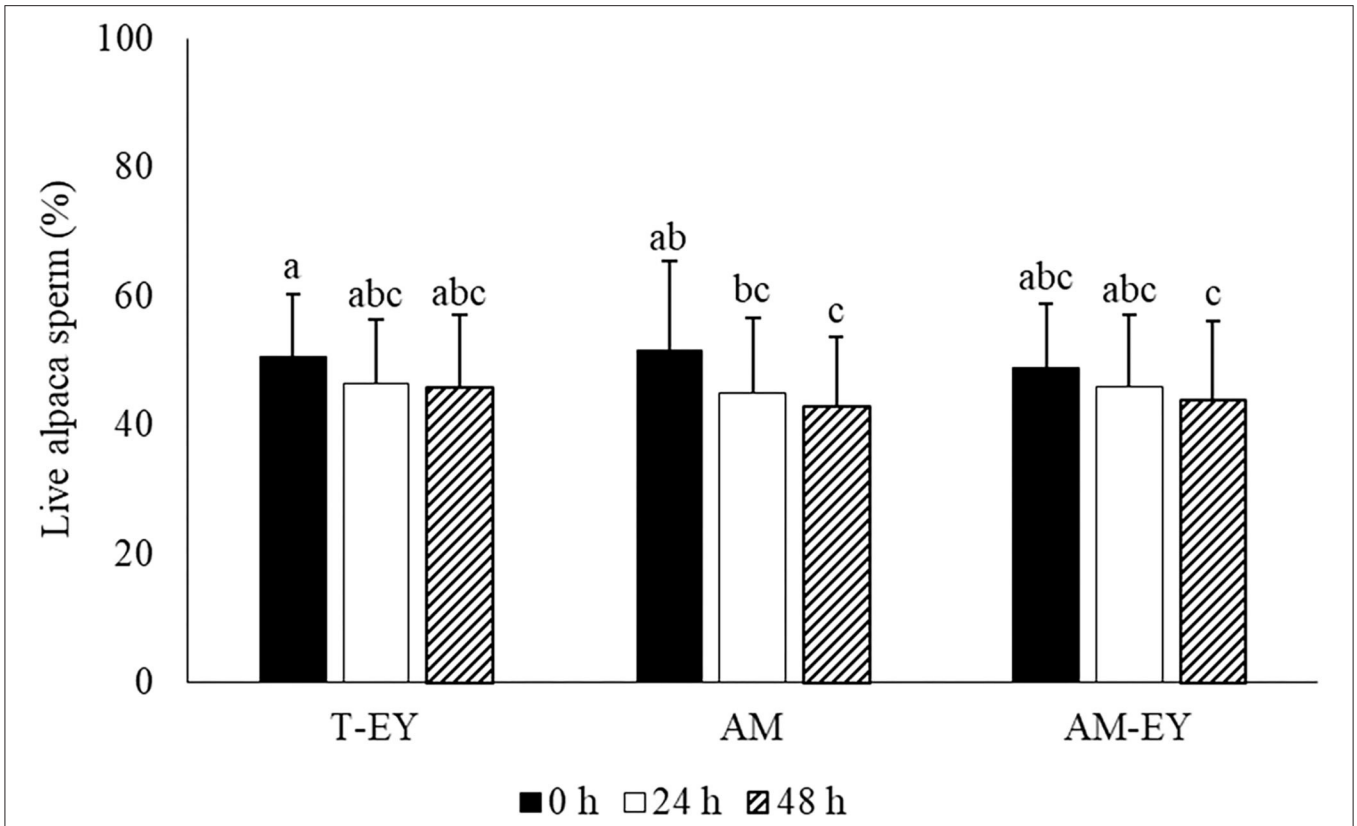


FIGURE 1 | Percentages of live sperm in alpaca samples collected from deviated deferent ducts and diluted (0 h) in Tris with 20% egg yolk (T-EY), Andromed® (AM) and AM with 20% egg yolk (AM-EY) evaluated at 24 and 48 h of storage at 5°C ($n = 15$). [a,b,c]Different letters indicate significant differences between samples and evaluation times ($p < 0.05$).

These authors do not mention the proportion of EY they added to the Tris extender and perhaps the differences in results could be attributed to a different percentage of EY or simply to a different response between individuals to cooling and/or interaction of sperm with the extender used. However, despite the different results obtained in these two studies, both the present one and that of Chahuayo show that the addition of EY is important for preserving motility in alpaca sperm.

In addition to enabling a greater contact of sperm with the extenders, due to the absence of seminal plasma, the deviated deferent ducts technique allows one to obtain sperm with progressive motility. It is known that SAC ejaculated sperm present very low or no progressive motility, with oscillatory motility being the main pattern observed (7, 23, 24). It has also been reported that the absence or presence of seminal plasma influences the patterns of llama sperm motility (25, 26). It was noteworthy in the current study that despite starting off with a sample that had PM, the aliquots that were diluted in AM adopted an oscillatory pattern of movement, mainly after 48 and 72 h of cooling, with this pattern of motility constituting the highest proportion of the TM observed at both these evaluation times. Although the reason for this change in motility pattern is unknown, it could be related to small particles of the extender adhering to the sperm membrane and limiting their movements. This change was not observed in the samples cooled with AM-EY, despite the basic extender being the same, so possibly this difference could be attributed to the presence of EY in the medium, as it has been hypothesized that egg yolk would induce llama sperm, obtained by electroejaculation, to attain progressive motility (11, 27). Likewise, Miceli et al. (28) observed that specific surface proteins on human spermatozoa were phosphorylated to a greater extent in sperm incubated with TEST buffer with egg yolk (TYB) than those incubated in other media without EY. Results of this study also indicated that TYB proteins could also be phosphorylated by the spermatozoa during the incubation and attach to the sperm membranes. Taking into account that initiation and maintenance of progressive sperm motility seem to be regulated by protein phosphorylation (29, 30), it would be necessary to conduct studies in SAC sperm to evaluate if the mechanism of egg yolk inducing PM is through the phosphorylation of proteins.

Regarding viability, we observed between 43 and 45% live sperm in all extenders at 48 h of conservation at 5°C. These percentages signify only a 10 to 15% decrease in viability compared to time 0 h and a 20 to 24% when compared to the collected samples. This is relevant because when planning an AI protocol, the percentage of live spermatozoa is essential. Most of the studies that compare different extenders in alpaca

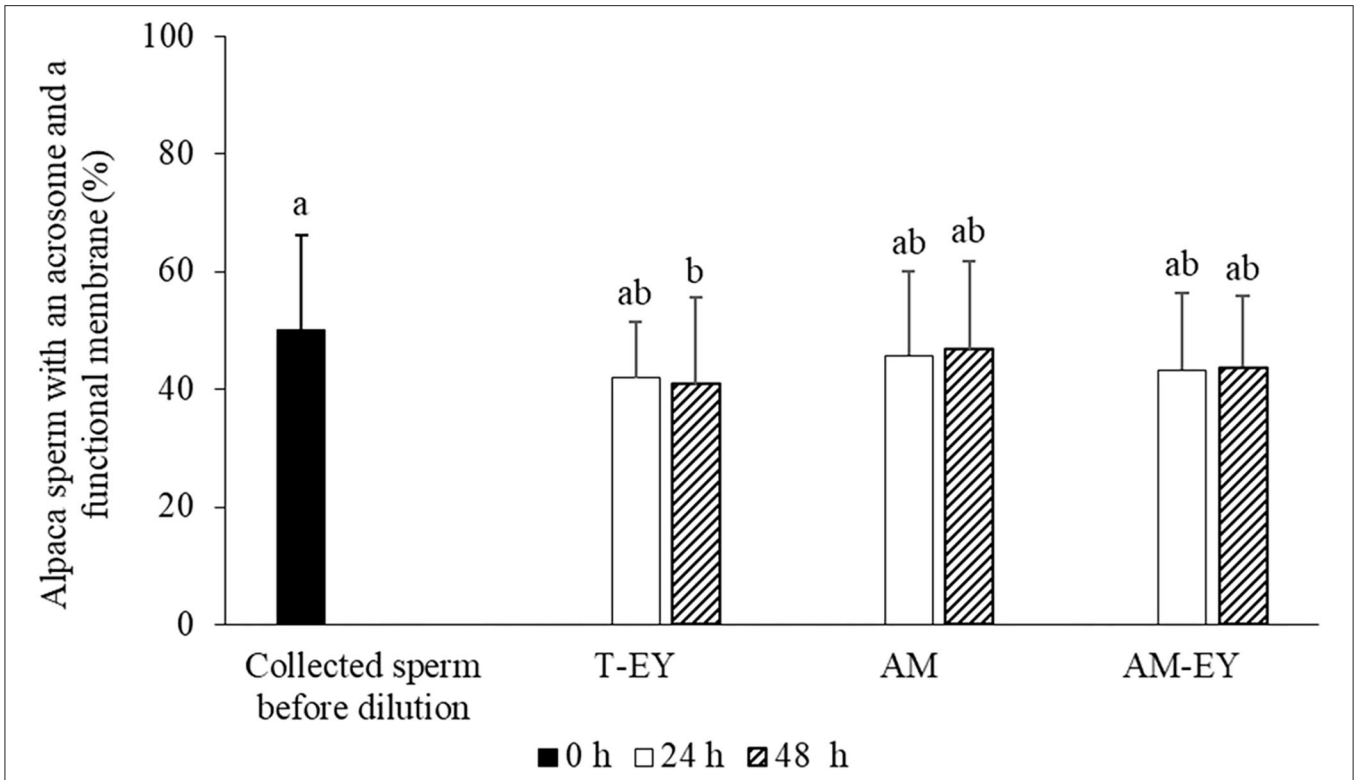


FIGURE 2 | Percentages of sperm with an acrosome and a functional membrane in alpaca samples collected from deviated deferent ducts and cooled to 5°C in Tris with 20% egg yolk (T-EY), Andromed® (AM) and AM with 20% egg yolk (AM-EY), evaluated at 24 and 48 h of storage ($n = 15$). ^{a,b}Different letters indicate significant differences between samples and evaluation times ($p < 0.05$).

TABLE 2 | Sperm morphology in samples collected from alpaca deviated deferent ducts and in samples cooled to 5°C in Tris with 20% egg yolk (T-EY), Andromed® (AM) and AM with 20% egg yolk (AM-EY) evaluated after 24 and 48 h of storage.

	Normal sperm (%)	Abnormal heads (%)	Detached heads (%)	Abnormal tails (%)	Abnormal midpieces (%)	Citoplasmic droplets (%)
Collected sperm before dilution	29.3 ± 9.5^{a}	14.4 ± 4.1^{a}	11.1 ± 5.7^{a}	16.7 ± 7.4^{a}	14.4 ± 3.2^{a}	14.1 ± 3.7^{a}
Tris-EY 24 h	30.4 ± 11.6^{a}	16.0 ± 3.0^{a}	9.5 ± 7.0^{a}	17.8 ± 8.9^{a}	14.3 ± 3.4^{a}	12.0 ± 3.7^{ab}
AM 24 h	29.7 ± 10.3^{a}	16.5 ± 5.2^{a}	10.7 ± 8.1^{a}	16.2 ± 5.3^{a}	13.2 ± 2.0^{a}	13.7 ± 4.3^{a}
AM-EY 24 h	29.4 ± 9.0^{a}	17.5 ± 4.5^{a}	11.4 ± 8.8^{a}	16.6 ± 7.7^{a}	13.8 ± 3.5^{a}	11.3 ± 4.2^{ab}
Tris-EY 48 h	29.5 ± 9.0^{a}	17.9 ± 5.3^{a}	12.8 ± 11.3^{a}	17.2 ± 5.5^{a}	12.8 ± 3.8^{a}	9.8 ± 3.7^{b}
AM 48 h	29.1 ± 7.4^{a}	15.6 ± 5.5^{a}	11.6 ± 11.2^{a}	16.8 ± 6.3^{a}	12.9 ± 4.7^{a}	14.0 ± 7.4^{a}
AM-EY 48 h	29.7 ± 8.4^{a}	16.8 ± 6.2^{a}	11.9 ± 11.1^{a}	15.4 ± 6.3^{a}	15.2 ± 4.8^{a}	11.0 ± 4.1^{ab}

Values are expressed as mean ± SD (n =15). ^{a,b}Different letters between rows indicate significant differences for each variable analyzed (p <0.05).

semen focus on evaluating sperm motility, rather than viability. Chahuayo and Paytán (14) obtained lower viability results than those observed in the present study: 4 and 20% live sperm after 48 h of cooling in AM and T-EY, respectively, signifying in this case, a 95 and 77% decrease, respectively compared to their time 0 h. As far as other species of SACs, such as llamas, Giuliano et al. (2) published similar results to the current study but with a non-commercial extender composed of lactose and 20% egg yolk, observing 48% live sperm after 24 h of cooling to 5°C. Carretero et al. (31) reported slightly higher values (55–60% viability) when cooling llama sperm for 24 h with and without seminal plasma in a lactose based extender with EY. Although no reports of cooling llama sperm using commercial extenders have been published, it would be interesting to do so to be able to compare between phylogenetically close species and perhaps even make some extrapolations.

Regarding acrosome integrity, results in this study were similar to those reported for alpaca sperm obtained by artificial vagina and diluted in commercial and non-commercial extenders, where no significant differences were observed in

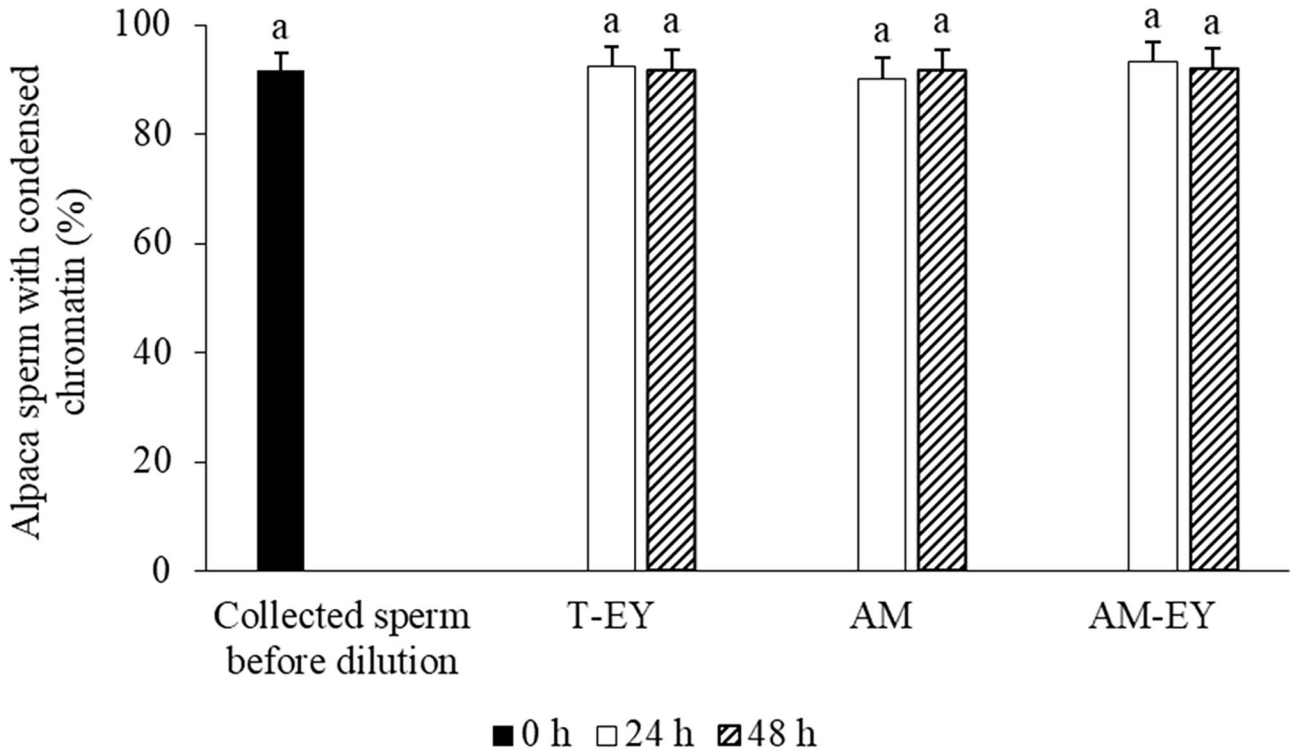


FIGURE 3 | Percentages of sperm with condensed chromatin in alpaca samples collected from deviated deferent ducts and cooled to 5°C in Tris with 20% egg yolk (T-EY), Andromed® (AM) and AM with 20% egg yolk (AM-EY) evaluated after 24 and 48 h of storage ($n = 15$). [a]Indicates absence of significant differences ($p > 0.05$).

acrosome integrity at the evaluation times studied (12). Again, this is relevant when planning an AI protocol. In addition, in this study this parameter was evaluated in conjunction with membrane function using a simple technique that is applicable in the field and which has previously been used to evaluate equine, porcine, donkey and llama sperm (21, 32, 33). The fact that the technique is simple and uses inexpensive solutions and dyes that are easy to acquire, make it possible to use it in AI campaigns in the field. Furthermore, as the samples are fixed, it allows them to be partially processed on site and evaluation can be finished once in the laboratory. This versatility and the good results obtained with this technique make this an interesting tool to implement when working with these species and under conditions that many times are far from ideal.

The percentage of normal spermatozoa in the samples diluted in any of the extenders was not altered by the cooling process. Similarly, Fumuso et al. (11, 27) observed that sperm morphology was conserved in llama frozen-thawed samples. Both these results and the ones from this study would seem to indicate that cryopreservation of SAC sperm does not affect sperm morphology in these species, at least not significantly.

Finally, sperm chromatin condensation was not affected over time with any of the extenders assayed nor did this parameter differ from that observed in collected sperm. These results contrast with those reported by Carretero et al. (31) in llama cooled sperm obtained by electroejaculation, as these authors observed a decrease in the percentage of sperm with condensed chromatin in samples cooled without seminal plasma, whereas the samples cooled with seminal plasma in the medium did not differ from raw semen. However, it is worth noting that in this latter study, the percentage of sperm with condensed chromatin in raw semen was lower than that registered in this study (74.8 vs. 91.8%) and this could have influenced the results.

CONCLUSIONS

In conclusion, the Andromed® commercial extender with the addition of 20% EY could be an alternative option for cooling alpaca sperm obtained from deviated deferent ducts assayed in this study, not only because it preserves all sperm characteristics for 48 h of storage, but also because being a commercial extender, it is easy for the producer to prepare, requires minimum equipment and supplies and allows more repeatable results to be obtained due to its less variable composition.

Therefore, both this extender and the amount of time sperm were maintained cooled with good viability results, could provide an interesting alternative to implement in future AI protocols using alpaca sperm collected from deviated deferent ducts.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary materials, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by Comité Institucional de Ética en Investigación (CIEI), Universidad Nacional del Altiplano Puno (UNAP).

AUTHOR CONTRIBUTIONS

MB carried out the study and wrote the manuscript. ET helped collect the samples. TH aided the collection of the data. DN critically read and translated the manuscript. MC designed and directed the study and critically read and corrected the manuscript. All authors contributed to the article and approved the submitted version.

ACKNOWLEDGMENTS

The authors wish to thank Dr. Susana Giuliano, for connecting us with other institutions and universities, thus giving us the opportunity to work together with international researchers in a different country.

Likewise, we thank Armando Nina, the person responsible for the semen lab of the experimental center, who accompanied us in all the work, both with the animals and in the laboratory.

Our gratitude also goes to all the technicians, shepherds, administrative staff and professionals at the CIP Quimsachata, who make the fundamental role of the experimental center a reality and without whose support this study would not have been possible.

Finally, we would like to make a special mention of recently deceased TH instrumental participation in this study as he generously opened to us the doors of the institution he led and aided the collection of the data. TH sadly was a victim of COVID-19 and thus was not able to see the fruit of this joint study published.

This research was supported by grants from the University of Buenos Aires (UBACyT 20020150200165BA) and from the Agencia Nacional de Promoción Científica y Tecnológica (PICT 2017-0394).

REFERENCES

1. Huanca W, Cordero A, Huanca T, Adams G. Biotecnologías reproductivas en camélidos sudamericanos domésticos: avances y perspectivas. *Arch Latinoam Prod Anim*. (2007) 15:195–201.
2. Giuliano S, Chaves MG, Trasorras V, Gambarotta M, Neild D, Director A, et al. Development of an artificial insemination protocol in llamas using cooled semen. *Anim Reprod Sci*. (2012) 131:204–10. doi: 10.1016/j.anireprosci.2012.03.010
3. Vaughan J, Galloway D, Hopkins D. Artificial insemination in alpacas (*Lama pacos*). Kingston: RIRDC Rural Industries Research and Development Corporation. (2003).
4. García W, Alarcón V, Bravo PW. Inseminación Artificial de alpacas con semen refrigerado y con inclusión de dos tipos de yema de huevo. *Rev Inv Vet Perú*. (2017) 28:337–44. doi: 10.15381/rivep.v28i2.13080
5. Murillo Y, Calsin B, Huanca T. *Tasa de Fertilidad a la Inseminación Artificial y Mérito Económico en Alpacas Huacaya*. Puno: Universidad Nacional del Altiplano, revista de investigaciones de la escuela de posgrado (2018) 7:717–27.
6. Bravo PV, Callo P, Garnica J. The effect of enzymes on semen viscosity in Llamas and Alpacas. *Small Rumin Res*. (2000) 38:91–5. doi: 10.1016/S0921-4488(00)00142-5
7. Giuliano SM, Carretero MI, Gambarotta MC, Neild DM, Trasorras VL, Pinto M, et al. Improvement of llama (*Lama glama*) seminal characteristics using collagenase. *Anim Reprod Sci*. (2010) 118:98–102. doi: 10.1016/j.anireprosci.2009.06.005
8. Casaretto C, Martínez Sarrasague M, Giuliano S, Rubin de Celis E, Gambarotta M, Carretero MI, et al. Evaluation of Lama glama semen viscosity with a cone-plate rotational viscometer. *Andrologia*. (2012) 44:335–41. doi: 10.1111/j.1439-0272.2011.01186.x
9. Kershaw CM, Evans G, Rodney R, Maxwell WM. Papain and its inhibitor E-64 reduce camelid semen viscosity without impairing sperm function and improve post-thaw motility rates. *Reprod Fertil Dev*. (2017) 29:1107–14. doi: 10.1071/RD15261
10. Carretero MI, Neild D, Ferrante A, Caldevilla M, Arraztoa C, Fumuso F, et al. Effect of cryoprotectant and equilibration temperature on *Lama glama* sperm cryopreservation. *Andrologia*. (2015) 47:685–93. doi: 10.1111/and.12319
11. Fumuso FG, Giuliano SM, Chaves MG, Neild DM, Miragaya MH, Carretero MI. Evaluation of the cryoprotective effect of seminal plasma on llama (*Lama glama*) spermatozoa. *Andrologia*. (2019) 51:e13270. doi: 10.1111/and.13270
12. Morton KM, Gibb Z, Bertoldo M, Chis Maxwell WM. Effect of diluent, dilution rate and storage temperature on longevity and functional integrity of liquid stored alpaca (*Vicugna pacos*) semen. *J Camelid Sci*. (2009) 2:15–25.
13. Pérez MG, Quintano J, Perez UH. Sobrevivencia espermática en refrigeración a 5°C recuperados del conducto deferente de alpaca en tres dilutores con dos protectores de membrana. *Spermova*. (2014) 4:153–8.
14. Chahuayo C, Paytán I. *Influencia de los dilutores Tris, SP-Talp y Andromed sobre la Viabilidad de Espermatozoides Epididimarios Refrigerados de Alpacas (Lama pacos)*. Huancavelica: Tesis para optar el título de ingeniero zootecnista, Universidad Nacional de Huancavelica (2013).
15. Foulkes JA. The separation of lipoproteins from egg yolk and their effect on the motility and integrity of bovine spermatozoa. *J Reprod Fertil*. (1977) 49:277–84. doi: 10.1530/jrf.0.0490277
16. Watson PF. Recent developments and concepts in the cryopreservation of spermatozoa and the assessment of their post-thawing function. *Reprod Fertil Dev*. (1995) 4:871–91. doi: 10.1071/RD9950871
17. Drobnis EZ, Crowe LM, Berger T, Anchordoguy TJ, Overstreet JW, Crowe JH. Cold shock damage is due to lipid phase transitions in cell membrane: a demonstration using sperm as a model. *J Exp Zool*. (1993) 265:432–7. doi: 10.1002/jez.1402650413
18. Pérez MG, Apaza EDH. Congelación de los espermatozoides procedentes de los conductos deferentes de camélidos. *Allpaqa, Revista de Investigación Del IIPC*. (2006) 11:17–23.
19. Pérez MG, Zevallos J, Perez UH. Recuperación de espermatozoides de alpacas del conducto deferente durante la época reproductiva. *Spermova*. (2014) 4:139–44.
20. Harrison RA, Vickers SE. Use of fluorescent probes to assess membrane integrity in mammalian spermatozoa. *J Reprod Fertil*. (1990) 88:343–52. doi: 10.1530/jrf.0.0880343
21. Carretero MI, Pigretti C, Bertuzzi ML, Fumuso FG. Test hipoósmotico combinado a la tinción de Coomassie Blue en espermatozoides de llama. *Spermova*. (2018) 8:129–32. doi: 10.18548/aspe/0006.10
22. Carretero MI, Giuliano SM, Casaretto CI, Gambarotta MC, Neild DM. Evaluación del ADN espermático de llamas utilizando azul de toluidina. *InVet*. (2009) 11:55–63.

23. Garnica J, Achata R, Bravo PW. Physical and biochemical characteristics of alpaca semen. *Anim Reprod Sci*. (1993) 32:85–90. doi: 10.1016/0378-4320(93)90059-Z
24. Kershaw-Young CM, Maxwell WMC. The effect of seminal plasma on alpaca sperm function. *Theriogenology*. (2011) 76:1197–206. doi: 10.1016/j.theriogenology.2011.05.016
25. Carretero MI, Fumuso F, Miragaya M, Herrera C, Giuliano S. Effect of seminal plasma in *Lama glama* sperm. *Reprod Fertil Dev*. (2015) 27:223. doi: 10.1071/RDv27n1Ab267
26. Fumuso FG, Giuliano SM, Chaves MG, Neild DM, Miragaya MH, Gambarotta MC, et al. Seminal plasma affects the survival rate and motility pattern of raw llama spermatozoa. *Anim Reprod Sci*. (2018) 192:99–106. doi: 10.1016/j.anireprosci.2018.02.019
27. Fumuso FG, Chaves G, Neild DM, Miragaya MH, Bertuzzi ML, Carretero MI. Incubation of frozen-thawed llama sperm with seminal plasma. *Andrologia*. (2020) 52:e13597 doi: 10.1111/and.13597
28. Miceli DC, Llanos R, Jimenez Díaz M, Peralta L. Phosphorylation of endogenous and TEST-yolk buffer proteins by intact human sperm. *Zygote*. (1998) 6:203–12. doi: 10.1017/S0967199498000136
29. Dey S, Roy D, Majumder GC, Bhattacharyya D. Extracellular regulation of sperm transmembrane adenylyl cyclase by a forward motility stimulating protein. *PLoS ONE*. (2014) 9:e110669. doi: 10.1371/journal.pone.0110669
30. Pereira R, Sá R, Barros A, Sousa M. Major regulatory mechanisms involved in sperm motility. *Asian J Androl*. (2017) 19:5–14. doi: 10.4103/1008-682X.167716
31. Carretero MI, Giuliano SM, Arraztoa CC, Santa Cruz RC, Fumuso FG, Neild DM. Comparison of two cooling protocols for llama semen: With and without collagenase and seminal plasma in the medium. *Andrologia*. (2017) 49:e12691. doi: 10.1111/and.12691
32. Ferrante A, Caldevilla M, Miragaya M. Combinación de la tinción de Azul de Coomassie con la prueba hipoosmótica para evaluar espermatozoides equinos y porcinos. *InVet*. (2017) 19:66.
33. Ferrante A, Baca Castex C, Bruno S, Arraztoa C, Plaza J, Neild D, et al. Comparison of whole and centrifuged egg yolk added to kenney's and lactose-EDTA extenders for donkey semen cryopreservation. *J Equine Vet Sci*. (2018) 65:12–8. doi: 10.1016/j.jevs.2018.02.024

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The handling editor declared a past co-authorship with the authors MC.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 01 December 2020
doi: 10.3389/fvets.2020.598117

Laterality of Ovulation and Presence of the Embryo Do Not Affect Uterine Horn Blood Flow During the First Month of Gestation in Llamas

Marcelo H. Ratto[1], *Felipe Urra*[2] *and Mauricio Silva*[3,4]*

[1] Faculty of Veterinary Sciences, Universidad Austral de Chile, Valdivia, Chile, [2] School of Graduate Studies, Universidad Austral de Chile, Valdivia, Chile, [3] Department of Veterinary Medicine and Public Health of Veterinary, Universidad Católica de Temuco, Temuco, Chile, [4] Núcleo de Investigación en Producción Alimentaria, Universidad Católica de Temuco, Temuco, Chile

We determined if laterality of ovulation and intrauterine embryo location differentially induces changes in the mesometrial/endometrial vascularization area (MEVA) between uterine horns, during and after embryo migration, elongation and implantation in llamas. Adult, non-pregnant and non-lactating llamas ($n = 30$) were subjected to daily B-mode ultrasound scanning of their ovaries. Llamas with a growing follicle ≥8 mm in diameter in the left ($n = 15$) or right ($n = 15$) ovary were assigned to a single mating with an adult fertile or vasectomized male. Power-doppler ultrasonography was used to determine the MEVA in a cross section of the middle segment of both uterine horns. MEVA was determined by off-line measurements using the ImageJ software. MEVA measurements were performed before mating (day 0) and on days 5, 10, 15, 20, 25, and 30 after mating in pregnant [llamas with left- ($n = 6$) or right-sided ($n = 6$) ovulations] and non-pregnant [llamas with left- ($n = 6$) or right-sided ($n = 6$) ovulations] females. Ovulation was confirmed by the disappearance of a follicle (≥8 mm) detected previously. Pregnancy was confirmed by the presence of the embryo proper. MEVA was analyzed by one-way ANOVA for repeated measures using the MIXED Procedure in SAS. If significant ($P \leq 0.05$) main effects or interactions were detected, Tukey's *post-hoc* test for multiple comparisons was used. Ovulation rate did not differ ($P = 0.4$) between females mated to an intact or vasectomized male and between right- or left-sided ovulations. Three females mated to the intact and 3 to the vasectomized male did not ovulate and were excluded of the study. First observation of fluid inside the gestational sac and of embryo proper, were made exclusively in the left uterine horn, on day 15.8 ± 3.8 and 22 ± 2.7, and 16.7± 2.6 and 27.5 ± 2.8 for pregnant llamas ovulating in the right and left ovary, respectively. Although the MEVA of both uterine horns was affected by time ($P < 0.05$), it was not affected by physiological status (pregnant vs. non-pregnant; $P = 0.9$) or laterality of ovulation ($P = 0.4$). Contrary to expectations, regardless of the laterality of ovulation, in pregnant llamas the left horn did not display a greater MEVA before or after embryo arrival, a trend that was observed during the first 30 days of gestation.

Keywords: llamas, ovulation, embryo, gestation, uterine vascularization

OPEN ACCESS

Edited by:
Regiane R. Santos, Schothorst Feed Research, Netherlands

Reviewed by:
Firdous Ahmad Khan, St. George's University, Grenada
Deborah Neild, University of Buenos Aires, Argentina

***Correspondence:**
Mauricio Silva
masilva@uct.cl

Specialty section:
This article was submitted to Animal Reproduction - Theriogenology, a section of the journal Frontiers in Veterinary Science

Received: *23 August 2020*
Accepted: *02 November 2020*
Published: *01 December 2020*

Citation:
Ratto MH, Urra F and Silva M (2020) Laterality of Ovulation and Presence of the Embryo Do Not Affect Uterine Horn Blood Flow During the First Month of Gestation in Llamas. Front. Vet. Sci. 7:598117. doi: 10.3389/fvets.2020.598117

INTRODUCTION

Llamas and alpacas have several unique reproductive characteristics, one of which is the establishment of embryo implantation and gestation exclusively in the left uterine horn, regardless of laterality of ovulation (1–3). Females from both species have a bicornate uterus that presents a clear asymmetry between uterine horns, with the left horn being larger than its right counterpart (4, 5). This asymmetry is not only observed in pluriparous and pregnant females but also in nulliparous and even in female fetuses, therefore it is not induced by pregnancy (5). Also, the arterial irrigation and venous drainage differ between both uterine horns in llamas. The presence of a prominent cross-over arterial branch extending from the right uterine artery to the left horn suggests that this is irrigated with a greater blood flow (4).

Besides, llamas, and alpacas present a peculiar pattern of intrauterine embryo migration. Although ovulation occurs with the same frequency in the left and right ovary (2, 6), embryos originated from right-ovary ovulations must migrate into the left uterine horn before the day of the beginning of luteolysis (Day 9 after ovulation) for the pregnancy to be successfully established (3, 7).

In most mammalian species significant changes in uterine vascular irrigation are observed during gestation, which are required to initially sustain embryo implantation (8) and latter fetal supply of nutrients and oxygen (9). This phenomenon has been mostly studied in females presenting a symmetric uterus, such as cows (10, 11), buffalo (12) sheep (13–15), goats (15), and mares (16–20), but only few studies have evaluated uterine vascularization during the first month of gestation (11, 16, 17, 20).

In horses and cattle (10, 20) the establishment of pregnancy gradually increases uterine blood flow in close relationship with embryo/fetal growth during gestation. These hemodynamic changes begin before embryo implantation occurs (11, 16, 17) and exponentially increase thereafter (10). Interestingly, the increase in uterine blood flow begins before an intimate contact between the embryo and the endometrium is established (17), and is closely influenced by embryo location (11, 16, 17). Embryo location induces significant differences in blood flow between both uterine horns in cows (10) and mares (17, 20), generating an asymmetrical blood flow in the former and a symmetrical blood provision in the latter before embryo fixation/implantation as a consequence of different intrauterine embryo migration patterns.

As mentioned before, more than 98% of gestations in llamas are carried out in the left uterine horn, therefore embryos originated from right ovulations must migrate to the left horn in order to achieve a successful pregnancy. The striking features of embryo migration and the special uterine vascular arrangement make this species an interesting model to study uterine vascular perfusion and pregnancy development. Therefore, the goal of this study was to determine if intrauterine embryo location differentially induces changes in mesometrial/endometrial vascularization (MEVA) between the right and left uterine horn, during embryo migration, elongation and implantation in llamas. Since an adequate endometrial blood supply is essential for a successful embryo implantation and survival (8, 21, 22), studies on the spatial relationship between the location of the early embryo/conceptus and the degree of uterine vascular perfusion in llamas may shed some light into the mechanisms controlling embryo implantation in the left uterine horn.

MATERIALS AND METHODS

The present study was conducted during the breeding season (November–January) at the Universidad Católica de Temuco, Temuco, Chile (38° 45′S−72° 40′W and 122 m above sea level). All procedures were reviewed and approved by the University Bioethics Committee and were performed in accordance with the animal care protocols established by the same institution.

Animals

Adult non-pregnant, non-lactating llamas [$n = 30$; age: 5–8 y; weight: 120.5 ± 14.1 Kg; mean Body Condition Score: 3.5 out of 5 (range: 3.0–4.0); parity: 3 ± 2] were maintained on pasture supplemented with hay and water *ad libitum*. Llamas were housed indoors at night and offered 250 g/animal of a commercial diet supplement containing 140 g/kg crude protein and 150 g/kg crude fiber. Also, one intact fertile and one vasectomized adult male (ages: 3 and 5 y; weight: 147.5 ± 8.1 Kg; Body Condition Score: 4 and 5, respectively) were kept under similar conditions as the females, but separate at all times from the female herd. Male-female contact was only allowed during the supervised matings. Vasectomy was performed by a standard surgical procedure 1 year before the start of the present experiment in the context of a previous study.

Experimental Design

Females were examined once daily by transrectal ultrasonography to monitor follicular growth and then by simple randomization were assigned to the following treatment groups: (a) presence of a growing follicle ≥8 mm in diameter in the right ovary and mating with an intact male ($n = 8$), (b) presence of a growing follicle ≥8 mm in diameter in the left ovary and mating with an intact male ($n = 7$), (c) presence of a growing follicle ≥8 mm in diameter in the right ovary and mating with a vasectomized male ($n = 8$), or (d) presence of a growing follicle ≥8 mm in diameter in the left ovary and mating with a vasectomized male ($n = 7$). Mating was validated only if the receptive female adopted the prone position soon after contact with the male and if intromission and copula lasted more than 5 min. After mating, females were examined using B-mode transrectal ultrasonography every 12 h until ovulation or 48 h, whichever came first. Ovulation was confirmed by the sudden disappearance of a follicle (≥8 mm) detected during previous examinations and only ovulated females were incorporated for the transrectal Power-doppler ultrasound examination.

Power-Doppler Ultrasonographic Evaluation

The area of mesometrial/endometrial vascularization of both uterine horns was evaluated by Power-doppler ultrasonography

in all ovulated females using a 5.0 MHz lineal array transducer coupled to a ultrasound monitor (Sonosite M-Turbo, USA) before mating (Day 0 = Day of mating) and on days 5, 10, 15, 20, 25, and 30 between 08:00 a.m. and 12:00 p.m. as described previously (11, 16, 17). In brief, the transducer was placed over a cross section of the middle segment of each uterine horn where a 10 s video-clip was registered. The area of mesometrial/endometrial vascularization was objectively assessed by off-line measurements of the number of colored pixels as an indicator of blood flow area. Three still images of each horn were selected by a blind procedure, and then used for the determination of the number of colored pixels, and the average was used for the statistical analyses. Power Doppler images were selected based on two criteria: (a) proper cross section of the uterine horn and, (b) absence or minimal presence of Power-doppler noise interference. Then, images were recorded, edited, and analyzed using the ImageJ software (NIH open access, USA). A female was considered pregnant when the gestational sac and the embryo proper were detected by ultrasonography.

Statistical Analyses

Statistical analyses were performed using the Statistical Analysis System software package SAS Learning Edition, version 4.1 (SAS Institute Inc., Cary, NC, USA, 2006). Serial data were compared by analysis of variance for repeated measures (Proc-mixed procedure) to determine the effects of female physiological status (pregnant vs. non-pregnant), laterality of ovulation (right or left ovary), time and treatments-by time interaction on left and right uterine horn MEVA. If significant ($P \leq 0.05$) main effects or interactions were detected, Tukey's *post-hoc* test for multiple comparisons was used to locate differences. All data are reported as mean ± SEM, and probabilities ≤ 0.05 were considered significant.

RESULTS

There was not a significant difference ($P = 0.4$) in ovulation rate between llamas mated with an intact fertile or vasectomized male. Six out of 8 and 6/7 llamas with a preovulatory follicle ≥8 mm diameter located in either the right or left ovary ovulated and became pregnant after mating with the intact fertile male. Similarly, 6/8 and 6/7 llamas with a preovulatory follicle ≥8 mm diameter located either in the right or left ovary ovulated after mating with the vasectomized male. In pregnant females the earliest ultrasound signs of gestations were observed exclusively in the middle section of the left uterine horn. First observations of fluid inside the gestational sac (i.e., embryonic vesicle) and the embryo proper were recorded on day 15.8 ± 3.8 and 22 ± 2.7, and 16.7± 2.6 and 27.5 ± 2.8, for pregnant llamas ovulating in the right and left ovary, respectively. Representative images of MEVA in gravid uterus (i.e., left uterine horn) 30 days after mating for 6 different females are shown in **Figure 1**.

There was an effect of time ($P < 0.05$) on the MEVA of both uterine horns, but this parameter was not affected by physiological status of the female (pregnant vs. non-pregnant; $P = 0.9$), laterality of ovulation ($P = 0.4$), nor by interactions between any of the variables measured. In pregnant and non-pregnant llamas with left-ovary ovulations the mean MEVA of right uterine horn displayed a significant ($P < 0.05$) decrease, compared to basal value, on day 10. On the contrary, in non-pregnant llamas with right-ovary ovulations the MEVA of the left uterine horn displayed a significant ($P < 0.05$) increase on day 20. The mean MEVA for both uterine horns, in pregnant and non-pregnant llamas with right or left ovary ovulations, during the entire period of evaluation are shown separately in **Figure 2**.

DISCUSSION

In the present study regardless of laterality of ovulation, intrauterine embryo location did not induce changes in mesometrial/endometrial vascularization area between the right and left uterine horn, during the phases of embryo migration, elongation and implantation in llamas.

The measurement of MEVA has been reported to be a reliable and sensitive tool to evaluate uterine blood flow during early gestation in mares and heifers (11, 16, 17). Also, using this ultrasonographic method our research group has demonstrated in previous studies (23, 24) that significant changes in uterine blood flow and vascularization area occur in llamas during the follicular growth phase or after mating. However, in the present study MEVA was similar for pregnant and non-pregnant females and between right and left uterine horns during the evaluation period.

A macroscopic anatomical study of uterine vascularization in llamas (4) has described the presence of a peculiar arrangement involving a prominent cross-over arterial branch extending from the right uterine artery to the left uterine horn, which could suggest that the left uterine horn is irrigated with a greater blood flow. However, the results of the present study were not able to detect a differential vascularization between uterine horns, regardless of the of female's physiological status or laterality of ovulation, therefore, not supporting the previously cited study (4).

Moreover, a significant individual variation regarding basal MEVA (i.e., pre mating) was observed among female llamas; however, no trend favoring a greater vascularization toward the left uterine horn was established. This great individual variation in uterine hemodynamic parameters has been reported by other studies in mares and it was not related to the stage of the cycle, age or parity (18, 20). Although several studies have described the hemodynamic changes during gestation in a variety of farm animal species, only a few (11, 16, 17) were conducted to evaluate hemodynamic changes during the embryonic peri implantation period.

In mares and cows (10, 20) the establishment of pregnancy gradually increases uterine arterial blood flow in accordance with embryo/fetal growth during the entire length of gestation period. These modifications seem to begin during the pre-implantation phase of embryo development (11, 16, 17) and exponentially increase thereafter (10). Interestingly, these modifications in uterine and endometrial vascular irrigation already begin during the histotrophic phase of embryo nutrition (17), when there is no intimate contact between the embryo and the endometrium, and are closely related to embryo location (11, 16, 17).

In the bovine, a species that does not present intrauterine embryo migration (i.e., the embryo remains in the uterine

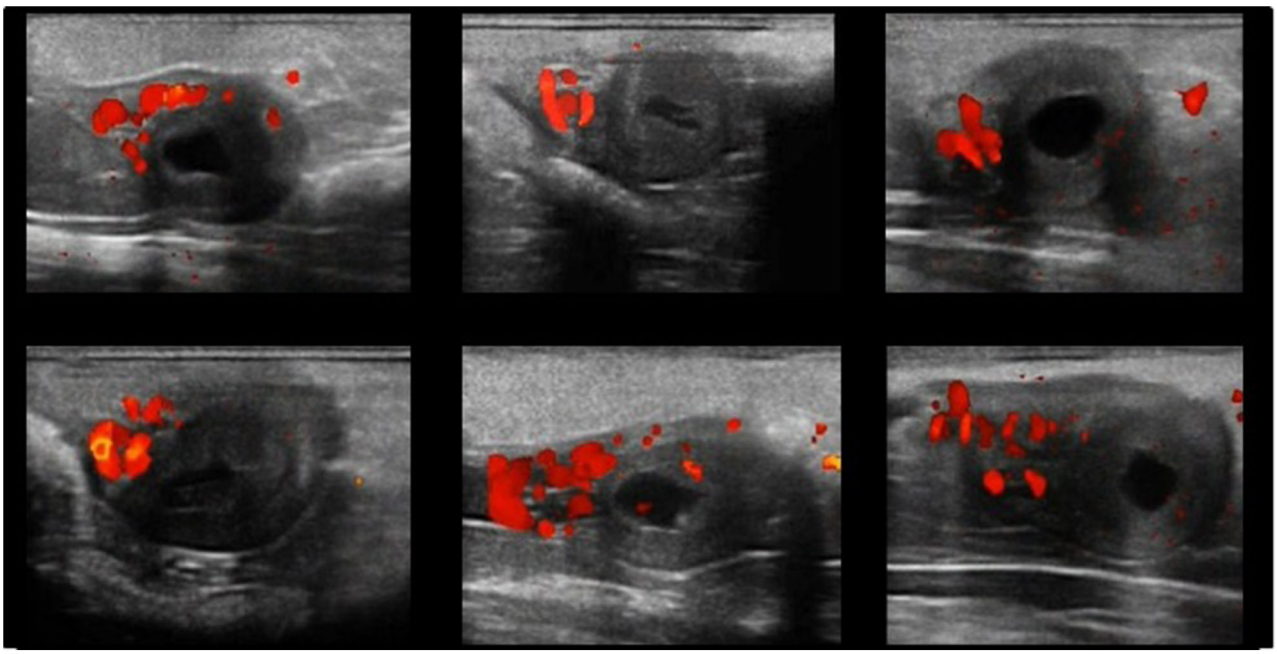


FIGURE 1 | Representative Power-doppler ultrasound images of mesometrial/endometrial vascularization area in gravid uterus (left uterine horn) 30 days after mating in llamas. Each image represents a different female.

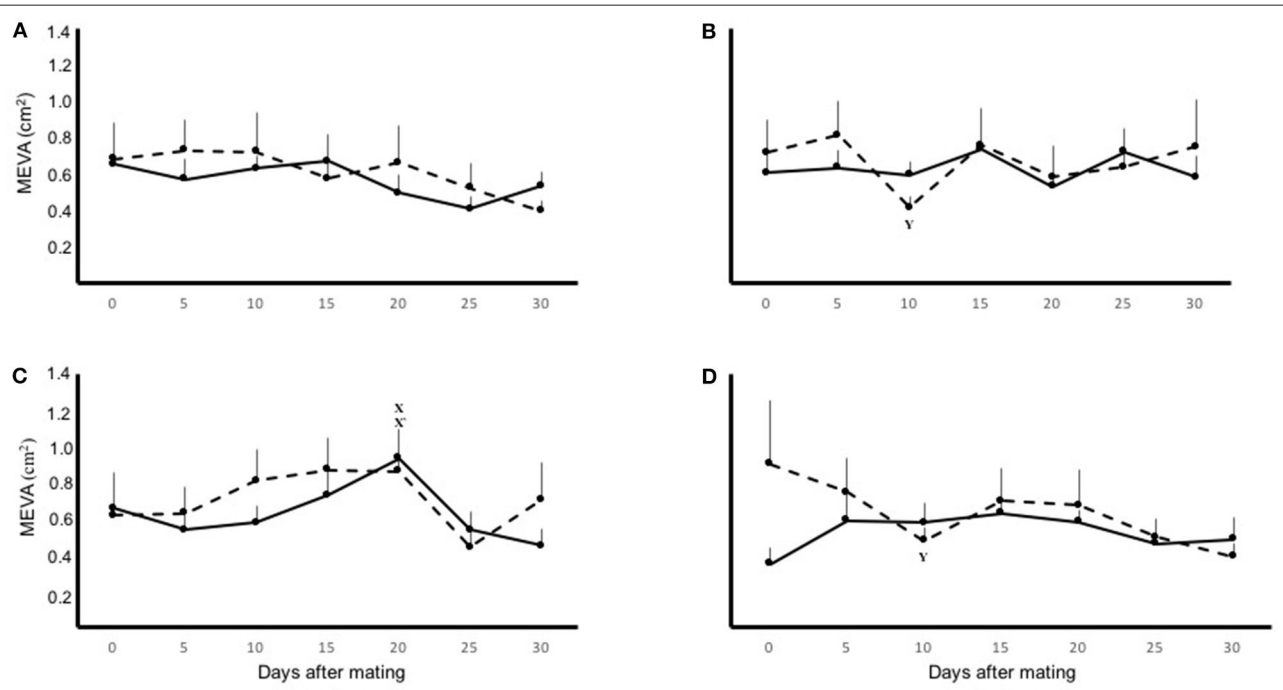


FIGURE 2 | Mean ± SEM mesometrial/endometrial vascularization area (MEVA) on the left (solid line) and right (dashed line) uterine horn, in pregnant **(A,B)** and non-pregnant **(C,D)** llamas with right- **(A,C)** or left-sided **(B,D)** ovulations, during the 30 day period of evaluation. Effect of: physiological status ($P = 0.9$); laterality of ovulation ($P = 0.4$); time ($P < 0.05$). $^{x,x'}$Within uterine horn, the first significant increase from basal MEVA ($P < 0.01$). ^{y}Within uterine horn, the first significant decrease from basal MEVA ($P < 0.01$).

horn ipsilateral to the ovary from which ovulation occurred) compared to the equine, there is a clear increase in blood flow in the uterine artery ipsilateral to the uterine horn containing the embryo during the first weeks of gestation (11, 25). In heifers, the increase in uterine blood flow is directly correlated to subtler changes in endometrial vascular perfusion, and it begins as early as Day 13 (25) or 18 (11) of pregnancy. This last study demonstrated a temporal synchrony between the increase in uterine/endometrial vascular irrigation and embryo elongation, which in turn is closely related to the beginning of adhesiveness of the chorion to the endometrium (Day 20; 11), suggesting that the direct contact of the embryo with the endometrium induces local changes in uterine/endometrial blood flow.

On the contrary, in mares in which pre-implantation embryo develops an intense intrauterine migration before fixation occurs (26), endometrial vascular irrigation increased in an alternate manner between uterine horns, which was tightly synchronized with embryo location. Accordingly, during the period of intense intrauterine migration even the presence of the embryo for periods of 7 min, or longer in one location, determined a localized increase in endometrial vascular perfusion (16); thus, during the pre-implantation phase embryo-induced changes in endometrial vasculature parallel embryo migration between uterine horns (16, 17). However, shortly after embryo fixation the increase in endometrial blood flow was only observed in the endometrium surrounding the fixed embryonic vesicle (16). Moreover, from fixation day onwards, the blood flow of the uterine artery ipsilateral to the horn containing the embryo increased drastically compared to its contralateral counterpart (20).

However, in the present study the MEVA was similar in all the categories evaluated. There was no change in uterine vascularization between uterine horns in pregnant and non-pregnant females or between those whose embryos were originated from left- or right-sided ovulations. Furthermore, MEVA did not increase significantly over time during the first 30 days of gestation in the pregnant group as was described for the bovine, where it increased from Day 13 or 18 of pregnancy (11, 25). The reasons for these differences with observations made in other species could be due to a slower rate of llama embryo/fetus development during the first 3 months of gestation, as measured by crown-rump length, compared to cattle, sheep and horses (27, 28).

The vast differences in uterine and endometrial vascular irrigation during the early phase of embryo development, between species that display different embryonic strategies to signal its presence to the dam, could be related to the secretion of vascular stimulants into the uterine lumen by the embryo (17). In this regard, several studies have demonstrated that the Day 16 bovine embryo (29), and specially the equine embryo, as early as Day 12 (30, 33) produce and secrete estrogen, a molecule involved in inducing uterine contractility (26) and significant increases in uterine blood flow (31). Thus, during pre-implantation embryo development, in the bovine this molecule would be secreted into just one uterine horn, while in the mare it would be evenly distributed between both horns and the uterine body, inducing the previously described vascular changes. Despite the fact that estradiol has also been suggested as the most probable signaling candidate responsible for maternal recognition of pregnancy and intrauterine migration for the llama blastocyst (32), our results do not show an effect of embryo signaling on uterine blood flow. Larger quantities of estradiol secreted by the equine blastocyst compared to the llama embryo (32, 33), could explain the described effect in mare uterine blood flow and the absence of it in llamas.

Although in the present study embryo location did not induce changes in MEVA between the right and left uterine horn during the first month of gestation in llamas, there was an effect of time on uterine horn blood flow. Considering the slower rate of development of llama embryo/fetus during the first months of gestation, future investigations should consider a longer observational period to determine potential interactions between embryo and uterine blood flow and should increase the number of animals per group.

Finally, similar to our results, Travassos-Beltrame et al. (13) did not find differences between hemodynamic parameters between left and right uterine horns in pregnant sheep. Even though they started the Doppler ultrasonographic evaluation after pregnancy diagnosis was made on Day 28, hemodynamic variables were not affected by uterine horn nor single vs. multiple gestations.

Contrary to expectations, based on our results we can conclude that regardless of laterality of ovulation, in pregnant llamas the left horn did not display a greater MEVA before or after embryo arrival, a trend that was observed during the first 30 days of gestation.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Universidad Católica de Temuco.

AUTHOR CONTRIBUTIONS

MS and MR designed the experiment and wrote the manuscript. MS and FU developed the field work and analyzed the data. All authors contributed to the article and approved the submitted version.

FUNDING

This study was supported by the Chilean National Science and Technology Research Council (Fondecyt 11140396) awarded to MS.

REFERENCES

1. Fernandez-Baca S, Hansel W, Novoa C. Embryonic mortality in the alpaca. *Biol Reprod.* (1970) 3:243–51. doi: 10.1093/biolreprod/3.2.243
2. Fernandez-Baca S, Sumar J, Novoa C, Leyva V. Relación entre la ubicación del cuerpo lúteo y la localización del embrión en la alpaca. *Rev Inv Pec (IVITA) Univ Nac San Marcos.* (1973) 2:131–5.
3. Sumar J, Adams GP. Reproductive anatomy and life cycle of the male and female llama and alpaca. In: Younquist RS, Threlfall WR, editors. *Current Therapy in Large Animal Theriogenology*. St. Louis, MO: Saunders Elsevier (2007). doi: 10.1016/B978-072169323-1.50118-5
4. Del Campo MR, Del Campo CH, Ginther OJ. Vascular provisions for a local utero-ovarian cross-over pathway in new world camelids. *Theriogenology.* (1996) 46:983–91. doi: 10.1016/S0093-691X(96)00262-2
5. Del Campo MR, Vasquez MS, Del Campo CH. Macroscopic and microscopic characteristics of the female llama (Lama glama) fetal, infantile, and adult genital tracts. *Reprod Fert Dev.* (2009) 22:227. doi: 10.1071/RDv22n1Ab137
6. Sumar J, Leiva V. *Relación Entre la Ubicación del CL y la Localización del Embrión en la Llama (Lama glama)*. Viedma: Anales III Convención Internancional Sobre Camelidos Sudamericanos (1979).
7. Aba MA, Kindahl H, Forsberg M, Quiroga M, Auza N. Levels of progesterone and changes in prostaglandin F(2alpha) release during luteolysis and early pregnancy in llamas and the effect of treatment with flunixin meglumine. *Anim Reprod Sci.* (2000) 59:87–97. doi: 10.1016/S0378-4320(00)00068-3
8. Nakamura H, T Hosono, K Minato, Hamasaki T, Kumasawa K, Kimura T. Importance of optimal local uterine blood flow for implantation. *J Obstet Gynaecol Res.* (2014) 40:1673–88. doi: 10.1111/jog.12418
9. Osol G, Mandala M. Maternal uterine vascular remodeling during pregnancy. *Physiology.* (2009) 24:58–71. doi: 10.1152/physiol.00033.2008
10. Bollwein H, Baumgartner U, Stolla R. Transrectal Doppler sonography of uterine blood flow in cows during pregnancy. *Theriogenology.* (2002) 57:2053–61. doi: 10.1016/S0093-691X(02)00706-9
11. Silva LA, Ginther OJ. Local effect of the conceptus in uterine vascular perfusion during early pregnancy in heifers. *Reproduction.* (2010) 139:453–63. doi: 10.1530/REP-09-0363
12. Varughese EE, Brar PS, Dhindsa SS. Uterine blood flow during various stages of pregnancy in dairy buffaloes using transrectal Doppler ultrasonography. *Anim Reprod Sci.* (2013) 140:34–9. doi: 10.1016/j.anireprosci.2013.05.011
13. Travassos-Beltrame R, Covre C, Buss Littig L, de Barros Martins A, Quirino CR, Bartholazzi Junior A, et al. Transrectal Doppler sonography of uterine blood flow in ewes during pregnancy. *Theriogenology.* (2017) 91:55–61. doi: 10.1016/j.theriogenology.2016.12.026
14. Turna Yilmaz Ö, Can Gündüz M, Evkuran Dal G, Ucmak M, Günay Ucmak Z, Karacam E, et al. Evaluation of changes in Doppler ultrasonography indices and levels of maternal serum angiogenic factors throughout pregnancy in ewes. *Theriogenology.* (2017) 89:183–91. doi: 10.1016/j.theriogenology.2015.09.010
15. Elmetwally M, Rohn K, Meinecke-Tillmann S. Noninvasive color Doppler sonography of uterine blood flow throughout pregnancy in sheep and goats. *Theriogenology.* (2016) 85:1070–9. doi: 10.1016/j.theriogenology.2015.11.018
16. Silva LA, Gastal EL, Beg MA, Ginther OJ. Changes in vascular perfusion of the endometrium in association with changes in location of the embryonic vesicle in the mare. *Biol Reprod.* (2005) 72:755–61. doi: 10.1095/biolreprod.104.036384
17. Silva LA, Klein C, Early AD, Sharp DC. Conceptus-mediated endometrial vascular changes during early pregnancy in mares: an anatomic, histomorphometric, and vascular endothelial growth factor receptor system immunolocalization and gene expression study. *Reproduction.* (2011) 142:593–603. doi: 10.1530/REP-11-0149
18. Bollwein H, Maierl J, Maier R, Stolla R. Transrectal Color Doppler sonography of the a. uterina in cyclic mares. *Theriogenology.* (1998) 49:1483–8. doi: 10.1016/S0093-691X(98)00094-6
19. Bollwein H, Weber F, Kolberg B, Stolla R. Uterine and ovarian blood flow during the estrous cycles in mares. *Theriogenology.* (2002) 57:2129–38. doi: 10.1016/S0093-691X(02)00703-3
20. Bollwein H, Maier R, Stolla R. Transrectal Doppler Sonography of uterine blood flow during early pregnancy in mares. *Theriogenology.* (2003) 60:597–605. doi: 10.1016/S0093-691X(03)00080-3
21. Raine-Fenning N. Doppler assessment of uterine artery blood flow for the prediction of pregnancy after assisted reproduction treatment. *Ultrasound Obstet Gynecol.* (2008) 31:371–5. doi: 10.1002/uog.5321
22. Hung Yu Ng E, Chi Wai Chan C, Shan Tang O, Shu Biu Yeung W, Chung Ho P. The role of endometrial and subendometrial vascularity measured by three-dimensional Power Doppler Ultrasound in the prediction of pregnancy during frozen-thawed embryo transfer cycles. *Hum Reprod.* (2006) 21:1612–7. doi: 10.1093/humrep/dei502
23. Silva M, Urra F, Ratto MH. Uterine endometrial vascularization during ovarian follicular growth in llamas: the effect of estradiol plasma concentration. *Theriogenology.* (2018) 106:164–9. doi: 10.1016/j.theriogenology.2017.10.027
24. Urra F, Ratto MH, Silva M. Evaluation of the effect of mating, intrauterine deposition of raw seminal plasma or seminal plasma purified β-NGF on endometrial vascularization in llamas. *Theriogenology.* (2019) 125:18–23. doi: 10.1016/j.theriogenology.2018.10.007
25. Ford SP, Chenault JR, Echternkamp SE. Uterine blood flows of cows during the oestrus cycle and early pregnancy: effect of the conceptus on the uterine blood supply. *J Reprod Fertil.* (1979) 56:53–62. doi: 10.1530/jrf.0.0560053
26. Leith GS, Ginther OJ. Characterization of intrauterine mobility of the early conceptus. *Theriogenology.* (1984) 22:401–8. doi: 10.1016/0093-691X(84)90460-6
27. Montelli S, Stelletta C, Ruiz J, Ballarin C, Cozzi B, Peruffo A. Mapping of the early intrauterine morphogenesis in the alpaca (*Vicugna pacos*): external features and development of the cephalic vesicle in comparison with the progressive Carnegie scale. *Anat Rec.* (2019) 302:1226–37. doi: 10.1002/ar.23980
28. McGeady TA, Quinn PJ, Fitzpatrick ES, Ryan MT, Cahalan S. *Veterinary Embryology*. Wiley-Blackwell (2013).
29. Chenault JR. Steroid metabolism by the early bovine conceptus –I. 5β-Reduction of neutral C19-steroids. *J Steroid Biochem.* (1980) 13:499–506. doi: 10.1016/0022-4731(80)90205-8
30. Raeside JL, Christie HL, Renaud HL, Waelchli RO, Betteridge KJ. Estrogens metabolism in the equine conceptus and endometrium during early pregnancy in relation to estrogen concentrations in yolk-sac fluid. *Biol Reprod.* (2004) 71:1120–7. doi: 10.1095/biolreprod.104.028712
31. Ford SP. Control of uterine and ovarian blood flow throughout the estrus cycle and pregnancy of ewes, sows and cows. *J Anim Sci.* (1982) 55:32–42.
32. Powell SA, Smith BB, Timm KI, Menino AR Jr. Estradiol production by preimplantation blastocyst and increased serum progesterone following estradiol treatment in llamas. *Anim Reprod Sci.* (2007) 102:66–75. doi: 10.1016/j.anireprosci.2006.10.002
33. Raeside JI, Christie HL, Waelchli RO, Betteridge KJ. Estrogen metabolism by the equine embryo proper during the fourth week of pregnancy. *Reproduction.* (2009) 138:953–60. doi: 10.1530/REP-09-0235

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 03 December 2020
doi: 10.3389/fvets.2020.581877

Birth of a Live Cria After Transfer of a Vitrified-Warmed Alpaca (*Vicugna pacos*) Preimplantation Embryo

Jennifer C. Lutz[1], *Susan L. Johnson*[1], *Kimberly J. Duprey*[1], *Paul J. Taylor*[2], *Henry William Vivanco-Mackie*[3], *Daniel Ponce-Salazar*[3], *Marlene Miguel-Gonzales*[3] *and Curtis R. Youngs*[4]*

[1] Cas-Cad-Nac Farm, Perkinsville, VT, United States, [2] GeneSearch, Inc., Bozeman, MT, United States, [3] Vivanco International SAC, Lima, Peru, [4] Animal Science Department, Iowa State University, Ames, IA, United States

The alpaca (*Vicugna pacos*) is an important species for the production of fiber and food. Genetic improvement programs for alpacas have been hindered, however, by the lack of field-practical techniques for artificial insemination and embryo transfer. In particular, successful techniques for the cryopreservation of alpaca preimplantation embryos have not been reported previously. The objective of this study was to develop a field-practical and efficacious technique for cryopreservation of alpaca preimplantation embryos using a modification of a vitrification protocol originally devised for horses and adapted for dromedary camels. Four naturally cycling non-superovulated Huacaya females serving as embryo donors were mated to males of proven fertility. Donors received 30 μg of gonadorelin at the time of breeding, and embryos were non-surgically recovered 7 days after mating. Recovered embryos ($n = 4$) were placed individually through a series of three vitrification solutions at 20°C (VS1: 1.4 M glycerol; VS2: 1.4 M glycerol + 3.6 M ethylene glycol; VS3: 3.4 M glycerol + 4.6 M ethylene glycol) before loading into an open-pulled straw (OPS) and plunging directly into liquid nitrogen for storage. At warming, each individual embryo was sequentially placed through warming solutions (WS1: 0.5 M galactose at 37°C; WS2: 0.25 M galactose at 20°C), and warmed embryos were incubated at 37°C in 5% CO_2 in humidified air for 20–22 h in 1 ml Syngro® holding medium supplemented with 10% (v/v) alpaca serum to perform an initial *in vitro* assessment of post-warming viability. Embryos whose diameter increased during culture ($n = 2$) were transferred individually into synchronous recipients, whereas embryos that did not grow ($n = 2$) were transferred together into a single recipient to perform an *in vivo* assessment of post-warming viability. Initial pregnancy detection was performed ultrasonographically 29 days post-transfer when fetal heartbeat could be detected, and one of three recipients was pregnant (25% embryo survival rate). On November 13, 2019, the one pregnant recipient delivered what is believed to be the world's first cria produced from a vitrified-warmed alpaca embryo.

Keywords: cryopreservation, galactose, South American camelid, vitrification, hatched blastocyst

OPEN ACCESS

Edited by:
Marcelo H. Ratto, Austral University of Chile, Chile

Reviewed by:
Virginia Luz Trasorras, University of Buenos Aires, Argentina
Divakar Justus Ambrose, University of Alberta, Canada

***Correspondence:**
Curtis R. Youngs
cryoungs@iastate.edu

Specialty section:
This article was submitted to Animal Reproduction - Theriogenology, a section of the journal Frontiers in Veterinary Science

Received: *10 July 2020*
Accepted: *19 October 2020*
Published: *03 December 2020*

Citation:
Lutz JC, Johnson SL, Duprey KJ, Taylor PJ, Vivanco-Mackie HW, Ponce-Salazar D, Miguel-Gonzales M and Youngs CR (2020) Birth of a Live Cria After Transfer of a Vitrified-Warmed Alpaca (Vicugna pacos) Preimplantation Embryo. Front. Vet. Sci. 7:581877. doi: 10.3389/fvets.2020.581877

INTRODUCTION

The alpaca (*Vicugna pacos*) is a member of the South American Camelid (SAC) family consisting of alpacas, llamas, vicuñas, and guanacos. The SACs, which number more than 9.1 million head globally (1), convert low-quality roughages into fiber and meat in a variety of different production and management conditions. Particularly in parts of South America, alpacas are often a major source of income for farmers in remote rural areas (2).

In contrast with other livestock species such as cattle and sheep, alpaca genetic improvement programs seldom utilize artificial insemination or embryo transfer because those reproductive biotechnologies are not yet well-developed for alpacas (3, 4). Of special note with respect to embryo transfer technology is the difficulty in cryopreserving alpaca embryos (5) using either slow cooling or vitrification methods (6). Possible reasons for the difficulty in cryopreserving alpaca preimplantation embryos include the high lipid content of cells comprising the embryo (7) and the large number of cells present when embryos enter the uterus as hatched blastocysts (8). This challenge with cryopreservation of large diameter embryos is not unique to alpacas, as large diameter equine blastocysts have proven challenging to cryopreserve, especially using slow cooling techniques (9).

Pregnancies have been reported from the transfer of alpaca preimplantation embryos cryopreserved via slow cooling (10, 11) or vitrification (10). However, no live births were reported from those studies. The overall objective of this study was to develop a field-practical and efficacious protocol for vitrification of alpaca preimplantation embryos. The specific objective was to test a modification of a vitrification protocol originally devised for horses (12, 13) and modified for use in dromedary camels (14) because this protocol had proven successful in two different species—one of which is a relative of the alpaca (15).

MATERIALS AND METHODS

Experimental Animals and Their Management

This field study was conducted at a privately owned Huacaya alpaca farm near Perkinsville, Vermont, USA (43.3737° N latitude and 72.5137° W longitude; elevation of 175 m above sea level). The coldest month is January (mean temperature of −7.8°C), and the warmest month is July (mean temperature of 20.3°C). Average annual precipitation is 1,022 mm. Animals were managed in groups of ∼20 females in indoor pens measuring ∼42 m^2 with access to outside grazing pastures. Animals were hand-fed once daily a commercial 15% crude protein pellet (Poulin Grain Alpaca Milk & Cria Pellet, Newport, Vermont, USA). Animals had *ad libitum* access to fresh water, orchard grass hay, and a mineral mix specially formulated for alpacas (Stillwater Mineral Formula 104, Paoloa, Kansas, USA).

Donor Selection

Embryo donors were evaluated for ovarian follicular development using a 7.5-MHz ultrasound transducer (Aloka SSD-500V). Females with ovarian follicles 7–10 mm in diameter were behavior tested to determine their sexual receptivity (16), and receptive females were naturally mated to a male alpaca of proven fertility. At the time of breeding (Day 0), donors ($n = 4$) were given 30 μg gonadorelin (Factrel®, Zoetis, Kalamazoo, MI USA) intramuscularly to aid in the induction of ovulation.

Recipient Selection

Potential recipient females ($n = 12$) were evaluated ultrasonographically as described above. Females with ovarian follicles 7–10 mm in diameter were behavior tested, and sexually receptive females ($n = 5$) received 30 μg gonadorelin 6 days prior to embryo transfer. Final selection of suitable recipients was based on ultrasonographic confirmation of a corpus luteum and non-receptive behavior when exposed to a male 24 h prior to transfer on Day 6. We chose to perform 1-day asynchronous transfers (17, 18). Pregnancy testing of recipients was performed via transrectal ultrasonography (19) 29 days post-transfer (to visualize fetal heartbeat) and again at 52, 70, 84, 109, and 177 days post-transfer (to monitor the pregnancy and to enable the determination of the approximate timing of fetal death loss were it to occur).

Media Preparation

Alpaca serum was prepared by collection of whole blood from four non-pregnant females at unknown stages of the estrous cycle into a 20-cc syringe. Blood was transferred into red-top Vacutainer® tubes (BD, Franklin Lakes, New Jersey, USA) before centrifugation at 3500 RPM for 20 min. Serum was sterile-filtered using a 0.22-μm low-protein binding Acrodisc® filter. Fresh serum was used to prepare the base medium and the culture medium. The culture medium and excess serum were frozen at −29°C until needed; the culture medium was thawed by placement onto a slide warmer at 37°C.

Glycerol and ethylene glycol were obtained from Fisher Scientific Company. All other chemicals were obtained from Sigma Aldrich Chemical Company. The base medium (BM) consisted of Dulbecco's phosphate buffered saline (D-PBS) supplemented with 0.3 mM sodium pyruvate, 3.3 mM galactose, and 20% (v/v) alpaca serum. Three vitrification solutions (VS) and two warming solutions (12–14) were subsequently prepared using BM. Vitrification solution 1 (VS1) was 1.4 molar (M) glycerol, VS2 was 1.4 M glycerol + 3.6 M ethylene glycol, and VS3 was 3.4 M glycerol + 4.6 M ethylene glycol. Warming solution 1 (WS1) was 0.5 M galactose, and WS2 was 0.25 M galactose. The culture medium consisted of Syngro® holding medium (Vetoquinol USA, Ft. Worth, Texas) supplemented with 10% (v/v) alpaca serum.

Embryo Collection, Processing, and Transfer

Embryos were non-surgically collected 7 days postbreeding via uterine lavage using a 14-Fr 5cc Foley catheter. Harvested embryos were transferred from the room temperature flushing medium (ViGRO™ Complete Flush, Vetoquinol USA, Ft. Worth, Texas) to an embryo holding medium, and their diameter was measured using an eyepiece reticle that had been inserted

into one eyepiece of a stereomicroscope and calibrated with a 25-mm stage micrometer (Klarmann Rulings, Inc., Litchfield, New Hampshire, USA). After measurement of the diameter, embryos were washed six times in holding medium supplemented with 10% (v/v) alpaca serum. Embryos were handled at 20°C unless otherwise noted.

Embryos were moved individually through a series of VS in 35-mm petri dishes: 500 µl drops of VS1 for 5 min, 500 µl drops of VS2 for 5 min, 5 µl drops of VS3 for 20 s, and 5 µl drops of VS3 for ≤20 s, while embryos were loaded into an open-pulled straw (OPS; Minitube USA, Verona, Wisconsin). Each OPS was plunged directly into liquid nitrogen where it was stored until recipient females were available (29 days).

At warming, each OPS was removed from liquid nitrogen, and its tip was submerged into a 1-ml drop of WS1 at 37°C. The embryo was expelled from the OPS into WS1 where it remained for 1 min before transfer into WS2 for 5 min at room temperature (20°C). Embryos were then transferred into a holding medium augmented with 10% (v/v) alpaca serum, and after 5 min, the embryo size was measured. Embryos were subsequently cultured at 37°C in 1 ml of culture medium in 4-well plates for 20–22 h in a gaseous environment of 5% CO_2 in humidified air.

At the end of the culture period, embryo size was measured to enable assessment of embryo growth by comparing changes in the embryo size from the post-collection to the post-warming to the post-incubation measurement periods. Embryos were loaded individually into sterile 0.25-ml straws and placed into a conventional bovine embryo transfer gun, covered with a sterile sheath and chemise, for non-surgical embryo transfer. Embryos were transferred transcervically to the uterine horn ipsilateral to the ovary possessing a corpus luteum (17, 18).

DNA Testing for Parentage Verification

On the private farm where this field study was conducted, it is standard operating procedure to perform DNA analysis on all animals to be registered in the alpaca breed association (Alpaca Owners Association, Inc.; formed from the merger of the Alpaca Owners & Breeders Association, Inc. and the

TABLE 1 | Size (in µm) of alpaca preimplantation embryos ($n = 4$) measured post-collection, post-warming/pre-incubation, and post-culture/pre-transfer.

Donor ID tag	Post-collection (fresh embryo)	Post-warming	Post-culture/ pre-transfer	Recipient ID tag	Uterine horn of embryo deposition[a]	Pregnancy outcome
687	375 × 400	350 × 375	725 × 775	1505	Left	Pregnant
943	550 × 575	575 × 575	625 × 675	1356	Left	Not pregnant
1615	275 × 275	200 × 200	200 × 250	1038[b]	Right	Not pregnant
1501	325 × 400	300 × 350	300 × 350			

[a] *In all instances, embryos were transferred non-surgically to the uterine horn ipsilateral to the ovary that possessed a corpus luteum.*
[b] *This recipient received two embryos.*

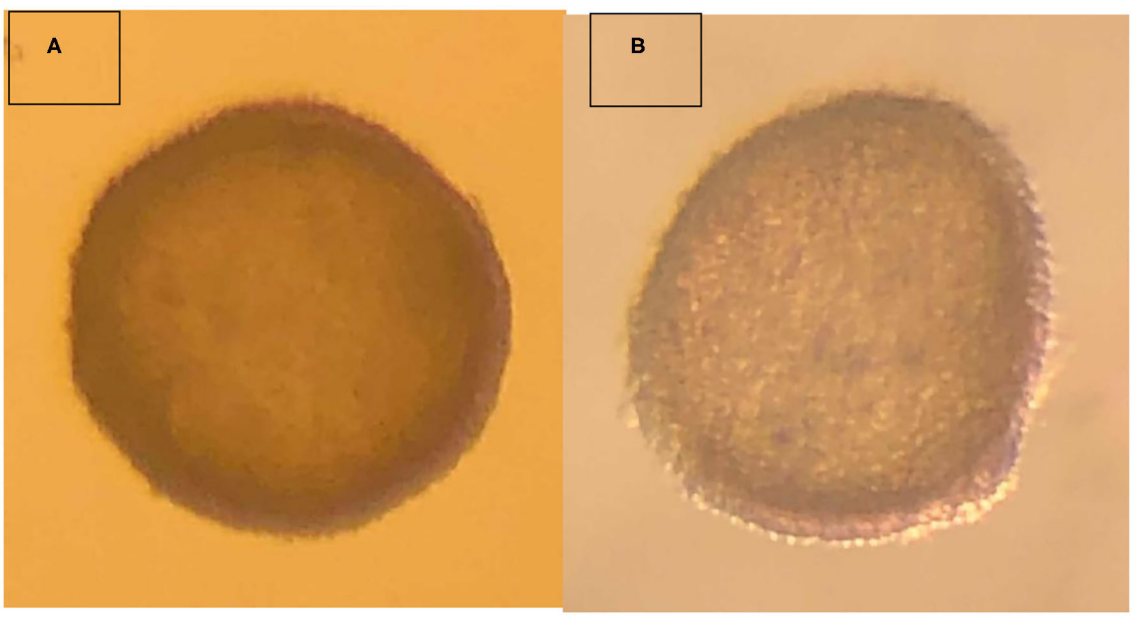


FIGURE 1 | Vitrified-warmed alpaca preimplantation embryo that resulted in the birth of a live cria. **(A)** depicts the freshly collected embryo, and **(B)** depicts the embryo post-warming.

Alpaca Registry, Inc.). Due to contractual agreements, the Alpaca Registry, Inc. and the Alpaca Owners Association, Inc. used different commercial DNA testing laboratories.

The DNA testing of the donor female was performed in November 2006 by the University of California–Davis veterinary genetics laboratory (Davis, California, USA). A whole blood sample was collected using a 3-cc syringe and then transferred into a 2-ml lavender top blood collection tube containing liquid EDTA as an anticoagulant. The blood tube was mailed to The Alpaca Registry, Inc. who subsequently forwarded it to the commercial DNA testing laboratory for analysis.

The DNA testing of the breeding sire and the recipient female was performed in January 2016 by DDC DNA Diagnostics Center (Fairfield, Ohio, USA), and the same laboratory performed DNA analysis of the cria produced after transfer of a vitrified-warmed embryo in November 2019. Blood samples were collected using a 3-cc syringe, and a few drops of blood were placed on a Whatman® FTA® card (Cytiva, Little Chalfont, UK) and allowed to dry before shipment via mail to the commercial DNA testing laboratory.

RESULTS

One embryo was recovered from each of the four donors, and all recovered embryos were hatched blastocysts with a mean diameter of 412 ± 62 μm (range of 275–575 μm). Embryo diameter was also measured post-warming and post-incubation/pre-transfer (**Table 1**). One embryo failed to increase in diameter during *in vitro* culture, while the remaining embryos

FIGURE 2 | Recipient female and cria resulting from the transfer of a vitrified-warmed alpaca preimplantation embryo. The cria was born November 13, 2019.

TABLE 2 | DNA verification of the parentage of the cria produced from a vitrified-warmed embryo.

	Donor female[a]		Breeding sire[b]		Recipient female[b]		Cria[b]	
DNA marker	**A1[c]**	**A2**	**A1**	**A2**	**A1**	**A2**	**A1**	**A2**
LCA5	192[d]	202	190	202	202	202	202	202
LCA8	231	241	237	259	237	243	237	241
LCA19	102	112	100	102	102	102	102	102
LCA37	n.d.	n.d.	134	134	134	158	134	156
LCA56	n.d.	n.d.	202	202	**198[e]**	**200**	202	226
LCA66	226	236	227	236	227	230	227	236
LPAC3	313	313	313	313	313	323	313	313
LPAC9	286	290	292	292	286	290	290	292
LPAC18	n.d.	n.d.	266	274	**268**	**268**	274	284
LPAC23	124	124	124	124	124	128	124	124
LPAC25	129	131	123	131	123	129	123	131
LPAC39	284	284	290	297	**285**	**293**	284	297
VOLP32	n.d.	n.d.	211	245	231	245	211	245
YWLL08	142	180	128	168	**136**	**160**	142	168
YWLL29	219	227	219	219	217	219	219	227
YWLL36	150	150	150	150	**164**	**170**	150	150
YWLL40	188	188	180	186	180	188	186	188

[a] *DNA testing of donor female performed by the University of California–Davis Veterinary Genetics Laboratory in November 2006.*

[b] *DNA testing performed by DDC DNA Diagnostics Center in January 2016, January 2016, and November 2019 for the breeding sire, recipient female, and cria, respectively.*

[c] *A1 denotes the allele inherited from one parent, whereas A2 denotes the allele inherited by the other parent.*

[d] *The number for each DNA marker represents identification of the specific allele; n.d. denotes a DNA marker that was not detected because it was not a part of the testing panel.*

[e] *Recipient female DNA markers LCA56, LPAC18, LPAC39, YWLL08, and YWLL36 (shown in bold font) demonstrate that the cria is genetically unrelated to the recipient.*

had a minor (<25%), moderate (>25%, but <50%), or large (>50%) increase in volume during incubation.

Embryos that grew during culture ($n = 2$) and that were considered highly viable based on their growth during *in vitro* culture were transferred individually into synchronous recipients, whereas embryos whose post-culture diameter was similar or exhibited a minor increase in their pre-vitrification diameter ($n = 2$) were transferred together into a single recipient (with the hopes of sending a stronger signal for maternal recognition of pregnancy).

Among the three recipients, only one pregnancy was detected (in the recipient that received the vitrified-warmed embryo which exhibited a large amount of growth in culture post-warming). The overall embryo survival rate was 25% (one of four embryos).

The embryo (**Figure 1**) that nearly doubled in size during *in vitro* culture gave rise to a viable offspring. On November 13, 2019, a healthy female cria (**Figure 2**) weighing 8.1 kg was born after a gestation of 353 days. Parentage of the embryo transfer cria was confirmed through DNA testing (**Table 2**). The cria has grown normally and, at 7.5 months of age, is free of any detectable abnormalities (**Figure 3**).

DISCUSSION

To the authors' knowledge, this birth represents the world's first cria produced after the transfer of a cryopreserved alpaca embryo. Preimplantation embryos recovered non-surgically from the uterus of alpaca donor females typically are hatched blastocysts > 400 μm in diameter (8), and this relatively large size poses a significant challenge to adequate removal of osmotically free water from the embryonic cells during the cryopreservation process. The high lipid content of alpaca embryos (7) also makes it difficult to achieve adequate embryo dehydration because the heads of phospholipids are hydrophilic.

Vitrification utilizes an ultrarapid cooling rate that causes intracellular water to transition from liquid to solid so quickly that ice crystal formation cannot occur (6). Vitrification has proven quite useful for large diameter equine blastocysts (13), as well as for high lipid content porcine (20) and dromedary camel embryos (21). We successfully employed a modified vitrification protocol to overcome the challenges to cryopreservation typically associated with large diameter and/or high lipid content embryos. The modifications we made to the previously published vitrification protocol (12–14) included the following: (1) replacement of glucose with galactose in the base medium, (2) replacement of fetal bovine serum with alpaca serum in all solutions containing serum, (3) use of a commercially available embryo holding medium for post-warming/pre-transfer temporary holding, (4) performing asynchronous embryo transfer (no more than 24 h asynchronous; standard operating procedure for embryo transfer on this farm) as is common in pigs (22) and horses (23), and (5) an overnight *in vitro* culture period to enable post-warming observation of greater duration (because embryos that initially appear viable shortly after warming often fail to produce pregnancies after embryo transfer); this enables a more accurate *in vitro* assessment of post-warming embryo viability.

We speculate that one or more of three factors likely contributed to our success in this study, although the small number of embryos used in this field study precludes an exact determination. Firstly, the vitrified-warmed embryo that produced the live cria was a relatively small hatched blastocyst (400 × 375 μm). Water removal from smaller diameter blastocysts should be easier than with larger diameter blastocysts (9). However, the effect of the size of camelid embryos on post-transfer pregnancy rate is unclear. In dromedary camels, embryos between 250 and 500 μm in diameter were more tolerant to vitrification than embryos <250 μm in diameter (24), yet embryo diameter had no effect in another study (21).

Secondly, we used galactose rather than glucose in the base medium used to prepare vitrification solutions, as had been done previously for bovine *in vitro* produced (25) and equine embryos (26). We also used galactose rather than sucrose as the non-permeating compound in the warming solutions as was done previously with equine (12, 13, 26), bovine *in vitro* produced (27), ovine (28), and dromedary camel embryos (14). The reasons for the greater apparent tolerance of alpaca embryos to the monosaccharide galactose

FIGURE 3 | Cria (medium fawn color, in foreground) produced from a vitrified-warmed alpaca preimplantation embryo at ∼7.5 months of age, demonstrating the normalcy of postnatal development.

vs. the disaccharide sucrose (14) are not known at present. Galactose and glucose are structural isomers (they have identical molecular formulas but different structures), whereas sucrose consists of glucose and fructose (a structural isomer of both galactose and glucose) held together by a glycosidic bond. Perhaps galactose is more beneficial than glucose in the initial pre-cooling dehydration of the embryo prior to vitrification. Alternatively, galactose may provide enhanced protection of cell membranes during vitrification and warming or may facilitate greater removal of permeating cryoprotectant from the embryo post-warming.

Thirdly, the recipient females in this study were housed in a herd that is accustomed to daily contact with humans. We believe that this low-stress environment may have enabled not only establishment of the pregnancy but also maintenance of the pregnancy to term. Previous work by several members of this same research team (10, 11) had resulted in several pregnancies with cryopreserved alpaca embryos; however, all pregnancies were lost within the first 4 months of gestation. Pregnancy loss in extensively managed alpacas is quite common and can range from 50 to 70% (29).

This report with alpacas follows a limited number of reports of successful cryopreservation of llama (30, 31) and dromedary camel embryos (21), as well as reports of pregnancies with frozen-thawed (32) and vitrified-warmed (33) alpaca embryos that apparently did not result in live births. In the llama

research in Argentina (30), hatched blastocysts collected on Days 8.0–8.5 were vitrified in 0.25-ml straws in VS containing glycerol, ethylene glycol, sucrose, glucose, and polyethylene glycol (PEG). Eight vitrified-warmed embryos were transferred into four recipients, two of which became pregnant. Llama embryo cryopreservation research conducted in the United States (31) examined vitrification of Day 7 hatched blastocysts which had undergone pre-vitrification blastocoele cavity collapse and were subsequently vitrified in 0.25-ml straws using VS comprised of glycerol, butanediol, PEG, sucrose, and fetal bovine serum. The reduction of blastocoele fluid volume led to a higher pregnancy rate when compared with intact blastocysts. Those two llama studies differed from the present study in a multitude of ways: different VS, different WS, vitrification in straws vs. OPS, intact vs. collapsed blastocyst, and differences in post-warming incubation (0 or 2.5 h vs. 20–22 h in the present study). Dromedary research conducted in the United Arab Emirates (21) used a commercial camel embryo vitrification kit, presumably based on a previously published protocol (14), that was tested with or without bovine serum albumin supplementation. That study was comparable to ours, except that all warmed embryos were transferred as pairs. The 60-day pregnancy rate achieved was 37.5% (15 pregnancies/40 transfers), with an overall embryo survival rate of 19%.

The successful production of a live cria from the transfer of a vitrified-warmed alpaca embryo in this study gives great hope for further development and use of this reproductive biotechnology in genetic improvement programs for alpacas. We have produced a second live cria in Peru using the same protocol (Vivanco et al., unpublished data), which lends credence to our technique. Further research with a larger number of animals, however, is needed to ensure that our technique is robust.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

Ethical review and approval was not required for this animal study because embryo collection and transfer, which were implemented in 2011 at Cas-Cad-Nac Farm, are routine animal husbandry procedures on this farm. This project involved only client-owned animals, all animal-related work was done by the animal owner, and best practices for veterinary care were followed. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

The idea for this experiment originated with JL. The experimental approach was developed by JL, CY, and PT using the methodology developed in part by HV-M, DP-S, and MM-G. The experiment was conducted by JL, SJ, and KD. The manuscript was written and/or edited by all authors.

FUNDING

This study was funded by Cas-Cad-Nac Farm.

REFERENCES

1. FAOSTAT (2018). Available online at: http://www.fao.org/faostat/en/#data/QA (accessed October 13, 2020).
2. Haudry De Soucy R. *Investing in the development of South American campesino camelid economies: the experience of the international fund for agricultural development* (IFAD). In: Pérez-Cabal MÁ, Gutiérrez JP, Cervantes I, Alcalde MJ, editors. *Fibre Production in South American Camelids and Other Fibre Animals*. Wageningen Academic Publishers: Wageningen (2011) 195–99. doi: 10.3920/978-90-8686-727-1_25
3. Stuart CC, Bathgate R. Advancing assisted reproductive technologies in camelids (especially the alpaca). *Rural Industries Research & Development Corporation Publication Number 15/067*. (2015). p. 36.
4. Palomino JM, Jones L, Vanhanen T, Mastromonaco GF, Busato R, Adams GP. Alpaca embryo transfer on a private Canadian farm. *Can Vet J*. (2018) 59:631–4.
5. Trasorras VL, Carretero MI, Neild DM, Chaves MG, Giuliano SM, Miragaya MH. Production, preservation, and transfer of South American camelid embryos. *Front Vet Sci*. (2017) 4:190. doi: 10.3389/fvets.2017.00190
6. Youngs CR, Leibo SP, Godke RA. Embryo cryopreservation in domestic mammalian livestock species. *CAB Rev*. (2010) 5:11. doi: 10.1079/PAVSNNR20105060
7. von Baer A, Del Campo MR, Donoso X, Toro F, von Baer L, Montecinos S, et al. Vitrification and cold storage of llama (*Lama glama*) hatched blastocysts. *Theriogenology*. (2002) 57:489.
8. Picha Y, Tibary A, Memon M, Kasimanickam R, Sumar J. Chronology of early embryonic development and embryo uterine migration in alpacas. *Theriogenology*. (2013) 79:702–8. doi: 10.1016/j.theriogenology.2012.11.027
9. Stout TAE. Cryopreservation of equine embryos: current state-of-the-art. *Reprod Dom Anim Suppl*. (2012) 3:84–9. doi: 10.1111/j.1439-0531.2012.02030.x
10. Vivanco-Mackie HW, Salazar MDP, Miguel M, Youngs C, Asparrin M. Embryo survival to calving according to type of embryo and embryo transfer method in alpacas. *Reprod Fertil Dev*. (2015) 27:173. doi: 10.1071/RDv27n1Ab164
11. Vivanco-Mackie HW, Ponce-Salazar MD, Miguel-Gonzales M, Youngs CR, Jara C, Asparrin M. Comparative study between slow freezing and vitrification on the survival rate of cryopreserved alpaca embryos post-transfer. *Reprod Fertil Dev*. (2019). 31:182. doi: 10.1071/RDv31n1Ab112
12. Eldridge-Panuska WD, Caracciolo di Brienza V, Seidel GE, Squires EL, Carnevale EM. *Establishment of pregnancies after serial dilution or direct transfer by vitrified equine embryos. Theriogenology*. (2005) 63:1308–19. doi: 10.1016/j.theriogenology.2004.06.015
13. Diaz F, Bondioli K, Paccamonti D, Gentry GT. Cryopreservation of day 8 equine embryos after blastocyst micromanipulation and vitrification. *Theriogenology*. (2016) 85:894–903. doi: 10.1016/j.theriogenology.2015.10.039
14. Herrid M, Billah M, Skidmore JA. Successful pregnancies from vitrified embryos in the dromedary camel: avoidance of a possible toxic effect of sucrose on embryos. *Anim Reprod Sci*. (2017) 187:116–23. doi: 10.1016/j.anireprosci.2017.10.015
15. Wu H, Guang X, Al-Fageeh M, Wang J. Camelid genomes reveal evolution and adaptation to desert environments. *Nat Commun*. (2014) 5:5188. doi: 10.1038/ncomms6188
16. Sumar J, Bravo PW, Foote WC. Sexual receptivity and time of ovulation in alpacas. *Small Ruminant Res*. (1993) 11:143–50. doi: 10.1016/0921-4488(93)90147-A

17. Vaughan J, Mihm M, Wittek T. Factors influencing embryo transfer success in alpacas—a retrospective study. *Anim Reprod Sci*. (2013) 136:194–204. doi: 10.1016/j.anireprosci.2012.10.010
18. Trasorras V, Chaves MG, Neild D, Gambarotta M, Aba M, Agüero A. Embryo transfer technique: factors affecting the viability of the corpus luteum in llamas. *Anim Reprod Sci.* (2010) 121:279–85. doi: 10.1016/j.anireprosci.2010.06.004
19. Parraguez VH, Cortéz S, Gazitúa FJ, Ferrando G, MacNiven V, Raggi LA. Early pregnancy diagnosis in alpaca *(Lama [sic] pacos) and llama (Lama glama)* by ultrasound. Anim Reprod Sci. (1997) 47:113–21. doi: 10.1016/S0378-4320(96)01630-2
20. Cuello C, Martinez C, Nohalez A, Parrilla I, Roca J, Gil MA, et al. Effective vitrification and warming of porcine embryos using a pH-stable, chemically defined medium. *Sci Rep*. (2016) 6:33915. doi: 10.1038/srep 33915
21. Skidmore JA, Vaughan JL, Herrid M. Successful vitrification of dromedary camel embryos using a novel embryo vitrification kit. *Anim Reprod Sci*. (2020) 218:106483. doi: 10.1016/j.anireprosci.2020.106483
22. Youngs CR. Factors influencing the success of embryo transfer in the pig. *Theriogenology*. (2001) 56:1311–20. doi: 10.1016/S0093-691X(01) 00632-X
23. Sieme H, Rau J, Tiedemann H, Oldenhof L, Barros R, Sanchez M, et al. Equine embryo transfer. In: Niemann H, Wrenzycki C, editors. *Animal Biotechnology* Cham (2018). p. 179–92. doi: 10.1007/978-3-319-92327-7_8
24. Herrid M, Billah M, Malo C, Skidmore JA. Optimization of a vitrification protocol for hatched blastocysts from the dromedary camel (*Camelus dromedarius*). *Theriogenology*. (2016) 85:585–90. doi: 10.1016/j.theriogenology.2015.09.048
25. Leibo SP, Martino A, Kobayashi S, Pollard JW. Stage-dependent sensitivity of oocytes and embryos to low temperatures. *Anim Reprod Sci.* (1996) 42:45–53. doi: 10.1016/0378-4320(96)01543-6
26. Young CA, Squires EL, Seidel GE, Kato H, McCue PM. Cryopreservation procedures for Day 7–8 equine embryos. *J Equine Vet Sci.* (1997) 29:98–102. doi: 10.1111/j.2042-3306.1997.tb05111.x
27. Kaidi S, Van Langendonckt A, Massip A, Dessy F, Donnay I. Cellular alteration after dilution of cryoprotective solutions used for the vitrification of *in vitro*-produced bovine embryos. *Theriogenology*. (1999) 52:515–25. doi: 10.1016/S0093-691X(99)00148-X
28. Baril G, Traldi AL, Cognié Y, Leboeuf B, Beckers JF, Mermillod P. Successful direct transfer of vitrified sheep embryos. *Theriogenology*. (2001) 56:299–305. doi: 10.1016/S0093-691X(01)00564-7
29. Raggi LA. Advances in research and management of llamas (*Lama glama*) and alpaca [sic] (*Lama* [sic] *pacos*) in chile. *Cien Investig Agr*. (2020) 27:27–37. doi: 10.7764/rcia.v27i1.994
30. Aller JF, Rebuffi GE, Cancino AK, Alberio RH. Successful transfer of vitrified llama (*Lama glama*) embryos. *Anim Reprod Sci*. (2002) 73:121–7. doi: 10.1016/S0378-4320(02)00120-3
31. Taylor P, Taylor S, Sansinena M, Godke R. Llama (*Lama glama*) pregnancies from vitrified/warmed blastocysts using a novel coaxial cryoprotectant microinjection system. *Reprod Fertil Dev*. (2005) 18:164. doi: 10.1071/RDv18n2Ab112
32. Paredes A, Deza H, Perez UH, Pérez MG. Viabilidad *in vitro* y *in vivo* de embriones de alpaca congelados/descongelados en dos agentes crioprotectores [Viability *in vitro* and *in vivo* of frozen/thawed alpaca embryos in two cryoprotectants agents] (2015). *Spermova. 5:115–8*. doi: 10.18548/aspe/0002.26
33. Cassa Salas A. *Vitrificación y Calidad de Embriones de Alpaca (Vicugna pacos) Recuperados por Superovulación Utilizando la Hormona Gonadotropina Coriónica Equina (eCG)*. Abancay: Tesis para Médico Veterinario y Zootecnista, Universidad Nacional Micaela Bastidas de Apurímac (2018). p. 105.

Conflict of Interest: The authors declare that this research was financed by and conducted at Cas-Cad-Nac Farm. JL, SJ, and KD are affiliated with or employed by Cas-Cad-Nac Farms. PT is affiliated with GeneSearch, Inc. HV-M, DP-S, and MM-G are affiliated with or employed by Vivanco International SAC. Although these results directly benefit Cas-Cad-Nac Farm, GeneSearch, Inc, and Vivanco International SAC, these results also benefit alpaca breeders throughout the world because they are being published in an open access journal.

The remaining author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 10 December 2020
doi: 10.3389/fvets.2020.597952

Air-Drying Llama Sperm Affects DNA Integrity

María Ignacia Carretero[1,2,3]*, *María Graciela Chaves*[1,2], *Claudia Cecilia Arraztoa*[1,2], *Fernanda Gabriela Fumuso*[1,2,3], *Mariana Carla Gambarotta*[1] and *Deborah Margarita Neild*[1,2]

[1] Facultad de Ciencias Veterinarias, Universidad de Buenos Aires, Buenos Aires, Argentina, [2] Instituto de Investigación y Tecnología en Reproducción Animal, Universidad de Buenos Aires, Buenos Aires, Argentina, [3] Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Buenos Aires, Argentina

The objective of this study was to evaluate the effects of air-drying preservation on llama sperm DNA. Semen collections were carried out using electroejaculation under general anesthesia. A total of 16 ejaculates were processed from 4 males ($n = 4$, $r = 4$). Each sample was diluted 4:1 in a collagenase solution in TALP media, then incubated and centrifuged at 800 g for 8 min. The pellet was re-suspended to a concentration of 20 million sperm/ml in TALP. Then the samples were placed onto sterile slides forming lines and were left to dry under laminar flow for 15 min. After this, the slides were placed into Falcon centrifuge tubes and kept at 5°C. Sperm characteristics (motility, membrane function, viability and morphology) were evaluated in raw semen and in the air-dried samples kept at 5°C for 30 min. DNA evaluation (integrity and degree of chromatin condensation) was carried out in raw semen and in the air-dried samples after 30 min, 7, 14, 21, 30, and 60 days after preservation. To compare raw semen to the air-dried samples, a Wilcoxon test was used for all sperm characteristics except for DNA, where a paired Student *t*-test was applied. A split plot design was used to compare chromatin condensation between the different periods of preservation and a Kruskal Wallis test was used to compare DNA integrity. Motility, membrane function, viability and sperm with intact DNA decreased in the air-dried samples ($p < 0.05$), while morphology and chromatin condensation were not affected ($p > 0.05$). No significant differences were observed in the percentage of sperm with condensed chromatin between the different periods of preservation ($p > 0.05$). On the other hand, a significant decrease in the percentage of sperm with intact DNA was observed as from day 7 of preservation ($p < 0.05$). In conclusion the air-drying process has a negative effect on llama sperm DNA, hence the media used will need to be improved to protect DNA and be able to implement this technique in this species.

Keywords: air-dried, chromatin, DNA, llama, preservation, sperm

OPEN ACCESS

Edited by:
Marcelo H. Ratto,
Austral University of Chile, Chile

Reviewed by:
Fabiana Souza,
São Paulo State University, Brazil
Abouzar Najafi,
University of Tehran, Iran

***Correspondence:**
María Ignacia Carretero
ignaciacarretero@gmail.com;
ignaciacarretero@fvet.uba.ar

Specialty section:
This article was submitted to Animal Reproduction - Theriogenology, a section of the journal Frontiers in Veterinary Science

Received: *22 August 2020*
Accepted: *19 November 2020*
Published: *10 December 2020*

Citation:
Carretero MI, Chaves MG, Arraztoa CC, Fumuso FG, Gambarotta MC and Neild DM (2020) Air-Drying Llama Sperm Affects DNA Integrity. Front. Vet. Sci. 7:597952. doi: 10.3389/fvets.2020.597952

INTRODUCTION

Artificial insemination (AI) with cryopreserved semen is a very useful tool for applying genetic improvement in herds; however, this biotechnology has not been effective in South American camelids (SACs). Attempts to extrapolate successful protocols in other species without considering the distinctive reproductive characteristics of SACs have led to errors and a slow development of AI in these species, obtaining pregnancy rates between 0 and 33% with cryopreserved semen (1–7).

Alternative preservation methods such as dehydration or air-drying sperm have been explored in different species [man: (8); mice: (9); and horses: (10, 11)]. These non-conventional techniques have the disadvantage that sperm become immotile and have impaired membranes, making it necessary to recur to intracytoplasmic sperm injection (ICSI) to obtain embryos. However, dehydration or air-drying have the advantages of easy preparation and low cost preservation of the samples, especially valuable for use in the field under difficult and often precarious conditions, as is the case of most of the work carried out in SACs and especially the wild species. In addition, with these methods the samples can be transported at 5°C, on slides or in tubes, without the need for cryoprotectants or liquid nitrogen and can also be used to conserve sperm recovered from the epididymis of post mortem individuals, allowing the conservation of genetic material of wild and/or endangered species.

Despite the various advantages of these alternative techniques, it is still unknown whether the sperm genetic material suffers any alterations over storage time. Alonso et al. (11) observed a higher percentage of DNA fragmentation in equine air-dried sperm compared to the control (cooled sperm), but the number of the samples was too low for a statistical analysis. Is interesting to highlight that sperm DNA quality is a very important parameter to evaluate because assisted reproductive techniques make it possible for sperm with damaged DNA to fertilize (12). Although the oocyte can repair a certain degree of DNA damage after fertilization, once this threshold is exceeded one or two effects are seen: (1) the percentage of embryo development decreases, known as embryo development block (13), and/or (2) early embryo loss is observed, also identified as a late paternal effect (14, 15). The ability to repair this damage would seem to be related to the genomic and cytoplasmic quality of the oocyte and to the amount of chromatin damage of the spermatozoa (13). Therefore, it is impossible to ignore the role of paternal DNA in early embryo development (16) and consequently, sperm DNA evaluation becomes important.

In this context, the objective of this study was to evaluate the effect of air-drying on llama sperm DNA.

MATERIALS AND METHODS

Reagents

Collagenase, 6-carboxyfluorescein diacetate, dimethyl sulfoxide, propidium iodide, toluidine blue, agaroses and the reagents for the TALP medium were purchased from Sigma Chemicals (Sigma Aldrich, Buenos Aires, Argentina). Type I collagenase (*Clostridium peptidase A from Clostridium histolyticum*) was used. The TALP medium was prepared according to Parrish et al. (17): NaCl (114 mM), KCl (3.1 mM), $NaHCO_3$ (2 mM), NaH_2PO_4 (0.3 mM), Na lactate (10 mM), $CaCl_2$ (2 mM), $MgCl_2$ (0.5 mM), Pyruvate (0.2 mM), HEPES (10 mM) and bovine serum albumin (3 mg ml/1).

Abbreviations: SACs, South American Camelids; SCD, Sperm Chromatin Dispersion assay; TB, Toluidine Blue.

Animals and Location

The study was carried out at the Faculty of Veterinary Sciences of the University of Buenos Aires, in Buenos Aires, Argentina. The city is situated at sea level, latitude 34° 36′ and longitude 58° 26′.

For the study, 4 male *Lama glama* ranging between 7 and 11 years of age and weighing 140.75 ± 18.32 kg (mean ± SD) were used. Animals were kept out at pasture in pens and supplemented with alfalfa pellets, fresh water *ad libitum*. All males were shorn during the month of November.

Semen Collection

Semen collections were carried out between the months of April and October using electroejaculation (EE) under general anesthesia according to Director et al. (18). The frequency of collection for each male was determined randomly. As EE requires general anesthesia, this method was not used on the same male at an interval of < 15 days and the collection frequency for each male was between 15 and 21 days. All procedures were approved by the Committee for the Use and Care of Laboratory Animals (CICUAL) of the Faculty of Veterinary Sciences of the University of Buenos Aires (protocol 2017/84).

Llama Sperm Preservation Using Air-Drying

A total of 16 ejaculates from 4 males ($n = 4$, $r = 4$) were processed according to Giuliano et al. (19). Briefly, each sample was diluted 4:1 in a 0.1% collagenase solution (1 mg/ml) in TALP medium. Samples were subsequently incubated 4 min at 37°C and then centrifuged 8 min at 800 *g*. The pellet was re-suspended to a concentration of 20 million sperm/ml in TALP. Then the samples were placed onto sterile slides forming lines and were left to dry under laminar flow for ∼15 min. After this, the slides were placed into Falcon centrifuge tubes covered with aluminum foil and were kept at 5°C until evaluation. To carry out the evaluations, TALP medium was placed on the slides and then aspirated with a micropipette and placed in a microcentrifuge tube (Eppendorf® AG, Germany).

Evaluation of Sperm Characteristics

Seminal characteristics (motility, morphology, membrane function and integrity or viability) were evaluated in raw semen and in air-dried samples preserved at 5°C for 30 min.

Sperm motility was evaluated using a phase contrast microscope (100 x magnification) and a warm stage (37°C). Sperm numbers were calculated using a Neubauer hemocytometer. The hypoosmotic swelling (HOS) test for evaluating membrane function, and the fluorochromes 6-carboxyfluorescein diacetate (CFDA) and propidium iodide (PI) for evaluating membrane integrity or viability, were used according to Giuliano et al. (20). Briefly, for the HOS test, samples were incubated (37°C) in a hypoosmotic solution containing fructose and sodium citrate (50 mOsmol/L). After incubation, a minimum of 200 spermatozoa were evaluated using a phase contrast microscope (400 x magnification). Sperm showing the characteristic swelling of the tail were classified as HOS positive, having a functional plasma membrane. For evaluating membrane integrity or viability, samples were incubated (37°C)

with CFDA and PI in an isotonic saline solution described by Harrison and Vickers (21) (NaCl 140 mM, glucose 10 mM, ClK 2.5 mM, polyvinyl-pyrrolidone 0.5 mg/ml and HEPES 20 mM). A minimum of 200 spermatozoa were evaluated per sample using an epifluorescence microscope with a rhodamine and standard fluorescein filter set (400 x magnification). Spermatozoa that fluoresced green throughout their length were classified as being viable (intact plasma membrane) while sperm nuclei that fluoresced red were classified as non-viable (damaged plasma membrane).

Sperm morphology was assessed placing a micro droplet between a slide and coverslip and using a phase contrast microscope (1,000 x magnification). For each sample, 200 spermatozoa were evaluated, and were classified into one of the following categories: normal, abnormal head, detached head, abnormal tail and cytoplasmic droplet. Thus, percentages of spermatozoa with normal or altered morphology were determined.

Sperm DNA Evaluation

DNA evaluation was carried out in raw semen and in air-dried samples preserved for 30 min and for 7, 14, 21, 30, and 60 days, using two methods.

Toluidine Blue (TB) Stain

The TB stain was carried out according Carretero et al. (22) to evaluate the degree of condensation/decondensation of llama sperm chromatin. Briefly, smears were made on clean, non-greasy slides and once dry were submerged for 2 min in ethanol 96° to fix them. Then slides were covered with 2 ml of a working solution of TB (0.02%). Preparations were observed directly under immersion oil (1,000 x magnification) evaluating a minimum of 200 spermatozoa per smear. Sperm were classified into three groups according to the degree of chromatin condensation: light blue (negative, condensed chromatin), light violet (intermediate, some degree of decondensation) and dark blue-violet (positive, high degree of decondensation). Positive and intermediate sperm were considered to have altered chromatin condensation. Dithiothreitol (DTT) 1% in distilled water was used as a positive control for sperm DNA decondensation.

Sperm Chromatin Dispersion Assay (SCD)

The SCD assay was carried out according to Carretero et al. (23) to evaluate the degree of DNA fragmentation. Briefly, aliquots of 50 μl of sperm suspension (5 million sperm/ml in PBS) were mixed with low-melting-point aqueous agarose and pipetted onto a glass slide previously coated with normal-melting-point aqueous agarose, covered with a coverslip and left to solidify at 4°C for 10 min. Then, each slide was incubated in different lysing solutions, dehydrated in sequential ethanol baths and stained with Giemsa. Preparations were observed directly under immersion oil (1,000 x magnification) evaluating a minimum of 200 spermatozoa per sample. Sperm were classified into the following categories: intact DNA (nuclei with large DNA dispersion halos + nuclei with medium-sized halos) and fragmented DNA (nuclei with small halos + nuclei with no halo). Samples incubated with NaOH 0.3 M during 30 min were used as a positive control of sperm DNA fragmentation.

Statistical Analysis

Statistical analysis was carried out using the InfoStat software. Normal distribution and homogeneity of variances of the data was checked by Shapiro-Wilk Normality test and an ANOVA, respectively. The level of significance was set at 0.05 for all analysis.

A Wilcoxon test was used to compare sperm motility, morphology, plasma membrane function and integrity and a paired Student *t*-test was used to compare chromatin condensation and DNA integrity, between raw semen and samples air-dried for 30 min. A split plot design was used to compare the degree of chromatin condensation between the different periods of preservation of the air-dried samples, blocking the males. Whereas, a Kruskal Wallis test was used to evaluate DNA fragmentation (SCD test) between the air-dried samples.

RESULTS

Sperm Characteristics

Air-dried sperm were immotile in all samples. The percentages of sperm with membrane integrity (viable sperm) and with functional membranes (HOS positive) were significantly lower in air-dried samples compared to raw semen ($p < 0.05$). Sperm morphology was not altered due to the process of air-drying (**Table 1**).

Sperm DNA Condensation

There was no significant difference for chromatin condensation either between raw semen and samples air-dried for 30 min or between the different periods of preservation of air-dried samples (**Figure 1**). In addition, 100% of the sperm incubated with DTT (control) were TB positive.

TABLE 1 | Seminal characteristics evaluated in raw semen and air-dried llama sperm preserved at 5°C during 30 min.

Sperm characteristics	Raw semen(%)	Air-dried sperm (%)
Motility	22.0 ± 10.9[a]	0.0[b]
Membrane function (HOS test)	36.8 ± 13.0[a]	2.2 ± 1.3[b]
Membrane integrity (viability)	60.1 ± 24.2[a]	0.2 ± 0.5[b]
Normal morphology	65.1 ± 14.3[a]	66.0 ± 4.7[a]
Abnormal heads	12.2 ± 2.8[a]	18.0 ± 2.7[a]
Detached heads	2.0 ± 1.6[a]	3.0 ± 0.5[a]
Abnormal tails	7.4 ± 2.4[a]	10. ± 6.1[a]
Cytoplasmatic droplets	13.1 ± 13.9[a]	3.0 ± 2.4[a]

Values are expressed as mean ± SD (n = 4; r = 4).
a, b Different letters between columns indicate significant differences for each sperm characteristic evaluated (p < 0.05).

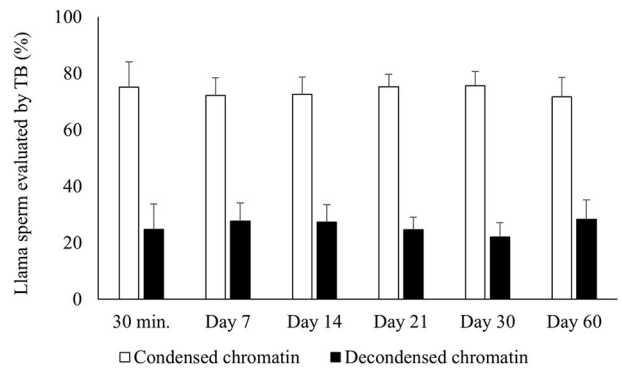


FIGURE 1 | Percentages of air-dried llama sperm with condensed (TB negative) and decondensed chromatin (TB intermediate + positive). Air-drying was evaluated after different times of preservation (30 min and 7, 14, 21, 30, and 60 days) ($n = 4$, $r = 4$). TB, Toluidine blue stain for evaluating sperm chromatin condensation. No significant differences were observed between the periods of preservation within each category ($p > 0.05$).

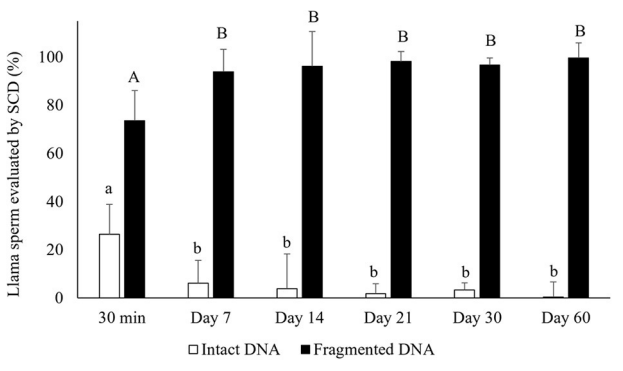


FIGURE 2 | Percentages of sperm with intact DNA (large dispersion halos + medium sized halos) and fragmented DNA (small halos + nuclei with no halo) in air-dried llama sperm samples. The effect of air-drying on sperm was evaluated after different periods of preservation (30 min and 7, 14, 21, 30, and 60 days) ($n = 4$, $r = 4$). SCD, Sperm Chromatin Dispersion test, used for evaluating sperm DNA fragmentation. [a,b]Different letters indicate significant differences in percentages of intact DNA between periods of preservation ($p < 0.05$). [A,B]Different letters indicate significant differences in percentages of fragmented DNA between periods of preservation ($p < 0.05$).

Sperm DNA Fragmentation

Air-drying significantly decreased the percentages of sperm with intact DNA compared to raw semen (intact DNA: 26.4 ± 14.4 and 82.5 ± 12.5% for air-dried sperm for 30 min and raw semen, respectively, mean ± SD). The period of preservation of air-dried samples affected the percentages of sperm with intact DNA, observing a significant decrease from day 7 of preservation ($p < 0.05$) (**Figure 2**). In addition, 100% of the sperm incubated with NaOH (control) presented fragmented DNA (no halos).

DISCUSSION

This is the first study to evaluate the simple, inexpensive method of air-drying to preserve llama spermatozoa parameters. Not surprisingly, llama air-dried sperm were immotile. The loss of motility of lyophilized rabbit sperm has been reported (24), as well as that of desiccated rhesus macaque semen submitted to nitrogen gas (25). Sitaula et al. (26) reported that changes in cell volume due to exposition to hyper or hypo-osmotic solutions results in an irreversible loss of motility, showing that one of the main factors that contributes to this loss is an osmotic effect. Similarly, viability or membrane integrity of air-drying llama sperm was very low (0.2 ± 0.5%), coinciding with reports from other authors evaluating freeze-dried mice, rabbit and bull sperm (24, 27, 28). Hence, the osmotic changes that sperm undergo, both during air-drying and when the samples are re-hydrated, could be the cause of the lack of motility and the extremely low percentages of viable sperm observed in this study. However, embryos have been obtained using ICSI with both non-motile and dead sperm in other species (9, 27).

Sperm morphology was not altered by the process of air-drying. Due to the large morphological variability of human spermatozoa (29) the influence of sperm morphology on ICSI results has been extensively studied in men, though with contradictory results (30–33). However, despite similarly variable morphology of llama sperm and the reported success in obtaining llama embryos using ICSI with ejaculated sperm (34, 35), this possible influence has not been studied in SACs. It has been proposed that subtle sperm organellar malformations cannot be detected either by the morphologist at 1,000 x magnification or by the embryologist at 200x−400x magnification. In this context, a new method for the detailed morphological evaluation of motile spermatozoa in real time: the motile sperm organellar morphology examination (MSOME) has been developed, achieving a magnification of 6,300x (36). Using MSOME, human sperm nucleus morphology has positively correlated with fertilization, implantation and pregnancy by ICSI (36, 37). These results further highlight the importance of the sperm nucleus in Assisted Reproductive Techniques (ART), especially ICSI.

With regard to the sperm nucleus, two different aspects were evaluated: the degree of chromatin condensation using TB and the degree of DNA fragmentation using the SCD test. In this study, the process of air-drying did not affect llama chromatin condensation, however, a high percentage of sperm with fragmented DNA was observed in the air-dried samples, as early as 30 min after preservation. This was surprising as we assumed that both aspects (chromatin condensation and DNA integrity) would be similarly affected. Previously, when adapting the SCD test to evaluate llama sperm, we proposed that sperm with fragmented DNA fail to produce a halo because the DNA fragments could be interacting within the llama sperm head by complementary bases (adenine-thymine; guanine-cytosine) making cohesive ends and as a result do not disperse around the nucleus core (23). It is conceivable that despite DNA being fragmented after air-drying, the fragments remained linked at their complementary bases and thus the sperm possibly did not have sites for TB to bind. Additionally, we have previously observed that llama sperm subjected to incubation at 100°C, exposed to UV or incubated with NaOH showed 100% DNA

fragmentation in SCD test, but the same samples stained with TB, did not show increased percentages of sperm with decondensed chromatin compared to untreated samples (unpublished data). Those results coincide with what we observed in this study, where despite DNA integrity being significantly decreased (SCD test), chromatin condensation (TB stain) was not affected. If this hypothesis proves to be correct, this could also be an indication that perhaps the SCD test is a more sensitive indicator of DNA alteration than the TB stain.

Although various reports have studied DNA fragmentation in sperm preserved using these alternative methods, to our knowledge there are no reports that evaluate their degree of chromatin condensation. Similar to our results, Klooster et al. (25) desiccated monkey sperm and reported high percentages of sperm with fragmented DNA using the TUNEL technique (93.2–95.5%) in samples preserved during 7 to 10 days at room temperature and at −80°C. Also, Alonso et al. (11) observed that equine sperm DNA fragmentation, evaluated with SCD, increased as the period of air-dried preservation increased (from 32 to 53% in air-dried sperm preserved during 2 days and 4 weeks, respectively). Two possible explanations for the increase in the percentages of sperm with fragmented DNA observed in llama air-dried samples could be a rise in reactive oxygen species (ROS) production and/or the release of endonucleases. The generation of low levels of ROS by spermatozoa plays an important role in different physiological events such as sperm capacitation, acrosome reaction, hyperactivation and sperm-oocyte fusion (38–41). However, high levels of ROS have been associated with cell damage, such as lipid peroxidation and DNA fragmentation (42, 43). Burnaugh et al. (44) incubated equine spermatozoa for 15 min at 38°C under hyperosmotic or hypoosmotic solutions and reported an increase in superoxide production. The changes in osmolarity that occur during the air-drying process could induce an increase in ROS levels and consequently alter sperm DNA. Regarding endonucleases, these sperm enzymes are leaked from plasma membrane-damaged spermatozoa during freeze-drying or freezing without cryoprotectants (27), are activated by divalent cation Ca^{2+} and Mg^{2+} (45) and have been proposed by Nakai et al. (46) to be one the causes of DNA fragmentation in lyophilized (freeze-dried) boar spermatozoa. Taking this into account, Nakai et al. (46) suggested that the use of chelating agents could decrease sperm DNA damage by chelating divalent cations that activate endonucleases. These authors observed that porcine spermatozoa, freeze-dried in the presence of chelating agents such as ethylenediaminetetraacetic acid (EDTA) and ethylene glycoltetraacetic acid (EGTA), presented lower DNA fragmentation values than the control group (4.1 *vs.* 12.2%). Likewise, Sitaula et al. (26) hypothesized that the addition of sugars, antioxidants and chelators to the media could help reduce oxidative stress, thereby minimizing membrane and mitochondrial damage during desiccation. With this in mind and considering the importance of developing a simple method of gamete conservation which would facilitate working with wild species under precarious conditions, it would be interesting to assay the use of media with the addition of sugars, antioxidants and/or chelating agents to evaluate if these substances have a beneficial effect on sperm air-drying and are able to preserve SAC sperm DNA integrity.

CONCLUSIONS

The air-drying process has a negative effect on llama sperm DNA, therefore the media used will need to be improved to protect DNA and be able to implement this technique in these species.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Committee for the Use and Care of Laboratory Animals (CICUAL) of the Faculty of Veterinary Sciences of the University of Buenos Aires (protocol 2017/84).

AUTHOR CONTRIBUTIONS

MIC designed, carried out the study, and wrote the manuscript. MGC, CA, and FF helped collect the samples, critically read, and corrected the manuscript. MG performed the statistical analysis. DN designed and directed the study and critically read and corrected the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This research was supported by grants from the University of Buenos Aires (UBACyT V805 and 20020170200113BA) and from the Agencia Nacional de Promoción Científica y Tecnológica (PICT 2017-0394).

REFERENCES

1. Bravo PW, Skidmore JA, Zhao XX. Reproductive aspects and storage of semen in Camelidae. *Anim Reprod Sci*. (2000) 62:173–93. doi: 10.1016/S0378-4320(00)00158-5
2. Aller JF, Rebuffi GE, Cancino AK, Alberio RH. Influencia de la criopreservación sobre la movilidad, viabilidad y fertilidad de espermatozoides de llama (*Lama glama*). *Arch Zootecnia*. (2003) 52:15–23.
3. Vaughan J, Galloway D, Hopkins D. *Artificial Insemination in Alpacas (Lama pacos)*. Kingston, VIC: RIRDC Rural Industries Research and Development Corporation. (2003).
4. Giuliano S, Chaves MG, Trasorras V, Gambarotta M, Neild D, Director A, et al. Development of an artificial insemination

protocol in llamas using cooled semen. *Anim Reprod Sci*. (2012) 131:204–10. doi: 10.1016/j.anireprosci.2012.03.010
5. García W, Alarcón V, Bravo PW. Inseminación Artificial de alpacas con semen refrigerado y con inclusión de dos tipos de yema de huevo. *Rev Inv Vet Perú*. (2017) 28:337–44. doi: 10.15381/rivep.v28i2.13080
6. Fumuso FG, Arraztoa CC, Chaves MG, Neild DM, Giuliano SM, Miragaya MH, et al. Inseminación artificial de llamas con semen congelado. *Res Prelimi Invet*. (2018) 20:127.
7. Stuart CC, Vaughan JL, Kershaw CM, de Graaf SP, Bathgate R. Effect of diluent type, cryoprotectant concentration, storage method and freeze/thaw rates on the post-thaw quality and fertility of cryopreserved alpaca spermatozoa. *Sci Rep*. (2019) 9:12826. doi: 10.1038/s41598-019-49203-z
8. Imoedemhe D, Oliva N, Adam A. Embryonic development following ICSI with human air dried sperm. *ESHRE Ann Meet*. (2003) 42:178.
9. Van Thuan N, Wakayama S, Kishigami S, Wakayama T. New preservation method for mouse spermatozoa without freezing. *Biol Reprod*. (2005) 72:444–50. doi: 10.1095/biolreprod.104.034678
10. Alonso A, Miragaya M, Losino L, Herrera C. Intracytoplasmatic sperm injection of equine oocytes using air-dried sperm or sperm stored in high osmolarity medium. *Reprod Fertil Dev*. (2007) 19:301. doi: 10.1071/RDv19n1Ab371
11. Alonso A, Baca Castex C, Ferrante A, Pinto M, Castaneira C, Trasorras V, et al. *In vitro* equine embryo production using air-dried spermatozoa, with different activation protocols and culture systems. *Andrologia*. (2015) 47:387–94. doi: 10.1111/and.12273
12. Genesca A, Caballin MR, Miro R, Benet J, Germa JR, Ecozcue J. Repair of human sperm chromosome aberrations in the hamster egg. *Hum Genet*. (1992) 89:181–6. doi: 10.1007/BF00217120
13. Sakkas D, Alvarez J. Sperm DNA fragmentation: mechanisms of origin, impact on reproductive outcome, and analysis. *Fertil Steril*. (2010) 93:1027–36. doi: 10.1016/j.fertnstert.2009.10.046
14. Tesarik J, Greco E, Mendoza C. Late, but not early, paternal effect on human embryo development is related to sperm DNA fragmentation. *Hum Reprod*. (2004) 19:611–5. doi: 10.1093/humrep/deh127
15. Borini A, Tarrozzi N, Bizzaro D, Bonu MA, Fava L, Flamigni C, et al. Sperm DNA fragmentation: paternal effect on early post-implantation embryo development in ART. *Hum Reprod*. (2006) 21:2876–81. doi: 10.1093/humrep/del251
16. Henkel R, Hajimohammad M, Stalf T, Hoogendijk C, Mehnert C, Menkeveld R. Influence of deoxyribonucleic acid damage on fertilization and pregnancy. *Fertil Steril*. (2004) 81:965–72. doi: 10.1016/j.fertnstert.2003.09.044
17. Parrish JJ, Susko-Parrish J, Winer MA, First LN. Capacitation of bovine sperm by heparin. *Biol Reprod*. (1988) 38:1171–80. doi: 10.1095/biolreprod38.5.1171
18. Director A, Giuliano S, Trasorras V, Carretero MI, Pinto M, Miragaya M. Electroejaculation in llama (*Lama glama*). *J Camel Pract Res*. (2007) 14:203–6.
19. Giuliano SM, Carretero MI, Gambarotta MC, Neild DM, Trasorras VL, Pinto M, et al. Improvement of llama (*Lama glama*) seminal characteristics using collagenase. *Anim Reprod Sci*. (2010) 118:98–102. doi: 10.1016/j.anireprosci.2009.06.005
20. Giuliano S, Director A, Gambarotta M, Trasorras V, Miragaya M. Collection method, season and individual variation on seminal characteristics in the llama (*Lama glama*). *Anim Reprod Sci*. (2008) 104:359–69. doi: 10.1016/j.anireprosci.2007.02.016
21. Harrison RA, Vickers SE. Use of fluorescent probes to assess membrane integrity in mammalian spermatozoa. *J Reprod Fertil*. (1990) 88:343–52. doi: 10.1530/jrf.0.0880343
22. Carretero MI, Giuliano SM, Casaretto CI, Gambarotta MC, Neild DM. Evaluación del ADN espermático de llamas utilizando azul de toluidina. *InVet*. (2009) 11:55–63.
23. Carretero MI, Lombardo D, Arraztoa CC, Giuliano SM, Gambarotta MC, Neild DM. Evaluation of DNA fragmentation in llama (*Lama glama*) sperm using the sperm chromatin dispersion test. *Anim Reprod Sci*. (2012) 131:63–71. doi: 10.1016/j.anireprosci.2012.02.008
24. Liu JL, Kusakabe H, Chang CC, Suzuki H, Schmidt DW, Julian M, et al. Freeze-dried sperm fertilization leads to full-term development in rabbits. *Biol Reprod*. (2004) 70:1776–81. doi: 10.1095/biolreprod.103.025957
25. Klooster KL, Burruel VR, Meyers SA. Loss of fertilization potential of desiccated rhesus macaque spermatozoa following prolonged storage. *Cryobiology*. (2011) 62:161–6. doi: 10.1016/j.cryobiol.2011.02.002
26. Sitaula R, Elmoazzen H, Toner M, Bhowmick S. Desiccation tolerance in bovine sperm: A study of the effect of intracellular sugars and the supplemental roles of an antioxidant and a chelator. *Cryobiology*. (2009) 58:322–30. doi: 10.1016/j.cryobiol.2009.03.002
27. Kusakabe H, Szczygiel MA, Whittingham DG, Yanagimachi R. Maintenance of genetic integrity in frozen and freeze-dried mouse spermatozoa. *Proc Natl Acad Sci USA*. (2001) 98:13501–6. doi: 10.1073/pnas.241517598
28. Martins CF, Báo SN, Dode MN, Correa GA, Rumpf R. Effects of freeze-drying on cytology, ultrastructure, DNA fragmentation, and fertilizing ability of bovine sperm. *Theriogenology*. (2007) 67:1307–15. doi: 10.1016/j.theriogenology.2007.01.015
29. Ombelet W, Menkveld R, Kruger TF, Steeno O. Sperm morphology assessment: historical review in relation to fertility. *Human Reprod Update*. (1995) 1:543–57. doi: 10.1093/humupd/1.6.543
30. Nagy ZP, Liu J, Joris H, Verheyen G, Tournaye H, Camus M, et al. Andrology: the result of intracytoplasmic sperm injection is not related to any of the three basic sperm parameters. *Human Reprod*. (1995) 10:1123–9. doi: 10.1093/oxfordjournals.humrep.a136104
31. De Vos A, De Velde HV, Joris H, Verheyen G, Devroey P, Van Steirteghem A. Influence of individual sperm morphology on fertilization, embryo morphology, and pregnancy outcome of intracytoplasmic sperm injection. *Fertil Steril*. (2003) 79:42–8. doi: 10.1016/S0015-0282(02)04571-5
32. Riqueros MI, Florensa M, Esbert M, Calderon A, Ballesteros A, Calderon G. Does sperm morphology affect the outcome of ICSI (intracytoplasmic sperm injection) cycles?. *Fertil Steril*. (2007) 88:S160. doi: 10.1016/j.fertnstert.2007.07.556
33. Pereira N, Neri QV, Lekovich JP, Spandorfer SD, Palermo GD, Rosenwaks Z. Outcomes of intracytoplasmic sperm injection cycles for complete teratozoospermia: a case-Control study using paired sibling oocytes. *BioMed Res Intern*. (2015) 6:470819. doi: 10.1155/2015/470819
34. Sansinena M, Taylos S, Taylor P, Schmidt E, Denniston R, Godke R. *In vitro* production of llama (*Lama glama*) embryos by intracytoplasmatic sperm injection: Effect of chemical activation treatments and culture conditions. *Anim Reprod Sci*. (2007) 99:342–53. doi: 10.1016/j.anireprosci.2006.05.020
35. Conde P, Herrera C, Chaves M, Giuliano S, Director A, Trasorras V, et al. *In vitro* production of llama embryos by IVF and ICSI with fresh semen. *Anim Reprod Sci*. (2008) 109:298–308. doi: 10.1016/j.anireprosci.2007.10.004
36. Bartoov B, Berkovitz A, Eltes F, Kogosowski A, Menezo Y, Barak Y. Real-time fine morphology of motile human sperm cells is associated with IVF-ICSI outcome. *J Androl*. (2002) 23:1–8. doi: 10.1002/j.1939-4640.2002.tb02595.x
37. Berkovitz A, Eltes F, Yaari S, Katz N, Barr I, Fishman A, et al. The morphological normalcy of the sperm nucleus and pregnancy rate of intracytoplasmic injection with morphologically selected sperm. *Human Reprod*. (2005) 20:185–90. doi: 10.1093/humrep/deh545
38. de Lamirande E, Gagnon C. Human sperm hyperactivation in whole semen and its association with low superoxide scavenging capacity in seminal plasma. *Fertil Steril*. (1993) 59:1291–5. doi: 10.1016/S0015-0282(16)55992-5
39. de Lamirande E, Gagnon C. A positive role for the superoxide anion in hyperactivation and capacitation of human spermatozoa. *Int J Androl*. (1993) 16:2–25. doi: 10.1111/j.1365-2605.1993.tb01148.x
40. de Lamirande E, Lamothe G. Reactive oxygen-induced reactive oxygen formation during human sperm capacitation. *Free Radic Biol Med*. (2009) 46:502–10. doi: 10.1016/j.freeradbiomed.2008.11.004
41. Baumber J, Sabeur K, Vo A, Ball BA, Reactive oxygen species promote tyrosine phosphorylation and capacitation in equine spermatozoa. *Theriogenology*. (2003) 60:1239–47. doi: 10.1016/S0093-691X(03)00144-4
42. Agarwal A, Said TM, Bedaiwy MA, Banerjee J, Alvarez JG. Oxidative stress in an assisted reproductive techniques setting. *Fertil Steril*. (2006) 86:503–12. doi: 10.1016/j.fertnstert.2006.02.088

43. Iommiello VM, Albani E, Di Rosa A, Marras A, Menduni F, Morreale G, et al. Ejaculate oxidative stress is related with sperm DNA fragmentation and round cells. *Intern J Endocrin*. (2015) 2015:321901. doi: 10.1155/2015/321901
44. Burnaugh L, Ball BA, Sabeur K, Thomas AD, Meyers SA. Osmotic stress stimulates generation of superoxide anion by spermatozoa in horses. *Anim Reprod Sci*. (2010) 117:249–60. doi: 10.1016/j.anireprosci.2009.05.014
45. Sotolongo B, Huange TTF, Isenberger E, Ward WS. An endogenous nuclease in hamster, mouse, and human spermatozoa cleaves DNA into loop-sized fragments. *J Androl*. (2005) 26:272–80. doi: 10.1002/j.1939-4640.2005.tb01095.x
46. Nakai M, Kashiwazaki N, Takizawa A, Maedomari N, Ozawa M, Noguchi J, et al. Effects of chelating agents during freeze-drying of boar spermatozoa on DNA fragmentation and on developmental ability *in vitro* and *in vivo* after intracytoplasmic sperm head injection. *Zygote*. (2007) 15:15–24. doi: 10.1017/S0967199406003935

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 16 December 2020
doi: 10.3389/fvets.2020.598147

Effect of Different Levels of Energy Diet Restriction on Energy Balance, Leptin and CL Development, Vascularization, and Function in South American Camelids

Cecilia Norambuena[1,2]*, *Francisca Hernandez*[1], *Jorge Alfaro*[1], *Gonzalo Silva*[1], *Shirley Topp*[1] and *Marcelo Ratto*[3]

[1] Department of Veterinary Science and Public Health, Faculty of Natural Resources, Catholic University of Temuco, Temuco, Chile, [2] Nucleus of Research in Food Production, Faculty of Natural Resources, Catholic University of Temuco, Temuco, Chile, [3] Institute of Animal Science, Faculty of Veterinary Science, Austral University, Valdivia, Chile

OPEN ACCESS

Edited by:
Rosa Maria Garcia-Garcia, Complutense University of Madrid, Spain

Reviewed by:
Jamie L. Stewart, Virginia-Maryland College of Veterinary Medicine, United States
Maria Florencia Gallelli, University of Buenos Aires, Argentina

***Correspondence:**
Cecilia Norambuena
mcnorambuena@uct.cl

Specialty section:
This article was submitted to Animal Reproduction - Theriogenology, a section of the journal Frontiers in Veterinary Science

Received: 23 August 2020
Accepted: 13 November 2020
Published: 16 December 2020

Citation:
Norambuena C, Hernandez F, Alfaro J, Silva G, Topp S and Ratto M (2020) Effect of Different Levels of Energy Diet Restriction on Energy Balance, Leptin and CL Development, Vascularization, and Function in South American Camelids. Front. Vet. Sci. 7:598147. doi: 10.3389/fvets.2020.598147

The objective was to determine the effect of energy diet restriction on energy balance, systemic leptin and corpus luteum (CL) vascularization, development, and function in South American camelids. In experiment 1, adult llamas were randomly assigned to receive a diet of 70% of their maintenance energy requirements (MER) (Restricted group, $n = 7$) or fed *ad libitum* (Control group, $n = 7$) during 28 days. Body live weight (BLW) and body condition score (BCS) were recorded, blood samples were collected every 2 weeks to measure plasma leptin concentrations, and energy metabolites were quantified. In experiment 2, adult alpacas were randomly assigned to receive a diet of 40% MER for 21 days (Restricted group, $n = 7$) or fed *ad libitum* (Control group, $n = 7$). Then, ovulation was induced with gonadorelin acetate (day = 0), and trans-rectal ultrasonography (7.5 MHz) was performed using B and Doppler mode to record the diameter of the pre-ovulatory follicle, ovulation, CL diameter, and vascularization from Days 0 to 13. Blood samples were collected every 48 h from Days 1 to 13 to quantify plasma leptin and progesterone concentrations. In experiment 1, energy diet restriction of 70% MER did not affect plasma leptin concentration and metabolic parameters of the Restricted group. In experiment 2, the Restricted group had a lower BCS ($p < 0.001$), a smaller diameter of the CL on Days 5 and 7 ($p < 0.05$), and a smaller maximum diameter of the CL (10.2 ± 0.6 mm) than the Control group (12.1 ± 0.6 mm; $p = 0.04$). Low energy restriction of 70% MER for 28 days did not affect the energy balance of llamas (Experiment 1). Moderate energy restriction of 40% MER for 21 days negatively affected energy balance (BCS), and CL development but not its vascularization, leptin, and progesterone concentrations. These species must be submitted to longer periods or a higher level of energy restriction to impair ovarian function.

Keywords: diet restriction, leptin, corpus luteum, llama, alpaca, progesterone

INTRODUCTION

Members of the Camelidae family are recognized for their ability to survive and reproduce in extreme environments (1). In Chile, the arrival of ruminants during the Spanish conquest decimated the population of camelids and displaced the domestic (*Lama glama*, *Vicugna pacos*) and wild species (*Vicugna vicugna*, *Lama guanicoe*) toward the marginal lands of the country (2, 3). Low fertility rates of highland camelid herds have been described [about 50%, (4)]. In a previous alpaca study (5), we have documented an overall embryo loss of 45.4% (49/108), during the 35 days after mating, which was close to the 50–58% reported in earlier studies (4, 6). Although the main factors related to embryo loss in these species are unknown, the poor nutritional status of the High Andean herds could be related (7).

Leptin, an adipocyte-derived hormone, acts as a critical metabolic signal linking nutrition and reproductive function (8). The rapid decrease in leptinemia in underfed animals could be translated as an acute signal to stimulate re-feeding behavior and glucocorticoid secretion; to decrease thyroid activity, energy expenditure, insulin sensitivity, and protein synthesis; and to block reproduction (9). Leptin modulates GnRH secretion in the hypothalamus indirectly through stimulation of neuropeptide Y and kisspeptin (10, 11). Systemic administration of leptin increased gonadotropin secretion in fasted ruminants and rats (12, 13). Leptin receptor, OB-R, has been characterized in the granulosa and theca cells, luteal cells, the ovarian stroma, and in endothelial cells in various species (14, 15) and recently in alpacas (16). These evidences support the notion that leptin may have a potential role in the maturation and selection of developing follicles, corpus luteum (CL) formation, function, and regression during estrus cycle via an autocrine/paracrine mechanism in several species (15).

In a previous study (17), severe long-term nutritional restriction negatively affected leptin concentration, the diameter of the pre-ovulatory follicle, and corpus luteum (CL) and progesterone production in llamas. The day-by-day profile of plasma leptin concentration was correlated with the CL growth during the entire luteal phase suggesting a potential local role of leptin on CL development in llamas. Kumar et al. (18) demonstrated that the leptin mRNA highest levels were in mid and late luteal stages consistent with *in vivo* luteinization of buffalo CL and declined coincidental to luteal regression. Also, the increased expression of steroidogenic enzymes (StAR, P450scc, HSD) has been correlated with the OB gene and OBR receptor activity in mid luteal phase suggesting a key role of leptin in progesterone production (18).

The mechanism of action of leptin on CL development is unknown. Wiles et al. (19) have demonstrated that the addition of leptin in an *in vitro* culture of goats luteal cells increased the expression of angiogenic factors such as angiotensin I (Ang1), fibroblast growth factor 2 (FGF2), and vascular endothelial growth factor (VEGF). In fact, CL vascularization did increase in alpacas previously treated with leptin during follicular phase (pre-ovulatory follicle $>$ 7 mm of diameter) and given GnRH for ovulation induction, however, CL diameter, plasma luteinizing hormone (LH), and progesterone concentration were similar between leptin and non-leptin-treated females (20).

The objectives of this study were to determine the effect of low energy diet restriction on energy balance in llamas (Experiment 1) and to evaluate the effect of moderate energy restriction on energy balance on leptin concentration and CL vascularization, development, and function in alpacas (Experiment 2).

MATERIALS AND METHODS

Experimental procedures were reviewed and approved by the Bioethical Committees of the Universidad Austral de Chile and Universidad Católica de Temuco and were performed in accordance with the animal care protocols established by the same institutions and in accordance with Chilean Animal Protection Act (2009).

Experiment 1: Effect of Low Energy Diet Restriction (70% of the Maintenance Energy Requirements) on Energy Balance in Llamas

Animals and Nutrition Management

This study was conducted in the llama farm at the University Austral of Chile located in Valdivia (39°38′S, 72°35′W). Mature non-lactating, non-pregnant, female llamas ($n = 14$), 6–9 years old, with a body condition score (BCS) of 3–3.5 [scale 1–5, (21)], were randomly assigned to an energy Restricted or a Control group ($n = 7$ per group). The Control group, weighting 149 $\pm$ 6 kg (mean $\pm$ SEM), was fed *ad libitum* with *Ballica* sp. hay [4.4% crude protein (CP); 5.7% total ashes (TA), and 1.8 Mcal/kg metabolic energy (ME), on dry matter base] and 300 g day^{-1} of commercial pelleted concentrate (16% CP; 5.4% TA, and 3.0 Mcal/kg ME; Cosetán®, Suralim, Osorno). Llamas of the Restricted group, weighting 146 $\pm$ 4 (mean $\pm$ SEM), were individually fed with the same diet as that of the Control group (3 hay:1 pellet, on the basis of total energy intake), but the amount of food provided 70% of maintenance energy requirements (MER) of the initial body live weigh (BLW). MER was calculated for each female using the following equation: 61.2 * $BLW^{0.75}$ (22). Llamas were kept indoors and fed under nutritional management during 28 days.

Llamas received water and mineral salts (Usablock®, Sweetlix) *ad libitum*, and they were dewormed using Sofomax® (Intervet). BLW (kg) and BCS were assessed weekly. Blood samples (4 ml) were collected by jugular venipuncture into heparinized tubes every 2 weeks to determine metabolic markers and plasma leptin concentration. Plasma was separated by centrifugation at 1,500*g* for 15 min and stored at −80°C until assayed.

Metabolic Markers

Metabolites such as glucose, triglycerides, non-esterified fatty acids, beta-hydroxybutyrate, cholesterol, and urea were analyzed as previously described (17, 23). In brief, glucose concentration was measured using a portable equipment (One touch®,

LifescanInc, USA). Triglycerides plasma concentration (GPO-PAD enzymatic method), non-esterified fatty acids (NEFA; ASC-ACOD enzymatic colorimetric method), beta-hydroxibutirato (BHB; 3 HBDH dependent enzymatic method), cholesterol (CHOD-PAD enzymatic method), and urea (kinetic glutamic-dehydrogenase method) were determined using Cobas-Miras-Plus® autoanalyzer (Roche D-10587, Berlin, Germany).

Leptin Concentration Analysis

Plasma leptin concentration was determined by radioimmunoassay (RIA; multi-species leptin kit®, Millipore, USA), using a 1470 Wallac Wizard Gamma counter (PerkinElmer Inc, USA). The limit of sensitivity and intra-assay coefficient of variation were 2.5 ng/ml and 10%, respectively.

Experiment 2: Effect of Moderate Energy Diet Restriction (40% of the Maintenance Energy Requirements) on Energy Balance and Luteal Function in Alpacas

Animals, Nutrition Management, and Experimental Design

The experiment was conducted in the alpaca farm at the Universidad Católica de Temuco, located in Temuco (38°46′S, 72°38′W). Non-pregnant non-lactating adult alpacas ($n = 14$) aged 3 to 9 years old with a BCS of 3 to 3.5 (scale 1–5; 20) were used in the study. Females were randomly assigned to the Restricted or Control group ($n = 7$ per group). The Control group weighting was fed *ad libitum* with *Ballica* sp. hay (4.5% CP, 1.2% TA, 2.6 Mcal/kg ME, on dry matter base) and 200 g day^{-1} of commercial pelleted concentrate (17% CP, 6% TA, 3 Mcal/kg ME on dry matter base; Cosetán®, Iansagro SA, Chile). Alpacas in the Restricted group were individually fed twice daily, with hay and pellets (3:1 on the basis of total energy intake), but the amount of food was reduced to 40% MER of each alpaca. The MER was calculated using the equation 66* initial BLW$^{0.75}$ (7). Alpacas were kept indoors and fed under nutritional management for 21 days (Day −21 to Day −1, **Figure 1**). They received water and mineral salts (Usablock®, Sweetlix) *ad libitum*, and they were dewormed using Sofomax® (Intervet). On the fourth day after the starting of the diet, alpacas received an intravaginal progesterone device (CIDR-B®, Easy-Breed, 0.3 g, Lab. Pfizer NZ) for 7 days to synchronize follicular wave emergence as previously described (24). All alpacas were examined daily by transrectal ultrasonography using a 7.5 MHz linear array transducer coupled to a monitor (Sonovet r3, Samsung Madison, USA) after the day of progesterone removal to determine the diameter of the pre-ovulatory follicle. Ovulation was induced 10 days after progesterone removal with an intramuscular administration of 50 μg of gonadorelin acetate (GnRH, Gonasyl®, Virbac, Day 0 = GnRH treatment, **Figure 1**). The ovaries were examined every other day from Day 1 to Day 13 to determine ovulation, CL diameter, and vascularization using B and Doppler mode. Ovulation was defined as the disappearance of a large follicle (≥7 mm) that had been detected in the previous examination.

Alpacas from the Restricted group were switched from 40% MER of the initial BLW to 100% MER of the BLW recorded at Day 0 (GnRH treatment, see **Figure 1**). This change of diet restriction was conducted in order to maintain the new BLW recorder at Day 0 until Day 13 during CL assessment. BLW and BCS were recorded twice in alpacas of both groups, at the beginning and at the end of the restricted period. BLW was recorded every other day from Day 1 to Day 13 using a digital scale (±0.1 kg).

B and Power Doppler Ultrasonography and Image Analysis

CL diameter and vascularization were recorded using B and Power Doppler mode from Day 1 to Day 13 by an operator who was blinded to the treatments and according to previous South American camelid studies (25, 26). In brief, the echotexture of the CL was measured in B mode at its maximum diameter using horizontal and vertical calipers. In Power Doppler mode, cineloops (10 s in length) of the CL vascularization were recorded during Power Doppler imaging and downloaded into VLC media player (www.videolan.org, Version 2.0, Boston, USA). Cineloops were examined frame by frame to select three images that represented the maximum vascular signal at the maximum cross-sectional area of the CL. Images were saved in JPG format with minimal compression and analyzed by ImageJ software (National Institute of Health, Maryland, Washington DC, USA) to determine the area of vascularization of the CL. The area of vascularization was estimated by measuring the area (cm^2) of the vascular flow signals (Power-Doppler) overlaying the B-mode image of the CL; the average of the three images was taken as the value for a given animal on a given day.

Leptin and Progesterone Concentration Analysis

Blood samples (4 ml) were collected into heparinized tubes to determine plasma leptin and progesterone concentration from Day 1 to Day 11 (Day 0 = GnRH treatment) and Day 1 to Day 13, respectively. Plasma leptin concentration was determined using the Sheep Leptin ELISA Kit® (MyBiosource, USA) which was validated previously (20). In brief, serially diluted plasma from alpacas containing high concentrations of leptin produced a displacement curve parallel to the standard curve. Intra- and inter-coefficients of variation were 5.0 and 7.2%, respectively, and the limit of sensitivity was 2.5 ng/ml. Plasma progesterone concentration was determined using a commercial, double-antibody radioimmunoassay kit (COAT-A COUNT 17A-OH Progesterone, Siemens, USA). The intra-assay coefficient of variation varied between 0.5 and 3.5%, and the inter-assay coefficient of variation was between 0.5 and 2.1%. The limit of sensitivity was 0.61 ng/ml. Analyses were performed at the Universidad de Concepción, Chile.

Statistical Analyses

Normality and homoscedasticity were assessed by Kolmogorov-Smirnov and Levene test, respectively, Log_{10-} transformations were performed whenever necessary. Paired or unpaired Student

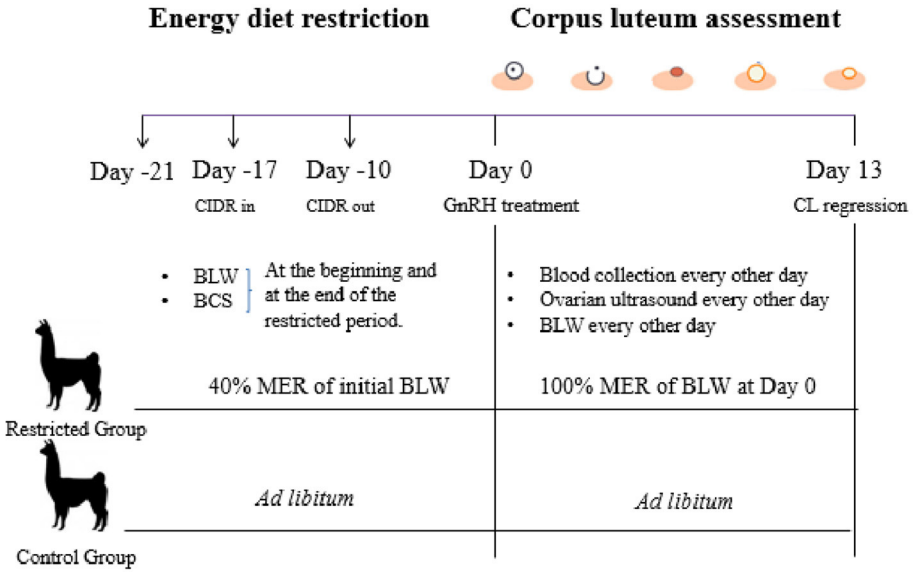


FIGURE 1 | Experimental design of Experiment 2.

t test were used to compare non-serial data as BLW, BCS, pre-ovulatory follicle diameter, and maximum CL diameter between groups. A mixed ANOVA with time points as repeated measurements was used to compare serial data (BLW, leptin and progesterone plasma concentration, CL diameter, and vascularization area). If significant main effects or interactions were detected ($p < 0.05$), ANOVA pairwise comparisons with Bonferroni adjustment were used. Pearson's correlation was used to determine relationships between CL diameter, vascularization area, and plasma progesterone concentration. Statistical analyses were done using SPSS Program (V 20). Data are reported as mean ± SEM.

RESULTS

Experiment 1

Plasma leptin concentration, metabolite markers, BLW, and BCS did not differ between the Restricted and Control groups (**Table 1**). The initial and final BLW and BCS of the Restricted group did not differ ($p = 0.06$ and $p = 0.9$, respectively). The Restricted group lost 9 kg, equivalent to 6% of its initial BLW. Initial and final BLW and BCS of Control group also were similar ($p = 0.4$ and $p = 0.9$, respectively).

Experiment 2

The Restricted and Control groups had similar BLW and BCS at the beginning of the diet restricted period (**Table 2**). Although BLW was not different between groups, BCS was higher in the Control than that of the Restricted group after 21 days of 40% MER. The Restricted group reduced the BLW and BCS significantly during the metabolic trial, but Control group did not (**Table 2**). There were no significant differences in BLW between groups during the corpus luteum assessment ($p > 0.05$). The diameter of the preovulatory follicle at the time of GnRH treatment was similar between groups: 8.4 ± 0.7 and 10 ± 0.8 mm for Restricted and Control groups, respectively ($p = 0.9$). Similarly, the proportion of ovulated animals (7/7 and 7/7, $p = 0.9$) was not different between Restricted and Control groups, respectively. There was an effect of treatment ($p = 0.01$) and day ($p = 0.001$) but not interaction ($p = 0.5$) on CL diameter between groups. Diameter of CL was greater in the Control than that of the Restricted group at Days 5 and 7 after GnRH treatment (**Figure 2A**). Maximum CL diameter was lower ($p < 0.04$) in the Restricted (10.2 ± 0.6) than that of the Control group (12.1 ± 0.6 mm). Maximum CL vascularization was not different between Restricted and Control groups (0.37 ± 0.26 vs. 0.42 ± 0.25 cm^2, respectively; $p = 0.3$, **Figure 2B**). However, there was an effect of time on CL vascularization ($p < 0.001$). Plasma progesterone and leptin concentrations were not affected by the treatment ($p = 0.7$ and $p = 0.7$, respectively, **Figures 2C,D**). Daily mean values of plasma leptin concentration during the luteal phase ranged from 6.9 ± 1.7 to 8.0 ± 1.6 ng/ml in both groups. There was no correlation between plasma leptin concentration on CL diameter, vascularization area, and plasma progesterone concentration ($p > 0.05$). The diameter of CL was positively correlated with vascularization area ($r = 0.6$; $p < 0.001$) and plasma progesterone concentration ($r = 0.3$; $p < 0.01$).

DISCUSSION

Based on the results of the present study, low energy restriction at 70% MER in llamas did not affect BLW, BCS, plasma leptin concentration, and metabolic parameters that may indicate a negative energy balance (Experiment 1). Moderate energy restriction at 40% MER in alpacas only reduced BCS and CL

TABLE 1 | Plasma leptin concentration, metabolites, body weight and body condition score (Mean ± SEM) of llamas fed 70% energy of maintenance requirements (Restricted group; $n = 7$), or fed *ad libitum* (Control group, $n = 7$) during 28 days.

Variable	Day 1			Day 14			Day 28		
	Restricted	Control	*P*	Restricted	Control	*P*	Restricted	Control	*P*
Leptin (ng/mL)	7.7 ± 1.4	5.5 ± 0.5	0.7	5.3 ± 0.4	7.5 ± 1.6	0.6	6.7 ± 1.2	5.5 ± 1.4	0.6
Glucose (mg/dL)	145 ± 5	140 ± 4.8	0.1	138 ± 5.9	151 ± 4.9	0.1	152 ± 4.6	142 ± 4.2	0.3
BHB (mmol/L)	0.07 ± 0.04	0.05 ± 0.03	0.8	0.004 ± 0.004	0.003 ± 0.03	0.3	0.07 ± 0.04	0.05 ± 0.04	0.7
NEFA (mmol/L)	0.05 ± 0.01	0.08 ± 0.01	0.3	0.17 ±0.03	0.09 ± 0.03	0.09	0.17 ± 0.03	0.2 ± 0.03	0.2
Cholesterol (mmol/L)	1.5 ± 0.4	1.8 ± 0.4	0.3	2.2 ± 0.4	2.6 ± 0.5	0.6	1.5 ± 0.3	1.8 ± 0.4	0.6
Urea (mmol/L)	7.9 ± 0.4	7.4 ± 0.4	0.6	5.7 ± 0.6	6.9 ± 0.7	0.2	6.2 ± 0.7	6.2 ± 0.7	0.9
Triglycerides (mml/L)	0.3 ± 0.05	0.4 ± 0.07	0.2	0.3 ± 0.06	0.4 ± 0.1	0.6	0.4 ± 0.05	0.5 ± 0.07	0.8
Total Protein (g/L)	101 ± 7.1	103 ± 2.4	0.9	69 ± 3.6	77 ± 2.4	0.1	73 ± 2.3	84 ± 5.2	0.06
BLW (kg)	146 ± 3.8	149 ± 6.2	0.7	137 ± 3.8	147 ± 6.5	0.7	137 ± 3.3	146 ± 4.8	0.2
BCS (1–5)	3.5 ± 0.1	3.5 ± 0.2	0.4	3.4 ± 0.1	3.5 ± 0.2	0.4	3.2 ± 0.1	3.5 ± 0.1	0.3

NEFA is non-esterified fatty acids; BLW is body live weight; BCS is Body condition score.

TABLE 2 | Values (Mean ± SEM) of body live weight (BLW) and body condition score (BCS) of alpacas fed 40% of maintenance energy requirements (restricted group; $n = 7$) or fed *ad libitum* (control group; $n = 7$) before and after the nutrition management for 21 days.

Variable	Control group	Restricted group	*P*-value
Before			
BLW (kg)	50.5 ± 2.6	52.6 ± 1.8^{a}	0.3
BCS (1-5)	3.1 ± 0.2	3.1 ± 0.2^{c}	0.9
After			
BLW (kg)	51.1 ± 2.5	49.3 ± 1.8^{b}	0.2
BCS (1-5)	3.1 ± 0.2^{x}	2.6 ± 0.2^{dy}	0.002

^{a,b}*different superscripts for BLW mean values differed within the group ($P < 0.01$).*
^{c,d}*different superscripts for BCS mean values differed within the group ($P < 0.001$).*
^{x,y}*different superscripts for BCS mean values differed between groups ($P < 0.01$).*

diameter, but no changes were detected in CL vascularization and plasma progesterone and leptin concentrations (Experiment 2).

Several studies have shown that energy status affects fertility in ruminant herds (27, 28). It has been described that nutritional restriction caused the absence or dysfunction of GnRH and gonadotrophic hormones secretion in sheep and cows (29–31). Hormonal dysfunction can result in the formation of a dominant follicle of smaller size and lower secretion of estradiol affecting estrus behaviors and the ovulatory process in cows (32, 33). Moreover, nutritional restriction reduced the diameter of the corpus luteum resulting in a lower production of progesterone which is associated with a higher rate of embryonic mortality and implantation /conception failures (32, 34). Negative energy balance has been associated to a dysfunction of various hormones and neuropeptides such as leptin, IGF-1, insulin, and neuropeptide Y that act at hypothalamic-pituitary-ovarian axis modulating GnRH secretion (11, 35).

In the present study, nutritional restriction at 70% MER for 28 days failed to induce negative energy balance in llamas (Experiment 1). However, females from the Restricted group lost 6% of their BLW; this loss can be partially attributed to a reduced gastrointestinal content that represents up to 11% of the BLW in llamas (36). Similar to our results, Bengoumi et al. (37) did not find any differences on body mass, hump volume, and lipid content after 2 months of restricted feeding at 68% MER in *Camelus dromedarius.* It has been described that the dromedary camel has the capability to reduce their energy expenditure up to 21% under fasting conditions (38). Llamas from the Restricted group maintained the homeostasis of proteins, lipids, carbohydrates, and energy; the values of metabolic markers were according to the referential values reported for camelids (39, 40). Negative energy balance (NEB) increased plasma concentrations of NEFA, BHB, or urea in previous studies conducted in llamas, alpacas, and vicuñas (17, 23, 41, 42). Starvation or dietary insufficiency results in mobilization of adipose stores, characterized by an increase in blood NEFA (over 0.6 mmol/L), and a mild increase in blood BHB (over 0.1 to 0.19 mmol/L) in camelids (43, 44). NEB induces amino acid breakdown to be used as energy substrate and possible gluconeogenesis that increases circulating urea (1, 17). The NEFA, BHB, and urea values reported in the study were lower than those cited before.

Sheep under energy diet restriction of 82% MER suffered a negative energy balance at Day 30 (45); apparently there must be a specific species response to the dietary restriction that also could be associated to the physiological and productive status of the animal.

In Experiment 2, although, alpacas fed 40% MER reduced their BLW up to 8%, it did not differ from the Control group. Nonetheless, BCS in alpacas from the Restricted group was lower than that observed in the Control Group indicating that negative energy balance caused lipolysis of fat reserves. Chagas et al. (35) suggested that measurement of BCS, unlike BLW, minimizes the influence of body size and gastrointestinal content. Leptin regulates energy balance, and its plasma concentration level correlates with fat reserves in several species (46) including BCS in llamas [$r = 0.8$, (23)]. Leptin exerts its effects through the leptin receptor (OB-R) which is highly expressed in the hypothalamus, where it is primarily responsible for suppression of food intake

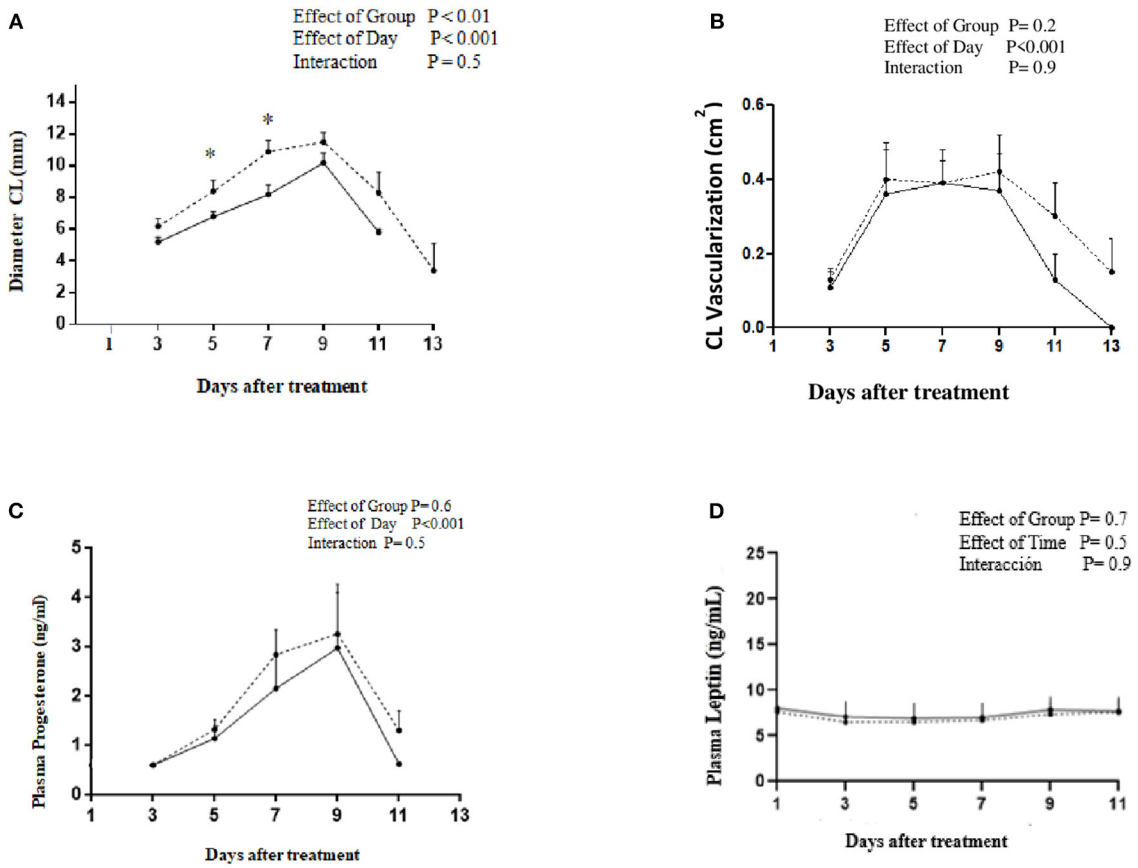


FIGURE 2 | Profiles of **(A)** CL diameter, **(B)** CL vascularization, **(C)** plasma progesterone concentration, and **(D)** plasma leptin concentration after treatment with GnRH to induce ovulation in alpacas under energy restriction 40% (_____, $n = 7$) or fed *ad libitum* (-------, $n = 7$).

and stimulation of energy expenditure (47). Nonetheless, the restricted period of 21 days was not enough to induce some changes in leptin concentration as mean values observed by Day 1 (Day 0 = GnRH treatment) were similar between groups (7.6 ± 1.5 vs. 8.0 ± 1.5 ng/ml for Restricted and Control groups, respectively, **Figure 2D**). Leptin values observed in the present study were lower than that previously reported (∼12 ng/ml) during the luteal phase in alpacas submitted to a fasting period during the pre-ovulatory stage (20).

The diameter of the pre-ovulatory follicle was similar between groups, and their sizes are similar to the mean values reported for non-pregnant, non-lactating alpacas (48). CL diameter in the Control group was significantly greater than that of the Restricted group. Indeed, CL diameter in the Restricted group was smaller than mean values (12–14 mm) reported in another study conducted in camelids (49). Corpus luteum formation involves the luteinization of granulosa and theca cells, increment of the ovarian blood flow, and the stimulation of a proliferation and differentiation process of endothelial and steroidogenic cells (50, 51). Luteinizing (LH) and growth hormones (GH) are the main luteotrophic hormones in bovines and along with insulin-like growth factor I (IGF-I) promote angiogenesis, mitosis, and progesterone production (52). Taking into account that 85% of the cells that proliferate in a developing CL are endothelial cells, it could be speculated that the greater size of CL observed in the Control group could be attributed to an increase of CL vascularization area (52), but although CL vascularization area was numerically greater in the Control than that of the Restricted group, these were not significantly different and so failed to explain CL size differences. The vascularization values observed in the present study were similar to those reported in other studies in alpacas and llamas (20, 25, 26).

It is well-known that negative energy balance (NEB) reduces systemic LH secretion in ruminants influencing CL development and progesterone production (32, 53). NEB also negatively affects the plasma concentration of IGF-I, a hormone that stimulates LH secretion at the hypothalamic and pituitary levels (11, 54). In addition, Insulin-like growth factor receptor has been detected in most of the luteal cells in alpacas suggesting that IGF-I may be involved in the CL steroid synthesis in camelids (16). It has been described that LH and IGF-I hormones also act synergistically stimulating the proliferation and differentiation of granulosa cells, as well as promoting steroidogenesis, vascularization, and oocyte health (16, 55). Nevertheless, the diameter of the pre-ovulatory follicles as well as the ovulatory rate of the Restricted

alpacas were not affected by NEB, which rules out a dysfunction of these hormones.

Despite the differences in CL diameter between groups, progesterone concentration was similar in both Restricted and Control groups; these values were similar to those reported in other studies in alpacas (56, 57). A positive correlation between CL vascularization and plasma progesterone concentration has been reported in camelid studies (20, 25). Then, the stability of the leptin plasma concentration of the Restricted group could stimulate an adequate vascularization of the CL to provide the precursors needed for progesterone production (19, 20).

The CL diameter and plasma progesterone concentration were positively correlated in the present study. Probably, a higher energy challenge could affect both parameters simultaneously, similarly as described in restricted llamas that lost near to 20% of BLW (17). Similarly, in ruminants, a decline in CL diameter and progesterone plasma concentration was observed in the two estrous cycles preceding anovulation, in heifers that lost 22% of BLW (53).

In ruminants, diets 40% MER for 14 days reduced leptinemia in sheep (58), and 60% MER for 21 days did it in a group of cows when compared to 130% MER (59). In addition, dietary restriction of 40% MER during 15 or 21 days induced anestrus in 60% of heifers, with reduced pre-ovulatory follicles or CL (29, 34). In this sense, the ability for camelids to maintain the energy balance and leptinemia may have comparative advantages of these species with respect to ruminants in terms of reproductive performances in the High Andes. The differential response between ruminants and camelids under nutritional restriction could be based on the following: (i) camelids are more efficient in reducing their basal metabolic rate during fasting (38), (ii) apparently, camelids use, to a greater extent, proteins as substrate for energy production and gluconeogenesis, being more efficient with the urea recycling (1, 7, 43).

We conclude that low energy restriction at 70% MER during 28 days in llamas did not affect BLW, BCS, plasma leptin concentration, and metabolic parameters that may indicate a negative energy balance (Experiment 1). The moderate energy restriction at 40% MER during 21 days in alpacas reduced BCS, BLW, and the CL diameter, but no changes were detected in CL vascularization, plasma progesterone, and leptin concentrations (Experiment 2). The results of the present study suggest that these species must be submitted to longer periods of nutritional restriction to induce a significant effect on systemic leptin concentration in order to impair follicular growth, CL vascularization, and progesterone production.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Bioethic Committee from Universidad Catolica de Temuco and Universidad Austral de Chile.

AUTHOR CONTRIBUTIONS

CN design the study and execute all the experiments, participate in data collection, data interpretation and writing the manuscript. FH, JA,GS, and ST collaboration in saplles collection, ultrasonography, and animal management. MR experimental design and writing of the manuscript, and direction of the study. All authors contributed to the article and approved the submitted version.

ACKNOWLEDGMENTS

This study was supported by the grant Fondecyt Iniciación 11130206 (Dr. Norambuena), Government of Chile and by the Nucleus of Research in Food Production, Faculty of Natural Resources, Catholic University of Temuco, Chile.

REFERENCES

1. Wenswoort J, Kyle D, Orskov E, Bourke D. Biochemical adaptation of camelids during periods where feed is withheld. *Revista Rangifer.* (2001) 21:45–8. doi: 10.7557/2.21.1.1527
2. Raggi SA, Ferrando R. Avances en fisiología y adaptación de camélidos sudamericanos. *Avances en Ciencias Veterinarias.* (1998) 13:1. doi: 10.5354/0719-5273.1998.4806
3. Marín JC, Zapata B, Gonzalez B, Bonacic C, Wheeler J, Casey C, et al. Sistemática, taxonomía y domesticación de alpacas y llamas: nueva evidencia cromosómica y molecular. *Revista Chilena de Historia Natural.* (2007) 80:121–40. doi: 10.4067/S0716-078X2007000200001
4. Fernández Baca S, Novoa C, Sumar J. Embryonic mortality in the Alpaca. *Biol Reprod.* (1970) 3:243–51. doi: 10.1093/biolreprod/3.2.243
5. Ratto M, Cervantes M, Norambuena MC, Silva M, Miragaya M, Huanca W. Effect of location and stage of development of dominant follicle on ovulation and embryo survival rate in alpacas. *Anim Reprod Sci.* (2011) 127:100–5. doi: 10.1016/j.anireprosci.2011.07.003
6. Bravo PW, Sumar J. *Factors that Determine Fertility in* Alpacas. In: *Proceedings of the International Convention on SAC.* Cusco, Perú (1985).
7. San Martin F, Van Saun R. Feeding management systems. In: Cebra C, Anderson DE, Tibary A, Van Saun RJ, Johnson LW, editors. *Alpaca and Llama Care*. Philadelphia, PA: Elsevier Inc. (2014). p. 91–100.
8. Donato J, Silva RJ, Sita LV, Lee S, Lee C, Lachinni S, et al. The ventral premammillary nucleus links fasting-induced changes in leptin levels and coordinated luteinizing hormone secretion. *J Neurosci.* (2009) 29:5240–50. doi: 10.1523/JNEUROSCI.0405-09
9. Chilliard Y, Delavaud C, Bonnet M. Leptin expression in ruminants: nutritional and physiological regulations in relation with energy metabolism. *Domest Anim Endocrinol.* (2005) 29:3–22. doi: 10.1016/j.domanied.2005.02.026
10. Henry BA, Goding JW, Tilbrook AJ, Dunshea FR, Clarke IJ. Intracerebroventricular infusion of leptin elevates the secretion of luteinising hormone without affecting food intake in long-term food-restricted sheep, but increases growth hormone irrespective of bodyweight. *J Endocrinol.* (2001) 168:67–77. doi: 10.1677/joe.0.1680067
11. García-García RM. Integrative control of energy balance in the female. *ISRN Vet Sci.* (2012) 1:1–13. doi: 10.5402/2012/121389
12. Amstalden M, García M, Stanko R, Nizielski S, Morrison C, Keisler D, et al. Central infusion of recombinant ovine leptin normalizes plasma

insulin and stimulates a novel hypersecretion of luteinizing hormone after short-term fasting in mature beef cows. *Biol Reprod.* (2002) 66:1555–61. doi: 10.1095/biolreprod66.5.1555
13. Zieba D, Amsteldan M, Maciel M, Keisle D, Raver N, Gertler A, et al. Divergent effects of leptin on luteinizing hormone and insuline secretion are dose dependent. *Exp Biol Med.* (2003) 228:325–30. doi: 10.1177/153537020322800312
14. Di Yorio MP, Bilbao MG, Pustovrh MC, Prestifilippo JP, Faletti AG. Leptin modulates the expression of its receptors in the hypothalamic-pituitary-ovarian axis in a differential way. *J Endocrinol.* (2008) 198:355–66. doi: 10.1677/JOE-07-0622
15. Sarkar M, Schilffarth S, Schams D, Meyer H, Berisha B. The expression of leptin and its receptor during different physiological stage in the bovine ovary. *Mol Reprod Dev.* (2010) 77:174–81. doi: 10.1002/mrd.21129
16. Gallelli MF, Bianchi C, Lombardo D, Rey F, Rodriguez FM, Castillo VA, et al. Leptin and IGF1 receptors in alpaca (*Vicugna pacos*) ovaries. *Anim Reprod Sci.* (2019) 200:96–104. doi: 10.1016/j.anireprosci.2018.12.001
17. Norambuena MC, Silva M, Urra F, Ullloa-Leal C, Fernández A, Adams G, et al. Effects of nutritional restriction on metabolic, endocrine, and ovarian function in llamas (*Lama glama*). *Anim Reprod Sci.* (2013) 138:252–60. doi: 10.1016/j.anireprosci.2013.01.019
18. Kumar L, Panda R, Hydera I, Yadav V, Sastry K, Sharma G, et al. Expression of leptin and its receptor in corpus luteum during estrous cycle in buffalo (Bubalus bubalis). *Anim Reprod Sci.* (2012) 135:8–17. doi: 10.1016/j.anireprosci.2012.08.030
19. Wiles JR, Katchko RA, Benavides EA, O'Gorman CW, Escudero JM, Keisler DH, et al. The effect of leptin on luteal angiogenic factors during the luteal phase of the estrous cycle in goats. *Anim Reprod Sci.* (2014) 148:121–9. doi: 10.1016/j.anireprosci.2014.05.002
20. Norambuena MC, Hernández F, Maureira J, Rubilar C, Alfaro J, Silva G, et al. Effects of leptin administration on development, vascularization and function of Corpus luteum in alpacas submitted to pre-ovulatory fasting. *Anim Reprod Sci.* (2017) 182:28–34. doi: 10.1016/j.anireprosci.2017.04.006
21. Van Saun RJ. Nutritional requirements and assessing nutritional status in camelids. *Vet Clin Anim Food Anim Pract.* (2009) 25:265–79. doi: 10.1016/j.cvfa.2009.03.003
22. Lopez A, Raggi LA. Requerimientos nutritivos de camélidos sudamericanos: llamas (*Lama glama*) y Alpacas (*Lama pacos*). *Archivos de Medicina Veterinaria.* (1992) 24:121–30.
23. Norambuena MC, Gómez Y, Ulloa-Leal C, Fernández A, Von Baer A, Ratto M. Relationship between systemic leptin concentration and reproductive state in llamas (*Lama glama*) from southern Chile. *Small Ruminant Res.* (2013) 113:402–4. doi: 10.1016/j.smallrumres.2013.04.008
24. Cavilla MV, Bianchi CP, Aguilera F, Hermida M, Aba MA. Hormonal changes and follicular activity after treatment with intravaginal progesterone-releasing devices in llamas. *Reprod Domest Anim.* (2016) 51:930–9. doi: 10.1111/rda.12762
25. Fernández A, Ulloa-Leal C, Silva M, Norambuena C, Adams G, Guerra M, et al. The effect of repeated administrations of llama ovulation-inducing factor (OIF/NGF) during the peri-ovulatory period on corpus luteum development and function in llamas. *Anim Reprod Sci.* (2014) 148:345–52. doi: 10.1016/j.anireprosci.2014.08.001
26. Ulloa-Leal C, Bogle OA, Adams GP, Ratto MH. Luteotrophic effect of ovulation-inducing factor/nerve growth factor present in the seminal plasma of llamas. *Theriogenology.* (2014) 81:1101–7. doi: 10.1016/j.theriogenology.2014.01.038
27. Patton J, Kenny DA, McNamara S, Mee JF, O'Mara FP, Diskin MG, et al. Relationships among milk production, energy balance, plasma analytes, and reproduction in Holstein-Friesian cows. *J Dairy Sci.* (2007) 90:649–58. doi: 10.3168/jds.S0022-0302(07)71547-3
28. Kim IH, Jeong JK. Risk factors limiting first service conception rate in dairy cows and their economic impact. *Asian-Australas J Anim Sci.* (2019) 32:519–26. doi: 10.5713/ajas.18.0296
29. Rhodes FM, Entwistle KW, Kinder JE. Changes in ovarian function and gonadotropin secretion preceding the onset of nutritionally induced anestrus in *Bos indicus* heifers. *Biol Reprod.* (1996) 55:1437–43. doi: 10.1095/biolreprod55.6.1437
30. Kiyma Z, Alexander BM, Van Kirk EA, Murdoch WJ, Hallford DM, Moss GE. Effects of feed restriction on reproductive and metabolic hormones in ewes. *J Anim Sci.* (2004) 82:2548–57. doi: 10.2527/2004.8292548x
31. Schneider J. Energy balance and reproduction. *Physiol Behav.* (2004) 81:289–317. doi: 10.1016/j.physbeh.2004.02.007
32. Diskin MG, Mackey DR, Roche JF, Sreenan JM. Effects of nutrition and metabolic status on circulating hormones and ovarian follicle development in cattle. *Anim Reprod Sci.* (2003) 78:345–70. doi: 10.1016/s0378-4320(03)00099-x
33. Diskin M. Reproductive management of dairy cows: a review (part I). *Ir Vet J.* (2008) 61:326–32. doi: 10.1186/s13620-017-0112-y
34. Mackey DR, Sreenan JM, Roche JF, Diskin MG. Effect of acute nutritional restriction on incidence of anovulation and periovulatory estradiol and gonadotropin concentrations in beef heifers. *Biol Reprod.* (1999) 61:1601–7. doi: 10.1095/biolreprod61.6.1601
35. Chagas LM, Bass JJ, Blache D, Burke CR, Kay JK, Lindsay DR, et al. Invited review: new perspectives on the roles of nutrition and metabolic priorities in the subfertility of high-producing dairy cows. *J Dairy Sci.* (2007) 90:4022–32. doi: 10.3168/jds.2006-852
36. Cristofanelli MA, Torres AD, Polidori C. Carcass characteristics of peruvian llama (*Lama glama*) and alpaca (*Lama pacos*) reared in the Andean highlands. *Small Ruminant Res.* (2005) 58:219–22. doi: 10.1016/j.smallrumres.2004.10.004
37. Bengoumi M, Faulconnier Y, Tabarani A, Sghiri A, Faye B, Chilliard Y. Effect of feeding level on body weight, hump size, lipid content and adiposyte volume in the dromedary camel. *Anim Res.* (2005) 54:383–93. doi: 10.1051/animres:2005029
38. Guerouali A, Zine-Filali R, Vermorel M, Wardeh M. Maintenance energy requirements and energy utilization by dromedary at rest. *Actes du Séminaire sur l'élevage et l'alimentation du dromadaire Douz Options Méditerranéennes, Série B.* (1995) 13:59–69.
39. Foster A, Bidewell C, Barnett J, Sayers R. Haematology and biochemistry in alpacas and llamas. *In Pract.* (2009) 31:276–81. doi: 10.1136/inpract.31.6.276
40. Husakova T, Pavlata L, Pechova A, Hauptmanova K, Pitropovska E, et al. Reference values for biochemical parameters in blood serum of young and adult alpacas (*Vicugna pacos*). *Animal.* (2014) 8:1448–55. doi: 10.1017/S1751731114001256
41. Norambuena MC, Hernández F, Alfaro J, Cárcamo G, Olavarría A, Velasco M. Relationship between the nutritional state before the breeding period and the reproductive success in alpacas (Vicugna pacos) from the Chilean Puna. *Austral J Vet Sci.* (2018) 50:55–7. doi: 10.4067/S0719-81322018000100110
42. Norambuena C, Mussa K, Hernández F, Alfaro J, Velasco M. Energy balance of pregnant vicuñas (*Vicugna vicugna*) in the Chilean High Andes. *Austral J Vet Sci.* (2019) 51:33–36. doi: 10.4067/S0719-81322019000100106
43. Cebra C. Hepatic, pancreatic, and metabolic disorders. In: Cebra C, Anderson DE, Tibary A, Van Saun RJ, Johnson LW, editors. *Alpaca and Llama Care*. Philadelphia, PA: Elsevier Inc. (2014). p. 537–52.
44. Van Saun R, Cebra C. Nutritional Diseases. In: Cebra C, Anderson DE, Tibary A, Van Saun RJ, Johnson LW, editors. *Alpaca and llama Care.* Philadelphia, PA: Elsevier Inc. (2014). p.124–39.
45. Sano H, Takebayashi Y, Kodama Y, Nakamura K, Ito H, Arino Y, et al. Effect of feed restriction and cold exposure on glucose metabolism in response to feeding and insulin in sheep. *J Anim Sci.* (1999) 77:2564–73. doi: 10.2527/1999.7792564x
46. Bonnet M, Delavaud C, Faulconnier Y, Leroux C, Djiane J, Bocquier F. Leptin in ruminants. Gene expression in adipose tissue and mammary gland, and regulation of plasma concentration. *Domest Anim Endocrinol.* (2001) 21:271–95. doi: 10.1016/s0739-7240(01)00124-2
47. Park H, Ahima R. Physiology of leptin: energy homeostasis, neuroendocrine function and metabolism. *Metabolism.* (2015) 64:24–34. doi: 10.1016/j.metabol.2014.08.004
48. Vaughan J, Macmillan K, D'Occhio MD. Ovarian follicular wave characteristics in alpacas. *Anim Reprod Sci.* (2004) 80:353–61. doi: 10.1016/j.anireprosci.2003.08.002
49. Abba M. Anatomy and physiology of Reproduction in the female alpaca. In: Cebra C, Anderson DE, Tibary A, Van Saun RJ, Johnson LW, editors. *Alpaca and Llama Care*. Philadelphia, PA: Elsevier Inc. (2014). p 140–150.

50. Acosta TJ, Miyamoto A. Vascular control of ovarian function: ovulation, corpus luteum formation and regression. *Anim Reprod Sci.* (2004) 82:127–40. doi: 10.1016/j.anireprosci.2004.04.022
51. Wiltbank MC, Salih SM, Atli MO, Luo W, Bormann CL, Ottobre JS, et al. Comparison of endocrine and cellular mechanisms regulating the corpus luteum of primates and ruminants. *Anim Reprod.* (2012) 9:242–59.
52. Schams D, Berisha B. Regulation of corpus luteum in cattle—an overview. *Reprod Domest Anim.* (2004) 39:241–51. doi: 10.1111/j.1439-0531.2004.00509.x
53. Bossis I, Welty SD, Wettemann RP, Vizcarra JA, Spicer LJ, Diskin MG. Nutritionally induced anovulation in beef heifers: ovarian and endocrine function preceding cessation of ovulation. *J Anim Sci.* (1999) 77:1536–46. doi: 10.2527/1999.7761536x
54. Vandehahar MJ, Sharma B-K, Fogwell RL. Effect of energy diet restriction on the expression of Insulin-like growth factor 1 in livers and corpus luteum of heifers. *J Dairy Sci.* (1995) 78:10–20. doi: 10.3168/jds.S0022-0302 (95)76695-4
55. Lucy MC. Regulation of ovarian follicular growth by somatotropin and insulin-like growth factors in cattle. *J Dairy Sci.* (2001) 83:1635–47. doi: 10.3168/jds.S0022-0302(00)75032-6
56. Bravo W, Pezo D, Alarcon B. Evaluation of early reproductive performance in the postpartum alpaca by progesterone concentrations. Animal Reproduction *Science.* (1995) 39:71–7.doi: 10.1016/0378-4320(94)01374-U
57. Echavarria L, Mendoza G, Evaristo R, Hinostroza E, Delgado A, Morán A, et al. Vías de señalización asociadas a la esteroidogénesis. *Revista Especializada en Ciencias Químico-Biológicas.* (2012) 15:24–36.
58. Delavaud C, Bocquier F, Baumont R, Chaillou E, Ban-Tokuda T, Chilliard Y. Body fat content and feeding level interact strongly in the short- and medium-term regulation of plasma leptin during underfeeding and re-feeding in adult sheep. *Br J Nutr.* (2007) 98:106–15. doi: 10.1017/S0007114507704968
59. Delavaud C, Ferlay A, Faulconnier Y, Bocquier F, Kann G, Chilliard Y. Plasma leptin concentration in adult cattle: Effects of breed, adiposity, feeding level, and meal intake. *J Anim Sci.* (2002) 80:1317–28. doi: 10.2527/2002.8051317x

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 05 January 2021
doi: 10.3389/fvets.2020.610597

Effects of NGF Addition on Llama (*Lama glama*) Sperm Traits After Cooling

Luciana M. Sari[1], *Renato Zampini*[1,2], *Francisco Gonzalez del Pino*[3], *Martin E. Argañaraz*[1,2], *Marcelo H. Ratto*[4] *and Silvana A. Apichela*[1,3]*

[1] Facultad de Bioquímica, Química y Farmacia, Instituto Superior de Investigaciones Biológicas (INSIBIO), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Instituto de Biología "Dr. Francisco D. Barbieri" Universidad Nacional de Tucumán (UNT), San Miguel de Tucumán, Argentina, [2] Cátedra de Biología Celular y Molecular, Facultad de Bioquímica, Química y Farmacia, Universidad Nacional de Tucumán, San Miguel de Tucumán, Argentina, [3] Cátedra de Zootecnia General I, Facultad de Agronomía y Zootecnia, Universidad Nacional de Tucumán, San Miguel de Tucumán, Argentina, [4] Facultad de Ciencias Veterinarias, Instituto de Ciencia Animal, Universidad Austral de Chile, Valdivia, Chile

To provide new insights into the mechanisms through which seminal plasma proteins can protect sperm from damage caused during refrigeration, we evaluate the possibility that β-NGF can contribute to the improvement of sperm quality after cooling. First, β-NGF was detected in refrigerated sperm and compared with unrefrigerated sperm by western blotting of the proteins adsorbed by sperm, showing that native β-NGF is still present even 24 h after cooling only as an active form. Then, the effect of exogenous β-NGF on sperm quality after cooling was evaluated. A total of 12 ejaculates from male llamas (three ejaculates per male), were obtained by electro-ejaculation, diluted 4:1 with buffer Hepes-balanced salt solution and centrifuged at 800 × g for 8 min to remove the seminal plasma. Sperm were suspended in Tris-citrate-fructose-egg yolk diluent for a final concentration of 30 × 10^6/ml and cooled at 5°C for 24 h. After refrigeration, the extended sperm were equilibrated for 5 min at 37°C and divided into the following subgroups: sperm samples without treatment (control) and sperm samples supplemented with exogenous human β-NGF (10, 100, and 500 ng/ml). At 5, 30, and 60 min of incubation sperm were evaluated for sperm viability (using eosin/nigrosin stain), sperm motility and vigor (observed under light microscopy), and mitochondrial activity (using the JC-1 fluorescent marker). Vigor data were analyzed with the nonparametric Kruskal-Wallis test. The rest of the variables were analyzed with a mixed models approach. Mean comparisons were performed using Fisher's LSD test with a confidence level of 95%. A principal components analysis was performed to analyze the relationships between variables. Treatment of 24 h cooled sperm with 10 or 100 ng/ml of human β-NGF increased the percentage of total motility and vigor ($p < 0.05$). Besides, an incubation time of 60 min would be adequate to improve sperm quality, since all variables are positively related. The significant improvement observed in the motility and vigor of post-refrigerated sperm suggests that supplementation with exogenous β-NGF may be profitable for the improvement of cooled llama sperm.

Keywords: β-nerve growth factor, seminal plasma, llama (*Lama glama*), sperm, cryopreservation, cooling

OPEN ACCESS

Edited by:
Regiane R. Santos, Schothorst Feed Research, Netherlands

Reviewed by:
Arlindo A. Moura, Federal University of Ceara, Brazil
Susana Giuliano, Universidad de Buenos Aires, Argentina

***Correspondence:**
Silvana A. Apichela
silvanaapichela@gmail.com

Specialty section:
This article was submitted to Animal Reproduction - Theriogenology, a section of the journal Frontiers in Veterinary Science

Received: *26 September 2020*
Accepted: *20 November 2020*
Published: *05 January 2021*

Citation:
Sari LM, Zampini R, Gonzalez del Pino F, Argañaraz ME, Ratto MH and Apichela SA (2021) Effects of NGF Addition on Llama (Lama glama) Sperm Traits After Cooling. Front. Vet. Sci. 7:610597. doi: 10.3389/fvets.2020.610597

INTRODUCTION

Artificial insemination (AI) is an important technique to ensure rapid genetic progress. To date, AI efficiency in domestic South American camelids is low (1, 2). Technical aspects of semen collection, dilution, and cryopreservation have been the main limitations for the development and use of AI in these species (1). Different protocols for sperm cooling and freezing, as well as semen extenders are currently under investigation. To date, higher pregnancy rates have been achieved using cooled sperm (3, 4) than with frozen-thawed semen (1, 5, 6).

A protocol for AI or cryopreservation of camelid semen should be designed taking into account the striking particularities of semen composition and spermatozoa. Camelid semen is highly viscous and form a threat when is pipetted (7). Unlike other domestic ruminants, the sperm movement is mainly oscillatory (8, 9), and the relationship between this characteristic and fertility has not been established yet. Seminal plasma composition seems also to be particular. Camelids account for a higher concentration of β-NGF when compared with other mammals (10). This factor is responsible for inducing ovulation in camelid females once it is deposited in the genital tract during copulation (11). In a previous study, we provided some evidence that seminal β-NGF may have roles in the regulation of llama sperm physiology (12).

When ejaculated and epididymal spermatozoa were analyzed, we found that β-NGF was present only in ejaculated sperm, indicating that this factor is provided by the seminal plasma during ejaculation (12). The presence of the main receptor of β-NGF, TrKA, in the sperm middle portion led us to propose that β-NGF might have an impact on sperm traits such as motility. Several studies performed in other species reported that the addition of β-NGF affects sperm traits. Treatment of frozen-thawed bull sperm cells with 40–80 ng/ml β-NGF increased both leptin secretion and sperm membrane integrity (13). In human sperm, addition of several concentrations of β-NGF (1–10 μM) for 30 min increased in a dose-dependent manner the sperm motility (14); and supplementation of freezing extender with β-NGF at 0.5 ng/ml improved sperm viability and motility and decreased DNA fragmentation after thawing (15, 16). The *in vitro* incubation of rabbit sperm with exogenous human β-NGF (100 ng/ml) maintained a high motility rate and track speed (17). Consistently, Bezerra et al. (18) reported a positive association between the β-NGF protein expression in seminal plasma and rabbit sperm motility.

To judge from the reports mentioned, β-NGF may be an optimal candidate protein to enhance sperm quality in camelids. Thus, the main objective of the current study was to evaluate the effect of exogenous human β-NGF on the semen quality of cooled llama sperm.

METHODS AND MATERIALS

Animals and Location

Four male llamas ≥4 years old were used. The study was carried out at the Experimental Center for Camelid Studies of the National University of Tucuman. The Experimental Center is situated 420 meters above sea level (masl), latitude 26°88′32″ S and longitude 65°38′43″ W. Animals were kept out at pasture in pens and supplemented with bales of alfalfa and water was provided *ad libitum*. All males were shorn during November.

Semen Collection

Semen was obtained during the winter-spring of 2019 at regular intervals of 14 days. Collections were performed under general anesthesia with 0.2 mg/kg of xylazine IV (Xilazina, Richmond, Argentina) and 1.5 mg/kg of ketamine IV (Ketamina, Holliday, Argentina). All procedures were in line with the UNT 002/18 Protocol approved by the Committee for the Care and Use of Laboratory Animals (CICUAL) from the Universidad Nacional de Tucumán, Argentina.

For electroejaculation, a stimulator similar to an Electrojac V (Sistel, Argentina) with a rectal probe with three linear electrodes was used. The probe was lubricated, gently inserted into the rectum, and oriented so that the electrodes were positioned ventrally to the prostate. According to Zampini et al. (19), the device was used in automatic mode, applying stimulation cycles of 2 s with 2 s intervals between stimuli. Voltage was increased one volt every five cycles (starting with 2 V) until erection occurred (∼8 V). Then, voltage was increased with 1 V increments every ten cycles until ejaculation.

Semen was collected in 50 ml Falcon tubes and immediately placed in a 37°C water bath.

Immediately after collection, semen samples were diluted with HBSS (25 mM Hepes, 130 mM NaCl, 5 mM KCl, 0.36 mM NaH_2PO_4, 0.49 mM $MgCl_2$, and 2.4 mM $CaCl_2$; pH 7.4, 290 mOsm/kg) at 37°C and centrifuged at 800 × g for 10 min at room temperature to remove the seminal plasma.

Sperm Cooling

Washed sperm were kept at 37°C and suspended in 200–300 μl of HBSS, and concentration, sperm viability, motility, and vigor were assessed as described below. Then, Tris citric acid fructose-egg yolk extender (TCF-EY: 250 mM Tris, 80 mM citric acid, 60 mM fructose, 20% egg yolk, 0.5% Equex, 80,000 IU Penicillin G sodium, and 0.1% Streptomycin sulfate) was added to obtain a final concentration of 30–40 × 10^6 spermatozoa/ml. Samples were subsequently placed in a 37°C water bath and then placed in a refrigerator. Temperature was monitored until reaching 5°C in ± 2.5 h (cooling rate 0.2°C/min). Samples were then maintained at 5°C for 24 h. After this period, the cooled sperm cells were warmed up to 37°C in a water bath.

Experiment 1: Detection of β-NGF in Cooled Llama Spermatozoa

Extraction of Sperm Adsorbed Protein

Sperm-adsorbed proteins (SAP) were obtained from fresh-washed sperm and 24 h cooled sperm, as previously described by Zampini et al. (20). Briefly, pools of six semen samples ($n = 3$) for each experimental group were diluted 5-fold and washed three times at 800 × g for 10 min with HBSS. The sperm pellets were suspended in HBSS with 1X protease inhibitor cocktail (Sigma). An equal volume of 1 M KCl in HBSS was added, and sperm proteins were extracted by gentle mixing on a rotary shaker for 1 h at 4°C. Spermatozoa were then removed by centrifugation at

6,000 × g for 10 min, and discarded. Extracts were clarified by filtration (0.2-μm cellulose acetate). A series of centrifugations in 3 kDa cellulose filters (Amicon, Lexington, MA) were performed at 9,000 × g at 4°C, re-diluting in HBSS every centrifugation to reduce the salts and to concentrate the sample. Protein content was determined using a BCA protein assay kit (Thermo Fisher Scientific, Rockford, IL) with a bovine serum albumin standard. Aliquots were stored at −70°C until needed.

Western Blotting

Western blotting was performed according to Sari et al. (12). Ten micrograms of total sperm-adsorbed proteins of fresh and cooled sperm ($n = 3$) obtained as indicated before, were subjected to 15% SDS-PAGE along with 100 ng of recombinant human β-NGF (R&D Systems, Minnesota, USA) in a separate well. Gels were run in a PROTEAN II xi Cell (Bio-Rad, Hercules, CA) at 150 V for 1.5 h at room temperature. Then, proteins were transferred onto PVDF membranes (Immobilon-P, Merck, Darmstadt, Alemania). Semi-dry electroblotting was carried out on a Trans-Blot SD semi-dry transfer cell (Bio-Rad; Richmond, CA) and run at 20 V for 30 min. The membranes were blocked in 5% BSA in HBSS-T (130 mM NaCl, 5 mM KCl, 1.36 mM Na_2HPO_4, 2.4 mM $CaCl_2$, 25 mM HEPES, 0.49 mM $MgCl_2$, 0.1% Tween 20) for 30 min at 37°C and then overnight at 4°C. Afterward, membranes were incubated with polyclonal antibodies against β-NGF (dilution 1:2,000, sc-548) (Santa Cruz Biotechnology) for 2 h at 37°C, followed by incubation with the secondary antibody for 1 h at 37°C (1:1,000) (biotinylated anti-rabbit IgG antibody, B8895, Sigma). Finally, membranes were incubated for 30 min with alkaline phosphatase-linked ExtrAvidin (1:5,000, Sigma), and protein bands were visualized using the SigmaFast substrate (BCIP/NBT, Sigma). Membrane digital images were obtained with a Pentax Optio M90 digital camera. Molecular weight marker proteins (Page Ruler Unstained Broad Range Protein Ladder, Thermo Fisher Scientific, Rockford, IL) were also run and transferred to determine the molecular weights of the labeled bands. The primary antibody omitted from the staining reaction was used as control.

Experiment 2: Effect of β-NGF Addition on Llama Sperm Traits

Sperm Preparation Samples

Twelve ejaculates were collected from 4 llamas ($n = 4$, $r = 3$). After 24 h of cooling, sperm samples were warmed at 37°C. Minimum viability of 55% of live sperm and absence of agglutinated sperm were verified before beginning the experiment. Then, each sperm sample was divided into four treatment groups: without addition of exogenous β-NGF (control group) and with addition of 10, 100, or 500 ng/ml of human recombinant β-NGF (R&D Systems, Minnesota, USA). Treatment groups were incubated at 37°C for 60 min. At 5, 30, and 60 min of incubation, aliquots were taken to assess the viability, motility, and vigor. Sperm mitochondrial function was evaluated after 60 min of β-NGF addition.

TABLE 1 | Sperm vigor score.

Score	Description
0	Sperm have no movement.
1	Total motility is poor. Very few sperm (about 10%) have weak movements.
2	Sperm have weak oscillatory movement. Progressive motility is not observed.
3	Sperm have mainly oscillatory movement, with slow beat frequency, and low progressive movement. Less than 40% of cells are motile.
4	Sperm have vigorous oscillatory movement, with fast beat frequency. About 40–50% of cells are motile.
5	Sperm have very fast oscillatory or progressive movement; 60% of cells are motile.

Assessment of Viability

The vital eosin-nigrosin staining was used for assessing sperm viability. Briefly, 10 μl of the sperm suspension were placed on a slide with an equal volume of dye (2 eosin: 3 nigrosin), mixed, and then smeared. Once dried, slides were examined under a bright-field microscope (Numak, model Zenith DO-1L, Buenos Aires, Argentina) at 400X. Live spermatozoa appeared unstained, whereas dead spermatozoa with disrupted membranes appeared stained in red. Two hundred spermatozoa where examined on each slide ($r = 2$), averaged, and expressed as percentage of live spermatozoa.

Assessment of Motility and Vigor

To evaluated sperm motility and vigor, 7 μl of the sperm suspension were placed in a slide under a heating stage, coved with a warmed coverslip (18 × 18 mm), and observed under a bright-field microscope (Numak, model Zenith DO-1L, Buenos Aires, Argentina) at 400X. Sperm motility was expressed as the percentage of total motility i.e., progressive plus oscillatory.

For assessment of vigor, sperm movement was scored from 0 to 5 following **Table 1** criteria, based on the number of sperm with movement, the frequency of oscillatory movement of the sperm, and the presence of progressive movement.

Assessment of Mitochondrial Activity

Mitochondrial membrane potential (MMP) was evaluated by using JC-1 dye (CAS 3520-43-2, Santa Cruz Biotechnology, USA). The JC-1 probe accumulates in the mitochondria as a fluorescent green monomer in inactive mitochondria, and when mitochondria exhibit high membrane potential (active mitochondria) the monomers form aggregates that shift to fluorescent orange.

For MMP assessment, 150 μl of each treatment group (30 × 10^6 sperm/ml) of were incubated with β-NGF as previously described. After 15 min of incubation, 3 μl of JC-1 (1.53 mM) was added and then incubated for a further 45 min. Then, the sperm were washed in HBSS and suspended in 150 μl of HBSS. Fluorescence was determined in a fluorometer (Perkin Elmer LS55) using 590-nm excitation/525-nm emission for the

J-aggregate of JC1, and 530-nm excitation/490-nm emission for the J-monomer. MMP was calculated as J-aggregate/J-monomer ratio.

Statistical Analysis

The effects of β-NGF and time on sperm viability, motility, and mitochondrial activity were determined using a linear mixed-effects model (21) with the R program through the graphical interface developed for Infostat (22). In the model, the llama was used as a random effect to account for the documented inter-llama variability (23). *Q-Q* plots were used to check for departures from the normal distribution. Differences between media treatments were compared with Fisher's least significant difference (LSD) test. The same effects on vigor were evaluated using the Kruskal-Wallis test. Data are presented as mean ± standard error (SE), except for vigor, which was expressed as the median, and the minimum significance level was $p < 0.05$.

A multivariate method of statistical analysis, principal component analysis (PCA), was applied in addition to the conventional tests used for univariate analysis. The PCA offers the advantage of allowing rapid visualization of the relevant information on a single scatter plot graph including all the sperm traits assayed and β-NGF concentrations at the same time. Separate models were used for 5, 30, and 60 min data. The results of the PCA were displayed in a two-dimensional graph with projections of the sperm traits variables (vectors) in the factor space in which the horizontal and vertical axis represents the principal factors. The angles between the vectors indicate the correlations between the variables, where the length of the vectors indicates the contribution of the variables to the total variance of the data matrix (24).

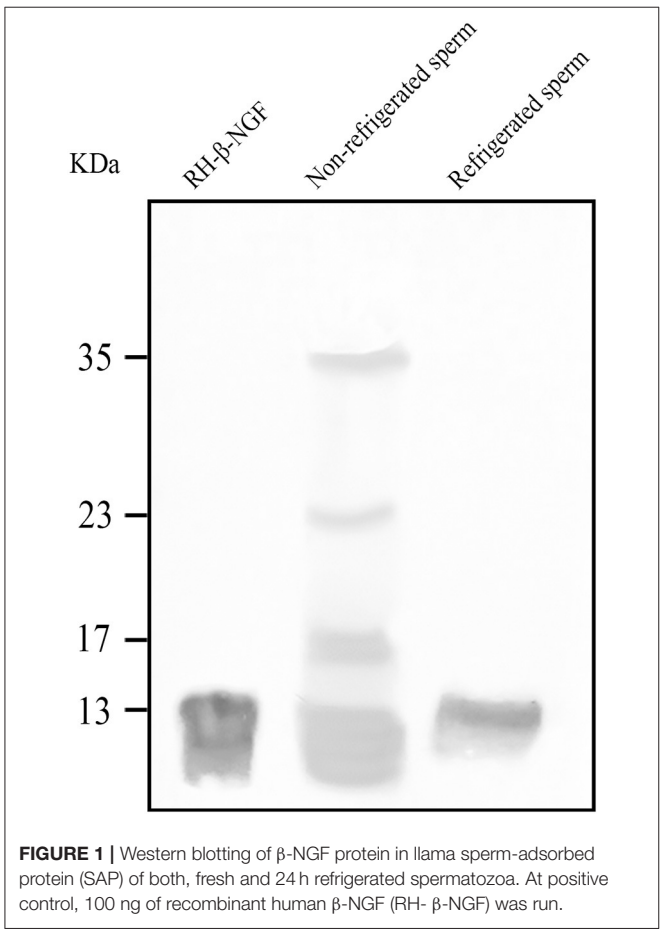


FIGURE 1 | Western blotting of β-NGF protein in llama sperm-adsorbed protein (SAP) of both, fresh and 24 h refrigerated spermatozoa. At positive control, 100 ng of recombinant human β-NGF (RH- β-NGF) was run.

RESULTS

Detection of β-NGF in Cooled Llama Spermatozoa

Western blotting revealed the presence of the β-NGF protein in the sperm adsorbed proteins of both, fresh and 24-h refrigerated spermatozoa (**Figure 1**). SAP of unrefrigerated samples presented four β-NGF species of 13, 17, 23, and 35 kDa, whereas refrigerated samples presented a unique protein band of 13 kDa.

Effect of β-NGF on Llama Sperm Traits

The effect of different concentrations of β-NGF on sperm viability was evaluated at three incubation times as shown in **Table 2**. At 5 min of incubation, no differences ($p > 0.05$) were observed for viability, whereas at 30 min of incubation, viability in control samples resulted higher than in 10 ng/ml β-NGF group ($p < 0.05$). Conversely, at 60 min after incubation, sperm samples with 10 ng/ml β-NGF added presented a higher percentage of live spermatozoa than those without β-NGF ($p < 0.05$).

Regarding sperm motility, the interaction between β-NGF concentration and time was not significant (interaction $p = 0.4940$) indicating that the effect of β-NGF addition on sperm motility is independent of the incubation time. Therefore, they were analyzed separately. Sperm motility parameters decreased over incubation time ($p < 0.05$) (**Figure 2A**). Media containing 10 and 100 ng/ml of β-NGF were superior showed a marked

TABLE 2 | Percentage of llama sperm viability.

Time (min) \ β-NGF (ng/ml)	0	10	100	500
5	74.70 ± 2.53^{a}	74.26 ± 2.50^{a,b}	71.90 ± 2.50^{a,b,c}	71.17 ± 2.50^{a,b,c,d}
30	71.90 ± 2.50^{a,b,c}	67.81 ± 2.50^{d,e,f}	69.81 ± 2.50^{c,d,e,f}	70.72 ± 2.50^{b,c,d}
60	66.44 ± 2.50^{f}	70.26 ± 2.50^{c,d,e}	68.17 ± 2.50^{d,e,f}	66.90 ± 2.50^{e,f}

Values are mean ± standard error for the concentration-time factor; n = 12 for each treatment.
^{a,b,c,d,e,f}Means with the same letter are not significantly different from each other (p > 0.05 Linear Mixed-Effects Models followed by LSD Fisher test).

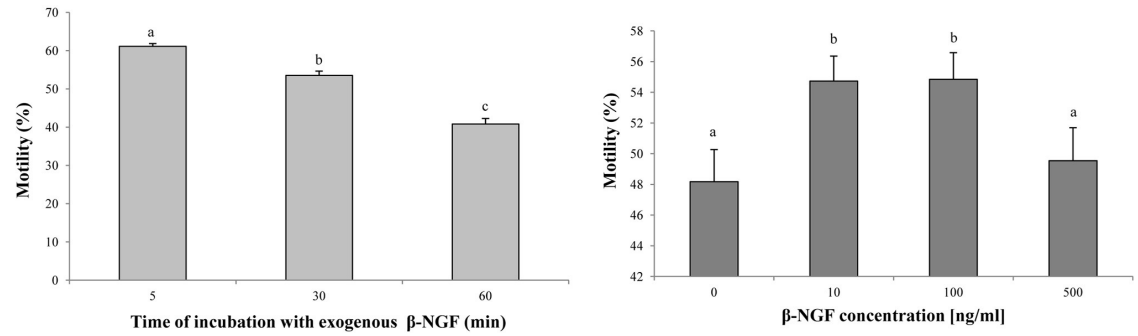


FIGURE 2 | Effect of β-NGF on llama sperm motility after cooling. **(A)** Incubation time-effect ($p < 0.05$, $n = 48$ for each time point). **(B)** Concentration effect ($p < 0.05$, $n = 36$ for each treatment). Results are expressed as mean ± SE. [a,b]Different letters indicate significant differences.

TABLE 3 | Percentage of llama sperm vigor (scale 0–5) after incubation with exogenous β-NGF.

β-NGF (ng/ml)	Time (min)		
	5	30	60
0	4 ± 0.30[a]	3 ± 0.23[a,b]	2 ± 0.16[a]
10	4 ± 0.28[a]	4 ±0.15[c]	3[a,b]
100	4 ± 0.16[a]	4 ± 0.12[c]	3 ± 0.19[b]
500	4 ± 0.21[a]	3 ± 0.14[b]	3 ± 0.21[a,b]

Data are expressed as median ± standard error.
[a,b,c]Within columns, different letters between rows indicate significant differences ($p < 0.05$, $n = 11$ per group).

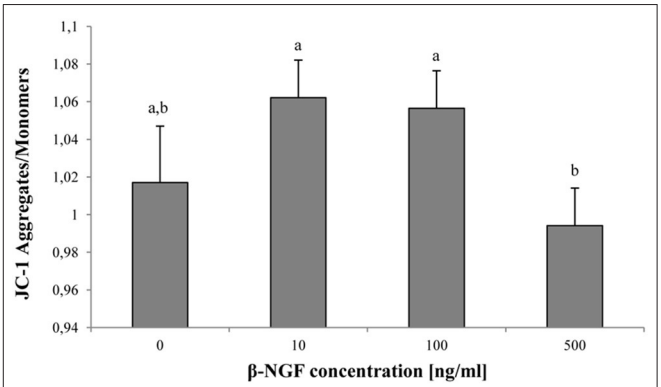


FIGURE 3 | Mitochondrial activity at 60 min of incubation with β-NGF. Bars represent the mean of JC-1 aggregate/monomer ratio ± SE, $n = 12$ for each treatment. [a,b]Different letters indicate significant differences ($p < 0.05$).

increase in total motility to the extenders containing 0 and 500 ng/ml of β-NGF ($p < 0.05$) (**Figure 2B**).

As shown in **Table 3**, the addition of 100 ng/ml of β-NGF increased significantly vigor after incubation for 30 or 60 min at 37°C when compared with the control group without β-NGF ($p < 0.05$).

Regarding mitochondrial activity, a decrease of MMP was observed when 500 ng/ml of β-NGF were added, compared with 10 or 100 ng/ml of β-NGF ($p < 0.05$). Nevertheless, no differences with the control were found (**Figure 3**).

The results of the PCA are shown in a two-dimensional graph (**Figure 4**). The length of the vectors represents the contribution of the variables to the total variance of each factor. When two variables were close to each other they were positively correlated. Otherwise, if they were orthogonal there was no correlation, if the variables were on the opposite side of the graph, they were negatively correlated. After 5 or 30 min from β-NGF addition, the motility and vigor of the sperm were close to each other in the right quadrant, indicating a positive interrelation between them, this association being closer after 30 min of incubation. It can also be appreciated that the 100 ng/ml dose of β-NGF induced the highest values of motility and vigor at these time points. Regarding sperm viability, the variable correlated negatively with vigor and motility at 5 and 30 min of β-NGF addition. At 60 min after sperm incubation with β-NGF, it is observed that all variables are positively correlated, and the values of these variables were higher when 10 or 100 ng/ml of β-NGF was added.

DISCUSSION

As described in the method section, before refrigeration the native source of β-NGF, the seminal plasma, was removed, and spermatozoa were extended in an appropriated diluent. Western blotting of sperm-adsorbed proteins revealed that after 24 h of cooling llama β-NGF was still adsorbed by spermatozoa, indicating a strong time-sustained affinity. However, there seems to be some degree of processing of the protein into an active form, since the pro-β-NGF isoforms are absent. Further studies would be necessary to define whether this factor remains functional. This is the first report about the presence of β-NGF adsorbed to cooled sperm conditions.

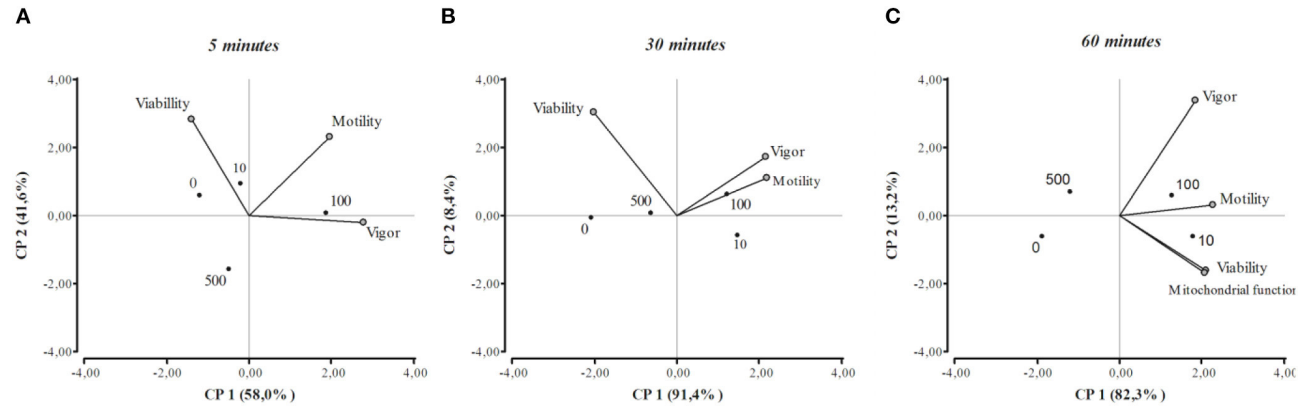


FIGURE 4 | Principal component analysis biplot at 5, 30, and 60 min of incubation with β-NGF **(A–C)**, showing the first two principal components (PC1 and PC2). Vectors indicate the relations between viability, motility, sperm vigor, and mitochondrial function. Each vector points in the direction of the increase in value. The dots represent the mean value for each concentration of β-NGF (0, 10, 100, 500 ng/ml).

To assess the effect of β-NGF supplementation, exogenous human β-NGF was added. Because the llama sperm TrKA-receptor was localized in the middle piece (12), it was hypothesized that β-NGF would directly affect the sperm motility improvement. To judge from the conditions in the present study, the addition of β-NGF improved the quality of cooled spermatozoa.

Motility of cooled llama sperm increased with the addition of 10 and 100 ng/ml of exogenous β-NGF, and this positive effect was independent of the incubation time. Several reports in other mammals indicate that β-NGF can promote sperm motility parameters of ejaculated sperm and in a time- and dose-dependent manner—human (14, 25); bull (13); rabbit (17). On the other hand, 100 ng/ml of β-NGF was the optimal dose to improve sperm motility in rabbits (17), and 10 ng/ml of β-NGF or less for human sperm (14, 15, 24). In South American camelids, ejaculates present mainly oscillatory sperm motility (23), and in some cases the differences between treatments can be reflected in the frequency of oscillatory movement (vigor) more than in the progressive movement. For this reason, sperm score vigor was included. As expected, β-NGF treatment enhanced sperm affected vigor, occurring consistently at 30 and 60 min of incubation with a concentration of 100 ng/ml of β-NGF.

With respect to viability, the effect of β-NGF addition was dose- and time-dependent since time is mainly responsible for sperm viability changes. β-NGF supplementation does not seem to negatively affect sperm viability in spermatozoa at any dose, although some statistically significant differences between treatments were observed. We found a positive effect at 60 min of incubation with 10 ng/ml of β-NGF. Improvement of sperm viability has been reported in other species. Bull spermatozoa presented increased sperm viability when 20, 80, or 120 ng/ml of β-NGF was added (13), whereas in rabbits 100 ng/ml of β-NGF increased sperm viability (17). This response would be mediated by β-NGF binding to the TrkA receptor (17).

As mentioned above, in the llama spermatozoa the TrKA receptor is located in the midpiece, suggesting that receptor localization is directly involved in mitochondrial function. In this study, the JC-1 dye was used due to its ability to discriminate high and low mitochondrial membrane potential (26). The addition of β-NGF (10, 100, 500 ng/ml) did not affect the MMP. Similarly, Li et al. (13) reported that increasing levels of β-NGF (from 20 to 120 ng/ml) did not affect the mitochondrial activity of bull spermatozoa. It has been proposed that a prolonged fall or increase in the MMP compared to normal levels can induce an unwanted loss of cell viability (27). Since no negative effects were observed in MMP as well as sperm viability, working concentrations of 10–500 ng/ml of β-NGF could be considered secure for the llama sperm in the conditions of the present study.

To clarify the role of β-NGF in llama sperm physiology, the relationship between sperm traits and β-NGF doses were characterized by principal component analysis. It was noticed that the relationship between viability, motility, sperm vigor, and mitochondrial function was positive only at 60 min of incubation with β-NGF, and improvement of all of these traits can be achieved with both 10 and 100 ng/ml of exogenous β-NGF. These results are encouraging for their application to artificial insemination. In this sense, it would be of interest to evaluate the sperm responsiveness to β-NGF when it is incubated for a longer time, as well as the effect on capacitation and acrosome reaction of having a better prediction of the β-NGF impact on the sperm-fertilizing capacity and then conducting artificial insemination experiments.

In conclusion, β-NGF increases sperm quality of cooled llama sperm. The addition of 10 or 100 ng/ml of human β-NGF would promote motility and vigor, while viability and mitochondrial activity are maintained. Supplementation with exogenous β-NGF may be profitable for successful refrigeration of llama sperm and assisted reproduction techniques.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary materials, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by Committee for the Care and Use of Laboratory Animals (CICUAL) from the Universidad Nacional de Tucumán, Argentina. UNT 002/18 Protocol.

AUTHOR CONTRIBUTIONS

SA, MR, and LS conceived and designed the study. SA and LS performed data interpretation and wrote the draft manuscript. RZ, LS, MA, and SA collected semen samples. LS performed western blot, semen refrigeration, sperm supplementation, MMP assessment, and the statistical analysis. FG and SA performed motility and vigor assessment. RZ and MA performed vitality assessment. RZ and LS performed protein extractions. MA, MR, and RZ revised and discussed the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by grant sponsor: UNT, grant number: PIUNT A616 and grant sponsor: ANPCyT, grant number: BID-PICT 2016-0145.

ACKNOWLEDGMENTS

We thank Magister Osvaldo Arce for his support with statistical analyses.

REFERENCES

1. Stuart CC, Vaughan JL, Kershaw CM, De Graaf SP, Bathgate R. Effect of diluent type, cryoprotectant concentration, storage method and freeze/thaw rates on the post-thaw quality and fertility of cryopreserved alpaca spermatozoa. *Sci Rep.* (2019) 9:12826. doi: 10.1038/s41598-019-49203-z
2. Fumuso FG, Giuliano SM, Chaves G, Neild DM, Miragaya MH, Bertuzzi ML, et al. Incubation of frozen-thawed llama sperm with seminal plasma. *Andrologia.* (2020) 52:e13597. doi: 10.1111/and13597
3. Giuliano SM, Chaves MG, Trasorras VL, Gambarotta, M, Neild D, et al. Development of an artificial insemination protocol in llamas using cooled semen. *Anim Reprod Sci.* (2012) 131:204–10. doi: 10.1016/j.anireprosci.2012.03010
4. García W, Alarcón V, Bravo PW. Inseminación artificial de alpacas con semen refrigerado y con inclusión de dos tipos de yema de huevo. *Rev Investig Vet Perú.* (2017) 28:337–44. doi: 10.15381/rivep.v28i213080
5. Aller JF, Rebuffi GE, Cancino AK, Alberio RH. Influencia de la criopreservación sobre la motilidad, viabilidad y fertilidad de espermatozoides de llama (*Lama glama*). *Arch Zoot.* (2003) 52:15–23.
6. Vaughan J, Galloway D, Hopkins D. *Artificial Insemination in Alpacas (Lama pacos).* Kingston, ACT: RIRDC Rural Industries Research and Development Corporation (2003).
7. Casaretto C, Martínez Sarrasague M, Giuliano S, Rubin de Celis E, Gambarotta M, Carretero I, et al. Evaluation of *Lama glama* semen viscosity with a cone-plate rotational viscometer. *Andrologia.* (2012) 44:335–41. doi: 10.1111/j.1439-0272.2011.01186x
8. Morton KM, Thomson PC, Bailey K, Evans G, Maxwell WMC. Quality parameters for alpaca (*Vicugna pacos*) semen are affected by semen collection procedure. *Reprod Dom Anim.* (2010) 45:637–43. doi: 10.1111/j.1439-0531.2008.01321x
9. Fumuso FG, Giuliano SM, Chaves MG, Neild DM, Miragaya MH, Gambarotta, et al. Seminal plasma affects the survival rate and motility pattern of raw llama spermatozoa. *Anim Reprod Sci.* (2018) 192:99–106. doi: 10.1016/j.anireprosci.2018.02019
10. Druart X, Rickard JP, Mactier S, Kohnke PL, Kershaw-Young CM, Bathgate R, et al. Proteomic characterization and cross species comparison of mammalian seminal plasma. *J Proteom.* (2013) 91:13–22. doi: 10.1016/j.jprot.2013.05029
11. Ratto MH, Leduc YA, Valderrama XP, van Straaten KE, Delbaere LT, Pierson RA, et al. The nerve of ovulation-inducing factor in semen. *Proc Natl Acad Sci USA.* (2012) 109:15042–47. doi: 10.1073/pnas1206273109
12. Sari LM, Zampini R, Argañaraz ME, Carretero MI, Fumuso FG, Barraza DE, et al. Expression of β-NGF and high-affinity NGF receptor (TrKA) in llama (Lama glama) male reproductive tract and spermatozoa. *Mol Reprod Dev.* (2018) 85:934–44. doi: 10.1002/mrd23075
13. Li C, Sun Y, Yi K, Ma Y, Zhang W, Zhou X. Detection of nerve growth factor (NGF) and its specific receptor (TrkA) in ejaculated bovine sperm, and the effects of NGF on sperm function. *Theriogenology.* (2010) 74:1615–22. doi: 10.1016/j.theriogenology.2010.06033
14. Lin K, Ding XF, Shi CG, Zeng D, QuZong S, Liu SH, et al. Nerve growth factor promotes human sperm motility in vitro by increasing the movement distance and the number of A grade spermatozoa. *Andrologia.* (2015) 47:1041–6. doi: 10.1111/and12375
15. Saeednia S, Bahadoran H, Amidi F, Asadi MH, Naji M, Fallahi P, et al. Nerve growth factor in human semen: effect of nerve growth factor on the normozoospermic men during cryopreservation process. *Iran J Basic Med Sci.* (2015) 18:292. doi: 10.22038/IJBMS.2015.4134
16. Saeednia S, Nashtaei MS, Bahadoran H, Aleyasin A, Amidi F. Effect of nerve growth factor on sperm quality in asthenozoosprmic men during cryopreservation. *Reprod Biol Endocrinol.* (2016) 14:29. doi: 10.1186/s12958-016-0163-z
17. Castellini C, Mattioli S, Dal B.osco A, Collodel G, Pistilli A, Stabile AM, et al. *In vitro* effect of nerve growth factor on the main traits of rabbit sperm. *Reprod Biol Endocrinol.* (2019) 17:1–11. doi: 10.1186/s12958-019-0533-4
18. Bezerra MJB, Arruda-Alencar JM, Martins JAM, Viana AGA, Neto AV, Rêgo JPA, et al. Major seminal plasma proteome of rabbits and associations with sperm quality. *Theriogenology.* (2019) 128:156–66. doi: 10.1016/j.theriogenology.2019.01013
19. Zampini R, Castro Gonzaléz XA, Sari LM, Martin A, Diaz AV, Argañaraz ME, et al. Effect of cooling and freezing on llama (*Lama glama*) sperm ultrastructure. *Front Vet Sci.* (2020) 7:842. doi: 10.3389/fvets.2020587596
20. Zampini R, Sequeira S, Argañaraz ME, Apichela SA. SL15: a seminal plasma-derived lectin from the sperm of llama (*Lama glama*). *Mol Reprod Dev.* (2017) 84:576–84. doi: 10.1002/mrd22816
21. Pinheiro JC, Bates DM. Linear mixed-effects models: basic concepts and examples. In: *Mixed-Effects Models in S and S-Plus.* New York, NY: Springer - Verlag (2000). p. 3–56.
22. Di Rienzo AJ, Macchiavelli R, Casanoves F. *Modelos Mixtos en Infostat.* Córdoba: Tutorial, Grupo InFoStat, FCA, Universidad Nacional de Córdoba (2009).
23. Giuliano S, Director A, Gambarotta M, Trasorras V, Miragaya M. Collection method, season and individual variation on seminal characteristics in the llama (*Lama glama*). *Anim Reprod Sci.* (2008) 104:359–69. doi: 10.1016/j.anireprosci.2007.02016
24. De Bruin JP, Dorland M, Spek ER, Posthuma G, Van Haaften M, Looman CWN, et al. Ultrastructure of the resting ovarian follicle pool in healthy

young women. *Biol Reprod.* (2002) 66:1151–60. doi: 10.1095/biolreprod66.41151
25. Shi CG, Lin K, Xu XB, Zhang SC, Wang N, Fan M. Evidence for the involvement of NGF in human sperm motility. *J Biomed Sci Eng.* (2012) 5:534–41. doi: 10.4236/jbise.201259066
26. Smiley ST, Reers M, Mottola-Hartshorn C, Lin M, Chen A, Smith TW, et al. Intracellular heterogeneity in mitochondrial membrane potentials revealed by a J-aggregate-forming lipophilic cation JC-1. *Proc Natl Acad Sci USA.* (1991) 88:3671–5. doi: 10.1073/pnas.88.93671
27. Zorova LD, Popkov VA, Plotnikov EY, Silachev DN, Pevzner IB, Jankauskas SS, et al. Mitochondrial membrane potential. *Anal Biochem.* (2018) 552:50–9. doi: 10.1016/j.ab.2017.07009

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 21 January 2021
doi: 10.3389/fvets.2020.594926

Use of Androcoll-E™ to Separate Frozen-Thawed Llama Sperm From Seminal Plasma and Diluent

Crissthel Yverlin Guillén Palomino[1], *Fernanda Gabriela Fumuso*[2,3], *Mariana Lucía Bertuzzi*[2,3], *Susana María Giuliano*[2], *Nicolás Velásquez González*[2], *Maria Victoria Bariani*[2] *and María Ignacia Carretero*[2,3]*

[1] *Laboratorio de Biotecnología Reproductiva, Estación Experimental Agraria Canaán, Instituto Nacional de Innovación Agraria (INIA-Ayacucho), La Molina, Perú,* [2] *Cátedra de Teriogenología, Facultad de Ciencias Veterinarias, Instituto de Investigación y Tecnología en Reproducción Animal, Universidad de Buenos Aires, Buenos Aires, Argentina,* [3] *Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Buenos Aires, Argentina*

It is not easy to separate frozen-thawed South American camelid sperm from seminal plasma (SP) and diluents to be used for *in vitro* embryo production. The objective of this study was to evaluate Androcoll-E™ (AE) efficiency to separate llama sperm from SP and freezing extender in frozen-thawed semen. A total of 22 ejaculates from five *Lama glama* males were collected using electroejaculation. After performing semen analysis (sperm motility, concentration, viability, membrane function, and acrosome integrity), samples were cryopreserved with a diluent containing lactose, ethylenediaminetetraacetic acid (EDTA), egg yolk, and 7% dimethylformamide. After thawing, samples were divided in aliquots, one of which was used as a control and the others processed by AE. *Experiment 1* (12 ejaculates): 100 μl of frozen-thawed semen was placed on top of 1,000 μl AE column and centrifuged at 800 *g* for 10 min. *Experiment 2* (10 ejaculates): two samples of 100 μl of frozen-thawed semen were placed on two columns of 500 μl AE each, and both were centrifuged at 800 *g* for 10 and 20 min, respectively. Pellets were resuspended in Tyrode's albumin lactate pyruvate (TALP) medium, and sperm parameters were evaluated. A significant decrease in all sperm parameters was observed in thawed samples compared to raw semen. AE allowed the separation of frozen-thawed sperm from SP and freezing extender independently from the height of the column used and time of centrifugation assayed. Although no significant differences were found between AE columns, higher sperm recovery was observed with 500 μl of AE coupled with 20 min of centrifugation. Despite the significant decrease observed in sperm motility in AE samples, no changes in sperm viability, membrane function, and acrosome integrity were observed when comparing control thawed semen with the sperm recovered after AE ($p > 0.05$). The use of AE columns, either 500 or 1,000 μl, allows the separation of frozen-thawed llama sperm from SP and freezing extender, preserving the viability, membrane function, and acrosome integrity. Of the protocols studied, 800 *g* centrifugation during 20 min using a 500 μl column of AE would be the method of choice to process frozen-thawed llama semen destined for reproductive biotechnologies.

Keywords: Androcoll, colloid, cryopreservation, diluent, llama, semen, seminal plasma

OPEN ACCESS

Edited by:
Marcelo H. Ratto,
Austral University of Chile, Chile

Reviewed by:
Marc Yeste,
University of Girona, Spain
Alfredo Ramirez,
Austral University of Chile, Chile

****Correspondence:***
María Ignacia Carretero
ignaciacarretero@gmail.com;
ignaciacarretero@fvet.uba.ar

Specialty section:
This article was submitted to Animal Reproduction - Theriogenology, a section of the journal Frontiers in Veterinary Science

Received: *14 August 2020*
Accepted: *24 November 2020*
Published: *21 January 2021*

Citation:
Guillén Palomino CY, Fumuso FG, Bertuzzi ML, Giuliano SM, Velásquez González N, Bariani MV and Carretero MI (2021) Use of Androcoll-E™ to Separate Frozen-Thawed Llama Sperm From Seminal Plasma and Diluent. Front. Vet. Sci. 7:594926. doi: 10.3389/fvets.2020.594926

INTRODUCTION

It is well-known that South American camelid (SAC) semen presents special rheological characteristics such as high structural viscosity (1) and the capacity to form a thread when handled (2). Furthermore, ejaculated sperm of these species have a particular motility pattern, they possess oscillatory motility with practically no progressive motility (2–4). Due to these characteristics, several *in vitro* SAC embryo production studies have reported the use of sperm recovered from animals with deviated deferent ducts or collected from the epididymis after death or castration, since those samples are free of seminal plasma (SP) and have sperm with progressive motility (5–13). Moreover, the absence of high viscosity and thread formation are advantages when processing these SP-free samples. However, protocols designed for epididymal sperm will not necessarily have similar outcomes when applied in raw semen samples that have SP, and the surgical intervention performed to deviate the deferent ducts renders the animal useless for future breeding.

Also, SAC embryos have been obtained *in vitro* using spermatozoa from enzymatically treated ejaculates subsequently processed with colloids such as Percoll® or Androcoll-E™ (AE) (14, 15). Although enzymatic treatment reduces thread formation of camelid semen and facilitates manipulation of samples, the effect that it could induce in sperm membrane components is still unknown. In addition, it has been suggested that Percoll® could be potentially dangerous to sperm cells (16). Thus, it is recommended to wash and centrifuge sperm after using this colloid, consequently increasing the time required for preparation of samples (17) and producing cell damage due to the rise in production of reactive oxygen species (18).

The use of frozen-thawed semen for *in vitro* embryo production in llamas is a very interesting tool, since it would allow the use of sperm at a later date and thus become independent from the day of sample collection, permitting a better planning of *in vitro* fertilization (IVF) timing. Additionally, it would allow the use of ejaculates from males of high genetic value that are no longer available to work with or even after their death. Moreover, survival of SAC cryopreserved sperm is very low, obtaining very poor pregnancy rates when artificial insemination (AI) is performed with frozen-thawed semen in these species (19–22). The results have been related to the rheological characteristics previously mentioned, which prevent accurate homogenization of ejaculates with the diluents and probably affecting proper penetration of cryoprotectants across sperm cell membranes. However, cryopreservation of llama sperm in the absence of SP does not improve sperm survival (23, 24). Considering all these facts, selection of suitable frozen-thawed sperm from samples where the majority are dead could be an alternative to SAC. In other species, greater percentages of motile, viable, and morphologically normal sperm with intact acrosomes and intact DNA have been reported in frozen-thawed samples treated with Androcoll [horse: (25, 26); dog: (27, 28); donkey: (29, 30); brown bear: (31)]. Recently, AE allowed the separation of llama sperm from SP in raw semen samples, avoiding the use of enzymatic treatments (32). However, this colloid has not yet been used with llama frozen-thawed semen samples. In this context, the objective of this study was to evaluate the efficiency of AE to separate llama frozen-thawed sperm from SP and the freezing extender.

Abbreviations: SACs, South American camelids; SP, seminal plasma; AE, Androcoll-E™.

MATERIALS AND METHODS

Reagents

Propidium iodide (PI), 6-carboxyfluorescein diacetate, dimethyl sulfoxide, and the reagents for the Tyrode's albumin lactate pyruvate (TALP) medium and for the hypoosmotic swelling (HOS) test were purchased from Sigma Chemicals (Sigma Aldrich, Buenos Aires, Argentina). Coomassie Blue (CB) was purchased from Bio-Rad, California, United States.

Animals and Location

The study was carried out at the Faculty of Veterinary Sciences of the University of Buenos Aires in Buenos Aires, Argentina. The city is situated at sea level, latitude 34°36′ and longitude 58°26′.

For the study, five male *Lama glama* ranging between 7 and 11 years of age and weighing 142.6 ± 19.2 kg (mean ± SD) were used. Animals were kept out at pasture in pens and supplemented with bales of alfalfa; they also had free access to fresh water throughout the study. All males were shorn during the month of November.

Experimental Design

Semen collections were carried out between April and October using electroejaculation (EE) under general anesthesia according to the technique described by Director et al. (33). The frequency of collection for each male was determined randomly. As EE requires general anesthesia, this method was not used on the same male at an interval of <15 days. All procedures were approved by the Committee for the Use and Care of Laboratory Animals (CICUAL) of the Faculty of Veterinary Sciences of the University of Buenos Aires (protocol 2019/22).

A total of 22 ejaculates from five adult llama males were processed. After the evaluation of semen characteristics, samples were cryopreserved with a diluent containing lactose, ethylenediaminetetraacetic acid (EDTA), egg yolk, and 7% of dimethylformamide. Briefly, ejaculates were diluted to a final concentration of 40×10^6 spermatozoa/ml or, if initial concentration was lower, a 1:1 (semen:extender) dilution was carried out. Then, samples were equilibrated for 20 min at room temperature, loaded into 0.25-ml straws and frozen using a manual method (34). Briefly, temperature descent was carried out in three phases by placing the straws, together with a digital thermometer, submerged in a mixture of ethanol:acetone (1:1) in a bronze canister with a graduated handle and holding over liquid nitrogen vapors inside a 10-L nitrogen tank. Temperature phases were as follows: (i) from room temperature to −15°C (temperature descent at a rate of 10°C−12°C min^{-1}), (ii) from −15°C to −120°C at a rate of 25°C−40°C min^{-1}, and (iii) the straws were plunged into liquid nitrogen at −196°C. The samples were thawed in a water bath at 37°C for 60 s. After

thawing, semen samples were divided into aliquots, one of which was used as a control (unprocessed sample) and the others were processed through AE according to the experiment. *Experiment 1* (12 ejaculates): 100 μl of frozen-thawed semen was placed on top of 1,000 μl AE column and centrifuged at 800 *g* for 10 min. *Experiment 2* (10 ejaculates): two samples of 100 μl of frozen-thawed semen were placed on top of two columns of 500 μl AE each, and both were centrifuged at 800 *g* for 10 and 20 min, respectively. In both experiments, after centrifugation through AE, pellets were resuspended in TALP medium and the sperm were evaluated.

Sperm Evaluations

Each ejaculate was evaluated for sperm quality before freezing (raw semen), after thawing (frozen-thawed semen control), and after processing by AE (frozen-thawed AE samples).

Volume of the ejaculates was evaluated using a micropipette.

Thread formation was evaluated indirectly according to the capacity of the samples to form a thread when 20 μl of the sample was pipetted onto a slide using a micropipette. This parameter was classified as either present or absent.

Sperm motility was evaluated using a phase contrast microscope (100×) and a warm stage (37°C). The patterns observed were oscillatory motility and progressive motility. In addition, total sperm motility was calculated (total motility = oscillatory + progressive).

Sperm concentration was calculated using a Neubauer hemocytometer chamber.

The 6-carboxyfluorescein diacetate (CFDA) and PI stains were used for assessing membrane integrity (viability) according to Giuliano et al. (35). Briefly, samples (12.5 μl) were incubated at 37°C for 10 min in 127 μl of staining medium. This medium contained 2 μl of a solution of CFDA (0.5 mg ml^{-1} in dimethyl sulfoxide) and 125 μl of saline medium [described by Harrison and Vickers (36)]. After the first 10 min of incubation, 2 μl of a solution of PI (0.5 mg ml^{-1} in isotonic saline) was added and the samples were incubated for another 10 min at 37°C. A minimum of 200 spermatozoa were evaluated per sample using an epifluorescence microscope (400×) (Leica®, 134 DMLS, Heerbrugg, Switzerland). Spermatozoa that fluoresced green throughout their length were classified as being viable (intact membrane), while sperm nuclei that fluoresced red were classified as non-viable (damaged membrane).

The HOS test in combination with CB staining, according to Carretero et al. (37), was performed to evaluate sperm membrane function and acrosome integrity. Briefly, for the HOS test, 12.5 μl of samples were incubated at 37°C for 20 min in 50 μl of a hypoosmotic solution containing fructose and sodium citrate (50 mOsml l^{-1}). After the incubation, 62.5 μl of 4% paraformaldehyde in PBS was added and samples were incubated for 4 min at room temperature, with subsequent centrifugation at 800 *g* for 10 min. Pellets were then resuspended in 100 μl of PBS. Next, small drops of the sample were placed on microscope slides, within previously marked wells using a hydrophobic barrier pen, and were left to dry at room temperature. Samples were stained with 0.22% CB for 5 min and were examined using an optical microscope at 1,000× magnification. Sperm cells that showed the characteristic tail swelling were classified as HOS positive (HOS+), while sperm cells without tail swelling were classified as HOS negative (HOS-). At the same time, sperm cells were classified according to CB acrosome staining as follows: purple-stained acrosomes (CB+; acrosome present) or absence of acrosome staining (CB-; acrosome absent). Finally, 200 spermatozoa from each sample were analyzed and classified into the following categories: (1) sperm with functional plasma membranes and presence of acrosomes (HOS+/CB+), (2) sperm with functional plasma membranes and without acrosomes (HOS+/CB–), (3) sperm with non-functional plasma membranes and presence of acrosomes (HOS–/CB+), and (4) sperm with non-functional plasma membranes and without acrosomes (HOS–/CB–).

Statistical Analysis

Statistical analyses were performed using InfoStat software (Student Version) (https://www.infostat.com.ar/index.php?mod=page&id=15). In all cases, normal distribution and homogeneity of variances of the data were corroborated using the Shapiro–Wilk's normality test and a Bartlett's test, respectively. The level of significance was set at 0.05 for all analyses.

To compare raw and frozen-thawed semen, a paired Student *t*-test was used to assess sperm viability, membrane function, and acrosome integrity; because sperm motility did not show a normal distribution, this was analyzed by Wilcoxon test.

To compare frozen-thawed semen (control) and frozen-thawed samples processed by AE, a paired Student *t*-test (experiment 1) and a factorial design with a Tukey test (experiment 2) were used to evaluate sperm concentration, viability, membrane function, and acrosome integrity. Because sperm motility did not show a normal distribution, these data were analyzed by a Wilcoxon test (experiment 1) and a Kruskal–Wallis test (experiment 2).

RESULTS

Raw Semen vs. Frozen-Thawed Semen (Control)

The volume of the ejaculates was 1.2 ± 0.6 ml (mean ± SD). Thread formation was observed in 15 of the 22 ejaculates that were collected (68.2%), which was maintained in nine of the 22 frozen-thawed semen samples (40.9%).

In both experiments (1 and 2), a significant decrease ($p < 0.05$) in all sperm parameters, except progressive motility, was observed in frozen-thawed semen samples (untreated controls) compared to raw semen (**Table 1** for experiment 1 and **Table 2** for experiment 2).

Frozen-Thawed Semen (Control) vs. Frozen-Thawed Samples Processed by Androcoll-E™

The use of AE allowed separation of frozen-thawed llama sperm from SP and from the freezing extender independently of the height of AE column (500 and 1,000 μl) and time

TABLE 1 | Sperm parameters evaluated in raw semen and frozen-thawed (FT) llama semen (untreated control) in ejaculates used for experiment 1.

Sperm (%)	Raw semen	FT semen (control)
Oscillatory motility	51.3 ± 8.6[a]	7.3 ± 7.2[b]
Progressive motility	2.7 ± 4.6[a]	1.1 ± 2.6[a]
Total motility	54.0 ± 13.0[a]	8.4 ± 6.6[b]
Viability	69.2 ± 11.2[a]	13.5 ± 9.5[b]
HOS+CB+	60.0 ± 10.5[a]	14.2 ± 4.8[b]

Values are expressed as means ± SD (n = 12 ejaculates). HOS+CB+: percentage of sperm positive to the hypoosmotic swelling test (HOS+) with an acrosome present (CB+). [a,b]For each sperm parameter, different letters between columns indicate significant differences (p < 0.05).

TABLE 2 | Sperm parameters evaluated in raw semen and frozen-thawed (FT) llama semen (untreated control) in ejaculates used for experiment 2.

Sperm (%)	Raw semen	FT semen (control)
Oscillatory motility	41.3 ± 19.7[a]	6.1 ± 6.0[b]
Progressive motility	3.1 ± 4.5[a]	3.0 ± 3.8[a]
Total motility	44.4 ± 19.1[a]	9.1 ± 5.8[b]
Viability	51.6 ± 16.8[a]	17.3 ± 6.8[b]
HOS+CB+	44.7 ± 11.9[a]	22.3 ± 8.9[b]

Values are expressed as means ± SD (n = 10 ejaculates). HOS+CB+: percentage of sperm positive to the hypoosmotic swelling test (HOS+) with an acrosome present (CB+). [a,b]For each sperm parameter, different letters between columns indicate significant differences (p < 0.05).

TABLE 3 | Sperm concentration, motility (oscillatory, progressive, and total), viability, and spermatozoa with functional membranes and an acrosome present (HOS+CB+) in frozen-thawed (FT) llama semen (untreated control) and FT samples centrifuged 20 min through 1,000 μl of Androcoll-E™ columns (FT-AE).

	FT semen (control)	FT-AE (1,000 μl)
Concentration (×10^6 sperm/ml)	30.0 ± 6.1[a]	4.4 ± 9.5[b]
Oscillatory motility (%)	7.3 ± 7.2[a]	1.4 ± 2.0[b]
Progressive motility (%)	1.1 ± 2.6[a]	0.4 ± 1.0[a]
Total motility (%)	8.4 ± 6.6[a]	1.8 ± 2.1[b]
Viability (%)	13.5 ± 9.5[a]	12.1 ± 10.0[a]
HOS+/CB+	14.2 ± 4.8[a]	11.0 ± 5.5[a]

Values are expressed as means ± SD (Experiment 1, n = 12 ejaculates). [a,b]For each sperm parameter, different letters between columns indicate significant differences (p < 0.05).

TABLE 4 | Sperm concentration, motility (oscillatory, progressive, and total), viability, and spermatozoa with functional membranes and an acrosome present (HOS+CB+) in frozen-thawed (FT) llama semen (untreated control) and FT samples submitted to different centrifugation times (10 and 20 min) through 500 μl of Androcoll-E™ columns (FT-AE).

	FT semen (control)	FT-AE (500 μl) 10 min	FT-AE (500 μl) 20 min
Concentration(×10^6 sperm/ml)	33.8 ± 10.0[a]	5.1 ± 4.1[b]	7.4 ± 4.5[b]
Oscillatory motility (%)	6.1 ± 6.0[a]	1.0 ± 1.1[b]	1.0 ± 1.6[b]
Progressive motility (%)	3.0 ± 3.8[a]	1.0 ± 1.7[a]	0.7 ± 1.6[a]
Total motility (%)	9.1 ± 5.8[a]	2.0 ± 2.6[b]	1.7 ± 3.2[b]
Viability (%)	17.3 ± 6.8[a]	21.5 ± 9.8[a]	24.7 ± 11.9[a]
HOS+/CB+	22.3 ± 8.9[a]	15.0 ± 8.9[a]	15.8 ± 9.2[a]

Values are expressed as means ± SD (Experiment 2, n = 10 ejaculates). [a,b]For each sperm parameter, different letters between columns indicate significant differences (p < 0.05).

of centrifugation used (10 and 20 min.). Although it is not possible to statistically compare experiments 1 and 2, the highest sperm recovery was observed in samples processed with 500 μl AE columns combined with 20 min of centrifugation at 800 *g* (**Tables 3, 4**).

None of the sperm pellets resuspended in TALP medium after AE treatment presented thread formation. In both experiments, a significant decrease ($p < 0.05$) in total and oscillatory sperm motility was observed in frozen-thawed samples centrifuged through AE compared to untreated control frozen-thawed semen. However, no significant differences were observed in sperm viability, membrane function, and acrosome integrity (HOS+CB+) between processed and control semen samples (**Table 3** for experiment 1 and **Table 4** for experiment 2). In addition, no statistical differences were observed in the other HOS/CB categories (HOS+/CB–, HOS–/CB+, and HOS–/CB–) between frozen-thawed samples centrifuged through AE and untreated control frozen-thawed semen (data not shown).

DISCUSSION

To our knowledge, this is the first study that evaluated the efficiency of a single-layer centrifugation (SLC) through AE to separate frozen-thawed llama sperm from SP and the freezing extender.

A significant decrease in all sperm parameters (total and oscillatory motility, viability, sperm membrane function, and acrosome integrity) was observed after thawing all samples. Several authors have observed the same results, not only in llamas and alpaca (20, 24, 34, 38–40) but also in thawed semen samples from other species [horse: (41); ram: (42, 43); dog: (28); bull: (44); donkey: (29, 30); brown bear: (45)]. The main concern in SACs is the greater loss in sperm quality after thawing (between 65 and 85%) compared to other species, in which the expected decrease in sperm survival is around 50% of the initial value (46). Accordingly, frozen-thawed SAC semen samples harbor a high percentage of damaged and dead cells; therefore, selecting good-quality sperm would be a useful procedure to implement.

Although sperm recovery in frozen-thawed samples processed by AE was low, it was possible to obtain llama sperm free of SP and freezing extender. In contrast, Giuliano et al. (47) observed that the use of AE colloid was unable to separate cooled llama sperm from the egg yolk extender used to preserve the samples. Bertuzzi et al. (32), on the other hand, successfully achieved the separation of llama sperm from SP in raw semen without previous enzymatic treatment using AE, but similarly

to the present study, obtained low sperm recovery. In our opinion, it would be possible to obtain greater numbers of recovered sperm by increasing the number of columns used and process the whole ejaculate (and not only an aliquot). In this study, a small volume of sample (100 μl) was placed over the AE column, but considering that the total volume of llama frozen-thawed semen varies between 0.25 and 5 ml, multiple columns could be used to process the whole frozen ejaculate, thus increasing the efficiency of the protocol in terms of sperm recovery. Furthermore, it has been suggested that the initial presence of thread formation in llama ejaculates would seem to play an essential role in the percentage of sperm recovery after the AE treatment (32). These authors observed a lower sperm recovery in samples that showed thread formation compared to the ones that did not. Thus, the same influence could be occurring in llama frozen semen since 40% of thawed samples presented thread formation. In addition, the presence of egg yolk in the diluent could further increase thread formation and exacerbate the situation.

Although total and oscillatory motility decreased in samples processed with AE, sperm viability, membrane function, and acrosome integrity were preserved. The presence of immotile sperm with intact and functional membranes in raw and frozen-thawed llama semen has been reported previously (24, 34, 35, 40). In contrast to our results, Giuliano et al. (47) observed that cooled llama sperm processed with Percoll® maintained the percentages of total sperm motility, while viable sperm decreased in comparison with the untreated cooled semen samples. The differences between the studies could be attributed to the different sperm preservation methods used (freezing vs. cooling) and also to the different colloids applied to process the samples (Androcoll™ vs. Percoll®).

Notably, in the present study and the one by Giuliano et al. (47), the use of AE and Percoll®, respectively, did not accomplish sperm selection, in consequence, the seminal quality of llama cryopreserved samples did not improve. In other species, greater percentages of motile, viable, and morphologically normal sperm with intact acrosomes and DNA have been reported in frozen-thawed samples treated with Androcoll [horse: (25, 26); dog: (27, 28); donkey: (29, 30); brown bear: (31)]. Differences in our results could be due to the fact that these authors used a species-specific Androcoll (Androcoll-E, Androcoll-P, Androcoll-C, and Androcoll-bear). It is worth noting that, currently, there are no reports on the use of a specific type of Androcoll for SACs. In a study performed in dromedary semen, the incorporation of SLC prior to freezing improved post-thaw sperm variables; however, these authors omitted the type of Androcoll used (48). The use of AE in the present study was decided because this treatment proved to be effective as part of a protocol to obtain llama embryos by IVF using raw llama semen previously treated with collagenase and then selected by AE (15). Other differences between the abovementioned studies and ours are the colloid:semen ratio and the centrifugation speed used. Regarding the colloid:semen relation, a similar proportion to the one used in Experiment 2 was tested in stallions (25, 26), while in studies carried out in brown bears, the authors used columns with similar volumes to those used in our experiment 1 (31). On the other hand, in dogs and donkeys, higher volumes of semen were placed over the colloid compared to our study (27–30). Finally, concerning centrifugation velocities, in the species where sperm quality improved after the use of Androcoll columns, lower centrifugation speeds were used (300 *g* 20 min and 600 *g* 10 min) compared to the one applied in the present study (800 *g*). We decided that testing a higher centrifugation speed was a good strategy because, taking into account the rheological characteristics of SAC semen (high structural viscosity and thread formation) and the fact that we did not treat the samples enzymatically prior to centrifugation through Androcoll, we considered that a higher speed could counteract the possible interference of these seminal characteristics with the separation of sperm from SP. Besides, in a previous experiment, we evaluated a lower centrifugation speed (600 *g*) to treat raw llama semen with AE without improving the quality of the sperm obtained and with less sperm recovery compared to 800 *g* (32). These results would seem to indicate that not only is the use of a species-specific Androcoll important when processing the samples but, for the protocol be efficient, the colloid:semen ratio and the centrifugation speed used perhaps also need to be varied according to the species.

Further research needs to be conducted to determine which modifications in the protocol such as colloid density, columns heights, and centrifugation speeds and time are required to improve sperm recovery and to increase the quality of the sperm obtained. Additionally, raw semen samples could be treated with AE before freezing as it has been demonstrated in other species that sperm selection with a SLC prior to cryopreservation can increase post-thaw sperm quality [horse: (41); boar: (49); brown bear: (45)]. One possible disadvantage of AE treatment prior to llama semen cryopreservation could be the low sperm recovery and thus an inadequate sperm pellet for cryopreservation. Added to this, previous studies have shown that freezing llama sperm in the absence of SP does not improve post-thaw cryosurvival (23, 24).

CONCLUSIONS

The use of AE columns, either 500 or 1,000 μl, allowed the separation of cryopreserved llama spermatozoa from SP and freezing extender, preserving sperm viability, membrane function, and acrosome integrity. Of the protocols tested, centrifugation through a 500 μl column of AE at 800 *g* during 20 min would be the method of choice to process frozen-thawed llama semen bound for reproductive biotechnologies such as IVF or intracytoplasmic sperm injection (ICSI). However, different AE:semen ratios, as well as other colloids, should be assayed to improve sperm recovery and to increase the quality of the sperm obtained.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Committee for the Use and Care of Laboratory Animals (CICUAL) of the Faculty of Veterinary Sciences of the University of Buenos Aires (protocol 2019/22).

AUTHOR CONTRIBUTIONS

CG carried out the study and critically read the manuscript. MLB and NV helped collect the samples. FF helped collect the samples and critically read and corrected the manuscript. SG critically read and corrected the manuscript. MVB critically read and translated the manuscript. MC designed and directed the study and wrote the manuscript.

FUNDING

This research was supported by grants from the University of Buenos Aires (UBACyT 20020190200084BA) and the Agencia Nacional de Promoción Científica y Tecnológica (PICT 2017-0394).

ACKNOWLEDGMENTS

The authors thank Deborah Margarita Neild for her critical reading and correction of the manuscript.

REFERENCES

1. Casaretto C, Martínez Sarrasague M, Giuliano S, Rubin de Celis E, Gambarotta M, Carretero MI, et al. Evaluation of Lama glama semen viscosity with a cone-plate rotational viscometer. *Andrologia*. (2012) 44:335–41. doi: 10.1111/j.1439-0272.2011.01186.x
2. Giuliano S, Carretero M, Gambarotta M, Neild D, Trasorras V, Pinto M, et al. Improvement of llama (*Lama glama*) seminal characteristics using collagenase. *Anim Reprod Sci*. (2010) 118:98–102. doi: 10.1016/j.anireprosci.2009.06.005
3. Tibary A, Vaughan J. Reproductive physiology and infertility in male South American camelids: a review and clinical observations. *Small Rum Res*. (2006) 61:283–98. doi: 10.1016/j.smallrumres.2005.07.018
4. Fumuso FG, Giuliano SM, Chaves MG, Neild DM, Miragaya MH, Gambarotta MC, et al. Seminal plasma affects the survival rate and motility pattern of raw llama spermatozoa. *Anim Reprod Sci*. (2018) 192:99–106. doi: 10.1016/j.anireprosci.2018.02.019
5. Ratto MH, Gomez C, Berland M, Adams GP. Effect of ovarian superstimulation on COC collection and maturation in alpacas. *Anim Reprod Sci*. (2007) 97:246–56. doi: 10.1016/j.anireprosci.2006.02.002
6. Condori RL, Huanca W, Chileno M, Cainzo J, Valverde F, Becerra JJ, et al. Effect of follicle-stimulating hormone addition on *in vitro* maturation and cleavage of alpaca (*Vicugna pacos*) embryos. *Reprod Fertil Dev*. (2010) 23:224. doi: 10.1071/RDv23n1Ab252
7. Huanca W, Condori RL, Chileno MA, Cainzos J, Becerra JJ, Quintela LA, et al. *In vivo* maturation and *in vitro* fertilization of alpaca oocytes. *Reprod Fertil Dev*. (2010) 23:204. doi: 10.1071/RDv23n1Ab211
8. Berland MA, von Baer A, Ruiz J, Parraguez V, Morales P, Adams GP, et al. *In vitro* fertilization and development of cumulus oocyte complexes collected by ultrasoundguided follicular aspiration in superstimulated llamas. *Theriogenology*. (2011) 75:1482–8. doi: 10.1016/j.theriogenology.2010.11.047
9. Arqque Monzón DB. Influencia del fluido folicular y gonadotropinas en la maduración y fertilización de ovocitos de alpacas huacaya. Puno: Facultad de Medicina Veterinaria y Zootecnia de la Universidad Nacional del Altiplano. (2017). Available online at: http://repositorio.unap.edu.pe/handle/UNAP/8084
10. Bravo Z, Valdivia M. Effect of follicular fluid on sperm motility of alpaca *Vicugna pacos* (Linnaeus, 1758). *JSM In vitro Fertil*. (2017) 2:1013.
11. Pérez Durand MG, Zevallos Aragón JP, Perez Guerra UH. Comparación de sistemas de cultivo de embriones de alpacas. *Rev Investig Altoandin*. (2017) 19:157–64. doi: 10.18271/ria.2017.274
12. Pacompia Torres MH. *Efecto de Las Gonadotropinas en La Maduración Y Fertilización de Ovocitos En Alpacas (Vicugna pacos)*. Perú: Universidad Nacional del Altiplano. (2017). Available online: http://repositorio.unap.edu.pe/handle/UNAP/5115
13. Mamani-Mango G, Gonzales MM, Hidalgo MR, Mendoza Mallma J, Ruiz Bejar J, Rivas Palma V, et al. Effect of extender and freezing rate on quality parameters and *in vitro* fertilization capacity of alpaca spermatozoa recovered from cauda epididymis. *Biopreserv Biobank*. (2018) 21:39–45. doi: 10.1089/bio.2018.0021
14. Conde P, Herrera C, Chaves M, Giuliano S, Director A, Trasorras V, et al. *In vitro* production of llama embryos by IVF and ICSI with fresh semen. *Anim Reprod Sci*. (2008) 109:298–308. doi: 10.1016/j.anireprosci.2007.10.004
15. Trasorras V, Giuliano S, Chaves G, Neild D, Agüero A, Carretero M, et al. *In vitro* embryo production in llamas (*Lama glama*) from *in vivo* matured oocytes with fresh semen processed with Androcoll-E™ using defined embryo culture media. *Reprod Dom Anim*. (2012) 47:562–7. doi: 10.1111/j.1439-0531.2011.01917.x
16. Mortimer D. Sperm preparation methods. *J Androl*. (2000) 21:357–66.
17. Henkel R, Schill W. Sperm preparation for ART. *Reprod Biol Endocrinol*. (2003) 1:108. doi: 10.1186/1477-7827-1-108
18. Aitken RJ, Clarkson JS. Significance of reactive oxygen species and antioxidants in defining the efficacy of sperm preparation techniques. *J Androl*. (1988) 9:367–76. doi: 10.1002/j.1939-4640.1988.tb01067.x
19. Bravo PW, Skidmore JA, Zhao XX. Reproductive aspects and storage of semen in *Camelidae*. *Anim Reprod Sci*. (2000) 62:173–93. doi: 10.1016/S0378-4320(00)00158-5
20. Aller JF, Rebuffi GE, Cancino AK, Alberio RH. Influencia de la criopreservación sobre la movilidad, viabilidad y fertilidad de espermatozoides de llama (*Lama glama*). *Arch Zoo*. (2003) 52:15–23.
21. Vaughan J, Galloway D, Hopkins D. Artificial insemination in alpacas (*Lama pacos*). Kingston, NY: RIRDC Rural Industries Research and Development Corporation (2003).
22. Fumuso FG, Arraztoa CC, Chaves MG, Neild DM, Giuliano SM, Miragaya MH, et al. Inseminación artificial de llamas con semen congelado. *Res Prelimi Invet*. (2018) 20:124. doi: 10.15381/rivep.v28i2.13080
23. Carretero MI, Fumuso FG, Chaves MG, Miragaya MH, Neild DM, Cetica P, et al. Comparison of two freeze thawing protocols for llama semen: with and without collagenase and seminal plasma in the medium. *Prelim. Res InVet*. (2017) 19:57.
24. Fumuso FG, Giuliano SM, Chaves MG, Neild DM, Miragaya MH, Carretero MI. Evaluation of the cryoprotective effect of seminal plasma on llama (*Lama glama*) spermatozoa. *Andrologia*. (2019) 2019:e13270. doi: 10.1111/and.13270
25. Macías García B, González Fernández L, Morrell JM, Ortega Ferrusola C, Tapia JA, Rodriguez Martínez H, et al. Single-layer centrifugation through colloid positively modifies the sperm subpopulation structure of frozen–thawed stallion spermatozoa. *Reprod Dom Anim*. (2009) 44:523–6. doi: 10.1111/j.1439-0531.2008.01276.x
26. Macías García B, Morrell JM, Ortega-Ferrusola C, González-Fernández L, Tapia JA, Rodríguez-Martínez H, et al. Centrifugation on a single layer of colloid selects improved quality spermatozoa from frozen-thawed stallion semen. *Anim Reprod Sci*. (2009) 114:193–202. doi: 10.1016/j.anireprosci.2008.08.025
27. Dorado J, Gálvez MJ, Morrell JM, Alcaráz L, Hidalgo M. Use of single-layer centrifugation with Androcoll-C to enhance sperm quality

in frozen-thawed dog semen. *Theriogenology*. (2013) 80:955–62. doi: 10.1016/j.theriogenology.2013.07.027
28. Urbano M, Dorado J, Ortiz I, Morrel JM, Demyda-Peyrás S, Gálvez MJ, et al. Effect or cryopreservation and single layer centrifugation on canine sperm DNA fragmentation assessed by the sperm chromatin dispersión test. *Anim Reprod Sci*. (2013) 143:118–25. doi: 10.1016/j.anireprosci.2013.10.005
29. Ortiz I, Dorado J, Morrell JM, Crespo F, Gosálvez J, Gálvez MJ, et al. Effect of single-layer centrifugation or washing on frozen-thawed donkey semen quality: Do they have the same effect regardless of the quality of the sample? *Theriogenology*. (2015) 84:294–300. doi: 10.1016/j.theriogenology.2015.03.021
30. Ortiz I, Dorado J, Morrell JM, Diaz-Jimenez MA, Pereira B, Consuegra C, et al. Comparison of sperm selection techniques in donkeys: motile subpopulations from a practical point of view. *Anim Reprod*. (2019) 16:282–9. doi: 10.21451/1984-3143-AR2018-0133
31. Anel-Lopez L, Ortega-Ferrusola C, Álvarez M, Borragán S, Chamorro C, Peña FJ, et al. Improving sperm banking efficiency in endangered species through the use of a sperm selection method in brown bear (*Ursus arctos*) thawed sperm. *BMC Vet. Res*. (2017) 13:200. doi: 10.1186/s12917-017-1124-2
32. Bertuzzi ML, Fumuso FG, Giuliano SM, Miragaya MH, Gallelli MF, Carretero MI. New protocol to separate llama sperm without enzymatic treatment using Androcoll-E™. *Reprod Domest Anim*. (2020) 55:1154–62. doi: 10.1111/rda.13755
33. Director A, Giuliano S, Carretero M, Pinto M, Trasorras V, Miragaya M. Electroejaculation and seminal characteristics in llama (*Lama glama*). *J Camel Practice Res*. (2007) 14:203–6.
34. Carretero MI, Neild D, Ferrante A, Caldevilla M, Arraztoa C, Fumuso F, et al. Effect of cryoprotectant and equilibration temperature on *Lama glama* sperm cryopreservation. *Andrologia*. (2015) 47:685–93. doi: 10.1111/and.12319
35. Giuliano S, Director A, Gambarotta M, Trasorras V, Miragaya M. Collection method, season and individual variation on seminal characteristics in the llama (*Lama glama*). *Anim Reprod Sci*. (2008) 104:359–69. doi: 10.1016/j.anireprosci.2007.02.016
36. Harrison RA, Vickers SE. Use of fluorescent probes to assess membrane integrity in mammalian spermatozoa. *J Reprod Fertil*. (1990) 88:343–52. doi: 10.1530/jrf.0.0880343
37. Carretero MI, Pigretti C, Bertuzzi ML, Fumuso FG. Test hipoósmotico combinado a la tinción de coomassie blue en espermatozoides de llama. *Spermova*. (2018) 8:129–32. doi: 10.18548/aspe/0006.10
38. Santiani Acosta A, Evangelista Vargas S, Valdivia Cuya M, Risopatrón González J, Sánchez Gutierrez R. Effect of the addition of two superoxide dismutase analogues (tempo and tempol) to alpaca semen extender for cryopreservation. *Theriogenology*. (2013) 79:842–6. doi: 10.1016/j.theriogenology.2012.12.012
39. Stuart CC, Vaughan JL, Kershaw CM, de Graaf SP, Bathgate R. Effect of diluent type, cryoprotectant concentration, storage method and freeze/thaw rates on the post-thaw quality and fertility of cryopreserved alpaca spermatozoa. *Sci Rep*. (2019) 9:12826. doi: 10.1038/s41598-019-49203-z
40. Fumuso FG, Giuliano SM, Chaves G, Neild DM, Miragaya MH, Bertuzzi ML, et al. Incubation of frozen-thawed llama sperm with seminal plasma. *Andrologia*. (2020) 2020:e13597. doi: 10.1111/and.13597
41. Hoogewijs M, Morrell J, Van Soom A, Govaere J, Johannisson A, Piepers S, et al. Sperm selection using single layer centrifugation prior to cryopreservation can increase thawed sperm quality in stallions. *Equine Vet J*. (2011) 43:35–41. doi: 10.1111/j.2042-3306.2011.00489.x
42. Hernández PJE, Fernández RF, Rodríguez SJL, Juárez RE, Soto MYG, García RAD. Effect of cryopreservation of sheep semen related to its viability and acrosomal status. *Rev Salud Anim*. (2012) 34:78–83.
43. Jha PK, Shahi Alam MG, Mansur AAL, Naher N, Islam T, Bhuiyan MU, et al. Cryopreservation of Bangladeshi ram semen using different diluents and manual freezing techniques. *Cryobiology*. (2019) 89:35–41. doi: 10.1016/j.cryobiol.2019.06.001
44. Sathe S, Shipley CF. Cryopreservation of semen. In: Hopper RM, editors. *Bovine Reproduction. 1st Edition*. Iowa: John Wiley & Sons, Inc (2015).
45. Álvarez-Rodríguez M, Álvarez M, Anel-López L, López-Urueña E, Manrique P, Borragán S, et al. Effect of colloid (Androcoll-bear, percoll, and puresperm) selection on the freezability of brown bear (*Ursus arctos*) sperm. *Theriogenology*. (2016) 85:1097–105. doi: 10.1016/j.theriogenology.2015.11.021
46. Watson PF. The causes of reduced fertility with cryopreserved semen. *Anim Reprod Sci*. (2000) 60–1:481–92. doi: 10.1016/S0378-4320(00)00099-3
47. Giuliano SM, Santa Cruz R, Arraztoa CC, Fumuso FG, Bertuzzi ML, Carretero MI. Selección espermática de semen refrigerado de llama con diluyente a base de yema de huevo. *Spermova*. (2019) 9:35–41. doi: 10.18548/aspe/0007.05
48. Malo C, Crichton EG, Morrell JM, Pukazhenthi BS, Johannisson A, Splan R, et al. Colloid centrifugation of fresh semen improves post-thaw quality of cryopreserved dromedary camel spermatozoa. *Anim Reprod Sci*. (2018) 192:28–34. doi: 10.1016/j.anireprosci.2018.02.005
49. Martinez-Alborcia MJ, Morrell JM, Gil MA, Barranco I, Maside C, Alkmin V, et al. Suitability and effectiveness of single layer centrifugation using androcoll-P in the cryopreservation protocol for boar spermatozoa. *Anim Reprod Sci*. (2013) 140:173–9. doi: 10.1016/j.anireprosci.2013.06.015

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 22 January 2021
doi: 10.3389/fvets.2020.583369

New Insights Into the Role of β-NGF/TrKA System in the Endometrium of Alpacas During Early Pregnancy

*Daniela E. Barraza[1], Luciana M. Sari[1], Silvana A. Apichela[1,2], Marcelo H. Ratto[3] and Martin E. Argañaraz[1,4]**

[1] Instituto Superior de Investigaciones Biológicas (INSIBIO), Consejo Nacional de Investigaciones Científicas y Técnicas - Universidad Nacional de Tucumán (CONICET-UNT), and Instituto de Biología "Dr. Francisco D. Barbieri", Facultad de Bioquímica, Química y Farmacia, UNT, San Miguel de Tucumán, Argentina, [2] Cátedra de Zootecnia General I, Facultad de Agronomía y Zootecnia, UNT, San Miguel de Tucumán, Argentina, [3] Facultad de Ciencias Veterinarias, Universidad Austral de Chile, Valdivia, Chile, [4] Cátedra de Biología Celular y Molecular, Facultad de Bioquímica, Química y Farmacia, UNT, San Miguel de Tucumán, Argentina

OPEN ACCESS

Edited by:
Khalid El Allali,
Agronomic and Veterinary Institute Hassan II, Morocco

Reviewed by:
Rosa Maria Garcia-Garcia,
Complutense University of Madrid, Spain
Deborah Neild,
University of Buenos Aires, Argentina
Martin G. Maquivar,
Washington State University, United States
Fabio S. Lima,
University of California, Davis, United States

***Correspondence:**
Martin E. Argañaraz
martin.arganaraz@fbqf.unt.edu.ar

Specialty section:
This article was submitted to Animal Reproduction - Theriogenology, a section of the journal Frontiers in Veterinary Science

Received: 14 July 2020
Accepted: 07 December 2020
Published: 22 January 2021

Citation:
Barraza DE, Sari LM, Apichela SA, Ratto MH and Argañaraz ME (2021) New Insights Into the Role of β-NGF/TrKA System in the Endometrium of Alpacas During Early Pregnancy. Front. Vet. Sci. 7:583369. doi: 10.3389/fvets.2020.583369

One striking reproductive feature in South American camelids is that more than 90% of gestations are established in the left uterine horn (LUH). This phenomenon could be related to a differential vascular irrigation of the LUH. An increase of vascularization in llama endometrium was observed after systemic administration of Beta Nerve Growth Factor (β-NGF), a neurotrophin present in the uterus and placenta of various mammals that is involved in pregnancy development. We hypothesized that the β-NGF signaling pathway is related to embryo implantation in the LUH in camelids. The aim of this study was to characterize the spatial expression of β-NGF and its high-affinity receptor, TrKA, between LUH and right uterine horn (RUH) of non-pregnant (NP) and early pregnant alpacas (15 and 30 days of gestation, 15 and 30P, respectively). In addition, β-NGF, TrKA, and Vascular Endothelium Growth Factor A (VEGFA) temporal gene expression patterns and counting of blood vessels were evaluated among groups. The β-NGF and TrKA were localized in the luminal, glandular, and vascular epithelium of the alpaca uterus and in the embryonic membranes of the 30-days-old conceptus. β-NGF and TrKA immunosignal were stronger in 15P females than that of NP and 30P. In addition, TrKA signal was higher in the LUH luminal epithelium of NP and 15P alpacas than that of NP-RUH and 15P-RUH. β-NGF mRNA relative abundance was higher in the 30P-RUH than that of NP-RUH; whereas TrKA mRNA abundance only differed between 15P-RUH and NP-LUH. VEGFA mRNA relative abundance was higher in NP females compared to the LUH of 15P and 30P alpacas, and lower to their right counterparts. The number of vessels per field was higher in 15P than that of 30P. A positive correlation was observed between the number of vessels per field and β-NGF immunosignal in 15P-LUH.

In contrast, the area occupied by vessels was higher in 30P alpacas than of NP and 15P females. The changes of β-NGF/TrKA expression pattern in the peri-implantation endometria between LUH and RUH and their localization in the extraembryonic membranes support the implication of the neurotrophin during implantation and pregnancy development in South American Camelids.

Keywords: alpacas, embryo implantation, uterus, β-NGF, VEGF

INTRODUCTION

In the reproductive physiology of mammals, critical events related to embryo survival occur during early pregnancy, with the majority of embryo losses occurring in the first month of pregnancy (1, 2). In South American camelids (SACs), the pregnancy rate 30 days after mating is <50% (3), indicating that embryo losses are much higher in SACs than in other small ruminants (4). Particularly, in SACs, the embryos formed in the right oviduct must first migrate to the left uterine horn (LUH) to subsequently achieve their successful implantation. In alpacas, embryos reach the uterus on 6 days post ovulation (dpo) and by 9 dpo, 83% of embryos derived from right-ovary ovulations have arrived to the LUH. Maternal recognition of pregnancy occurs between 8 and 10 dpo, conceptus elongation between 10 and 15 dpo, and the apposition and implantation start around 20 dpo (5–7). If an embryo implants in the right uterine horn (RUH) gestation probably would not continue, since RUH would seem to be inadequate to sustain pregnancy (8, 9), (since 98% are carried out in LUH, a 2% do actually get to term in RUH) (10, 11). It has been proposed that this unique pattern of early embryo migration and implantation is influenced by a vascular asymmetry favoring the blood vessels that irrigate the LUH (12). Interestingly, Urra et al. (13) reported an increase in endometrial vascularization in the llama uterus, after the intrauterine administration of beta nerve growth factor (β-NGF).

The β-NGF, a member of the neurotrophin family, would seem to be a key factor for the success of pregnancy since it constitutes a functional link between the nervous, immune, and endocrine systems (14). The multiple effects of β-NGF are mediated through its receptors, the high-affinity membrane receptor tyrosine kinase A (TrkA) and the low-affinity receptor, the nerve growth factor receptor (NGFR or p75NTR) (15–17). The expression of β-NGF and its receptors in a wide variety of reproductive tissues, including different organs of the male and female genital tract, enables it to perform key reproductive functions.

This system is present in the uterus of several species such as rat (18), squirrel (15), ewe (19), horse, pig, human (20), and induced ovulatory species such as rabbits and camelids (17). Several reports suggest that this factor would participate in the endometrial prostaglandin synthesis during the maternal recognition of pregnancy, early development of the embryo, and conceptus immunotolerance (21, 22). Furthermore, in humans and rats, β-NGF has been shown to be a potent angiogenic factor, increasing the expression of vascular endothelial growth factor (VEGF) and promoting vascular cell proliferation in female reproductive organs (23, 24). The VEGFA, a member of the VEGF family, is a marker of uterine receptivity for implantation in humans and bovines. It mediates the establishment of an appropriate uterine environment through the regulation of endothelial cell growth, angiogenesis, and vascular permeability (25).

In camelids, β-NGF is present in the seminal plasma acting as an ovulation-inducing factor (26, 27). The factor is absorbed from the endometrium surface after copulation, rapidly entering systemic circulation to elicit GnRH secretion from the hypothalamus (28). Moreover, the factor increases uterus vessel formation and *corpus luteum* vascularization. In fact, β-NGF addition to llama cultured granulosa cells increased the expression of *VEGFA* after 48 h *in vitro* culture (29).

The mechanisms leading to embryo implantation and survival in SACs is far from being explained. Given this scenario, associated with the reports on the roles of β-NGF in the uterus during pregnancy, the objectives of this study were: (1) Characterize the spatial localization and expression pattern of β-NGF and TrkA in the endometrium of LUH and RUH in alpacas during the first month of gestation. (2) Describe the VEGFA gene expression pattern and count the number of associated blood vessels, and its association with β-NGF expression.

MATERIALS AND METHODS

Animals and Sampling

Twelve adult female Huacaya breed alpacas (*Vicugna pacos*), > 2 years of age, weighing 65–70 kg and that were destined for meat production, were used in this study. According to their reproductive status, females were divided into 3 experimental groups: non-pregnant (NP; $n = 4$), 15 days of pregnancy (15P; $n = 4$), and 1 month of pregnancy (30P; $n = 4$).

Non-pregnant and 15 days-pregnant alpacas were obtained from the Veterinary Research Center (IVITA), Universidad Nacional Mayor de San Marcos (UNMSM) in the province of Canchis, Perú (14°S, 71°W; 3698 m altitude). Virgin females, between 2 and 3 years of age, were mated once with a fertile adult male and they were slaughtered fifteen days after mating, in line with standards set by the local Committee of Animal Ethics and Welfare (Comité de Ética y Bienestar Animal; CEBA from the School of Veterinary Medicine of the UNMSM). Females with follicles of <7 mm in diameter in their ovaries were considered NP; while, those with a *corpus luteum* and embryos in the uterus were considered pregnant (**Table 1**).

Alpacas around 1 month of pregnancy (30P) were obtained from a slaughterhouse at Huancavelica, Perú (12°S, 74°W,

TABLE 1 | Reproductive features of alpaca's experimental groups.

Physiological status	*N*	Follicle/CL diameter (mm)	Left side follicle/CL (%)	Embryo (mm)	Left side pregnancy (%)
NP	4	8.5 ± 1.8	75	NA	NA
15P	4	14.5 ± 1.3	50	18.5 ± 1.1**	NA
30P	4	16.0 ± 1.0	50	16.5 ± 1.5*	100

NP, non-pregnant; 15P, 15 days-pregnant; 30P, 30 days-pregnant; CL, corpus luteum.
NA, not applicable.
***Elongated embryo.*
**Crown-rump length.*

3676 m altitude). The gestational age (GA) of these animals was calculated using embryo crown-rump length (CRL) and the formula GA= $(10.328*CRL)^{0.4427}$ (**Table 1**) as it was previously reported (6, 7, 30, 31). Based on previous studies maternal recognition of pregnancy occurs between 8 and 10 dpo and embryo apposition happens around 20 dpo (5, 30). Therefore, 15P samples corresponded to the pre-implantation stage; while in 30P samples the embryo was already implanted in the uterus.

Endometria from the midsection of the LUH and RUH were dissected in two segments of 10 mm, which were subsequently placed in 4% formaldehyde-PBS solution (pH 7.4) for immunohistochemical assays or RNAlater® (Ambion, Austin, USA) for RT-PCR assays. RNAlater®-embedded samples were transported on dry ice to the laboratory at INSIBIO, Argentina and stored at −80°C until further analysis.

RNA Isolation and cDNA Synthesis

Total RNA from endometria was extracted using the Spin Vacuum total RNA Isolation System™ (Promega, Madison, USA) according to the manufacturer's instructions. The RNA samples were quantified at 260 nm on a Shimadzu UV-spectrophotometer 1800 (Shimadzu Corporation, Japan) and its integrity was examined by electrophoresis on 1.5% agarose gels stained with Invitrogen™ SYBR™ Safe DNA Gel Stain (Carlsbad, USA). Finally, total RNA was stored at −80°C.

Reverse transcription of all of the RNA samples was carried out with M-MLV reverse transcriptase™ (Promega) and Oligo(dT)15-primer™ (Promega) in a 25 μl reaction mixture. Reactions were performed by incubating the mixture in a thermocycler (Techne™ TC-512 Gradient Thermal Cycler, Burlington, USA) at 42°C for 90 min followed by a reverse-transcriptase inactivation at 94°C for 5 min.

Semi-quantitative PCR of *β-NGF, TrKA, and VEGFA*

The relative abundance of *β-NGF, TrKA,* and *VEGFA* mRNA was analyzed by semi-quantitative PCR in NP ($n = 4$), 15P ($n = 4$), and 30P ($n = 4$) endometria using *ACTB* as reference gene as was described by Barraza et al. (32).

PCR amplifications were carried out in a final volume of 10 μl containing 0.5 μL of cDNA, 2 μL of 5X Green GoTaq® Reaction Buffer (Promega), 0.2 mM of dATP, dTTP, dCTP and dGTP (Promega), 2.5 units of GoTaq® DNA polymerase (Promega), and 1 μM of each primer pair: *β-NGF (5′-TGCTGGG AGAGGTGAACATT-3′, 5′-CGAAGGTGTGGGTTGTGGTA-3′), TrKA (5′-GCTTCATCTTCACCGAGTTCCT-3, 5′-TAGCCAG CAGCGTGTAGTTG-3′), VEGFA (5′-CGGTATAAATCCTGG AGCGT-3′, 5′-GCCTCGGCTTGTCACATCT-3′)* and *ACTB (5′-GCGGGACCACCATGTACC-3′, 5′-ACTCCTGCTTGCTGAT CCAC-3′)*. Oligonucleotides were synthetized at Thermo Fisher Scientific Custom Standard DNA Oligos Service (Buenos Aires, Argentina).

Different amplification settings were assayed to determine optimal PCR conditions: 94°C for 3 min, followed by 40 cycles at 94°C for 10 s, 60°C for 5 s, 72°C for 5 s, and a final extension at 72°C for 5 min. PCR products were analyzed with 1.5% agarose gel electrophoresis.

For semi-quantitative expression analysis, the 4 samples of each uterine horn and physiological status were amplified in duplicate ($n = 4$, $r = 2$). The amplification products were visualized in agarose gels and documented with a Pentax™Optio™ M90 digital camera (Milan, Italy), and the optical density of PCR products was quantified using ImageJ 1.42q software (National Institutes of Health, Bethesda USA). Data represent the average of the 2 PCR amplifications. The relative abundance of each transcript was normalized against that of *ACTB*, and the transcript/*ACTB* ratio was calculated.

Immunohistochemistry

Immune assays were performed as described by Sari et al. (33). Fixed tissues were dehydrated in ethanol, cleared in chloroform, and embedded in paraffin blocks. Five-micron sections were cut and mounted on Biotraza positively charged slides (Huida Medical Instruments CO., Jiangsu, China). After deparaffinization with xylene and rehydration, sections were subjected to antigen retrieval by incubating in Proteinase K (Sigma-Aldrich, St. Louis, USA) solution (10 μg/μL in Tris EDTA (TE) Buffer, 50 mM Tris Base, 1 mM EDTA, 0.5% Triton X-100, pH 8.0) for 20 min at 37°C in a humid chamber, followed by incubation in TE Buffer at room temperature (RT) for 10 min. The slides were then blocked with 1% Bovine Serum Albumin (Sigma-Aldrich) in PBS at room temperature for 30 min. Then, they were incubated at 37°C for 1 h with polyclonal antibodies against β-NGF (dilution 1:1,500, sc-548) or TrKA (dilution 1:100, sc-118, Santa Cruz Biotechnology, Santa Cruz, USA). After washing in 0.02% Tween-PBS, slides were incubated for 20 min at RT in a humidified chamber with a 1:200 dilution of the biotinylated anti-rabbit IgG antibody (B8895, Sigma-Aldrich). Subsequently, samples were incubated at RT for 30 min with

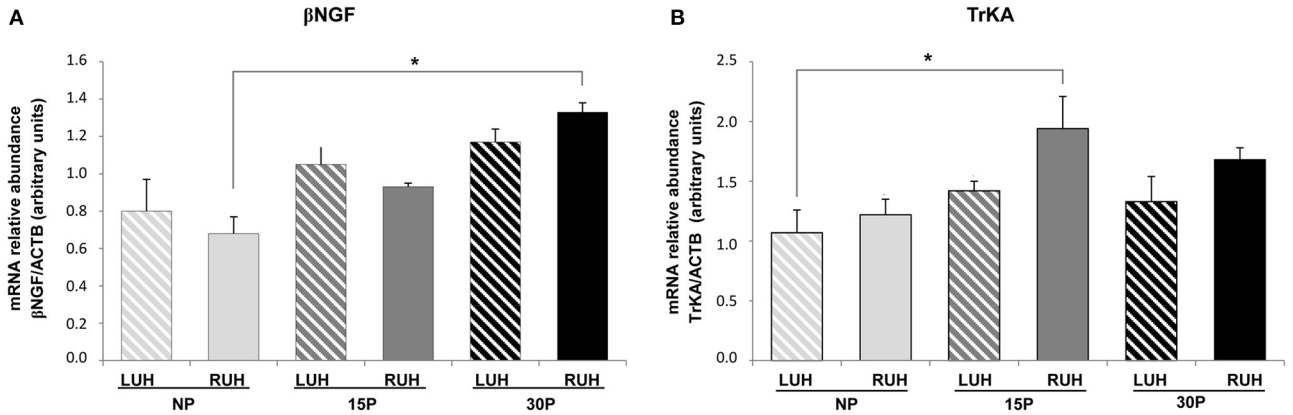


FIGURE 1 | Relative abundance of *β-NGF* **(A)** and *TrKA* **(B)** transcripts in the endometrium of non-pregnant (NP), 15 days-pregnant (15P), and 30 days-pregnant (30P) alpacas by RT-PCR. Histograms illustrate mean values ± SE. Asterisks denote significant differences (*$p < 0.05$). LUH (left uterine horn) and RUH (right uterine horn).

a solution 1:500 ExtraAvidin®-Alkaline Phosphatase (Sigma-Aldrich). Following three washes with 0.02% Tween in PBS, the sections were incubated with SIGMAFAST™ BCIP®/NBT (B5655, Sigma-Aldrich) until color was developed.

Finally, the sections ($n = 4$) were counterstained with Nuclear Fast Red (N3020, Sigma-Aldrich), then dehydrated and mounted with Entellan® (Merck, Darmstadt, Germany). Negative controls were performed by replacing the primary antibody with blocking buffer with 1% BSA-PBS with 0.02% Tween under the same experimental conditions.

Samples were observed under a Leica DM500™ light microscope, and images were captured with a Leica ICC50 ™ HD camera using LASZ Leica Inc. Software.

The ImageJ 1.42q software (National Institutes of Health, Bethesda USA) was used to measure the immunolabeled area of each sample according to Jensen (34). For this analysis, three different photographs (100X) of each section were used. Three rectangular regions of interest (ROI) were randomly selected in each image for its evaluation. First, the images were converted to 8 bits, then a specific threshold was determined and quantification was performed. Data were expressed as pixels/μm^2.

Histological Evaluation of Blood Vessels

The number and area occupied by vascular vessels were determined as follows: endometrial sections of 5 μm were stained with hematoxylin and eosin (BIOPUR, Rosario, Argentina). Then, images were captured with a DM500™ microscope coupled to a Leica ICC50™ HD camera, and the images were converted to 8 bits. Each vessel was marked in black and ImageJ v1.42q (National Institutes of Health, Bethesda USA) was used to analyze the number of marked vessels/field and the total area of vascularization (35). Nine representative microscopic fields of each uterine horn ($n = 4$) were randomly selected for all measurements. Data represent the average of the nine measurements at 100X magnification. Data were expressed as a percentage per field of endometrium.

Statistical Analysis

Statistical analysis was performed with InfoStat software (Universidad Nacional de Córdoba, Córdoba, Argentina, see http://infostat.com.ar). Gene relative abundance as well as immunohistochemistry staining were analyzed with One-way Analysis of Variance (ANOVA) followed by Tukey *post-hoc* test, which is a multiple comparison test. The power of each performed test with $\alpha = 0.050$ was between 1.0 and 0.8; 0.80 is the lowest desired power value. Less than desired power indicates there are less likely possibilities to detect a difference when one actually exists. On the other hand, the number of vessels and the vascularized area were evaluated using the Kruskal–Wallis test (a non-parametric, distribution free test). Results are expressed as the mean ± standard error (SE). In addition, correlation analysis between the variables was performed for each uterine horn, without discriminating between physiological status. Pearson Correlation Coefficient was calculated. In all the analyses, data were considered statistically significant at $p < 0.05$.

RESULTS

Relative Abundance of *β-NGF* and *TrKA* mRNA in Alpaca Endometrium

The presence of *β-NGF* and *TrKA* transcripts was found in the uterine horns of alpacas in both pregnant and non-pregnant females.

The relative abundance of *β-NGF* showed similar levels between the left and right uterine horns in all the experimental groups. When the physiological status was compared, the RUH of 30P animals displayed higher relative abundance levels than the RUH of NP females, $p = 0.008$. While *β-NGF* relative abundance in the LUH presented steady levels in all the groups (**Figure 1A**).

Regarding *TrKA,* transcript relative abundance was higher in the right uterine horn of 15P females compared to NP-LUH, $p = 0.025$ (**Figure 1B**).

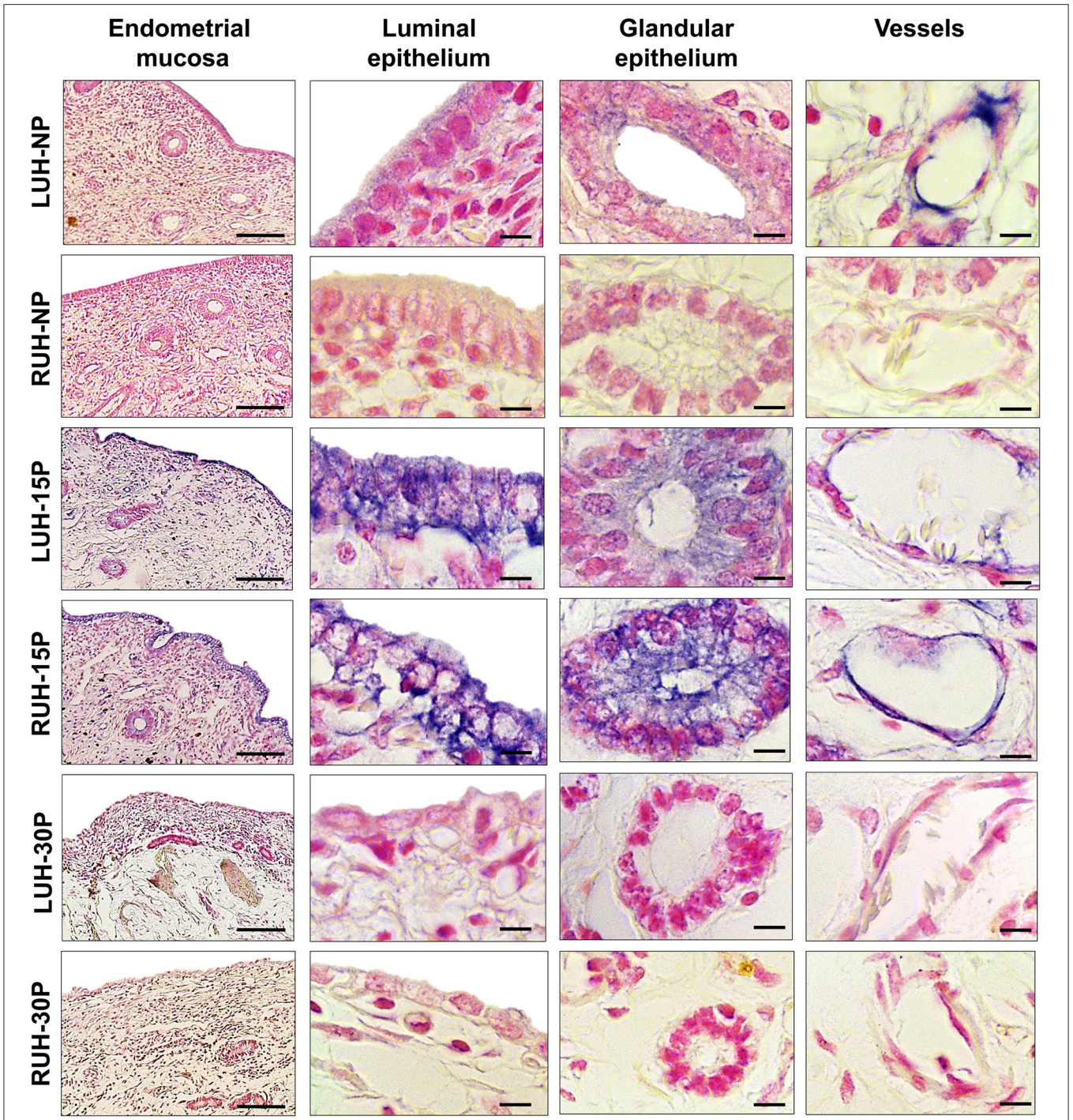


FIGURE 2 | Immunohistochemical localization of β-NGF in the endometrial mucosa of alpacas, positive labeling gives a blue-violaceous coloration. A 10X magnification of endometria of the left uterine horn (LUH) and right uterine horn (RUH) of non-pregnant females (NP); 15 days-pregnant (15P); and 30 days-pregnant females (30P) is presented (scale bar = 100 μm). Also, a 100X magnification of the luminal, glandular, and vascular endothelium of the different physiological status is shown (scale bar = 10 μm).

Immunolocalization of β-NGF and TrKA in Alpaca Endometrium

β-NGF tissue localization and immunolabeling intensity changed during the peri-implantation period; the signal was more intense in the uterine horns of 15P alpacas compared to the uterine horns of NP and 30P females. In non-pregnant females, the β-NGF mark was faint and located in the luminal and glandular epithelium as well as the endothelium of both uterine

TABLE 2 | Densitometry analysis of β-NGF immunolabeling in alpaca endometria.

β-NGF		Luminal epithelium	Glandular epithelium	Vascular epithelium	Total immunolabeling
NP	LUH	$30140 \pm 65.39^{b,A}$	29986 ± 29.78^{a}	19725 ± 29.18^{b}	$79851 \pm 97.13^{b,A}$
	RUH	$6214 \pm 9.37^{a,B}$	9433 ± 3.69^{a}	$8051 \pm 14.35^{a.b}$	$23698 \pm 10.52^{a,B}$
15P	LUH	78435 ± 67.39^{c}	81881 ± 33.12^{b}	60675 ± 43.54^{c}	220991 ± 104.71^{c}
	RUH	74510 ± 20.12^{c}	80920 ± 159.33^{b}	53315 ± 62.76^{c}	208745 ± 225.73^{c}
30P	LUH	5109 ± 2.08^{a}	3653 ± 2.57^{a}	3929 ± 8.46^{a}	12692 ± 8.57^{a}
	RUH	4687 ± 5.26^{a}	3427 ± 9.58^{a}	$4819.4 \pm 3.54^{a.b}$	12934 ± 8.03^{a}

**Mean values (pixels/μm^2) ± SE are presented, comparisons are made within each column. When the physiological status are compared the significant differences ($p < 0.05$) are indicated by different lowercase letters (a, b, c). The statistical differences ($p < 0.05$) between LUH and RUH within NP (non-pregnant), 15P (15 days-pregnant) and 30P (30 days-pregnant) status are indicated by different capital letters (A, B). LUH, Left uterine horn; RUH, right uterine horn.*

horns. NP-LUH showed a stronger immunosignal compared to NP-RUH at the luminal epithelium. In 15 day-pregnant alpacas, an intense immunolabel was observed mainly in the luminal epithelium. A mark was also present in the glandular epithelium and the endothelium. No differences were observed between the left and right uterine horns. In 30 day-pregnant animals, β-NGF staining was weak in the endometrium, no differences between LUH and RUH were detected (**Figure 2** and **Table 2**).

TrKA mark was co-localized with β-NGF. The receptor immunolabel was detected in the luminal, glandular, and vascular epithelium. The same distribution was observed in pregnant and non-pregnant alpacas and between left and right uterine horns (**Figure 3**). However, the mark was stronger in the uterine horns of 15P females compared to NP and 30P animals. Statistically significant differences between LUH and RUH were observed only at the luminal epithelium of NP and 15P alpacas, where the signal was more intense in the LUH (**Table 3**).

In 30 days-pregnant alpacas, a strong immunosignal was observed in the extraembryonic membranes of the conceptus for β-NGF (**Figures 4A,B**) and TrKA (**Figures 4C,D**).

Relative Abundance of *VEGFA* in Alpaca Endometrium

VEGFA transcripts were present in the endometrium of uterine horns from non-pregnant and pregnant alpacas (**Figure 5**). Differences between left and right uterine horns were observed only in pregnant alpacas (15 and 30P), in all cases, the RUH showed a higher relative abundance than the LUH ($p \leq 0.01$).

When the different physiological status were compared, only the LUH of non-pregnant females displayed a higher relative abundance of *VEGFA* than that of LUH of pregnant alpacas. The RUH presented similar relative abundance in all the experimental groups. No statistically significant association was observed between β-NGF expression and *VEGFA* relative abundance (**Table 4**).

Vessels Abundance and Distribution

The number of blood vessels was similar for LUH and RUH in the 3 groups studied. The endometrium of 30 days-pregnant alpacas showed fewer vessels than 15 day-pregnant females. 30P-RUH was significantly different from both 15P-RUH and 15P-LUH. Whereas, 30P-LUH presented a smaller number of vessels than 15-LUH (**Figure 6A**).

The area occupied by the vessels was similar between non-pregnant and 15 days-pregnant females. While 30 days-pregnant animals presented significantly higher values of the vascularized area compared to non-pregnant and 15 day-pregnant alpacas. No differences between left and right uterine horns were detected (**Figure 6B**).

LUH displayed a positive association between β-NGF immunolabelling and the number of vessels ($p = 0.034$); however, the correlation between β-NGF immunolabelling and the vascularized area of the endometrium was negative ($p = 0.027$). Regarding RUH, no statistically significant association was observed between β-NGF immunosignal and the number or area of vessels (**Table 4**).

DISCUSSION

The current study reveals for the first time the gene expression patterns and the protein localization of β-NGF and TrKA in the alpaca uterus during the peri-implantation period along with *VEGFA* gene expression patterns and the features of endometrium vasculature. These results support the implication of the β-NGF system during the early pregnancy period of South American camelids.

β-NGF and TrKA presence has been reported in the uterus of spontaneous and reflex ovulatory species, as well as in species with different placentation, such as humans, rodents, and rabbits with hemochorial placentation, goats and bovines with synepitheliochorial placentation, and pigs and horses with epitheliochorial placentation (17, 20, 36, 37). In all the species assayed, β-NGF immunoreactivity was detected in the luminal epithelium, glandular epithelium, and vascular tissue, this latter especially in pig and horse. The uterine expression of TrKA mirrored that of its ligand, it is mainly localized in the luminal, glandular epithelium, and vessels of the above-mentioned species (17, 20, 36, 37). Coincidently, the same distribution was observed for β-NGF system in alpaca oviducts (38), and uterine horns. In camelids, the establishment of pregnancies occurs almost exclusively in the LUH; at 15 days of pregnancy elongated embryos, that reach a length of 21 cm, are free within the uterine lumen until day 22–26 of gestation when the trophoblast

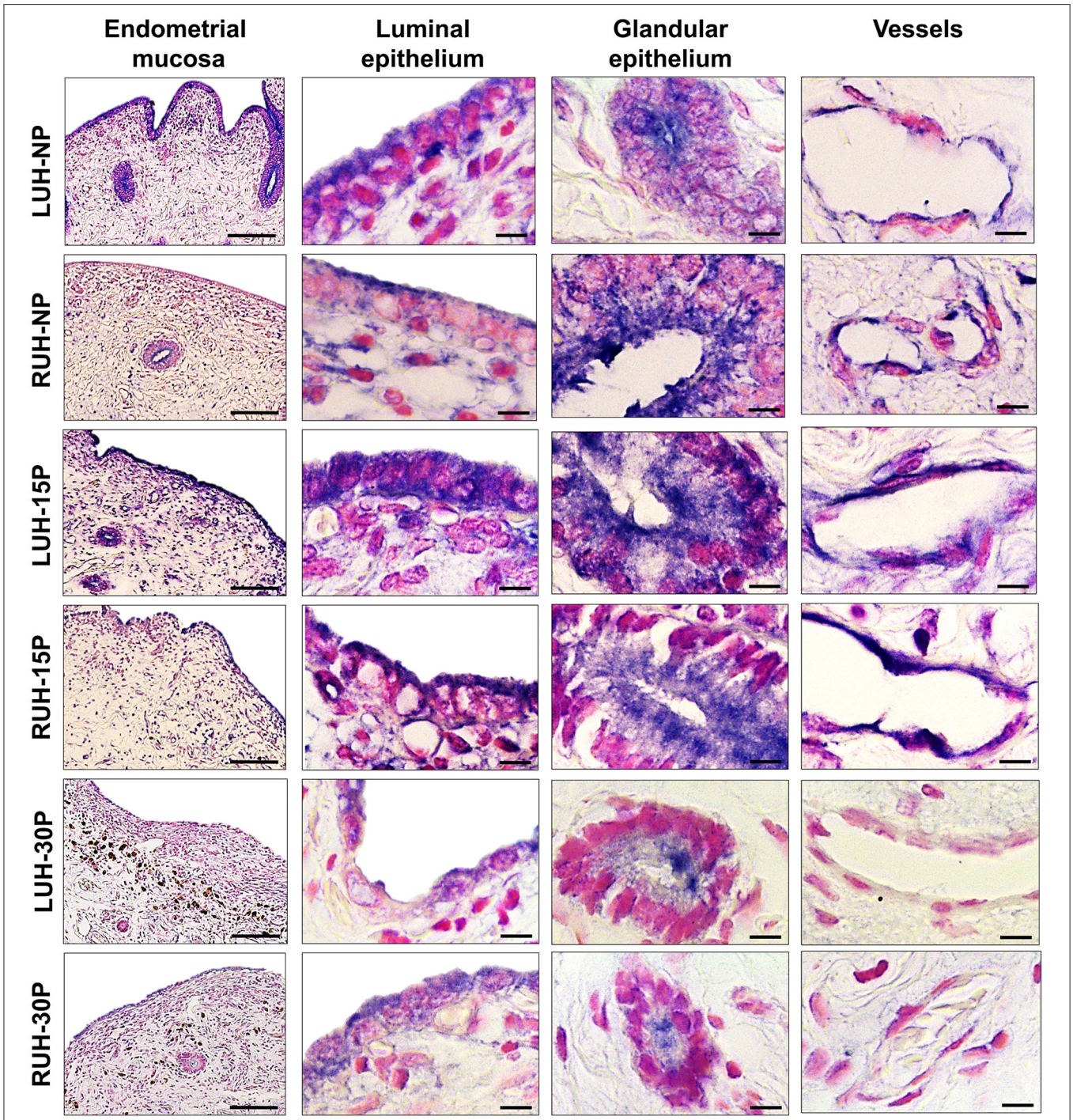


FIGURE 3 | Immunohistochemical localization of TrKA in the endometrial mucosa of alpacas, positive labeling gives a blue-violaceous coloration. A 10X magnification of endometria of the left uterine horn (LUH) and right uterine horn (RUH) of non-pregnant females (NP); 15 days-pregnant (15P); and 30 days-pregnant females (30P) is presented (scale bar = 100 μm). Also, a 100X magnification of the luminal, glandular and vascular endothelium of the different physiological status is shown (scale bar = 10 μm).

becomes apposed to the epithelial surface of the uterus (6, 7). Striking differences were determined in alpacas for β-NGF and TrKA immunolabeling among the different physiological status analyzed. The signal was remarkably strong in the uterus of 15 days-pregnant females when the embryo elongates and the endometrium prepares for implantation. In contrast, it was faint in non-pregnant and 30 days-pregnant alpacas. In squirrel (*Citellus dauricus Brandt*), β-NGF and TrKA proteins reached the

TABLE 3 | Densitometry analysis of TrKA immunolabeling in alpaca endometria.

TrKA		Luminal epithelium	Glandular epithelium	Vascular epithelium	Total immunolabeling
NP	LUH	$102691 \pm 58.71^{b.c,A}$	110213 ± 72.76^{a}	47758 ± 148.23^{b}	$260663 \pm 205.68^{b.c}$
	RUH	$61587 \pm 60.11^{a,B}$	89403 ± 45.13^{a}	47950 ± 27.85^{b}	$198940 \pm 90^{a.b}$
15P	LUH	$108196 \pm 78.62^{c,A}$	151973 ± 35.88^{b}	85953 ± 26.31^{c}	346123 ± 108.34^{d}
	RUH	$75620 \pm 58.33^{a,B}$	147180 ± 115.85^{b}	$64857 \pm 91.01^{b.c}$	$287657 \pm 258^{c.d}$
30P	LUH	67204 ± 24.84^{a}	89689 ± 60.97^{a}	3909 ± 3.89^{a}	160803 ± 82.38^{a}
	RUH	$79176 \pm 27.92^{a.b}$	101597 ± 79.04^{a}	3789 ± 6.26^{a}	151814 ± 68.71^{a}

**Mean values (pixels/μm^2) ± SE are presented, comparisons are made within each column. When the physiological status are compared the significant differences ($p < 0.05$) are indicated by different lowercase letters (a, b, c, d). The statistical differences ($p < 0.05$) between LUH and RUH within NP (non-pregnant), 15P (15 days-pregnant) and 30P (30 days-pregnant) status are indicated by different capital letters (A, B). LUH, Left uterine horn; RUH, right uterine horn.*

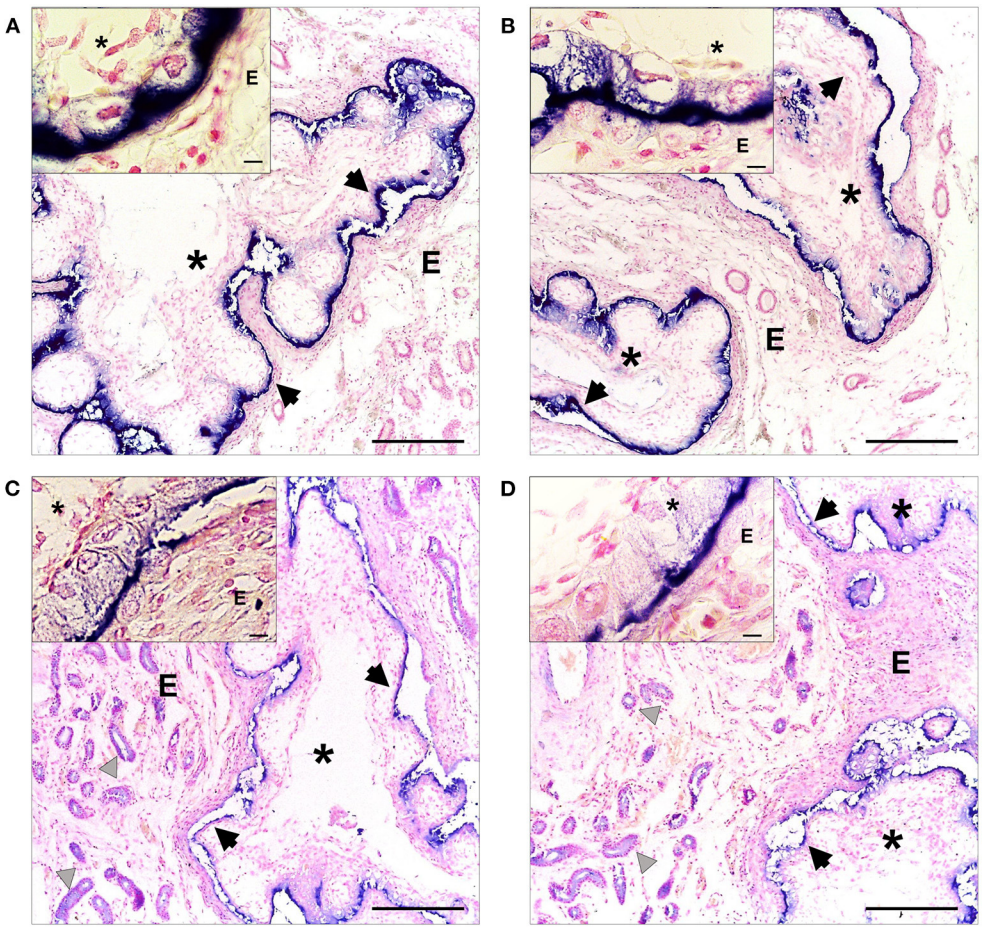


FIGURE 4 | Immunohistochemical localization of β-NGF **(A,B)** and TrKA **(C,D)** in the endometrial mucosa of 30 days-pregnant alpacas, positive labeling gives a blue-violaceous coloration. 100-fold magnification (bar = 100 μm) of left uterine horn **(A,C)** and right uterine horn **(B,D)**. In addition, inset shows a magnification of the extraembryonic membranes (bar = 10 μm). E: endometrium, asterisks: extraembryonic membranes, black arrows indicate the positive immunolabeling on the extraembryonic membranes, gray triangles indicate positive TrKA immunostaining in glandular epithelium and vessels.

highest immunoreactivity at the time of early pregnancy (15). In coincidence, Lobos et al. (18) reported higher amounts of mature β-NGF in the uterus from early pregnant rats compared to non-pregnant, middle, and late pregnant females. Interestingly, although β-NGF protein was almost imperceptible in the uterus of 30 days-pregnant alpacas, a strong signal was ascertained

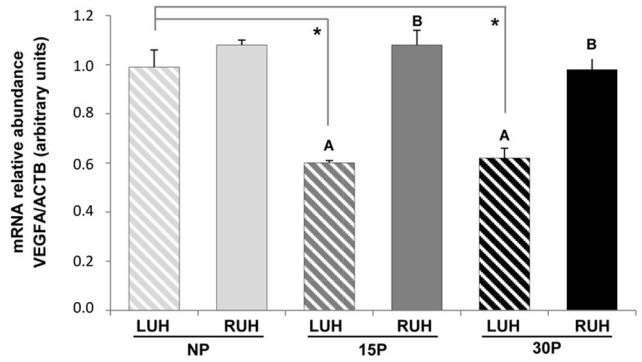


FIGURE 5 | Relative abundance of *VEGFA* mRNA in the endometrium of non-pregnant (NP), 15 days-pregnant (15P), and 30 days-pregnant (30P) alpacas by RT-PCR. Histograms illustrate mean values ± SE. Different uppercase letters **(A,B)** indicate significant differences between LUH (left uterine horn) and RUH (right uterine horn) within NP, 15P, and 30P status ($p < 0.05$). Asterisks indicate significant differences (*$p < 0.05$) between the physiological status.

in the extraembryonic membranes, as well as for TrKA signal. According to this, in humans, β-NGF was found in the cyto and syncytial trophoblast, chorionic mesodermic cells, and in decidua during the first trimester of gestation (39). Moreover, Coassin et al. (40) also reported the presence of β-NGF and TrKA in human amniotic membranes. In alpacas, even though no differences were observed in the β-NGF immunohistochemical patterns between the LUH and the RUH during pregnancy, TrKA immunolabeling was stronger in the LUH of non-pregnant and 15 days-pregnant alpacas. Maranesi et al. (17), demonstrated that treatment with TrKA inhibitor reduced the NGF-induced pathway in rabbit uterus. In addition, gene expression studies of matrix metalloproteinases (MMPs) during early pregnancy in alpacas (*Vicugna pacos*), demonstrated higher mRNA levels of MMP-2, which play an important role during embryo implantation in the uterine horn, in the LUH compared to the RUH of non-pregnant and 15 days-pregnant alpacas (41). Interestingly, MMP-2 activates pro-NGF into NGF (42). All these data would suggest that the NGF pathway is more active in the luminal epithelium of the left uterine horn, especially in the pre-implantation period, when the immunolabelling of β-NGF and TrKA was increased.

It has been suggested that β-NGF function in the uterus and placenta during pregnancy is to ensure adequate maternal immunomodulatory and developmental processes at the fetal-maternal interface. A well-balanced level of β-NGF is required for a successful pregnancy outcome as both insufficient or elevated levels of the factor may provoke fetal rejection (2, 21, 43). In alpacas the β-NGF system was not only detected in embryo membranes when the implantation has already occurred but also in the pre-implantation endometrium, during embryo elongation, suggesting a role during this period.

Intriguingly, in alpaca endometrium β-NGF and TrKA mRNA and protein expression were dissimilar, analogous observations were reported by Sari et al. (38) when analyzing gene and protein expression pattern in the llama oviduct. Many authors have postulated that regulation of β-NGF production does not occur solely at the level of transcription and that post-transcriptional mechanisms operate as well. Even more, there is a diverse post-transcriptional regulation between different cell types (44–46). For example, in rat uterus, alterations in NGF isoforms during pregnancy, accumulation of proNGF, and decreased ratios of mature β-NGF to proNGF were reported (18).

A rise in endometrial vascularization during pregnancy has been described in other mammals. Mares that were pregnant exhibited a greater area of the endometrium occupied by blood vessels compared with non-pregnant females and mares at 21 days of pregnancy exhibited the greatest area of endometrium occupied by blood vessels (47). Something similar occurs in cows, in which the number of blood vessels in the endometrium increased from day 15 to 18 of gestation, and pregnant cows had more blood vessels than non-pregnant cows on day 18 (48, 49). Likewise, in alpacas, a larger area of the endometrium was occupied by blood vessels at 30 days of gestation. It is known that intrauterine administration of β-NGF induces an increase of endometrial vascularization in both uterine horns (13) and it has been demonstrated that β-NGF acts as a pro-angiogenic factor that increases the expression of VEGFA in cultures of llama granulosa cells (29). In 15 days-pregnant alpacas, the increased β-NGF protein intensity was in correspondence with the elevated number of vessels in the endometrium. At 15 days of pregnancy, embryos occupied the entire uterine cavity and on day 45 the entire extent of the trophoblast was closely attached to the left and right uterine epithelium (6, 7). This could explain the vascular similarities between both uterine horns; nevertheless, a positive correspondence between β-NGF and the number of vessels was only observed in the left uterine horn.

The VEGF family is known to regulate vascular functions such as angiogenesis, vasculogenesis, vascular permeability, and lymphangiogenesis; and is composed of several subtypes including VEGFA, VEGFB, VEGFC, VEGFD, and placental growth factor (50). *VEGFA* mRNA can be alternatively spliced into five iso-forms representing proteins of 121, 145, 165, 189, and 206 amino acids (51). In alpacas, *VEGFA* mRNA was present in the uterus of pregnant and non-pregnant females, showing a higher expression in the endometrium of non-pregnant animals and differences between horns in pregnant females. In bovines, gene expression of *VEGFA* transcript variant 1 was higher in non-pregnant females (18th day of estrous cycle) compared with pregnant ones (18th day of pregnancy). Even more, *VEGFA* levels did not show changes during early pregnancy or during the peri-implantation period (49). Hayashi et al. (48) speculated that the lower peripheral estradiol concentrations in pregnant heifers explain these results. In addition to this, Johnson et al. (52) determined that the expression of *VEGFA* in the endometrium of ovariectomized sheep was stimulated by estradiol. Moreover, estradiol receptor, mRNA expression was lower in the ipsilateral uterine horn of non-pregnant sheep, cows and horses, and in pregnant mares (day 13), indicating changes in the sensitivity

TABLE 4 | Pearson correlation coefficient between β-NGF and VEGFA, blood vessel area and number for left and right uterine horns.

		VEGFA mRNA relative abundance		Area of blood vessels		Number of blood vessels	
		LUH	RUH	LUH	RUH	LUH	RUH
β-NGF mRNA relative abundance	LUH	−0.492	−0.201	0.446	0.475	0.092	−0.453
	RUH	−0.576	−0.290	0.816**	0.853**	−0.168	−0.430
β-NGF immunosignal density	LUH	−0.220	0.373	−0.633*	−0.632*	0.612*	0.578*
	RUH	−0.460	0.356	−0.411	−0.412	0.546	0.431

LUH, Left uterine horn; RUH, right uterine horn.
***p < 0.01 (bilateral).*
**p < 0.05 (bilateral).*

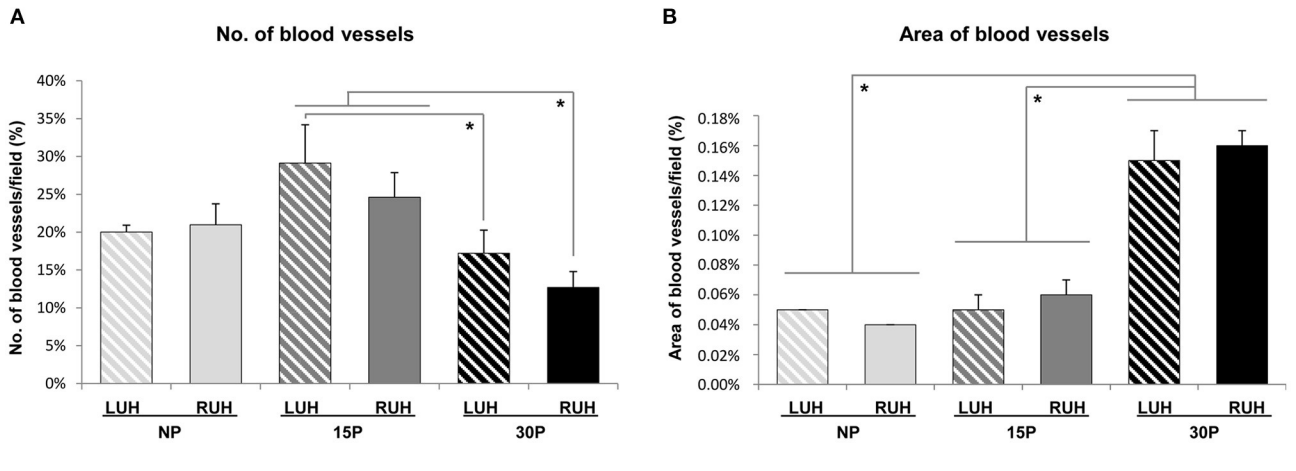


FIGURE 6 | Effect of pregnancy status and stage of pregnancy on the blood vessel number and area in the endometrium of alpacas. **(A)** Number of blood vessels in LUH (left uterine horn) and RUH (right uterine horn) of non-pregnant (NP), 15 days-pregnant (15P), and 30 days-pregnant (30P) alpacas. **(B)** Percentage of the endometrial area occupied by blood vessels in LUH and RUH. Histograms illustrate mean values ± SE. Asterisks indicate significant differences (*$p < 0.05$).

of the endometrium to estradiol (53). These findings could also explain the differences of *VEGFA* expression between uterine horns and physiological status in alpacas. In mares and pigs, *VEGFA* was upregulated during early pregnancy. In pigs *VEGFA* isoform 188 aa was specifically assayed (46); while in horses, the *VEGFA* transcript variant X1 was amplified (54). In alpacas, primers amplified *VEGFA* transcript variant X1(186 aa), X2 (180 aa), and X3 (162 aa), a fact that could also explain the differences between alpacas and other livestock species. Although the *VEGFA* gene expression pattern did not show drastic changes between pregnancy periods and was even decreased compared to non-pregnant alpacas, other members of the VEGF system could be involved in the angiogenesis changes observed in alpacas. Hayashi et al. (48), reported an increase of *VEGFB* on day 15 compared to days 18 and 27 of pregnancy, suggesting the possible involvement of VEGFB in endometrial receptivity for successful implantation at earlier stages in Japanese Black cows.

In summary, the endometrium expresses β-NGF, TrKA, and VEGFA in pregnant and non-pregnant alpacas. β-NGF differential protein pattern in alpaca endometrium during the peri-implantation period of pregnancy implies a precise regulation, which suggests a possible role in embryo development and implantation. The proangiogenic function of β-NGF could be responsible for the increase of endometrial vasculature, thereby supporting embryo implantation and survival. Nevertheless, further investigation on the β-NGF and VEGF signaling pathway is necessary to fully understand the differential implantation in South American camelids.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Committee of Animal Ethics and Welfare (Comité de Ética y Bienestar Animal (CEBA) from the School of Veterinary Medicine of the UNMSM. Written informed consent for participation was not obtained from the owners because the animals were destined for meat production and they were sent to a slaughterhouse, where samples were collected following the regulations of the abattoir.

AUTHOR CONTRIBUTIONS

DB and MA analyzed the data and wrote the manuscript. MA, LS, and DB performed the laboratory analysis and participated in the acquisition of the data. MA and SA designed the study. SA and MR discussed and revised the manuscript. All authors read and approved the manuscript for publication.

FUNDING

This work was partially supported by Universidad Nacional de Tucumán [Grant number PIUNT A616/3], Fondo para la Investigación Científica y Tecnológica, [Grant number BID-PICT 2017-0363 and BID-PICT 2016-0145].

ACKNOWLEDGMENTS

The authors of this manuscript thank Prof. Joel Pacheco (Instituto Veterinario de Investigaciones Tropicales y de Altura, Universidad Nacional Mayor de San Marcos) and Dr. Renato Zampini (Instituto Superior de Investigaciones Biológicas, CONICET-UNT) for their collaboration.

REFERENCES

1. Galina CY, Valencia J. *Reproducción de Animales Domésticos*. 2, ed Limusa, Noeniega editors. México (2006). p. 128, 130, 135, 136.
2. Tometten M, Blois S, Kuhlmei A, Stretz A, Klapp BF, Arck PC. Nerve growth factor translates stress response and subsequent murine abortion via adhesion molecule-dependent pathways. *Biol Reproduct*. (2006) 74:674–83. doi: 10.1095/biolreprod.105.044651
3. Sumar J. Removal of the ovaries or ablation of the corpus luteum and its effect on the maintenance of gestation in the alpaca and llama. *Acta Vet Scand Suppl*. (1988) 83:133–41.
4. Diskin MG, Morris DG. Embryonic and early foetal losses in cattle and other ruminants. *Reproduct Domestic Animals*. (2008) 43:260–7. doi: 10.1111/j.1439-0531.2008.01171.x
5. Aba MA, Kindahl H, Forsberg M, Quiroga M, Auza N. Levels of progesterone and changes in prostaglandin F(2alpha) release during luteolysis and early pregnancy in llamas and the effect of treatment with flunixin meglumine. *Anim Reprod Sci*. (2000) 59:87e97. doi: 10.1016/S0378-4320(00)00068-3
6. Olivera LVM, Zago DA, Jones CJP, Bevilacqua E. Develpmental changes at the materno-embryonic interface in early pregnancy of the alpaca, Lamos pacos. *Anat Embryol*. (2003) 207:317–31. doi: 10.1007/s00429-003-0346-1
7. Picha Y, Tibary A, Memon M, Kasimanickam R, Sumar J. Chronology of early embryonic development and embryo uterine migration in alpacas. *Theriogenology*. (2013) 79:702–8. doi: 10.1016/j.theriogenology.2012.11.027
8. Brown BW. A review on reproduction in South American camelids. *Animal Reproduct Sci*. (2000) 58:169–95. doi: 10.1016/S0378-4320(99)00081-0
9. Powell SA, Smith BB, Timm KI, Menino AR. Estradiol production by preimplantation blastocysts and increased serum progesterone following estradiol treatment in llamas. *Animal Reproduct Sci*. (2007) 102:66–75. doi: 10.1016/j.anireprosci.2006.10.002
10. Fernandez-Baca S, Hansel W, Saatman R, Sumar J, Novoa C. Differential luteolytic effects of right and left uterine horns in the alpaca. *Biol Reproduct*. (1979) 20:586–95. doi: 10.1095/biolreprod20.3.586
11. Vaughan J, Mihm M, Wittek T. Factors influencing embryo transfer success in alpacas—a retrospective study. *Animal Reproduct Sci*. (2013) 136:194–204. doi: 10.1016/j.anireprosci.2012.10.010
12. Del Campo MR, Del Campo CH, Ginther OJ. Vascular provisions for a local utero-ovarian cross-over pathway in new world camelids. *Theriogenology*. (1996) 46:983–91. doi: 10.1016/S0093-691X(96)00262-2
13. Urra F, Ratto MH, Silva M. Evaluation of the effect of mating, intrauterine deposition of raw seminal plasma or seminal plasma purified β-NGF on endometrial vascularization in llamas. *Theriogenology*. (2019) 125:18–23. doi: 10.1016/j.theriogenology.2018.10.007
14. Vandita D, Kilari A, Pisal H, Patil V, Mehendale S, Wagh G, et al. Maternal nerve growth factor levels during pregnancy in women with preeclampsia: a longitudinal study. *Int J Dev Neurosci*. (2015) 47:340–6. doi: 10.1016/j.ijdevneu.2015.08.003
15. Li B, Sheng X, Song M, Zhang H, Weng J, Zhang M, et al. Expression of nerve growth factor and its receptors TrkA and p75 in the uterus of wild female ground squirrel (Citellus dauricus Brandt). *General Comparat Endocrinol*. (2012) 176:62–9. doi: 10.1016/j.ygcen.2011.12.024
16. Mohamed R, El-Remessy AB. Imbalance of the nerve growth factor and its precursor: implication in diabetic retinopathy. *J Clin Exp Ophthalmol*. (2015) 6:483. doi: 10.4172/2155-9570.1000483
17. Maranesi M, Parillo F, Leonardi L, Rebollar PG, Alonso B, Petrucci L, et al. Expression of nerve growth factor and its receptors in the uterus of rabbits: functional involvement in prostaglandin synthesis. *Domestic Animal Endocrinol*. (2016) 56:20–8. doi: 10.1016/j.domaniend.2016.02.001
18. Lobos E, Gebhardt C, Kluge A, Spanel-Borowski K. Expression of nerve growth factor (NGF) isoforms in the rat uterus during pregnancy: accumulation of precursor proNGF. *Endocrinology*. (2005) 146:1922–9. doi: 10.1210/en.2004-0925
19. Mirshokraei P, Hassanpour H, Rahnama A, Foster WG. Gene expression of BDNF and its receptors, TrkB and p75 in the uterus and oviduct of pregnant and non-pregnant ewes. *Res Vet Sci*. (2013) 95:164–8. doi: 10.1016/j.rvsc.2013.03.009
20. Wessels JM, Wu L, Leyland NA, Wang H, Foster WG. The brain-uterus connection: brain derived neurotrophic factor (BDNF) and its receptor (Ntrk2) are conserved in the mammalian uterus. *PLoS ONE*. (2014) 9:e94036. doi.org/10.1371/journal.pone.0094036 doi: 10.1371/journal.pone.0094036
21. Tometten M, Blois S, Arck PC. Nerve growth factor in reproductive biology: link between the immune, endocrine and nervous system? *Chem Immunol Allergy*. (2005) 89:135–48. doi: 10.1159/000087962
22. Garcia-Garcia RM, Arias-Alvarez M, Sanchez-Rodriguez A, Lorenzo PL, Rebollar PG. Role of nerve growth factor in the reproductive physiology of female rabbits: a review. *Theriogenology*. (2020) 150:321–8. doi: 10.1016/j.theriogenology.2020.01.070
23. Julio-Pieper M, Lara HE, Bravo JA, Romero C. Effects of nerve growth factor (NGF) on blood vessels area and expression of the angiogenic factors VEGF and TGFbeta1 in the rat ovary. *Reproduct Biol Endocrinol*. (2006) 4:1–11. doi: 10.1186/1477-7827-4-57
24. Julio-Pieper M, Lozada P, Tapia V, Vega M, Miranda C, Vantman D, et al. Nerve growth factor induces vascular endothelial growth factor expression in granulosa cells via a trkA receptor/mitogen-activated protein kinase-extracellularly regulated kinase 2-dependent pathway. *J Clin Endocrinol Metabol*. (2009) 94:3065–71. doi: 10.1210/jc.2009-0542
25. Mona e Pinto J, Pavanelo V, Alves de Fátima L, Medeiros de Carvalho Sousa LM, Pacheco Mendes G, Machado Ferreira R, et al. Treatment with eCG decreases the vascular density and increases the glandular density of the bovine uterus. *Reproduct Domestic Animals*. (2014) 49:453–62. doi: 10.1111/rda.12307
26. Kershaw-Young CM, Druart X, Vaughan J, Maxwell WMC. β-Nerve growth factor is a major component of alpaca seminal plasma and induces ovulation in female alpacas. *Reproduct Fertility Dev*. (2012) 24:1093–7. doi: 10.1071/RD12039
27. Ratto MH, Leduc YA, Valderrama XP, Van Straaten KE, Delbaere LT, Pierson RA, et al. The nerve of ovulation-inducing factor in semen. *Proc Natl Acad Sci USA*. (2012) 109:15042–7. doi: 10.1073/pnas.1206273109

28. El Allali K, El Bousmaki N, Ainani H, Simonneaux V. Effect of the camelid's seminal plasma ovulation-inducing factor/β-NGF: a kisspeptin target hypothesis. *Front Vet Sci*. (2017) 4:99. doi.org/10.3389/fvets.2017.00099 doi: 10.3389/fvets.2017.00099
29. Valderrama XP, Goicochea JF, Silva ME, Ratto MH. The effect of seminal plasma β-NGF on follicular fluid hormone concentration and gene expression of steroidogenic enzymes in llama granulosa cells. *Reproduct Biol Endocrinol*. (2019) 17:60. doi: 10.1186/s12958-019-0504-9
30. Catone G, Basile M, Barbato O, Ayala C. Transvaginal embryo biometry in alpaca (Lama pacos): Preliminary report. In: Gerken M, Renieri C, Editors. *South American Camelids Research*. (2006) 1:99–105.
31. Argañaraz ME, Apichela SA, Zampini R, Vencato J, Stelletta C. Biochemical and protein profile of alpaca (Vicugna pacos) uterine horn fluid during early pregnancy. *Reprod Domest Anim*. (2015) 50:121–8. doi: 10.1111/rda.12460
32. Barraza DE, Zampini R, Apichela SA, Pacheco JI, Argañaraz ME. Changes in mucins and matrix metalloproteases in the endometrium of early pregnant alpacas (Vicugna pacos). *Acta Histochem*. (2018) 120:438–45. doi: 10.1016/j.acthis.2018.05.009
33. Sari LM, Zampini R, Argañaraz ME, Carretero MI, Fumuso FG, Barraza DE, et al. Expression of β-NGF and high-affinity NGF receptor (TrKA) in llama (Lama glama) male reproductive tract and spermatozoa. *Mol Reproduct Dev*. (2018) 85:934–44. doi: 10.1002/mrd.23075
34. Jensen EC. Quantitative analysis of histological staining and fluorescence using ImageJ. *Anatomical Record*. (2013) 296:378–81. doi: 10.1002/ar.22641
35. Woloszyk A, Buschmann J, Waschkies C, Stadlinger B, Mitsiadis TA. Human dental pulp stem cells and gingival fibroblasts seeded into silk fibroin scaffolds have the same ability in attracting vessels. *Front Physiol*. (2016) 7:140. doi: 10.3389/fphys.2016.00140
36. Ren L, Medan MS, Weng Q, Jin W, Li C, Watanabe G, et al. Immunolocalization of nerve growth factor (NGF) and its receptors (TrkA and p75LNGFR) in the reproductive organs of Shiba goats. *J Reproduct Dev*. (2005) 51:399–404. doi: 10.1262/jrd.16082
37. Jana B, Andronowska A. Expression of nerve growth factor and its receptors in the uterus of gilts with endometritis induced by infection with Escherichia coli. *J Comparat Pathol*. (2012) 147:522–32. doi: 10.1016/j.jcpa.2012.03.001
38. Sari LM, Zampini R, Argañaraz ME, Ratto MH, Apichela SA. Effect of mating on mRNA and protein expression of beta nerve growth factor and its receptor, TrKA, in the oviduct of llama (Lama glama). *Mol Reprod Dev*. (2020) 87:1133–40. doi: 10.1002/mrd.23426
39. Toti P, Ciarmela P, Florio P, Volpi N, Occhini R, Petraglia F. Human placenta and fetal membranes express nerve growth factor mRNA and protein. *J Endocrinol Invest*. (2006) 29:337–41. doi: 10.1007/BF03344105
40. Coassin M, Lambiase A, Micera A, Tirassa P, Aloe L, Bonini S. Nerve growth factor modulates in vitro the expression and release of TGF-β1 by amniotic membrane. *Graefe's Arch Clin Exp Ophthalmol*. (2006) 244:485–91. doi: 10.1007/s00417-005-0098-0
41. Barraza DE, Zampini R, Apichela SA, Pacheco JI, Argañaraz ME. Modifications of extracellular matrix features in the left and right uterine horns during the embryo pre-implantation period in Vicugna pacos. *Theriogenology*. (2020) 157:440–8. doi: 10.1016/j.theriogenology.2020.07.001
42. Saygili E, Schauerte P, Pekassa M, Saygili E, Rackauskas G, Schwinger RH, et al. Sympathetic neurons express and secrete MMP-2 and MT1-MMP to control nerve sprouting via pro-NGF conversion. *Cell Mol Neurobiol*. (2011) 31:17–25. doi: 10.1007/s10571-010-9548-2
43. Frank P, Barrientos GL, Tirado González I, Cohen M, Moschansky P, Peters EM, et al. Balanced levels of nerve growth factor are requiredfor normal pregnancy progression. *Reproduction*. (2014) 148:179–89. doi: 10.1530/REP-14-0112
44. Gonzalez D, Les Dees W, Hiney JK, Ojeda SR, Saneto RP. Expression of β-nerve growth factor in cultured cells derived from the hypothalamus and cerebral cortex. *Brain Res*. (1990) 511:249–58. doi: 10.1016/0006-8993(90)90169-C
45. Colangelo AM, Pani L, Mocchetti I. Correlation between increased AP-1?-NGF binding activity and induction of nerve growth factor transcription by multiple signal transduction pathways in C6-2B glioma cells. *Mol Brain Res. (*1996) 35:1–10. doi: 10.1016/0169-328X(95)00171-N
46. Sherer TB, Neff PS, Tuttle JB. Increased nerve growth factor mRNA stability may underlie elevated nerve growth factor secretion from hypertensive vascular smooth muscle cells. *Mol Brain Res*. (1998) 62:167–74. doi: 10.1016/S0169-328X(98)00247-2
47. Silva LA, Klein C, Ealy AD, Sharp DC. Conceptus-mediated endometrial vascular changes during early pregnancy in mares: an anatomic, histomorphometric, and vascular endothelial growth factor receptor system immunolocalization and gene expression study. *Reproduction*. (2011) 142:593–603. doi: 10.1530/REP-11-0149
48. Hayashi KG, Hosoe M, Fujii S, Kanahara H, Sakumoto R. Temporal expression and localization of vascular endothelial growth factor family members in the bovine uterus during peri-implantation period. *Theriogenology*. (2019) 133:56–64. doi: 10.1016/j.theriogenology.2019.04.021
49. Silva LA, Ginther OJ. Local effect of the conceptus on uterine vascular perfusion during early pregnancy in heifers. *Reproduction*. (2010) 139:453–63. doi: 10.1530/REP-09-0363
50. Girling JE, Rogers PA. Regulation of endometrial vascular remodelling: role of the vascular endothelial growth factor family and the angiopoietin-TIE signalling system. *Reproduction*. (2009) 138:883–93. doi: 10.1530/REP-09-0147
51. Zygmunt M, Herr F, Münstedt K, Lang U, Liang OD. Angiogenesis and vasculogenesis in pregnancy. *Eur J Obstetr Gynecol Reproduct Biol*. (2003) 110:S10–8. doi: 10.1016/S0301-2115(03)00168-4
52. Johnson ML, Grazul-Bilska AT, Redmer DA, Reynolds LP. Effects of estradiol-17beta on expression of mRNA for seven angiogenic factors and their receptors in the endometrium of ovariectomized (OVX) ewes. *Endocrine*. (2006) 30:333–42. doi: 10.1007/s12020-006-0012-5
53. Kalpokas I, Mattos RC, Cavestany D, Martínez MN, Perdigón F, Meikle A. Effect of side of the corpus luteum and pregnancy on estrogen and progesterone receptor expression and localization in the endometrium of mares. *Theriogenology*. (2018) 114:221–8. doi: 10.1016/j.theriogenology.2017.12.034
54. Welter H, Wollenhaupt K, Tiemann U, Einspanier R. Regulation of the VEGF-system in the endometrium during steroid-replacement and early pregnancy of pigs. *Exp Clin Endocrinol Diabetes*. (2003) 111:33–40. doi: 10.1055/s-2003-37498

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 02 February 2021
doi: 10.3389/fvets.2020.597921

Distribution of GnRH and Kisspeptin Immunoreactivity in the Female Llama Hypothalamus

Marco Berland[1†], *Luis Paiva*[2*†], *Lig Alondra Santander*[1] *and Marcelo Héctor Ratto*[2*]

[1] *Departamento de Ciencias Veterinarias y Salud Pública, Facultad de Recursos Naturales, Universidad Católica de Temuco, Temuco, Chile,* [2] *Instituto de Ciencia Animal, Facultad de Ciencias Veterinarias, Universidad Austral de Chile, Valdivia, Chile*

Llamas are induced non-reflex ovulators, which ovulate in response to the hormonal stimulus of the male protein beta-nerve growth factor (β-NGF) that is present in the seminal plasma; this response is dependent on the preovulatory gonadotrophin-releasing hormone (GnRH) release from the hypothalamus. GnRH neurones are vital for reproduction, as these provide the input that controls the release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the pituitary gland. However, in spontaneous ovulators, the activity of GnRH cells is regulated by kisspeptin neurones that relay the oestrogen signal arising from the periphery. Here, we investigated the organisation of GnRH and kisspeptin systems in the hypothalamus of receptive adult female llamas. We found that GnRH cells exhibiting different shapes were distributed throughout the ventral forebrain and some of these were located in proximity to blood vessels; sections of the mediobasal hypothalamus (MBH) displayed the highest number of cells. GnRH fibres were observed in both the organum vasculosum laminae terminalis (OVLT) and median eminence (ME). We also detected abundant kisspeptin fibres in the MBH and ME; kisspeptin cells were found in the arcuate nucleus (ARC), but not in rostral areas of the hypothalamus. Quantitative analysis of GnRH and kisspeptin fibres in the ME revealed a higher innervation density of kisspeptin than of GnRH fibres. The physiological significance of the anatomical findings reported here for the ovulatory mechanism in llamas is still to be determined.

Keywords: LHRH, metastin, OVLT, median eminence, induced ovulation, llama glama, camelids

OPEN ACCESS

Edited by:
Rosa Maria Garcia-Garcia, Complutense University of Madrid, Spain

Reviewed by:
Khalid El Allali, Agronomic and Veterinary Institute Hassan II, Morocco
Cecilia Dall'Aglio, University of Perugia, Italy

***Correspondence:**
Luis Paiva
luis.paiva@postgrado.uach.cl
Marcelo Héctor Ratto
marceloratto@uach.cl

[†] *These authors share first authorship*

Specialty section:
This article was submitted to Animal Reproduction - Theriogenology, a section of the journal Frontiers in Veterinary Science

Received: *22 August 2020*
Accepted: *30 November 2020*
Published: *02 February 2021*

Citation:
Berland M, Paiva L, Santander LA and Ratto MH (2021) Distribution of GnRH and Kisspeptin Immunoreactivity in the Female Llama Hypothalamus. Front. Vet. Sci. 7:597921. doi: 10.3389/fvets.2020.597921

INTRODUCTION

In female mammals, ovulation relies on the integration of different central and peripheral components that establish reciprocal interactions. At the pituitary level, the preovulatory release of the luteinizing hormone (LH) from gonadotroph cells is led by the gonadotrophin-releasing hormone (GnRH) released from the hypothalamus (1).

GnRH neurones originate in the olfactory placode in the early embryonic life and migrate through the vomeronasal axons to the basal forebrain during development, stopping their migratory journey 'randomly' along the basal forebrain (2). In the adult hypothalamus, GnRH cells are distributed in a bilateral long scattered continuum (2, 3) that exhibit distinct species-related number and distribution. The preoptic area (POA)–anterior hypothalamus and the mediobasal hypothalamus (MBH) are two regions known to harvest large numbers of GnRH somas (4),

but GnRH somas and fibres can also be found in other brain areas, such as the olfactory bulb and hippocampus (5). Approximately 50% of the GnRH cells send their fibres to the median eminence (ME) where nerve endings release their products into the hypophyseal portal system that transport GnRH molecules to the pituitary (6, 7). The activity of these hypophysiotropic GnRH cells is regulated by several inputs and factors (such as environmental cues) that vary in different species (8).

Kisspeptin neurones (encoding the gene *KISS1*) are recognised as the main input involved in the activation of GnRH cells that lead to the preovulatory GnRH/LH surge by relaying oestrogen signalling in spontaneous ovulators (9, 10). In seasonal breeders, kisspeptin cells have also been shown to be modulated by photoperiodic changes, and so they are directly involved in the control of reproductive seasonality in different species (10, 11). Two populations of kisspeptin cells located in the POA (or homologous areas) and the arcuate nucleus (ARC)–co-expressing neurokinin B and dynorphin in the latter, and so-called KNDy neurones–of the hypothalamus can be found in several species (12). So, when oestrogen concentrations rise, *KISS1* expression is upregulated in POA cells, and they become activated, prompting the activation of GnRH cells and their product release (13, 14). Evidence indicates that kisspeptin may act directly over GnRH somas (15, 16), but also, perhaps, at the ME in some species, including rats (17), sheep (18), and humans (19).

The physiological role of kisspeptin in induced ovulation species is not fully understood. In the musk shrew (*Suncus murinus*), *KISS1* expression seems to be like in spontaneous ovulators, but mating is reported as the critical cue for the activation (by means of c-Fos expression) of POA *KISS1* cells (20), indicating kisspeptin cells as a potential player involved in the ovulatory mechanism. Llamas are also induced ovulators, however, in this species, ovulation occurs in response to the hormonal [but not reflex (21)] stimulus of the protein beta-nerve growth factor (β-NGF) that is present in the male seminal plasma (22, 23); the resulting LH surge and ovulation have been shown to be dependent on GnRH release (24), implying the participation of a central mechanism. Although the involvement of kisspeptin in this response has been suggested (25), the organisation of

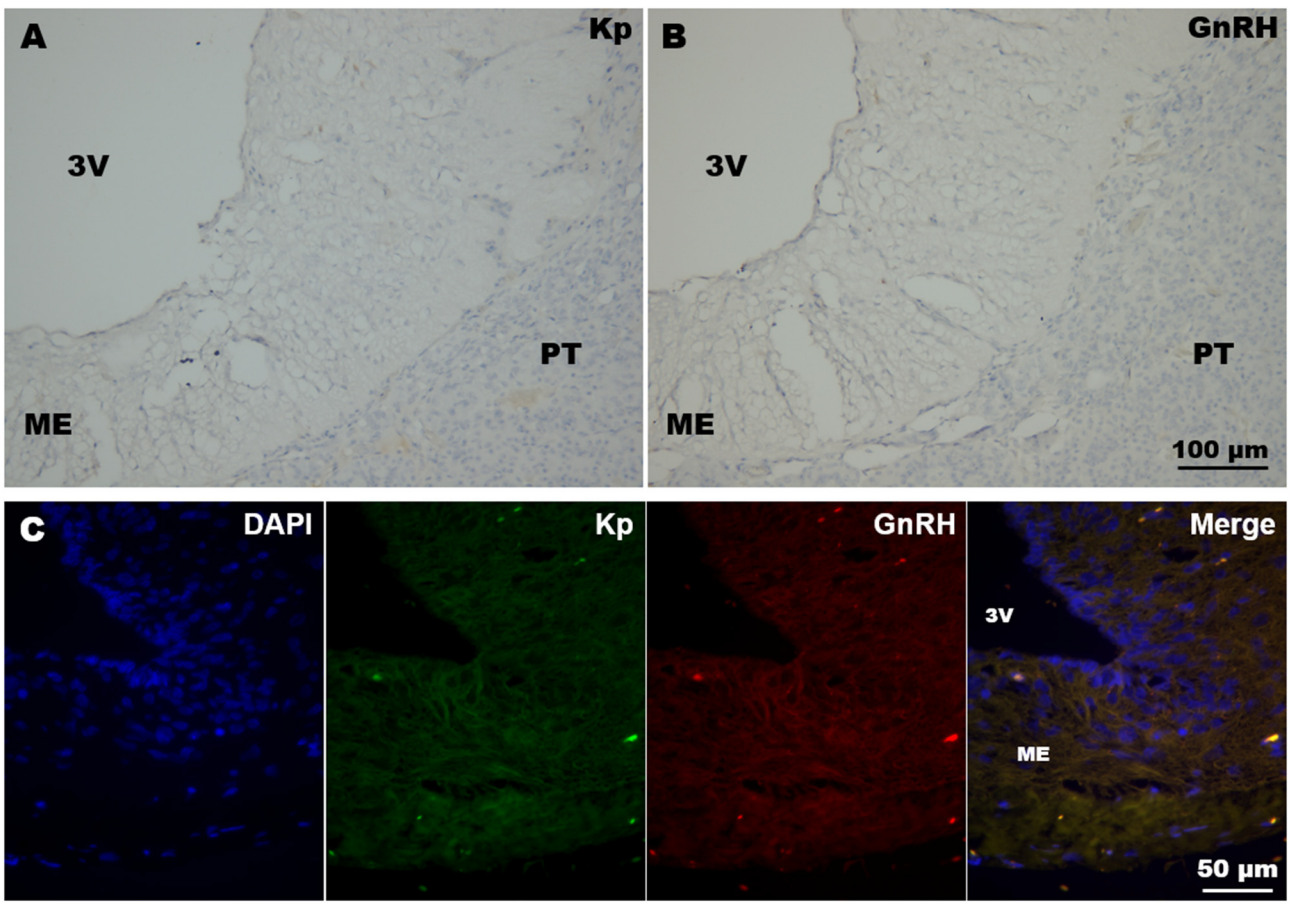


FIGURE 1 | Immunohistochemistry negative controls. **(A,B)** Coronal sections of llama ME showing DAB immunohistochemistry where **(A)** kisspeptin and **(B)** GnRH primary antibodies were omitted. **(C)** Example of double immunofluorescence in llama ME (coronal section) incubated without kisspeptin (Kp) and GnRH primary antibodies. 3v, third ventricle; ME, median eminence; PT, pituitary.

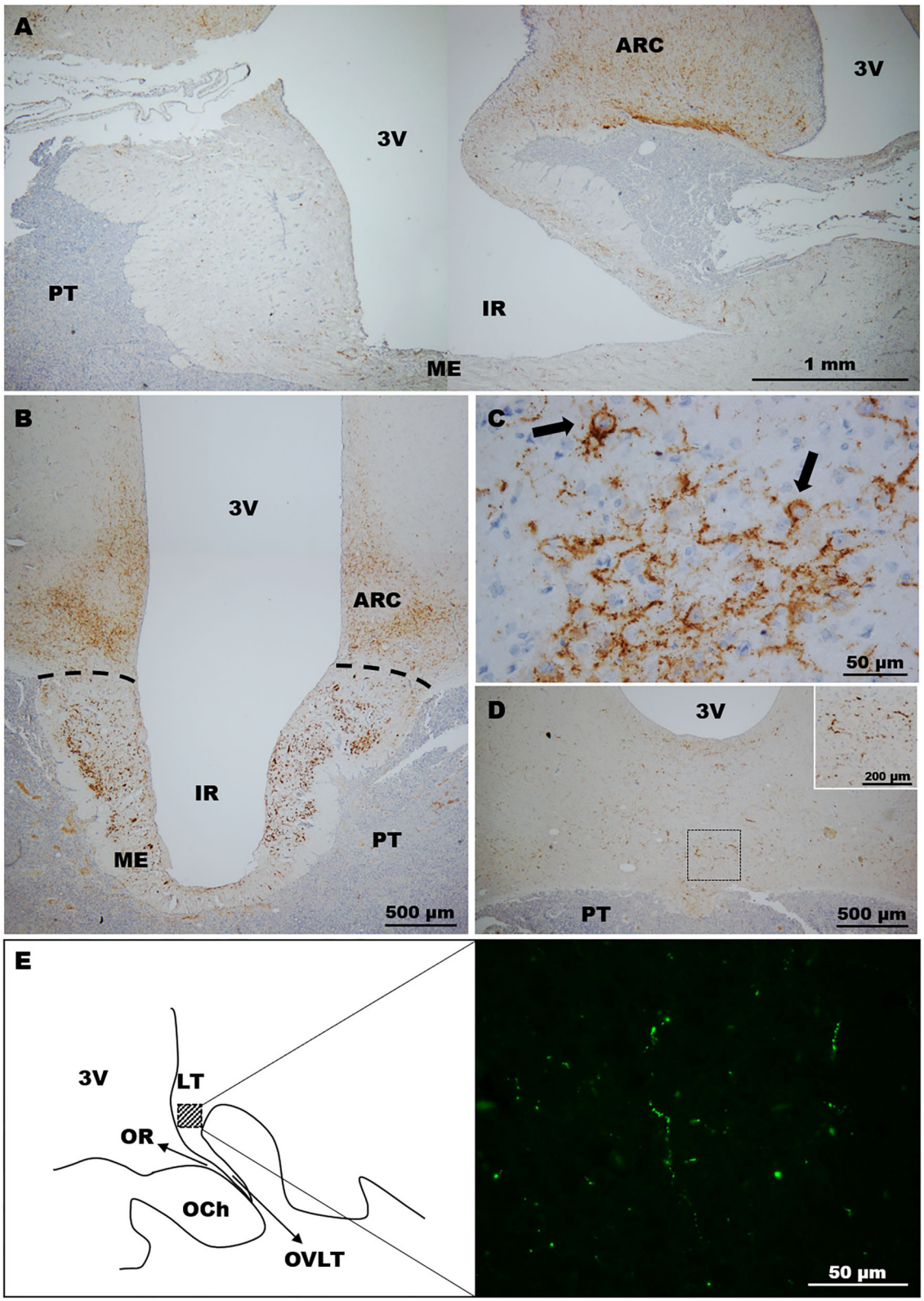


FIGURE 2 | Distribution of kisspeptin in the llama hypothalamus. **(A)** Sagittal image showing the distribution of kisspeptin fibres in the MBH. **(B)** Coronal section. Immunolabelled fibres were located mostly in the ARC and ME. **(C)** Image of the ARC showing (arrows) the labelling of kisspeptin somas. **(D,E)** Kisspeptin fibres were moderately found in some areas of the **(D)** anterior hypothalamus and **(E)** POA. **(E)** Left panel shows a schematic representation of the POA on a sagittal section

(Continued)

FIGURE 2 | indicating in the square area the high magnification picture (right panel) displaying kisspeptin immunolabelled fibres at this zone. 3v, third ventricle; ARC, arcuate nucleus; IR, infundibular recess; LT, laminae terminalis; ME, median eminence; OCh, optic chiasm; OR, optic recess; OVLT, organum vasculosum laminae terminalis; PT, pituitary.

kisspeptin systems and its physiological role is not clear.

In this study, we characterise the distribution of GnRH and kisspeptin cells and their fibres (i.e., axons and dendrites) in the hypothalamus of receptive female llamas using immunohistochemistry. We also carried out dual immunofluorescence in sections containing the ME to analyse the density of GnRH- and kisspeptin-containing fibres, which are next to fenestrated blood vessels of the hypophyseal portal system.

METHODS

Animals

Llamas were maintained in the llama research farm of the Universidad Austral de Chile, Valdivia, Chile (39°38′S, 73°5′W, and 19 m above sea level) and were given *ad libitum* access to water and pastures supplemented with hay and feed pellets (14% crude protein, 2.5% crude fat, 12% crude fibre); female llamas were maintained separately from males. Three (3) adult non-pregnant, non-lactating female llamas, weighing 120–140 kg (4–8 years old), were used in this study. These llamas were daily examined by transrectal ultrasonography using a 7.5 MHz transducer coupled with an Aloka SSD-500 scanner (Aloka Co., Ltd., Tokyo, Japan) to determine follicular growth; females exhibiting a follicle ≥8 mm in diameter that grew for 3 consecutive days were considered sexually receptive and euthanised. Experiments were conducted in June.

All the procedures were carried out in accordance with the Chilean Animal Protection Act (No. 20380; 2009) and the regulations and approved procedures (ref. 253/2015) by the University Bioethical Committee.

Tissue Collection and Immunohistochemistry

Llamas were terminally anesthetised by an injection of sodium pentobarbital (80 mg/kg i.v.), and then the jugular veins were cannulated for exsanguination. Once death was confirmed, the brain was quickly removed and dissected, and the hypothalamic chunk was fixed by immersion in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 72 h. The tissue was embedded in paraffin and then cut using a rotary microtome to obtain 5-μm serial sections; two hypothalami were cut coronally, and one hypothalamus was cut sagittally. One in every 30 sequentially cut sections (i.e., 150 μm) was mounted onto slides.

Conventional immunohistochemistry methods were used. Sections were washed in 0.02 M potassium phosphate-buffered saline (KPBS; pH 7.4) between steps. In brief, paraffin-embedded sections were dewaxed, and heat-induced epitope retrieval was performed for 20 min using 1× Tris–EDTA buffer pH 9.0 at 90°C. Then, the endogenous peroxidase activity was blocked by incubating the sections in 2% H_2O_2 in cold methanol for 20 min. To prevent the background staining caused by interactions of antibodies with the tissue, sections were incubated in blocking buffer solution containing 0.02 M KPBS + 0.5% Triton X-100 + 2% normal goat serum for 30 min. Sections were then incubated overnight at 4°C in blocking buffer containing either mouse monoclonal HU11B anti-GnRH-I antibody (cat. sc-32292; Santa Cruz Biotechnology Inc., Dallas, TX, USA) diluted at 1:1,000 or rabbit polyclonal anti-kisspeptin antibody (cat. AB9754; Millipore, Billerica, MA, USA) diluted at 1:1,000. Following this, sections were washed and incubated for 1 h at room temperature with secondary goat anti-mouse IgG or goat anti-rabbit IgG biotinylated antibodies (cat. 115-065-003 and 111-065-003, respectively; Jackson ImmunoResearch Inc., West Grove, PA, USA) diluted at 1:500 in 0.02 M KPBS and then incubated in Vectastain Elite ABC Kit (cat. PK-6100; Vector Laboratories Inc., Burlingame, CA, USA) following the manufacturer's instructions. The immunoreaction was revealed by a solution containing 0.05% diaminobenzidine + 0.015% H_2O_2 in 0.02 M KPBS. Then, sections were counterstained with haematoxylin, dehydrated in increasing concentrations of ethanol and coverslipped.

For double immunofluorescence, sections were treated and incubated simultaneously with primary antibodies as described above, but the immunoreactions were visualised by incubating the sections with donkey anti-mouse IgG Alexa Fluor 594 and donkey anti-rabbit IgG Alexa Fluor 488 (cat. A-21203 and A-21206, respectively; Invitrogen, Carlsbad, CA, USA) diluted at 1:500 in blocking buffer containing 2% donkey normal serum for 1 h at room temperature. Sections were counterstained using 4', 6-diamidino-2-phenylindole (DAPI).

In all immunohistochemistry and immunofluorescence experiments, the specificity of immunoreactions was tested by omission of primary antibodies in control sections containing the ME; no immunoreaction was detected in these sections (**Figure 1**).

Imaging and Data Analysis

Images were captured using a Nikon digital camera attached to an upright Nikon Eclipse i80 microscope (Nikon Instruments Inc., Tokyo, Japan). Since thin sections make it difficult to identify cell shapes, only clearly distinguishable cell somas exhibiting a single or more processes parallel to the coronal plane were used for description and illustration purposes. Dendrites and axons are irrespectively reported as fibres similarly as Witkin et al. (26). For quantification of GnRH and kisspeptin fibres in the ME, four sections per animal were analysed; in each of these sections, four digital images of non-overlapping microscopic fields at 40× magnification were captured at different sectors of the ME. The area (μm^2) covered by GnRH and kisspeptin fibres in each image was calculated using a PC running Fiji version 1.52p; briefly, red (i.e., GnRH) and green (i.e., kisspeptin) channels of image

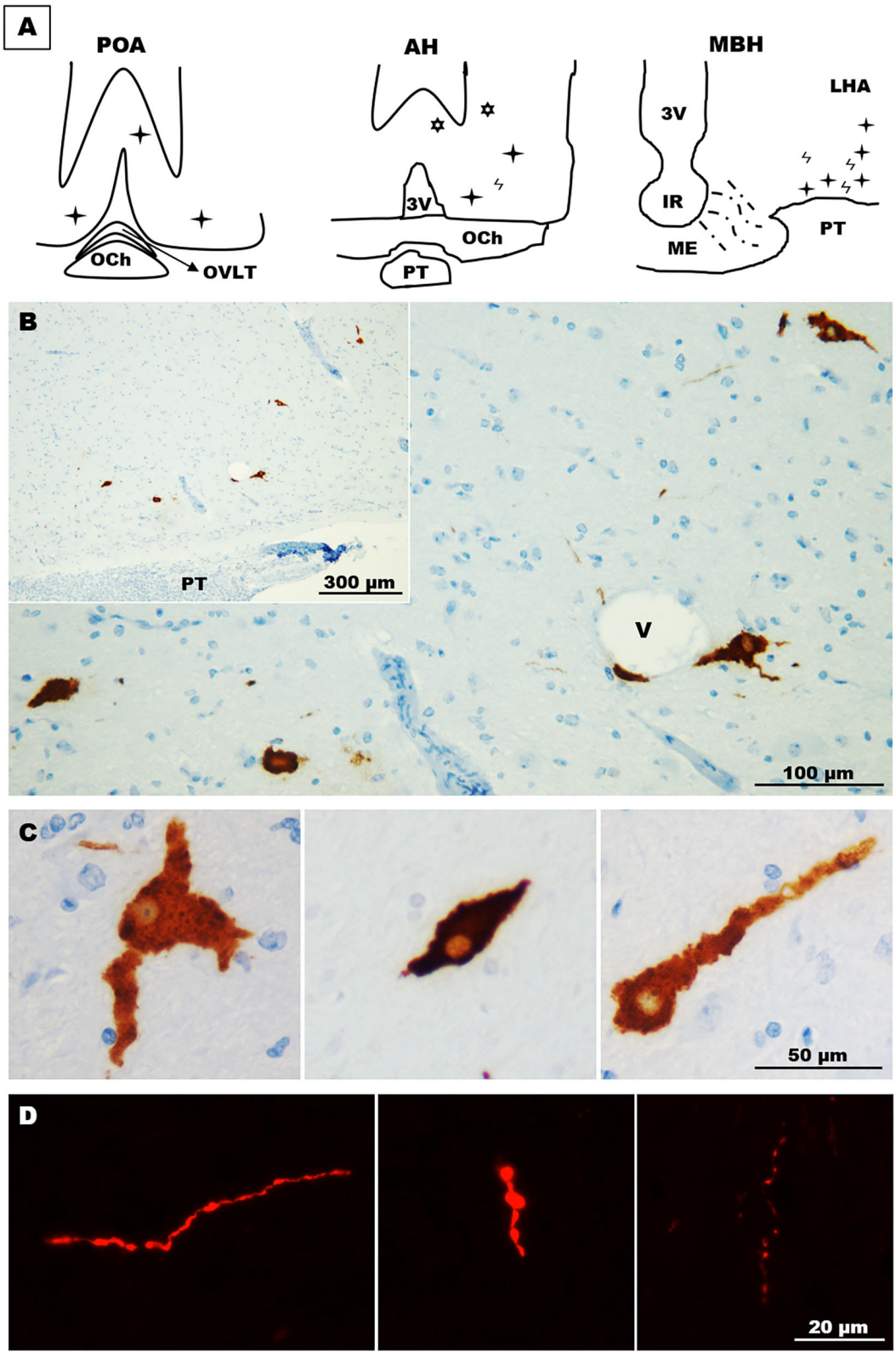


FIGURE 3 | Distribution and morphology of GnRH cells in the llama hypothalamus. **(A)** Schematic representation of the relative abundance of GnRH cells in the POA, anterior hypothalamus, and MBH. ϟ = fibres, ✦ = hypothalamic cells, ✡ = extrahypothalamic cells. **(B)** Coronal section showing scattered GnRH cells in the ventro-lateral area of the MBH. **(C)** GnRH cells exhibited different forms, including multipolar (left panel), fusiform (middle panel) and unipolar (right panel) shapes. **(D)**

(Continued)

FIGURE 3 | Immunofluorescences showing the different shapes and calibres of GnRH fibres found in the llama hypothalamus. 3v, third ventricle; AH, anterior hypothalamus; IR, infundibular recess; LHA, lateral hypothalamic area; MBH, mediobasal hypothalamus; ME, median eminence; OCh, optic chiasm; OVLT, organum vasculosum laminae terminalis; POA, preoptic area; PT, pituitary; V, blood vessel.

files were separately converted into 8-bit black and white images, thresholded using the same values, and the areas calculated using the Analyze Particle macro. The area calculated in each image was averaged for each section; the values in each section were averaged in each animal. The fibre density was calculated as the percentage of immunoreactive material covering the whole image area ($234 \times 292\ \mu m^2$). Values are expressed as the mean + SEM; only descriptive statistics were used in this study.

RESULTS

Kisspeptin

In llamas, immunohistochemistry revealed the abundant presence of varicose fibres exhibiting different calibres in the ARC region, and also the internal zone of ME (**Figures 2A,B**). In the latter, the abundant presence of kisspeptin-immunoreactive material was found in the lateral walls of the ME. Analysis of the ARC showed the presence of some somas (**Figure 2C**).

Moderate kisspeptin fibres were found in the ventral region of the anterior hypothalamus (**Figure 2D**). Sagittal sections revealed the presence of fibres in the POA (lamina terminalis; **Figure 2E**). Somas were not detected in sections containing the POA and anterior hypothalamus in the llamas analysed.

GnRH

Immunohistochemistry revealed GnRH somas located in different areas of the llama hypothalamus. Two (2) to 3 GnRH somas per section were found in sections containing the POA, which were mainly found in the diagonal band of Broca. In the anterior hypothalamus, somas (∼1–2) were found in the latero-ventral area, but also in extrahypothalamic sites near to the lateral ventricles (**Figure 3A**). Sections of the MBH exhibited the greatest number of GnRH somas, where 3–5 GnRH cells per section were detected in the latero-ventral area of the hypothalamus (**Figures 3A,B**); in some sections, GnRH cells were in close contact to blood vessels (**Figure 3B**). These neurones displayed different forms, including multipolar, bipolar, and monopolar as shown in **Figure 3C**. In most sections, different neural fibres were detected in the hypothalamic parenchyma, which exhibited different lengths and variable calibres (**Figure 3D**).

In the ME, immunolabelling of GnRH showed the moderate presence of GnRH fibres that were mainly located in the pre-infundibular and post-infundibular regions as shown in **Figure 4A**. Fibres were also found in the rostral and caudal walls of the ME, but they were rarely detected in the ventral wall of the ME. Fibres were detected in the internal zone of the ME, and some of these penetrated into the ME external zone; analysis of coronal sections revealed that most fibres were mainly located in the lateral walls of the ME (**Figure 4B**).

In the organum vasculosum laminae terminalis (OVLT), the presence of GnRH fibres varied in each section analysed; some of these displayed the abundant presence of fibres mainly located in the ventral OVLT, next to the narrowest area of the optic recess as presented in **Figure 4C**; coronal sections revealed that most of the fibres were located in the medial area of the OVLT (**Figure 4D**). These fibres exhibited different shapes, including long punctuate axon-like fibres, and thick processes (**Figure 4C**).

Kisspeptin and GnRH Fibre Relationship in the ME

The ME plays a pivotal role through the release of hypophysiotropic factors, including GnRH, which stimulates the preovulatory LH release from the pituitary gland. To determine the configuration of this site in receptive llamas, double immunofluorescence against kisspeptin and GnRH was conducted, showing that GnRH and kisspeptin fibres were closely distributed in the ME, but no obvious contacts between these fibres were observed. Analysis of high magnification images of different sectors of the ME (**Figure 5A**) showed a greater amount of kisspeptin than of GnRH immunoreactive material (2:1 ratio). Kisspeptin fibres covered an average area of $1{,}230.9 \pm 618.8$ vs. $708.9 \pm 98.8\ \mu m$ of GnRH fibres (**Figure 5B**); these values represent 1.8 ± 0.9 and $1.0 \pm 0.2\%$ of the total area (40× image) analysed, respectively.

DISCUSSION

In this study, we investigated the presence and distribution of kisspeptin and GnRH cells in the adult female llama hypothalamus; two crucial neuroendocrine systems involved in the LH surge and ovulation in mammals. We found that kisspeptin is abundantly expressed in the llama MBH, where kisspeptin cells were detected in the ARC similarly to that reported in other spontaneous and induced ovulators. We also found GnRH cells and fibres distributed throughout rostral and caudal regions of the llama hypothalamus.

GnRH cells originate in the olfactory placode and migrate through the basal forebrain stopping their migration in a 'random' manner, and so the somas display a scattered distribution in the vertebrate adult hypothalamus (27). Our results show that the llama GnRH cells distribute unevenly throughout the hypothalamus similarly as described in other induced (28–30) and also spontaneous ovulators (26, 31, 32). Although the number of GnRH cells was not quantified in this study because of the use of thin sections sampled every $150\ \mu m$, sections of MBH contained approximately twice the number of cells than rostral sections containing the POA–anterior hypothalamus. This result is consistent with Carrasco et al. (33), who reported a higher number of cells in the MBH than in the POA, showing a proportion of 2:1.

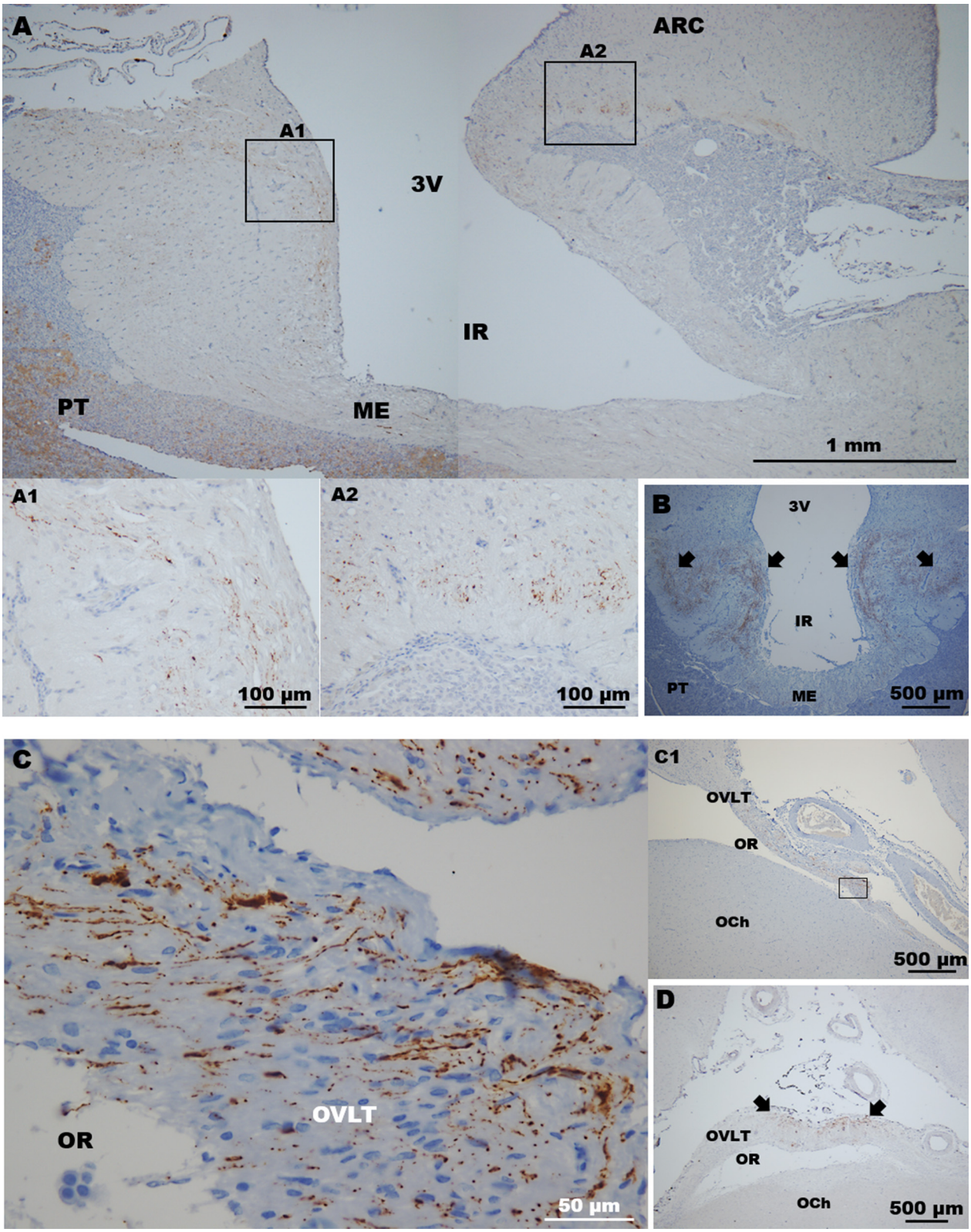


FIGURE 4 | Detection of GnRH fibres in circumventricular organs. **(A)** In sagittal sections, GnRH fibres were found in the **(A1)** rostral (pre-infundibular) and **(A2)** caudal (post-infundibular) areas of the ME. **(B)** Coronal section showing GnRH fibres in the lateral walls (arrows) of the ME surrounding the infundibulum; virtually none fibres were detected in the ventral wall of the ME. **(C)** High magnification image of **(C1)** a sagittal section showing GnRH fibres in the ventral area

(Continued)

FIGURE 4 | (square) of the OVLT. **(D)** Coronal section. Most of the fibres were found in the medial area (arrows) of the OVLT. 3v, third ventricle; ARC, arcuate nucleus; IR, infundibular recess; ME, median eminence; OCh, optic chiasm; OR, optic recess; OVLT, organum vasculosum laminae terminalis; PT, pituitary.

In the present study, we used the HU11B monoclonal antibody that is reported (34) to exhibit high-specificity for sequential, rather than conformational, GnRH decapeptide (cleaved) structure, and which is able to bind GnRH fragments comprising up to four NH_2-terminal amino acid residues. Furthermore, Urbanski (34) reports virtually no immunoactivity against salmon and chicken GnRH-I variants in which two and one amino acid residues of this fragment are exchanged, respectively. This implies that the cells labelled here are unlikely to include cells expressing other molecular GnRH forms than the encoded by the 'mammalian' gene (*GNRH1*) that is highly conserved among mammalian species except in guinea pig (35). Although originally identified in chicken, GnRH-II expressing cells have been found in the brain of macaques and humans, and also in the induced ovulator musk shrew (36). The GnRH-II peptide shares 70% of homology with the mammalian GnRH-I as the amino acid residues of positions 5, 7, and 8 next to the NH_2-terminus are substituted (35, 37). Whether this GnRH form coexists in the llama brain has not been determined so far.

Kisspeptin cells of the POA and ARC participate in the modulation of the activity of GnRH cells; the former population has been linked to the control of the preovulatory GnRH surge in spontaneous ovulators. Here, we fail to detect the presence of POA kisspeptin cells in llamas; it is not clear whether this result was a consequence of a lack of immunoreactivity of this population or the kisspeptin system displays a different distribution in this species, similarly as reported in mares (38). Recently, it has been reported the presence of a low number of kisspeptin cells in the POA–anterior hypothalamus regions; this number was only an eighth of the kisspeptin ARC cells detected in adult llamas exhibiting a preovulatory follicle (39). Although the discrepancy between these results is apparently difficult to conciliate, a plausible explanation could be that either POA cells were missed by the mapping method employed in this study or the immunoreactivity of this population might be influenced by seasonality.

In seasonal breeders, kisspeptin cells are reported to undergo variations in their product synthesis as a consequence of photoperiodic changes that affect both ARC and POA cell populations in a species-specific fashion (10). In line with this, dromedary camels (displaying reproductive seasonality between November and April in Morocco) exhibit twice the number of immunoreactive kisspeptin cells in both the POA and ARC during the breeding season than in the non-breeding season, and this increase is more marked in females (40). Conversely, female llamas–and other South American camelids–are not considered seasonal breeders, exhibiting ovarian cyclicity, conception, and labour all year round (41–43) similarly as observed in the llama herd involved in the present study; in their natural habitat, breeding seasonality is associated to management practices, as a short wet and warm season restricts the abundance of high-quality forage between December and March in the high Andes (44). Whether llama kisspeptin cells exhibit seasonal variations remains to be determined.

In this study, quantification of GnRH and kisspeptin fibres in the ME revealed a higher prevalence of kisspeptin than of GnRH peptide at this level. Even though several studies (17, 45, 46) have investigated the morphological distribution of GnRH and kisspeptin fibres, no studies have described the concurrent densities of fibre innervation in the ME. In female rats, immunoelectron microscopy has revealed occasional direct synaptic contacts of kisspeptin-containing fibres to GnRH fibres; these animals presented rich innervation of both kisspeptin and GnRH fibres located in the internal and external zones of the ME, respectively (17). Furthermore, Pompolo et al. (47) reported colocalisation of GnRH and kisspeptin peptides in cells and fibres of the POA and also the ME in ewes. In the present study, colocalisation of these peptides was not detected in any of the brain regions analysed. Similarly as found here, variation in kisspeptin fibre distribution in the ME has been reported within single animals (46).

In several species (17, 45, 48), it has been described the presence of kisspeptin fibres in the ME, and even the release of measurable quantities of kisspeptin in the portal blood has been reported in the sheep (49). Furthermore, kisspeptin administration has been shown to stimulate GnRH release from the ME *in vitro* (17) and *in vivo* (50). In addition to this apparent effect within the ME boundaries, the rich kisspeptin innervation of the llama ME found in this study also suggests a potential hypophysiotropic role. Interestingly, Smith et al. (49) reported that *in vitro* kisspeptin application to pituitary gonadotroph cells (that express the kisspeptin receptor) during the follicular, but not luteal, phase prompts LH release in ewes.

GnRH cells appear to be a vital player in the ovulatory mechanism of llamas, as blockade of its peripheral receptors prevents LH-dependent ovulation (24). Since in New and Old World camelids ovulation occurs in response to the exogenous hormonal male β-NGF stimulus (23), attention has been given to the potential mechanism(s) involved in this response. Recently, kisspeptin has been proposed (25, 39) to be the mediator involved in the llama ovulatory mechanism under the assumption that exogenous kisspeptin effects mimic the actions of brain kisspeptin systems on GnRH cells (39), and yet this does not prove the physiological role of kisspeptins in the llama brain. It is unlikely that kisspeptins–including short forms (51, 52)–penetrate the blood–brain barrier in neuroactive amounts, and so it is thought (53) that, when given systemically, circulating kisspeptin acts locally on the OVLT, where rodent GnRH cells have been shown to extend their processes (54) similarly as reported here. Since oestradiol concentrations affect the magnitude of LH released following β-NGF administration in llamas

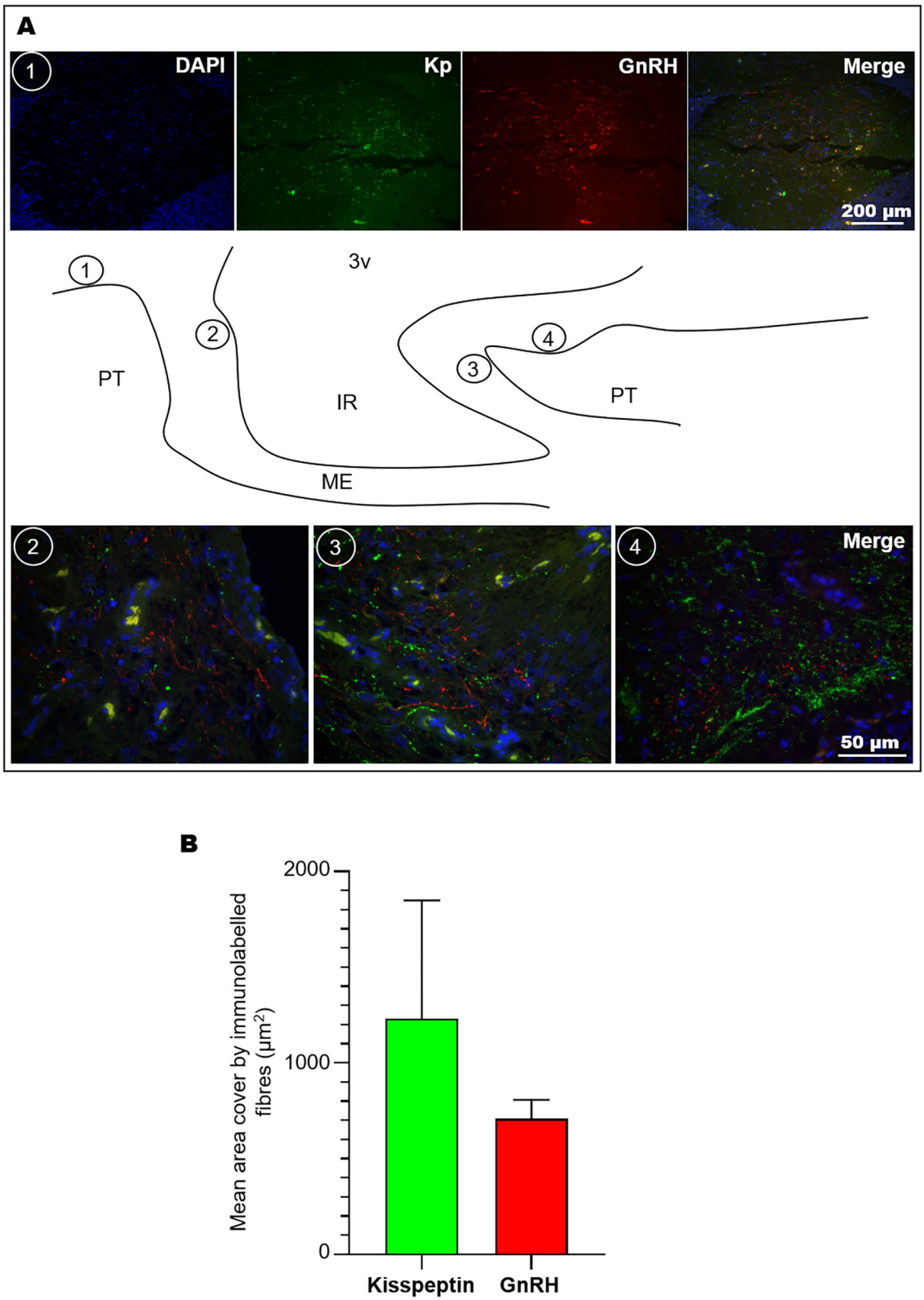


FIGURE 5 | Co-labelling of kisspeptin and GnRH fibres in the llama ME. **(A)** Coronal (1) and sagittal images (2, 3, 4) showing the abundance of kisspeptin (Kp) and GnRH fibres at different levels of the ME as indicated in the sagittal schematic representation; scarce fibres were found in the ventral walls of the ME. **(B)** Mean (+SEM) area covered by immunolabelled kisspeptin and GnRH fibres in 40× microphotographs. 3v, third ventricle; IR, infundibular recess; ME, median eminence; PT, pituitary.

(55), the rich kisspeptin innervation of the ME suggests that this is an area where oestrogen-sensitive kisspeptin cells might play a modulatory effect, for example, by affecting the amount of GnRH released in response to β-NGF. The physiological significance of the anatomical findings reported here for the ovulatory mechanism in llamas is still to be determined.

DATA AVAILABILITY STATEMENT

The datasets supporting the conclusions of the present study are available from the corresponding authors upon reasonable request. Requests to access the datasets should be directed to Luis Paiva, luis.paiva@postgrado.uach.cl; Marcelo Ratto, marceloratto@uach.cl.

ETHICS STATEMENT

The animal study was reviewed and approved by the Bioethical Committee of the Universidad Austral de Chile, reference no. 253/2015.

AUTHOR CONTRIBUTIONS

MB and MR performed the animal experiments and tissue collection. LS, MB, and LP performed the immunohistochemistry. LP and LS analysed the images and data. LP wrote the manuscript. LP and MR edited and reviewed the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the Agencia Nacional de Investigación y Desarrollo (ANID) of Chile; grants ANID FONDECYT Postdoctorado 3200439 (LP) and ANID FONDECYT Regular 1190980 (MR).

ACKNOWLEDGMENTS

We thank Prof. Iván Valdebenito from the Universidad Católica de Temuco for assisting and providing access to the imaging facilities.

REFERENCES

1. Clarke IJ, Cummins JT. The temporal relationship between gonadotropin releasing hormone (GnRH) and luteinizing hormone (LH) secretion in ovariectomized ewes. *Endocrinology*. (1982) 111:1737–9. doi: 10.1210/endo-111-5-1737
2. Wray S. From nose to brain: development of gonadotrophin-releasing hormone-1 neurones. *J Neuroendocrinol.* (2010) 22:743–53. doi: 10.1111/j.1365-2826.2010.02034.x
3. Cho HJ, Shan Y, Whittington NC, Wray S. Nasal placode development, GnRH neuronal migration and Kallmann syndrome. *Front Cell Dev Biol.* (2019) 7:121. doi: 10.3389/fcell.2019.00121
4. Herbison AE. Chapter 11–physiology of the adult gonadotropin-releasing hormone neuronal network. In: Plant TM, Zeleznik AJ, editors. *Knobil and Neill's Physiology of Reproduction*. 4th ed. San Diego, CA: Academic Press (2015). p. 399–467.
5. Merchenthaler I, Gorcs T, Setalo G, Petrusz P, Flerko B. Gonadotropin-releasing hormone (GnRH) neurons and pathways in the rat brain. *Cell Tissue Res.* (1984) 237:15–29. doi: 10.1007/BF00229195
6. Silverman AJ, Jhamandas J, Renaud LP. Localization of luteinizing hormone-releasing hormone (LHRH) neurons that project to the median eminence. *J Neurosci.* (1987) 7:2312–9.
7. Goldsmith PC, Thind KK, Song T, Kim EJ, Boggant JE. Location of the neuroendocrine gonadotropin-releasing hormone neurons in the monkey hypothalamus by retrograde tracing and immunostaining. *J Neuroendocrinol.* (1990) 2:157–68. doi: 10.1111/j.1365-2826.1990.tb00846.x
8. Herbison AE. Control of puberty onset and fertility by gonadotropin-releasing hormone neurons. *Nat Rev Endocrinol.* (2016) 12:452–66. doi: 10.1038/nrendo.2016.70
9. Piet R, de Croft S, Liu X, Herbison AE. Electrical properties of kisspeptin neurons and their regulation of GnRH neurons. *Front Neuroendocrinol.* (2015) 36:15–27. doi: 10.1016/j.yfrne.2014.05.006
10. Scott CJ, Rose JL, Gunn AJ, McGrath BM. Kisspeptin and the regulation of the reproductive axis in domestic animals. *J Endocrinol.* (2019) 240:R1–16. doi: 10.1530/JOE-18-0485
11. Simonneaux V, Ansel L, Revel FG, Klosen P, Pevet P, Mikkelsen JD. Kisspeptin and the seasonal control of reproduction in hamsters. *Peptides.* (2009) 30:146–53. doi: 10.1016/j.peptides.2008.06.006
12. Goodman RL, Lehman MN. Kisspeptin neurons from mice to men: similarities and differences. *Endocrinology*. (2012) 153:5105–18. doi: 10.1210/en.2012-1550
13. Smith JT, Popa SM, Clifton DK, Hoffman GE, Steiner RA. Kiss1 neurons in the forebrain as central processors for generating the preovulatory luteinizing hormone surge. *J Neurosci.* (2006) 26:6687–94. doi: 10.1523/JNEUROSCI.1618-06.2006
14. Clarkson J, d'Anglemont de Tassigny X, Moreno AS, Colledge WH, Herbison AE. Kisspeptin-GPR54 signaling is essential for preovulatory gonadotropin-releasing hormone neuron activation and the luteinizing hormone surge. *J Neurosci.* (2008) 28:8691–7. doi: 10.1523/JNEUROSCI.1775-08.2008
15. Smith JT, Coolen LM, Kriegsfeld LJ, Sari IP, Jaafarzadehshirazi MR, Maltby M, et al. Variation in kisspeptin and RFamide-related peptide (RFRP) expression and terminal connections to gonadotropin-releasing hormone neurons in the brain: a novel medium for seasonal breeding in the sheep. *Endocrinology*. (2008) 149:5770–82. doi: 10.1210/en.2008-0581
16. Yip SH, Boehm U, Herbison AE, Campbell RE. Conditional viral tract tracing delineates the projections of the distinct kisspeptin neuron populations to gonadotropin-releasing hormone (GnRH) neurons in the mouse. *Endocrinology.* (2015) 156:2582–94. doi: 10.1210/en.2015-1131
17. Uenoyama Y, Inoue N, Pheng V, Homma T, Takase K, Yamada S, et al. Ultrastructural evidence of kisspeptin-gonadotrophin-releasing hormone (GnRH) interaction in the median eminence of female rats: implication of axo-axonal regulation of GnRH release. *J Neuroendocrinol.* (2011) 23:863–70. doi: 10.1111/j.1365-2826.2011.02199.x
18. Smith JT, Li Q, Yap KS, Shahab M, Roseweir AK, Millar RP, et al. Kisspeptin is essential for the full preovulatory LH surge and stimulates GnRH release from the isolated ovine median eminence. *Endocrinology.* (2011) 152:1001–12. doi: 10.1210/en.2010-1225
19. Hrabovszky E, Ciofi P, Vida B, Horvath MC, Keller E, Caraty A, et al. The kisspeptin system of the human hypothalamus: sexual dimorphism and relationship with gonadotropin-releasing hormone and neurokinin B neurons. *Eur J Neurosci.* (2010) 31:1984–98. doi: 10.1111/j.1460-9568.2010.07239.x
20. Inoue N, Sasagawa K, Ikai K, Sasaki Y, Tomikawa J, Oishi S, et al. Kisspeptin neurons mediate reflex ovulation in the musk shrew (*Suncus murinus*). *Proc Natl Acad Sci USA*. (2011) 108:17527–32. doi: 10.1073/pnas.1113035108
21. Berland MA, Ulloa-Leal C, Barria M, Wright H, Dissen GA, Silva ME, et al. Seminal plasma induces ovulation in llamas in the absence of a

copulatory stimulus: role of nerve growth factor as an ovulation-inducing factor. *Endocrinology.* (2016) 157:3224–32. doi: 10.1210/en.2016-1310
22. Ratto MH, Berland MA, Silva ME, Adams G. New insights of the role of beta-NGF in the ovulation mechanism of induced ovulating species. *Reproduction.* (2019) 157:R199–R207. doi: 10.1530/REP-18-0305
23. Silva M, Paiva L, Ratto MH. Ovulation mechanism in South American Camelids: the active role of beta-NGF as the chemical signal eliciting ovulation in llamas and alpacas. *Theriogenology.* (2020) 150:280–7. doi: 10.1016/j.theriogenology.2020.01.078
24. Silva ME, Smulders JP, Guerra M, Valderrama XP, Letelier C, Adams GP, et al. Cetrorelix suppresses the preovulatory LH surge and ovulation induced by ovulation-inducing factor (OIF) present in llama seminal plasma. *Reprod Biol Endocrinol.* (2011) 9:74. doi: 10.1186/1477-7827-9-74
25. El Allali K, El Bousmaki N, Ainani H, Simonneaux V. Effect of the Camelid's seminal plasma ovulation-inducing factor/beta-NGF: a kisspeptin target hypothesis. *Front Vet Sci.* (2017) 4:99. doi: 10.3389/fvets.2017.00099
26. Witkin JW, Paden CM, Silverman AJ. The luteinizing hormone-releasing hormone (LHRH) systems in the rat brain. *Neuroendocrinology.* (1982) 35:429–38. doi: 10.1159/000123419
27. Duan C, Allard J. Gonadotropin-releasing hormone neuron development in vertebrates. *Gen Comp Endocrinol.* (2020) 292:113465. doi: 10.1016/j.ygcen.2020.113465
28. Foster WG, Younglai EV. An immunohistochemical study of the GnRH neuron morphology and topography in the adult female rabbit hypothalamus. *Am J Anat.* (1991) 191:293–300. doi: 10.1002/aja.1001910308
29. Dellovade TL, Rissman EF. Gonadotropin-releasing hormone-immunoreactive cell numbers change in response to social interactions. *Endocrinology.* (1994) 134:2189–97. doi: 10.1210/endo.134.5.8156921
30. Wersinger SR, Baum MJ. The temporal pattern of mating-induced immediate-early gene product immunoreactivity in LHRH and non-LHRH neurons of the estrous ferret forebrain. *J Neuroendocrinol.* (1996) 8:345–59. doi: 10.1046/j.1365-2826.1996.04623.x
31. Silverman AJ, Antunes JL, Ferin M, Zimmerman EA. The distribution of luteinizing hormone-releasing hormone (LHRH) in the hypothalamus of the rhesus monkey. Light microscopic studies using immunoperoxidase technique. *Endocrinology.* (1977) 101:134–42. doi: 10.1210/endo-101-1-134
32. Lehman MN, Robinson JE, Karsch FJ, Silverman AJ. Immunocytochemical localization of luteinizing hormone-releasing hormone (LHRH) pathways in the sheep brain during anestrus and the mid-luteal phase of the estrous cycle. *J Comp Neurol.* (1986) 244:19–35. doi: 10.1002/cne.902440103
33. Carrasco RA, Singh J, Adams GP. Distribution and morphology of gonadotropin-releasing hormone neurons in the hypothalamus of an induced ovulator–the llama (Lama glama). *Gen Comp Endocrinol.* (2018) 263:43–50. doi: 10.1016/j.ygcen.2018.04.011
34. Urbanski HF. Monoclonal antibodies to luteinizing hormone-releasing hormone: production, characterization, and immunocytochemical application. *Biol Reprod.* (1991) 44:681–6. doi: 10.1095/biolreprod44.4.681
35. Millar RP. GnRHs and GnRH receptors. *Anim Reprod Sci.* (2005) 88:5–28. doi: 10.1016/j.anireprosci.2005.05.032
36. Rissman EF, Alones VE, Craig-Veit CB, Millam JR. Distribution of chicken-II gonadotropin-releasing hormone in mammalian brain. *J Comp Neurol.* (1995) 357:524–31. doi: 10.1002/cne.903570404
37. Urbanski HF. Differential roles of GnRH-I and GnRH-ii neurons in the control of the primate reproductive axis. *Front Endocrinol (Lausanne).* (2012) 3:20. doi: 10.3389/fendo.2012.00020
38. Decourt C, Tillet Y, Caraty A, Franceschini I, Briant C. Kisspeptin immunoreactive neurons in the equine hypothalamus Interactions with GnRH neuronal system. *J Chem Neuroanat.* (2008) 36:131–7. doi: 10.1016/j.jchemneu.2008.07.008
39. Carrasco RA, Leonardi CE, Hutt K, Singh J, Adams GP. Kisspeptin induces LH release and ovulation in an induced ovulator. *Biol Reprod.* (2020) 103:49–59. doi: 10.1093/biolre/ioaa051
40. Ainani H, El Bousmaki N, Poirel VJ, Achaaban MR, Ouassat M, Piro M, et al. The dromedary camel displays annual variation in hypothalamic kisspeptin and Arg-Phe-amide-related peptide-3 according to sex, season, and breeding activity. *J Comp Neurol.* (2020) 528:32–47. doi: 10.1002/cne.24736
41. England BG, Cardozo AG, Foote WC. A review of the physiology of reproduction in the New World Cameiidae. *Int Zoo Yearb.* (1969) 9:104–10. doi: 10.1111/j.1748-1090.1969.tb02644.x
42. Sumar JB. Reproduction in female South American domestic camelids. *J Reprod Fertil Suppl.* (1999) 54:169–78.
43. Vaughan JL, Tibary A. Reproduction in female South American camelids: a review and clinical observations. *Small Rumin Res.* (2006) 61:259–81. doi: 10.1016/j.smallrumres.2005.07.015
44. Van Saun RJ. Effect of nutrition on reproduction in llamas and alpacas. *Theriogenology.* (2008) 70:508–14. doi: 10.1016/j.theriogenology.2008.04.025
45. Matsuyama S, Ohkura S, Mogi K, Wakabayashi Y, Mori Y, Tsukamura H, et al. Morphological evidence for direct interaction between kisspeptin and gonadotropin-releasing hormone neurons at the median eminence of the male goat: an immunoelectron microscopic study. *Neuroendocrinology.* (2011) 94:323–32. doi: 10.1159/000331576
46. True C, Kirigiti M, Ciofi P, Grove KL, Smith MS. Characterisation of arcuate nucleus kisspeptin/neurokinin B neuronal projections and regulation during lactation in the rat. *J Neuroendocrinol.* (2011) 23:52–64. doi: 10.1111/j.1365-2826.2010.02076.x
47. Pompolo S, Pereira A, Estrada KM, Clarke IJ. Colocalization of kisspeptin and gonadotropin-releasing hormone in the ovine brain. *Endocrinology.* (2006) 147:804–10. doi: 10.1210/en.2005-1123
48. Ramaswamy S, Guerriero KA, Gibbs RB, Plant TM. Structural interactions between kisspeptin and GnRH neurons in the mediobasal hypothalamus of the male rhesus monkey (*Macaca mulatta*) as revealed by double immunofluorescence and confocal microscopy. *Endocrinology.* (2008) 149:4387–95. doi: 10.1210/en.2008-0438
49. Smith JT, Rao A, Pereira A, Caraty A, Millar RP, Clarke IJ. Kisspeptin is present in ovine hypophysial portal blood but does not increase during the preovulatory luteinizing hormone surge: evidence that gonadotropes are not direct targets of kisspeptin *in vivo*. *Endocrinology.* (2008) 149:1951–9. doi: 10.1210/en.2007-1425
50. Keen KL, Wegner FH, Bloom SR, Ghatei MA, Terasawa E. An increase in kisspeptin-54 release occurs with the pubertal increase in luteinizing hormone-releasing hormone-1 release in the stalk-median eminence of female rhesus monkeys *in vivo*. *Endocrinology.* (2008) 149:4151–7. doi: 10.1210/en.2008-0231
51. Kinsey-Jones JS, Li XF, Luckman SM, O'Byrne KT. Effects of kisspeptin-10 on the electrophysiological manifestation of gonadotropin-releasing hormone pulse generator activity in the female rat. *Endocrinology.* (2008) 149:1004–8. doi: 10.1210/en.2007-1505
52. d'Anglemont de Tassigny X, Jayasena CN, Murphy KG, Dhillo WS, Colledge WH. Mechanistic insights into the more potent effect of KP-54 compared to KP-10 *in vivo*. *PLoS ONE.* (2017) 12:e0176821. doi: 10.1371/journal.pone.0176821
53. Prevot V. GnRH neurons directly listen to the periphery. *Endocrinology.* (2011) 152:3589–91. doi: 10.1210/en.2011-1544
54. Herde MK, Geist K, Campbell RE, Herbison AE. Gonadotropin-releasing hormone neurons extend complex highly branched dendritic trees outside the blood-brain barrier. *Endocrinology.* (2011) 152:3832–41. doi: 10.1210/en.2011-1228
55. Silva ME, Recabarren MP, Recabarren SE, Adams GP, Ratto MH. Ovarian estradiol modulates the stimulatory effect of ovulation-inducing factor (OIF) on pituitary LH secretion in llamas. *Theriogenology.* (2012) 77:1873–82. doi: 10.1016/j.theriogenology.2012.01.004

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 10 February 2021
doi: 10.3389/fvets.2021.611301

Spermatozoa Obtained From Alpaca *vas deferens*. Effects of Seminal Plasma Added at Post-thawing

Eduardo G. Aisen[1], Wilfredo Huanca López[2], Manuel G. Pérez Durand[3], Edita Torres Mamani[4], Juan C. Villanueva Mori[2], María J. Ousset[1], Víctor H. Medina[1], Uri H. Pérez Guerra[3] and Teodosio Huanca Mamani[4]*

[1] Laboratorio de Teriogenología "Dr. Héctor H. Morello," Instituto de Biotecnología del Comahue-Centro de Investigaciones en Toxicología Ambiental y Agrobiotecnología del Comahue (Consejo Nacional de Investigaciones Científicas y Técnicas-Universidad Nacional del Comahue), Facultad Cs. Agrarias, Universidad Nacional del Comahue, Cinco Saltos, Argentina, [2] Laboratorio de Reproducción Animal, Facultad de Medicina Veterinaria, Universidad Nacional Mayor de San Marcos, Lima, Peru, [3] Laboratorio de Reproducción Animal y Laboratorio de Biotecnología de la Reproducción, Facultad de Medicina Veterinaria y Zootecnia, Universidad Nacional del Altiplano, Puno, Peru, [4] Instituto Nacional de Innovación Agraria - Programa Nacional de Camélidos, Estación Experimental Quimsachata-ILLPA-Instituto Nacional de Innovación Agraria, Puno, Peru

OPEN ACCESS

Edited by:
Marcelo H. Ratto, Austral University of Chile, Chile

Reviewed by:
María Ignacia Carretero, University of Buenos Aires, Argentina
Alessandra Rota, University of Pisa, Italy

***Correspondence:**
Eduardo G. Aisen
eduardoaisen@hotmail.com

Specialty section:
This article was submitted to Animal Reproduction - Theriogenology, a section of the journal Frontiers in Veterinary Science

Received: *28 September 2020*
Accepted: *12 January 2021*
Published: *10 February 2021*

Citation:
Aisen EG, Huanca López W, Pérez Durand MG, Torres Mamani E, Villanueva Mori JC, Ousset MJ, Medina VH, Pérez Guerra UH and Huanca Mamani T (2021) Spermatozoa Obtained From Alpaca vas deferens. Effects of Seminal Plasma Added at Post-thawing. Front. Vet. Sci. 8:611301. doi: 10.3389/fvets.2021.611301

The viscous seminal plasma (SP) is currently a major impediment to the handling of ejaculate and the development of some biotechnologies in South American camelids. The *vas deferens*-collected spermatozoa of alpacas is a useful technique to avoid this problem. On the other hand, SP contains a large protein component that has been implicated in the function of spermatozoa within the female reproductive tract. In this sense, the low fertility achieved using transcervical insemination with frozen-thawed spermatozoa in alpacas could be improved by adding SP. This study aimed to evaluate the effect of the whole SP on some *in vitro* parameters of alpaca spermatozoa after the freezing-thawing-process and the fertility after artificial insemination. It would contribute to a better understanding of the interaction between thawed sperm cells and SP. Spermatozoa were obtained by surgically diverted *vas deferens*. The samples were diluted with a Tris-based extender, packaged in straws, and frozen. At thawing, each straw was divided into two post-thawing conditions: with the addition of 10% of PBS (control) or with 10% SP (treatment). The sperm cells were evaluated using dynamic parameters, sperm cell morphology, and morphometry. Fertility was assessed by an artificial insemination trial. All *in vitro* parameters were analyzed by ANOVA. A heterogeneity test was scheduled for the fertility trial. After the freezing-thawing process, motility and plasma membrane functionality was improved when SP was added. No differences were found for post-thaw viability between the control and treatment samples. The percentage of normal cells was higher with SP at post-thawing, and a decrease of the presence of bent tailed spermatozoa with a droplet in the SP group was observed. The length of the head spermatozoa was 3.4% higher in the samples with PBS compared to those in which SP was added. Females pregnant at day 25 post-insemination were 0/12 (with SP inside the

straw) and 1/10 (without SP inside the straw). In conclusion, the presence of 10% SP at post-thawing improves sperm cells' motility, functionality, and morphology, indicating that it would be beneficial to improve the frozen-thawed alpaca's physiology spermatozoa. More fertility trials must be developed to increase this knowledge.

Keywords: seminal plasma, alpaca spermatozoa, sperm cryopreservation, *vas deferens*, South American camelids

INTRODUCTION

The most remarkable characteristics of semen in South American camelids are its high structural viscosity and thread formation (1–3). These rheological characteristics impede the homogeneous mixing of semen with the extender, thereby limiting contact between the sperm cell membrane and cryoprotective compounds during the cooling and freezing processes (4–6), consequently hindering the development of artificial insemination technologies for this species (7).

To reduce the thread formation, semen from llamas was incubated in a 0.1% collagenase solution (1). Working with Bactrian camel semen, the ejaculates were treated with a magnetic stirrer at low speed (100 rpm) for 5 min (8), providing a liquefied homogenous specimen. Recently, the combination of the slow mechanical method (aspirating and expelling with a syringe or pipette) with low gravity acceleration (700 × g) allows obtaining SP without sperm cells [(9) for llamas; (10) for alpacas], avoiding the possible expelling of fluids (including proteins) from the damaged spermatozoa.

To avoid the problem of high viscosity, several authors decided to develop their experiments using epididymal or *vas deferens*-collected spermatozoa (11, 12). Pérez et al. (13) demonstrated that it is possible to obtain sperm cells from surgically diverted *vas deferens* in male alpacas and llamas, thus facilitating the evaluation of concentration, motility, abnormalities, and subsequent cryopreservation. This kind of collection may be useful for research purposes.

However, SP contains a large protein component that has been implicated in the function, transit, and survival of spermatozoa within the female reproductive tract (14). These authors identified a total of 10 alpaca SP proteins. Those that are in the 10–25 kDa range have an important modulation effect on sperm functionality. For example, RSVP14, a binder of sperm protein that can protect ram sperm membranes from cold shock (15), is present in alpaca SP, suggesting a common role of this seminal plasma protein on sperm functions in various species. In llamas, it was observed that sperm bind to N-acetylgalactosamine (GalNAc) on the surface of the oviductal epithelium. This condition is necessary to establish the oviductal sperm reservoir of South American camelids (16). More recently, seminal lectin SL15 was studied. This lectin is likely presented to sperm via seminal plasma since epididymal sperm cannot bind GalNAc, whereas ejaculated sperm does (17). These findings have great importance in explaining SP's relevance of the functions of ejaculated sperm and sperm cells obtained from epididymis or *vas deferens*.

Alpacas, like other Old and New World camelids, are classified as an induced ovulating species. They need external stimulation during the copulation to develop ovulation (18). Alpaca and camel SP contains a 13 kDa protein identified as beta nerve growth factor (β-NGF), which plays an important role in ovulation in this species (3). Although the effect of β-NGF on South American camelids sperm motility has not been studied (6), the co-location of β-NGF with tyrosine kinase receptor A (TrKA) in the middle piece of ejaculated and acrosome reacted llama sperm (19), suggest a possible action on sperm motility.

With several compounds, SP can both inhibit and stimulate sperm function and fertility through multifunctional actions (20). It is well known that some proteins of SP linked to spermatozoa are necessary to achieve fertility and oocyte binding. They were identified in alpaca SP and are similar to those reported in other species (5). The addition of SP to sperm, following cryopreservation, increased post-thawed motility and fertility in ram (21), enhanced post-thawed sperm function in boar (22), and increased artificial insemination fertility in stallion (23). In alpaca, it was demonstrated that 10% of SP incubated with sperm cells recovered from the epididymis preserve motility, acrosome, and viability of spermatozoa (24). In contrast, when SP was added to thawed llama semen, it was incapable of preserving motility or membrane function during 3 h of incubation (6). It was observed that SP from alpaca males with high semen freezability are related to specific protein fractions (14–15 kDa), which are absent in SP from males with low semen freezability (25). These findings allow us to conclude that SP is necessary to maintain the survival and fertilizing capacity of spermatozoa in camelids.

Furthermore, few trials use transcervical insemination with frozen-thawed spermatozoa in alpacas, and the fertility achieved is null or very low (26). Explanations for this low fertility include several factors such as the time between insemination and ovulation, the number of sperm cells, the volume of the dose, handling of the frozen-thawed semen, use of females with doubtful fertility, and others (27).

This study aimed to evaluate the effect of whole SP on some *in vitro* parameters of alpaca sperm cells after the freezing-thawing-process and the fertility rate achieved after an artificial insemination trial.

MATERIALS AND METHODS

Animals

Five adult alpaca males (>4 years old with proven fertility) were used to obtain sperm cells from *vas deferens*. Animal welfare conditions were ensured in accordance with institutional statements (approved by Ethics Committee, Facultad de

Veterinaria y Zootecnia, Universidad Nacional del Altiplano, Puno, Perú, 15° 49′ 34.5″ S, 70° 00′43.5″ W, 3 820 MSL). Spermatozoa were obtained by pipette aspiration of surgically diverted *vas deferens*. This technique is described by Pérez et al. (11, 13) and consists of five steps: (a) the males were fasted for 24 h before the surgery and were chemically restrained with Acepromazine maleate (0.1 mg/Kg BW); (b) the animals were placed in a supine position, and the surgical field was prepared in the inguinal region to perform local anesthesia; (c) a small incision (4 cm) was made in the skin over the penis; (d) the *vas deferens* of the right and left side were located and dissected in a length of 7 cm; (e) then, the dissected parts of *vas deferens* were redirected below the subcutaneous tissue and fixed to the skin of the internal face of the femoral region, and protected with a temporary patch.

Reagents

All chemical reagents employed were of the highest commercially available purity, purchased from Sigma–Aldrich Co. (Saint Louis, USA).

Sperm Cells Recovery and Process

Before each recovery, the fistula's opening and the skin near this hole were washed with sterile PBS. The recovery process was begun with a rectilinear massage of the *vas deferens* with the fingertips, allowing the sperm cells to move toward the outflow of the fistula. As the drops with spermatozoa appeared, they were aspirated by means of an automatic pipette with a tip moistened in a Tris-based extender. The drops (about 80–100 μL) containing the sperm cells were quickly diluted with a Tris-based solution and placed in a plastic tube at 37°C. The samples were assessed through total sperm cells (TS), sperm cell concentration, sperm motility (TM), and sperm cell morphometry (28). Only samples with TS higher than 20×10^6 spermatozoa and TM higher than 50% were used.

A total of 20 samples (five males × four repetitions) were performed. The collected and evaluated samples were diluted with Tris (240 mM), citric acid (76 mM), glucose (22 mM), glycerol (3% v/v), and egg yolk (10% v/v) to a final concentration of 50×10^6 spermatozoa/mL (29). After the dilution and cooling processes, the mixtures were stabilized at 5°C for 90 min. They were then packaged in 0.25-mL straws (IMV Technologies, L'Aigle, France) and frozen in liquid nitrogen vapor (−100°C). For the freezing process, the straws were placed on a rack inside a Styrofoam box. The height was graduated manually, remaining 10 min at 24 cm, 10 min at 12 cm, and 5 min at 3 cm above the liquid nitrogen level. Finally, the straws were plunged into the liquid nitrogen and stored in a nitrogen tank at −196°C [modified from (30)].

Sperm Cells Treatments

Thawing was performed by immersion in a water bath for 20 s at 37°C. Each straw was divided into two post-thawing conditions: with the addition of 10% PBS (control) or with 10% SP (treatment). Both fractions were incubated at 37°C for 20 min. Seminal plasma was obtained from ejaculates of six adult alpaca males, during the breeding season, under the same animal welfare conditions indicated previously. For this purpose, semen samples obtained by means of an artificial vagina were treated using a slow mechanical method (9, 31) to reduce thread formation and to obtain a more fluent sample. Briefly, each ejaculate was very slowly aspirated and expelled (avoiding foam formation) with a needle (0.5 mm inner diameter) attached to a syringe, repeating this action 10 times. Then, the ejaculates were centrifuged at 700 × g for 30 min (10), and the supernatant was recovered and centrifuged once again. SP was then recovered, and a drop was evaluated by microscopy to confirm the absence of cells (32). The SP obtained was polled and stored at −20°C before use.

Polyacrylamide Gel Electrophoresis (SDS-PAGE)

An electrophoresis was carried out to obtain an approximation of the molecular weight of the proteins present in the SP prepared for this experiment. Six sources (males) of SP were electrophoresed in 12% polyacrylamide gels containing sodium dodecyl sulfate (SDS-PAGE) at room temperature (Mini Protean 3-Bio-Rad Laboratories, Inc, California, USA), as described by Laemmli (33). The total amount of 11 μg of protein, quantified by the Bradford (34) method, was seeded in each lane. Molecular weight was estimated using protein molecular weight standards (PB-L Productos Bio-logicos, Buenos Aires, Argentina). The gels were stained with Coomassie blue and then washed, scanned (transmission acquisition), and observed.

In vitro Assessment

After thawing and incubation with PBS or SP, the samples were evaluated *in vitro* using an inverted microscope (Nikon Eclipse Ti-S, Tokyo, Japan).

Sperm motility was determined by placing 10 μL of a sperm sample on a warm slide and covering it with a warm coverslip, using a warm stage (37°C) phase-contrast optics and video-microscopy (35).

Sperm motility index (ISM) was calculated as TM × movement quality (36). In this way, the quantity (percentage of motility) and quality (vigor) of sperm movement could be considered in a single parameter.

Plasma membrane functional integrity was assessed by the hypoosmotic swelling test (HOST), using a modified method developed by Giuliano et al. (37). This method consists of the incubation (37°C, 20 min) of 50 μL of sperm samples in 200 μL of hypoosmotic solution (2.45 mg/mL of fructose and 4.5 mg/mL of sodium citrate, adjusted to 50 mOsm). The incubation was stopped with a hypoosmotic formol solution, and then 200 spermatozoa were evaluated.

Cell viability was determined with eosin-nigrosin staining (Eo-Ni), according to Bloom (38) and Aisen et al. (28). On a warm stage, 10 μL of the sperm sample and 10 μL of the supravital staining solution (5 g/L of eosin Y, 100 g/L of nigrosin, 29 g/L of sodium citrate) were mixed. After 30 s, the mixed drop was smeared by sliding a coverslip in front of it, dried on air, and observed by bright field microscopy (400 x).

Sperm cell morphology was performed using the slides stained with eosin-nigrosin and Barth and Oko (39) classification, modified by Medina et al. (40).

Sperm cell morphometry was developed as described by Aisen et al. (28). A high-resolution color digital camera (DS-Ri-U2, Nikon, Tokyo, Japan) and software for processing microscopic images (Nikon NIS Elements Advanced Research) were used. The head length (L-head) and head width (W-head) were measured, and ellipticity (E) was calculated, such as the L-head/W-head ratio.

Fertility Trial

Fertility was assessed by an artificial insemination trial (extensive field conditions). Spermatozoa from *vas deferens* (four males) were frozen into 0.5 mL-straws containing 20×10^6 total sperm cells in two different volumes. One batch was loaded only with sperm cells + diluent (0.4 mL). The other batch (SP treatment) was filled partially with SP (0.2 mL) and loaded behind an air bubble to separate the spermatozoa + extender (0.2 mL); those were loaded at the end (**Figure 1**).

Each straw was separately thawed in a water bath (37°C) for 20 s. They were managed carefully, especially those containing SP to keep the two parts separate. The straws were dried with absorbent paper and placed into a universal semen applicator (IMV Technologies, France). Finally, this AI device was protected with a sterile sheath before seeding the female tract.

A group of adult alpaca females (Huacaya breed) from the Quimsachata Research Station (ILLPA-INIA, Puno, Perú, 15°45′ S, 70°34′ W, 4200 MSL) were destined to induce ovulation as described by Adams et al. (32). Considering that South American camelids are species of induced ovulation, the animals were subjected to the following maneuvers. Alpaca females were selected according to their ovarian dynamics. For this purpose, the presence of a dominant follicle ≥ 7 mm, detected by transrectal ultrasonography (Esaote portable ultrasound, 5 MHz probe, Genoa, Italy), was the condition to include them in the test. After performing the ultrasound examination, ovulation was induced with 1 ml of SP given intramuscularly (32). For this purpose, semen from adult alpaca males, obtained with an artificial vagina, was centrifuged at 1,500 × g for 15 min, and the supernatant was observed for the absence of sperm cells. A 1:1 SP dilution with PBS + antibiotics (kanamycin sulfate 25 μg/ml) was stored at −18°C until the moment of ovarian stimulation.

Two groups (12 and 10 females, with or without SP into the straws, respectively) were inseminated around 26 h after seminal plasma stimulation. The animals were restrained in stocks, the rectum was emptied of feces, and the perineum was cleaned. A lubricated, gloved hand was placed in the rectum, and the anatomical structures were located (cervix and bifurcation of the uterine horns). With the other hand, the vulva labia were separated, and the AI device was introduced into the vagina at an angle of approximately 40° upwards. With the index finger guiding the semen applicator, the cervix was threaded and traversed. The device was then directed to the uterine horn corresponding to the side where an ovarian with a dominant follicle ≥ 7 mm was observed (7). The seeding of the straw's content was performed as deep as possible in the uterine horn (closest to the uterotubal junction), slowly depressing the injector plunger.

Pregnancy was diagnosed by ultrasonography on Day 25 post-insemination, using the same ultrasound equipment, to locate an anechoic structure in the uterus, compatible with the embryonic vesicle (41).

Statistical Analyses

All *in vitro* parameters were analyzed by ANOVA (main effects), with a Fisher–LSD *post hoc* test, using the software StatSoft, Inc. (2007). A heterogeneity test was planned for the fertility trial.

RESULTS

Assessment of Spermatozoa Obtained

The values of raw sperm cell samples obtained from *vas deferens,* expressed as means ± standard error of the mean, were TS = $35.53 \pm 8.44 \times 10^6$ spermatozoa; sperm concentration = $405.00 \pm 107.54 \times 10^6$ cells/mL; TM = 57.50 ± 6.86 %; L-head = 6.06 ± 0.06 μm; W-head = 3.67 ± 0.03 μm; E = 1.65 ± 0.02. TS and TM were strongly affected by male factor.

Identification of Molecular Weight of SP Proteins

The protein bands observed by SDS PAGE of SP are shown in **Figure 2**. Five single or group of bands were identified, with a molecular weight around 13, 14, 20, 25, and 60 kDa.

In vitro Effects of SP at Thawing

At thawing, no interactions between males, dates, and replicates were found. The sperm cell dynamic parameters at thawing are

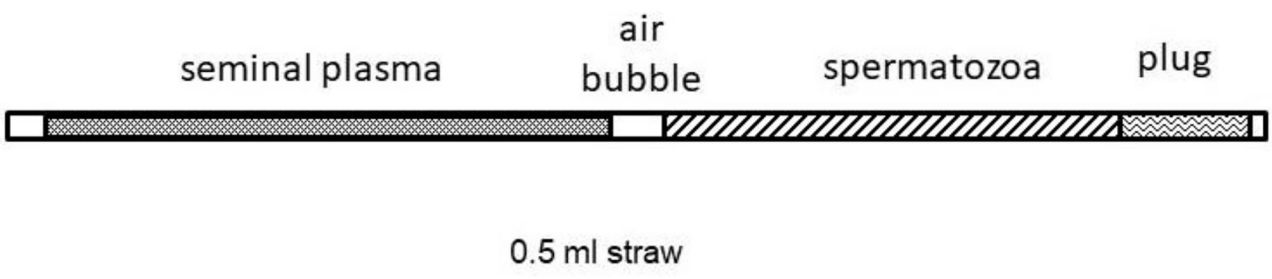


FIGURE 1 | Scheme of straw loading with seminal plasma and spermatozoa.

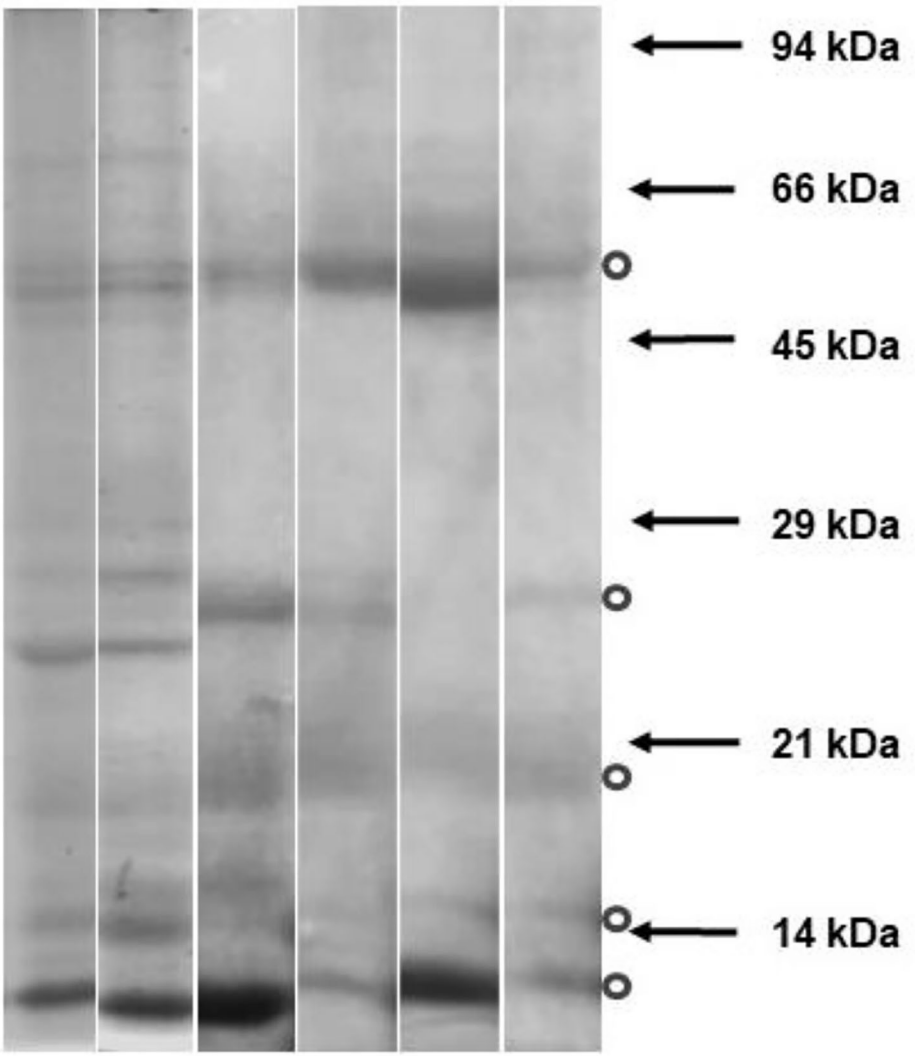


FIGURE 2 | Protein bands observed by SDS PAGE of Seminal plasma from six alpaca males. Six seminal plasmas were electrophoresed in polyacrylamide gels containing sodium dodecyl sulfate (Mini Protean 3-Bio-Rad Laboratories, Inc, California, USA). Molecular weight was estimated using protein molecular weight standards (positions indicated with arrows). The gels were stained with Coomassie blue. Dots indicate most frequent bands observed.

shown in **Table 1**. The presence of SP after the freezing-thawing improved the TM ($p = 0.035$), ISM ($p = 0.017$), and HOST ($p = 0.022$), compared to the PBS treatment. No differences were found for Eo-Ni with respect to the addition of SP.

The sperm cell morphology parameters are shown in **Table 2**. Normal sperm cell morphology was higher ($p = 0.022$) when SP was present at thawing, compared with PBS treatment (60.90 ± 1.40% vs. 54.60 ± 1.40%, respectively). A decrease in the presence of bent tailed with droplet spermatozoa was observed in the SP group with respect to the control group (0.45 ± 0.32% vs. 2.90 ± 1.03%, respectively).

The sperm head cell morphometry parameters are shown in **Table 3**. The L-head showed a highly significant increase of about 3.4% ($p = 0.0001$) in the control samples (with PBS) compared to those where SP was added at thawing (6.29 ± 0.03 μm vs. 6.08 ± 0.03 μm, respectively). W-head and E were both 2% higher in the control samples with respect to those with SP added, showing statistical differences ($p < 0.05$).

In vivo Effects of SP at Thawing

The females who were pregnant at Day 25 post-insemination were 0/12 (with SP inside the straw) and 1/10 (without SP inside the straw). No significant differences were found between the groups ($p = 0.26$).

DISCUSSION

The pipette aspiration of surgically diverted *vas deferens* seems to be a useful technique to obtain sperm cells from male alpacas, especially for the study of the freezing-thawing process (42). In

TABLE 1 | Effect of seminal plasma added at post-thawing on frozen-thawed spermatozoa obtained from alpaca *vas deferens*. Sperm parameters (total motility, sperm motility index, viability, and membrane function).

Treatment	TM (%)	ISM (%)	Eo-Ni (%)	HOST (%)
With PBS	18.3 ± 2.1	40.0 ± 3.2	61.4 ± 2.5	41.5 ± 1.3
With SP	25.8 ± 2.42	78.5 ± 9.0	63.4 ± 1.4	48.2 ± 1.7
p value	0.035	0.017	0.510	0.022

Values are mean ± standard error.
SP, seminal plasma; TM, total motility; ISM, sperm motility index; Eo-Ni, supravital stain; HOST, plasma membrane functionality. The parameters were analyzed by ANOVA (main effects), with Fisher–LSD post hoc test.

TABLE 2 | Effect of seminal plasma added at post-thawing on frozen-thawed spermatozoa obtained from alpaca *vas deferens*. Sperm morphology.

Sperm morphology category	Treatment		*p* value
	With PBS (%)	With SP (%)	
Normal sperm cells	54.60 ± 1.42	60.90 ± 1.40	0.022
Small head	19.45 ± 1.25	18.79 ± 1.65	0.754
Detached normal head	1.53 ± 0.59	2.22 ± 0.62	0.347
Detached abnormal head	0.13 ± 0.02	0.45 ± 0.02	0.229
Abaxial head	2.10 ± 0.59	1.27 ± 0.69	0.389
Bent tail with droplet	2.90 ± 1.03	0.45 ± 0.32	0.04
Bent tail	12.30 ± 1.88	11.53 ± 0.93	0.721
Coiled tail	1.25 ± 0.61	1.33 ± 0.61	0.91
Proximal droplet	3.91 ± 1.61	1.69 ± 0.81	0.247
Distal droplet	1.44 ± 0.91	0.56 ± 0.36	0.393
Fractured neck	0.80 ± 0.60	0.68 ± 0.54	0.787

Values are mean ± standard error.
SP, seminal plasma. The parameters were analyzed by ANOVA (main effects), with Fisher–LSD post hoc test.

TABLE 3 | Effect of seminal plasma added at post-thawing on frozen-thawed spermatozoa obtained from alpaca *vas deferens*. Sperm head morphometry.

Treatment	Head sperm cell morphometry		
	Length (μm)	Width (μm)	Ellipticity
With PBS	6.29 ± 0.03	3.70 ± 0.03	1.73 ± 0.01
With SP	6.08 ± 0.03	3.62 ± 0.02	1.70 ± 0.01
p value	0.0001	0.026	0.037

Values are mean ± standard error.
SP, seminal plasma. The parameters were analyzed by ANOVA (main effects), with Fisher–LSD post hoc test.

this experiment, a high TS variation between males was observed, going from 5×10^6 to 152×10^6 total sperm cells. For this reason, and because of a linked low volume recovery, several samples were discarded. Frequently, working with very small volumes at the time of collection makes it difficult to handle and quickly protect against thermal changes, triggering the spermatozoa's damage or death. Pérez et al. (13) reported that this collection could be performed twice a week, obtaining about 25×10^6 total spermatozoa at each collection, which is close to the value obtained in our work ($35.53 \pm 8.44 \times 10^6$ spermatozoa). Motility of the spermatozoa recovered (57.50 ± 6.86 %) was similar to that achieved by Kershaw-Young and Maxwell in 2011 (56.3 ± 2.80%) and other authors for epididymal alpaca spermatozoa [52.7 ± 3.3% by (12) and 56.8 ± 9.8% by (13)].

After the freezing-thawing process, TM and sperm morphology related to the sperm tail status were improved when SP was added after thawing. In this case, the recovery of frozen-thawed spermatozoa was better, indicating SP's direct effect on some aspects of sperm cell physiology. Regarding this finding, it could be connected with the high ISM achieved. Motility of ejaculated or epididymal spermatozoa obtained from male alpacas (raw state) was improved when SP (especially at 10%) was added during incubation at 37°C (24).

Although no significant differences were observed for Eo-Ni at post-thawing in this experiment, an increase in live ram spermatozoa has been reported when adding 20% of SP at thawing. This addition caused the best values in the sperm quality parameters studied (43). In alpaca, Kershaw-Young and Maxwell (24) observed that the viability of ejaculated sperm was better when incubated in 10 rather than 100% seminal plasma. The cause of this reduced viability is not known, but may be associated with increased osmotic stress and lower pH due to the higher proportion of SP in the sample with 100% SP, which could affect the sperm parameters. Fumuso et al. (6) observed that the percentages of total live sperm were preserved over 3 h of incubation in all SP final concentrations evaluated (0, 10, or 50%), and no significant differences were observed in total live spermatozoa between the SP concentrations assayed. Although there is a coincidence with what was observed in our study (0 or 10% of SP), this work's results are not entirely comparable since the studies were developed using ejaculated spermatozoa, which had already come into contact with SP.

Regarding the results in this experiment, higher preservation of the plasma membrane functionality was observed, shown by HOST, indicating a possible beneficial interaction between SP proteins and the plasmalemma of the sperm cell. There are a few references to studies researching SP's effect on HOST using spermatozoa collected from the *vas deferens*. Zea et al. (44), evaluating spermatozoa from alpaca, found no differences in the addition of SP at thawing for this parameter. However, the percentage of SP used was not indicated in this work.

A group of five common protein bands was identified by SDS PAGE. Those with a molecular weight of around 13 and 14 kDa would correspond to βNGF and RSVP14, respectively (14). The bands below 25 kDa correspond to several proteins with a modulation effect on sperm functionality. The 20 kDa proteins prevent cold shock sperm membrane damage and show seasonal differences in ram SP proteins (15). Some of these proteins specifically bound to the acrosomal region of the ram sperm surface (45), such as the Lactotransferrin, epididymal secretory protein E1, Synaptosomal-associated protein 29, and RSVP-20 present in this fraction. The 60 kDa band proteins were observed as the most abundant in all male alpacas, without diet influence (46). It was demonstrated that not all SP proteins bound specifically to the sperm surface and improved the thawed sperm

cells. These interacting seminal plasma proteins are not sufficient to emulate the effects of complete SP regarding sperm functional parameters (43). When certain proteins were incubated with frozen-thawed ram spermatozoa, they partially repaired semen cryodamage, protecting both the sperm motility and the ultrastructure (45). These authors demonstrated that sperm membrane was improved in frozen-thawed sperm cells treated for 15 min with SP proteins. These proteins could be sufficient to reverse molecular signals of capacitation caused by freezing, perhaps acting through the inhibition of the signal transduction pathways of capacitation (43). Some of these proteins are spermadhesins (heparin-binding proteins). They are the most likely protein fraction that binds to phospholipids on the sperm membrane upon ejaculation, stabilizing the sperm membrane, and preventing capacitation (14). Centurion et al. (47) incubated fresh boar spermatozoa with heparin-binding spermadhesins and non-heparin-binding spermadhesins. They found that non-heparin-binding spermadhesins contributes to maintaining sperm with high viability, motility, and mitochondrial activity for at least 5 h. They conclude that both spermadhesins exert antagonistic effects on the functionality of highly diluted boar spermatozoa. However, recent studies on llama ejaculates did not find improvements when SP was added after the freezing-thawing (6). When the results obtained in llamas are compared with those observed in our work on alpacas, it is important to consider the difference in the source of spermatozoa. In llama, ejaculated sperm had previous contact with SP, suggesting (but not reliably verified) that, in this case, the bounded proteins of the SP present in the semen at the time of ejaculation have previously exercised some protection against the cryopreservation. In the case of alpaca spermatozoa analyzed in our experiment, the first contact with SP was at post-thawing incubation.

There is a hypothesis (biphasic effect): SP interaction with spermatozoa could be beneficial in the short term in normal reproductive physiology but could be detrimental in the long-term preservation condition (48). Considering Kershaw-Young and Maxwell's (24) findings, most of the spermatozoa parameters incubated with SP measured at 0.5, or 1 h (motility, intact acrosomes, viability) showed the same behavior, predicting the dynamics during the incubation. However, most of the values of the parameters studied showed a strong decrease after 1 h. On the other hand, as indicated previously, ram sperm cells treated with SP for 15 min were sufficient at improving progressive motility and other parameters (45). For these reasons, we have considered exposing the sperm cell to the SP for a short time.

The morphology of frozen-thawed spermatozoa showed that the percentage of normal sperm cells was higher with SP at post-thawing. The principal differences were the values of the bent tailed with droplet cells (a secondary abnormality), indicating possible damage on tail plasma membrane permeability when the spermatozoa were incubated with PBS. Interestingly, the percentage of bent tails with droplets was close to zero when SP was present at post-thawing.

Continuing with sperm measurements, L-head was 3.4% higher in the control samples (with PBS) with respect to thawed samples with SP added. The analysis of SP's addition at post-thawing on the head morphometry allows supposing that, in this condition, SP modules the membrane permeability, slightly reducing the flow of water into the sperm cells because of the presence of glycerol at thawing (49). Due to the small capacity of the head sperm cell to change its volume, length may be the best parameter related to this modification compared to width. In ram spermatozoa, the ultrastructures below the plasma membrane (cytoskeleton included) seem to be less rigid at the acrosomal region than the equatorial segment or the post-acrosomal region (50, 51). It is also important to highlight that sperm cell morphometry parameters with SP at post-thawing (**Table 3**) were similar to those at the pre-freezing stage (L-head $= 6.06 \pm 0.06\,\mu m$; W-head $= 3.67 \pm 0.03\,\mu m$; E $= 1.65 \pm 0.02$), allowing the cell to maintain the best physiological condition.

For the artificial insemination trial, the straws corresponding to the treatment group were filled with 0.2 mL of SP and 0.2 mL of sperm + extender. This original 1:1 ratio in the straw would not be maintained during seeding. It was considered that, during insemination, the part of SP (which is expelled at first) would be deposited on the uterine mucosa, losing a part of it due to the dilution effect in the uterine fluids. The part of thawed sperm cells that was seeded later would be deposited on this portion of SP, reaching a real final SP/sperm ratio much <50%, emulating a little more to *in vitro* conditions. Apichela et al. (52) confirmed that the presence of viscous mucus (in particular secreted by the bulbourethral glands) in the utero-tubal junction in llamas was involved in the formation of the sperm reservoir. This seminal factor can be modified when ejaculates are diluted with extenders, resulting in a diminished adhesion of sperm to the oviductal mucosa and the viability in the female tract. For this reason, SP added into the straw could artificially restore (partially) the conditions that sperm cells have after natural mating. This experimental design attempted to offer to thawed sperm (with a lower fertilizing capacity compared to fresh sperm) a more appropriate media to maintain their functions until reaching the oviduct. However, this condition could not be demonstrated during this fertility test.

This artificial insemination trial was carried out in field conditions, where it was not possible to perform tracing of the ovarian follicle's development. The follicular size was used as a guide to select those females that presented favorable conditions to induce ovulation. In this sense, Adams et al. (32) showed that, by selecting llamas with follicles larger than ≥8 mm, ovulation was detected in 90% of females after i.m. administration of seminal plasma. Unlike our work, females were previously induced to synchronize the follicular-wave emergence among animals with LH. More recently, Ascencio et al. (53) studied the effect of the stage of development of the follicular wave prior to the natural mating on the ovulation rate and the recovery rate and embryo quality in alpacas. These authors found that there were no significant differences between groups by size of the dominant follicle (phase of follicular growth, regression, or static), ovulation rate, or size of the corpus luteum on the day of embryo recovery. The lack of greater monitoring of ovarian dynamics in our work could have influenced the pregnancy rates obtained. Due to the low pregnancy values obtained, other factors such as the number of motile sperm cells and relocation of liquid components inside the straws should be considered in

future experiments. The pregnancy diagnosis by ultrasonography was also performed at the 4th month, confirming that the only pregnant female remains in this state.

In conclusion, the presence of 10% SP at post-thawing improves sperm cell motility, plasma membrane functionality, and cell morphology, indicating that this condition would be beneficial to improve the physiology of the frozen-thawed alpaca spermatozoa. More *in vitro* and *in vivo* trials must be developed to increase knowledge of these findings.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study Plastic surgery of the vas deferens in South American camelids was previously reviewed and approved by the Institutional Animal Care and Use Ethics Committee of the Faculty of Veterinary Medicine and Zootechnics, Universidad Nacional del Altiplano (Perú), being applied to the present work.

AUTHOR CONTRIBUTIONS

EA: coordination, laboratory work and development of *in vitro* assays. WH: coordination and development of field trial. MP: development of surgically diverted vas deferens sperm recovery and development of *in vitro* assays. ET: surgically diverted vas deferens sperm recovery and laboratory work. JV: laboratory work and development of *in vitro* assays. MO: development of polyacrylamide gel electrophoresis (SDS-PAGE). VM: sperm morphology assessment. UP: laboratory work. TH: chief coordinator. All authors contributed to the article and approved the submitted version.

ACKNOWLEDGMENTS

The authors are grateful to the three Universities and the ILLPA-INIA for the contributions of material support and human resources that made it possible to meet the proposed objectives. Through this work, the authors wish to highlight the trust, deep and continuous work, support and openness to develop new lines of research in South American camelids that characterized Teodosio Huanca Mamani, recently deceased.

REFERENCES

1. Giuliano S, Carretero M, Gambarotta M, Neild D, Trasorras V, Pinto M, et al. Improvement of llama (*Lama glama*) seminal characteristics using collagenase. *Anim Reprod Sci*. (2010) 118:98–102. doi: 10.1016/j.anireprosci.2009.06.005
2. Casaretto C, Martínez Sarrasague M, Giuliano S, Rubin de Celis E, Gambarotta M, Carretero MI, et al. Evaluation of Lama glama semen viscosity with a cone-plate rotational viscometer. *Andrologia*. (2012) 44:335–41. doi: 10.1111/j.1439-0272.2011.01186.x
3. Kershaw-Young CM, Druart X, Vaughan J, Maxwell WM. β-Nerve growth factor is a major component of alpaca seminal plasma and induces ovulation in female alpacas. *Reprod Fertil Dev*. (2012) 24:1093–7. doi: 10.1071/RD12039
4. Huanca W, Cordero A, Huanca T, Adams GP. Biotecnologias reproductivas en Camélidos Sudamericanos Domésticos: avances y perspectivas. *Arch Latinoam Prod Anim*. (2007) 15:195–201. Available online at: http://www.bioline.org.br/pdf?la07052
5. Kershaw-Young CM, Maxwell WMC. Seminal plasma components in camelids and comparisons with other species. *Reprod Domest Anim*. (2012) 47:369–75. doi: 10.1111/j.1439-0531.2012.02100.x
6. Fumuso FG, Giuliano SM, Chaves G, Neild DM, Miragaya MH, Bertuzzi ML, et al. Incubation of frozen-thawed llama sperm with seminal plasma. *Andrologia*. (2020) 52:1–9. doi: 10.1111/and.13597
7. Huanca W, Adams GP. Semen collection and artificial insemination in llamas and alpacas. In: Youngquist R, Threlfall W, editors. *Current Therapy in Large Animal*. St. Louis, MO: Theriogenology. 2nd. ed Elsevier (2007). p. 869–73.
8. Mosaferi S, Niasari-Naslaji A, Abarghani A, Gharahdaghi AA, Gerami A. Biophysical and biochemical characteristics of bactrian camel semen collected by artificial vagina. *Theriogenology*. (2005) 63:92–101. doi: 10.1016/j.theriogenology.2004.03.021
9. Bérgamo NS, Medina VH, Martínez CY, Aisen EG. Reduction of thread formation in llama semen and its effects on sperm quality. In: *4th International Society of Camelid Research and Development-ISOCARD Conference, Almaty, Kazakhstan* (2015).
10. Reyna I, Huanca W, Ampuero A, Huanca T. Efecto de Cuatro Diluciones de Plasma Seminal sobre la Tasa de Ovulación, Tamaño de Cuerpo Lúteo y Perfil de Progesterona en Alpacas. *Rev Investig Vet Perú*. (2015) 26:614–20. doi: 10.15381/rivep.v26i4.11218
11. Pérez MG, Apaza E, Deza H. Congelación de los espermatozoides procedentes de los conductos deferentes de camélidos. *Allpaqa Rev Investig IIPC*. (2006) 11:17–23.
12. Morton KM, Evans G, Maxwell WMC. Effect of glycerol concentration, Equex STM supplementation and liquid storage prior to freezing on the motility and acrosome integrity of frozen-thawed epididymal alpaca (*Vicugna pacos*) sperm. *Theriogenology*. (2010) 74:311–6. doi: 10.1016/j.theriogenology.2010.02.015
13. Pérez MG, Quintano J, Pérez UH. Sperm survival in cooling a 5 °C recovered the vas deferens of alpaca in three extenders with two membrane protector. *Spermova*. (2014) 4:153–8.
14. Druart X, Rickard JP, Mactier S, Kohnke PL, Kershaw-Young CM, Bathgate R, et al. Proteomic characterization and cross species comparison of mammalian seminal plasma. *J Proteom*. (2013) 95:13–22. doi: 10.1016/j.jprot.2013.05.029
15. Cardozo JA, Fernández-Juan M, Cebrián-Pérez JA, Muiño-Blanco T. Identification of RSVP14 and RSVP20 components by two-dimensional electrophoresis and Western-blotting. *Reprod Dom Anim*. (2008) 43:15–21. doi: 10.1111/j.1439-0531.2006.00845.x
16. Apichela SA, Valz-Gianinet JN, Schuster S, Jiménez-Díaz MA, Roldán-Olarte EM, Miceli DC. Lectin binding patterns and carbohydrate mediation of sperm binding to llama oviductal cells *in vitro*. *Anim Reprod Sci*. (2010) 118:344–53. doi: 10.1016/j.anireprosci.2009.07.008
17. Zampini R, Sequeria S, Argañaraz M, Apichela SA. SL15: a seminal plasma-derived lectin from the sperm of llama (Lama glama). *Mol Reprod Dev*. (2017) 9999:1–9. doi: 10.1002/mrd.22816
18. San Martín M, Copaira M, Zuñoga J, Rodríguez R, Bustinza G, Acosta L. Aspects of reproduction in the alpaca. *J Reprod Fertil*. (1968) 16:395–9. doi: 10.1530/jrf.0.0160395
19. Sari LM, Zampini R, Argañaraz ME, Carretero MI, Fumuso FG, Barraza DE, et al. Expression of β-NGF and high affinity NGF receptor (TrKA) in llama (Lama glama) male reproductive tract and spermatozoa. *Mol Reprod Dev*. (2018) 85:934–44. doi: 10.1002/mrd.23075
20. Maxwell WMC, de Graaf SP, El-Hajj Ghaoui R, Evans G. Seminal plasma effects on sperm handling and female fertility. *Soc Reprod Fertil Suppl*. (2007) 64:13–38. doi: 10.5661/RDR-VI-13
21. Maxwell WM, Evans G, Mortimer ST, Gillan L, Gellatly ES, McPhie CA. Normal fertility in ewes after cervical insemination with frozen-thawed

spermatozoa supplemented with seminal plasma. *Reprod Fertil Dev*. (1999) 11:123–6. doi: 10.1071/RD99046
22. Okazaki T, Abe S, Yoshida S, Shimada M. Seminal plasma damages sperm during cryopreservation, but its presence during thawing improves semen quality and conception rates in boars with poor post-thaw semen quality. *Theriogenology*. (2009) 71:491–8. doi: 10.1016/j.theriogenology.2008.08.014
23. Alghamdi AS, Madill S, Foster DN. Seminal plasma improves fertility of frozen equine semen. *Anim Reprod Sci*. (2005) 89:242–5. doi: 10.1016/S0378-4320(05)00254-X
24. Kershaw-Young CM, Maxwell WMC. The effect of seminal plasma on alpaca sperm function. *Theriogenology*. (2011) 76:1197–206. doi: 10.1016/j.theriogenology.2011.05.016
25. Juyena NS, Stelletta C. Seminal plasma: an essential attribute to spermatozoa. *J Androl*. (2012) 33:536–51. doi: 10.2164/jandrol.110.012583
26. Bravo PW, Alarcón V, Baca L, Cuba Y, Ordoñez C, Salinas J, et al. Semen preservation and artificial insemination in domesticated South American camelids. *Anim Reprod Sci*. (2013) 136:157–63. doi: 10.1016/j.anireprosci.2012.10.005
27. Stuart CC, Vaughan JL, Kershaw CM, de Graaf SP, Bathgate R. Effect of diluent type, cryoprotectant concentration, storage method and freeze/thaw rates on the post-thaw quality and fertility of cryopreserved alpaca spermatozoa. *Sci Rep*. (2019) 9:12826. doi: 10.1038/s41598-019-49203-z
28. Aisen E, Turín Vilca J, Huanca Mamani T, Madrid Bury N, Villanueva JC, Medina V, et al. Efecto del ambiente sobre la morfometría de los espermatozoides de alpaca. In: *VII World Congress on South American Camelids. Puno, Perú* (2015).
29. Pelufo V, López Armengol MF, Malcotti V, Venturino A, Aisen EG. Effects of glycerol and sugar mixing temperature on the morphologic and functional integrity of cryopreserved ram sperm. *Theriogenology*. (2015) 83:144–51. doi: 10.1016/j.theriogenology.2014.09.007
30. Terreros M, Huanca W, Arriaga I, Ampuero A. Efecto de Tres Crioprotectores en la Criopreservación de Espermatozoides Epididimarios de Alpaca. *Rev Investig Vet Perú*. (2015) 26:420–6. doi: 10.15381/rivep.v26i3.11182
31. Raymundo F, Huanca W, Huanca T, Huerta S, Cordero A. Efecto de tres dilutores en la conservación del semen de alpacas. *Rev Investig Vet Perú*. (2006) 17:125–30. Available online at: http://www.scielo.org.pe/scielo.php?script=sci_arttext&pid=S1609-91172006000200007&lng=es&tlng=es
32. Adams G, Ratto M, Huanca W, Singh J. Ovulation-inducing factor in the seminal plasma of alpacas and llamas. *Biol. Reprod*. (2005) 73:452–7. doi: 10.1095/biolreprod.105.040097
33. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*. (1970) 227:680–5. doi: 10.1038/227680a0
34. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*. (1976) 72:248–54. doi: 10.1016/0003-2697(76)90527-3
35. Medina VH, Aisen EG, Turín Vilca J, Bérgamo N, Cuello S, Paredes E, et al. *Mejoramiento de los parámetros de eyaculados de Lama glama, mediante la utilización de un filtrado en el procesamiento del semen*. In: *VII World Congress on South American Camelids. Puno, Perú* (2015).
36. Turín Vilca J, Villanueva JC, Huanca López W, Medina VH, Huanca Mamani T, Aisen E. Effect of seminal plasma added before or after freezing-thawing on alpaca semen cryopreservation. In: *18th International Congress on Animal Reproduction, Tours (France). Abstracts*. (2016). 299 p.
37. Giuliano S, Director A, Gambarotta M, Trasorras V, Miragaya M. Collection method, season and individual variation on seminal characteristics in the llama (Lama glama). *Anim Reprod Sci*. (2008) 104:359–69. doi: 10.1016/j.anireprosci.2007.02.016
38. Blom E. A one-minute live-dead sperm stain by means of Eosin-Nigrosin. *Fertil Steril*. (1950) 1:176–7. doi: 10.1016/S0015-0282(16)30125-X
39. Barth AD, Oko RJ. *Abnormal Morphology of Bovine Spermatozoa*. 1st ed. Ames, IA: Iowa State University Press. (1989).
40. Medina V, Bérgamo N, Martínez C, Aisen E. Caracterización de morfoanomalías de espermatozoides de llama (Lama glama). In: *35° Congreso Argentino de Producción Animal, Córdoba, Argentina*. (2012). 100 p.
41. Cárdenas MO, Ratto M, Cordero A, Huanca W. Determinación de la fertilidad en llamas con un servicio, mediante conducta sexual y ecografía. *Rev Inv Vet Peru*. (2001) (Supl. 1) 12:467–9.
42. Gómez-Quispe OE, Pérez MG, Ojeda-Gutiérrez YG. Survival to freezing of sperm collected by vas deferens from *Vicugna pacos* with tempol antioxidant addition. *Asian J Anim Vet Adv*. (2016) 11:377–82. doi: 10.3923/ajava.2016.377.382
43. Ledesma A, Fernández-Alegre E, Cano A, Hozbor F, Martínez-Pastor F. Seminal plasma proteins interacting with sperm surface revert capacitation indicators in frozen-thawed ram sperm. *Anim Reprod Sci*. (2016) 173:35–41. doi: 10.1016/j.anireprosci.2016.08.007
44. Zea F, Pérez G, Maquera Z, Pacheco J. Restauración del plasma seminal en espermatozoides colectados del conducto deferente de alpaca (Vicugna pacos). In: *VII Congreso Mundial en Camélidos Sudamericanos Puno, Perú Abstracts*. (2015). 636 p.
45. Bernardini A, Hozbor F, Sánchez E, Fornes M, Alberio R, Cesari A. Conserved ram seminal plasma proteins bind to the sperm membrane and repair cryopreservation damage. *Theriogenology*. (2011) 76:436–47. doi: 10.1016/j.theriogenology.2011.02.020
46. Juyena NS, Vencato J, Pasini G, Vazzana I, Stelletta C. Alpaca semen quality in relation to different diets. *Reprod Fertil Dev*. (2012) 25:683–90. doi: 10.1071/RD12050
47. Centurión F, Vázquez JM, Calvete JJ, Roca J, Sanz L, Perilla I, et al. Influence of porcine spermadhesins on the susceptibility of boar spermatozoa to high dilution. *Biol Reprod*. (2003) 69:640–6. doi: 10.1095/biolreprod.103.016527
48. Druart X, de Graaf S. Seminal plasma proteomes and sperm fertility. *Anim Reprod Sci*. (2018) 194:33–40. doi: 10.1016/j.anireprosci.2018.04.061
49. Mazur P. A biologist's view of the relevance of thermodynamics and physical chemistry to cryobiology. *Cryobiology*. (2010) 60:4–10. doi: 10.1016/j.cryobiol.2009.12.001
50. Holt WV, North RD. Cryopreservation, actin localization and thermotropic phase transitions in ram spermatozoa. *J Reprod Fertil*. (1991) 91:451–61. doi: 10.1530/jrf.0.0910451
51. Aisen E, Quintana M, Medina V, Morello H, Venturino A. Ultramicroscopic and biochemical changes in ram spermatozoa cryopreserved with trehalose-based hypertonic extenders. *Cryobiology*. (2005) 50:239–49. doi: 10.1016/j.cryobiol.2005.02.002
52. Apichela SA, Argañaraz ME, Giuliano SM, Zampini R, Carretero MI, Miragaya M, et al. Llama oviductal reservoirs: involvement of bulbourethral glands. *Andrologia*. (2014) 46:290–5. doi: 10.1111/and.12080
53. Ascencio SJ, Huanca LW, Turín VJ, Mamani MC, Cordero RA, Hilari O, et al. Efecto del estadio de desarrollo de la onda folicular sobre la respuesta ovárica y tasa de recuperación y calidad de embriones en alpacas. *Rev Investig Vet Perú*. (2019) 30:745–52. doi: 10.15381/rivep.v30i2.16066

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 17 March 2021
doi: 10.3389/fvets.2021.597964

Rescue and Conservation of Male Adult Alpacas (*Vicugna pacos*) Based on Spermatogonial Stem Cell Biotechnology Using Atomized Black Maca as a Supplement of Cryopreservation Medium

Martha Valdivia[1*†], *Zezé Bravo*[1†], *Jhakelin Reyes*[1†] *and Gustavo F. Gonzales*[2†]

[1] *Laboratory of Reproductive Physiology, Research Institute "Antonio Raimondi," Zoology Department, Biological Sciences Faculty, Universidad Nacional Mayor de San Marcos, Lima, Peru,* [2] *Endocrine and Reproductive Laboratory, Department of Biological and Physiological Science, and Laboratory of Investigation and Development (LID), Faculty of Sciences and Philosophy, Universidad Peruana Cayetano Heredia, Lima, Peru*

OPEN ACCESS

Edited by:
Marcelo H. Ratto,
Austral University of Chile, Chile

Reviewed by:
Juan G. Maldonado-Estrada,
University of Antioquia, Colombia
Javiera Bahamonde,
Austral University of Chile, Chile

***Correspondence:**
Martha Valdivia
mvaldiviac@unmsm.edu.pe

†ORCID:
Martha Valdivia
orcid.org/0000-0002-8301-5536
Zezé Bravo
orcid.org/0000-0002-8539-9262
Jhakelin Reyes
orcid.org/0000-0001-9562-3099
Gustavo F. Gonzales
orcid.org/0000-0003-1611-2894

Specialty section:
This article was submitted to Animal Reproduction - Theriogenology, a section of the journal Frontiers in Veterinary Science

Received: 23 August 2020
Accepted: 18 January 2021
Published: 17 March 2021

Citation:
Valdivia M, Bravo Z, Reyes J and Gonzales GF (2021) Rescue and Conservation of Male Adult Alpacas (Vicugna pacos) Based on Spermatogonial Stem Cell Biotechnology Using Atomized Black Maca as a Supplement of Cryopreservation Medium. Front. Vet. Sci. 8:597964. doi: 10.3389/fvets.2021.597964

This is the first time that testicular tissue ($n = 44$) and isolated testicular cells ($n = 51$) were cryopreserved from alpaca testes 24 h postmortem. For this purpose, internally designed freezing media and cryopreservation protocols were used. Testicular tissue fragments (25 mg) and isolated testicular cells were frozen in MTDB (trehalose and black maca), MTD (trehalose), MSDB (sucrose and black maca), and MSD (sucrose) media. Isolated spermatogonial cells were cryopreserved in two ways, before and after proliferation *in vitro*. After cryopreservation, the percentage of cell viability in Group 1 (>50% of cell viability) by trypan blue did not show differences within each group ($p > 0.05$) but showed significant differences when comparing fragments with isolated cells ($p < 0.05$). Spermatogonial stem cells (SSC) were identified by flow cytometry as strong *Dolichos biflorus* agglutinin (sDBA) and mitochondrial activity of SSC as strongly positive for MitoSense (sMitoSense+) in intact mitochondria cells, weakly positive for MitoSense (wMitoSense+) in early apoptosis, and necrosis with 7-Aminoactinomycin-D positive (7-AAD). After freezing, in Group 1M (≥30% sMitoSense+), the fragments did not show differences between the media ($p > 0.05$), but in the isolated cells frozen in MSDB medium, 63.68 ± 8.90% ($p < 0.05$). In Group 2M (<30% sMitoSense+), necrosis (7AAD+) in MSDB medium was 27.03 ± 5.80%, and necrosis in isolated cells was 14.05 ± 9.3% with significant differences between these groups ($p < 0.05$); in sMitoSense+, the isolated cells (34.40 ± 23%) had a higher percentage than the fragments (12.4 ± 5.2) ($p < 0.05$). On the other hand, MSDB and MSD media were significantly higher for isolated cells than for fragments in sDBA+ ($p < 0.05$). On the other hand, the SSC (sDBA+) had significant differences ($p < 0.05$) between fresh cells 7.43 ± 1.3% (sDBA+) compared with those cryopreserved in MSDB medium 1.46 ± 0.34% (sDBA+). Additionally, the proliferated and cryopreserved SSC 6.29 ± 1.17% (sDBA+) did not show significant differences concerning the fresh cells ($p > 0.05$). In conclusion, the black maca showed

antioxidant properties when it was included in the freezing medium and, therefore, improved the SSC's conservation of the alpaca. Furthermore, the proliferation of isolated cells *in vitro* produces a higher amount of SSC after thawing them for further preclinical or clinical work.

Keywords: spermatogonia stem cell, alpaca, testicular biopsies, isolated spermatogonial cells, cryopreservation

INTRODUCTION

Spermatogonial stem cells (SSC) are present in minimal amounts in the seminiferous tubules of adult animals; in particular, it is estimated that only 0.03% of cells correspond to SSC (1, 2). SSC have the ability to self-renew, which guarantees balanced male fertility (3). This property makes them attractive as an advanced biotechnological tool for conserving genetic material from wildlife and elite animals, since the establishment of SSC banks (4).

In addition, testicular tissue preserved *ex vivo* through the freezing of testicular biopsies or isolated SSC from adult individuals could serve as a reservoir for the rescue and conservation of male fertility (4). In fact, the preservation of SSC allows for the rescue of important genetic material. Therefore, these cells can help to preserve male fertility in individuals from childhood to adulthood and in animals with good reproductive characteristics. Indeed, frozen and thawed testis tissues or isolated cells can be transplanted to the same individual from which the progenitor tissues were derived or to an individual of a lesser race, making the recipient individual produce male animal gametes from these sources (5).

Moreover, SSC cryopreservation would allow the study, rescue, and conservation of SSC of animals with high genetic value (6), including animals with a high economic impact in Peru, such as alpacas, and can thus be used for reproductive genetic management of Peruvian alpacas producing good fiber. In animal models, it has been described that frozen testis tissue can undergo differentiation after cryopreservation, thereby producing primary spermatocytes that eventually differentiate into round spermatocytes and ensuring the production of spermatozoa (1, 7, 8). Therefore, cryopreservation of testicular tissue shows excellent potential in assisting male fertility (9, 10) because spermatozoa and SSC can be rescued from testicular biopsies after thawing and be used for assisted reproduction techniques of high complexity, such as intracytoplasmic insemination (ICSI), with low abortion rates (11, 12).

Currently, several research groups are committed in developing biotechnologies in the fields of both isolation, cryopreservation, and transplantation of SSC, highlighting the possible applications of SSC (13). For example, extensive work has been carried out on humans (11), mice (14, 15), cattle (16), pigs (17), and alpacas (18), where it was possible to identify SSC as well as early differentiating SSC using molecular markers and *Dolichos biflorus* agglutinin (DBA) (19).

Cryopreservation of isolated SSC or SSC in the form of testicular biopsies has the potential, in the long term, to support highly efficient methods of reproductive biotechnology for conserving male genetic material and could lay the foundation for the creation of SSC banks for the Peruvian alpaca, generating potentially useful new alternatives to conserve male fertility in alpaca elites. Alpaca is one of the leading livestock species of the high Andean region in Peru, with great economic impact. Peru hosts more than 87% of the worldwide alpaca population. Distributed in a more significant proportion in Puno, Cuzco, and Huancavelica, 85% of the alpaca population corresponds to the Huacaya breed (with 95% white and 5% colored) and 15% to the Suri breed (7), whose productivity is currently declining. Peruvian alpacas have high genetic variability (20, 21); however, fiber productivity decreases annually by 2.3%.

Various research groups have started to study and characterize this species from the reproductive perspective, with genetic improvement as one of the main objectives (22, 23). Advances have been made in the cryopreservation of semen and epididymal sperm, artificial insemination, and embryo culture as well as in the evaluation of genetic diversity, in order to establish strategies for the selection of reproducers and the application of appropriate technologies for this species (24). However, alpaca reproduction still shows considerable difficulties, such as a long gestation time, difficult handling, and establishing artificial insemination programs. Indeed, this method is limited by semen collection or by low post-thawing motility of epididymal sperm (25). Unfortunately, adequate protocols for semen preservation, one of the most widely used techniques in reproductive biotechnology in other domestic species, have not yet been established for alpacas; therefore, it is necessary to study germ cells from different perspectives.

In adult alpacas, the presence of SSC was identified using molecular markers (Zbtb16, Integrin β1) and by flow cytometry (FC) after enzymatic isolation from testicles of adult alpacas and staining with DBA, as SSC are strongly DBA-positive (sDBA+) cells (18) that can proliferate and differentiate *in vitro* (26). Because of this property, freezing of gonadal tissue or isolated spermatogonial cells can facilitate the conservation of SSC as well as testicular sperm, which could be used in several reproductive procedures (6, 27, 28).

Unfortunately, it was never possible to freeze testicular biopsies or testicular fragments of alpaca with traditional cryopreservation media; however, in some species, such as mice, pigs, cattle, and humans, some freezing media and techniques have been optimized. For instance, vitrification-based freezing of human testicular biopsies has been described, with promising results for the cryopreservation of testicular tissue (9). Moreover, it has been observed that freezing testicular tissue of mice with dimethylsulfoxide (DMSO) and propanediol (PROH) preserves

the ability of testicular sperm to produce embryos *via* ICSI, which, when transferred to pseudopregnant females, generate new individuals (10).

Therefore, supplementation of the medium with different additives is a strategy to improve the efficiency of cryopreservation. For instance, the use of sugars, such as trehalose and sucrose, as non-permeable cryoprotectants is recommended to increase the recovery, viability, proliferative capacity, and colonization efficiency of undifferentiated spermatogonia after thawing in species, such as mice (15), pigs (17, 29), and cattle (16). In addition, cryopreservation media have also been supplemented with additives, such as collagen, laminin, and antioxidants. The latter can increase the survival of cells by scavenging the reactive oxygen species generated during the cryopreservation process, which can damage cellular structures, such as membranes or proteins, thereby the sensitivity to freeze/thaw processes, or DNA integrity, thus reducing the viability and proliferative capacity of SSC (13).

Indeed, there is an obvious need to preserve male fertility in individuals from childhood to adulthood (for example in oncology patients) and animals with some good reproductive characteristics that become evident during the adult stage. Therefore, conserving testicular tissue *ex vivo* through the freezing of testicular biopsies or isolated SSC of adult individuals could serve as a reservoir for the rescue and conservation of male fertility.

Nevertheless, alpaca sperm cryopreservation has not been used extensively because of its limiting step of semen collection. In addition, alpaca epididymal sperm presents low sperm motility after thawing, so it is impossible to use it in artificial insemination programs. On the other hand, freezing of gonadal tissue or isolated spermatogonial cells would facilitate the conservation of SSC and spermatozoa and contribute to the preservation fertility of alpacas.

Therefore, SSC are attractive as an advanced biotechnological tool for the conservation of male genetic material of wildlife and elite animals. SSC freezing/thawing would allow the study, rescue, and conservation of SSC of animals with high genetic value, including animals with a high economic impact in Peru, such as alpacas, and thus can be used for reproductive genetic management in Peruvian alpacas producing good fiber. Furthermore, in animal models, it has been described that differentiation of SSC ensures spermatozoa production (1, 7, 8). In adult alpacas, the presence of SSC has been identified using molecular markers (Zbtb16, Integrin β1) and by FC after enzymatic isolation from testicles of adult alpacas and staining with DBA, as SSC are sDBA+ cells [mean ± standard deviation (SD) of sDBA+] corresponding to SSC = 4.43 ± 0.68% (18), which can proliferate and differentiate *in vitro* (26, 30). Cryopreservation of testicular tissue shows promising applications in male fertility (9, 10). Indeed, testicular biopsies, spermatozoa, and SSC can be recovered after thawing and used in assisted reproduction techniques of high complexity, such as ICSI, with low abortion rates (12).

It has been observed that administering maca *in vivo* to mice with physically induced subfertility during 35-days treatments reduces sperm DNA fragmentation, from 11.1 ± 19.29 to 2.29 ± 2.30% (mean ± SD) while increasing sperm concentration and mobility (31). In addition, different biological responses to various ecotypes of maca have been observed (32). For example, maca can exert anti-stress energizing effects and scavenge free radicals and provide cytoprotection under oxidative stress conditions (33–35). Unfortunately, it was never possible to freeze testicular biopsies or testicular fragments of alpaca with traditional cryopreservation media.

After thawing human testes tissue, the testicular sperm can be used for ICSI with low abortion rates (12). Advances in tissue cryopreservation, as well as in germ cell transplantation and testicular biopsy technology, open new horizons for the preservation of male fertility (36, 37), offering alternatives to cancer patients affected in their fertility since childhood (38). Indeed, cryopreservation of testis biopsies can help children who are subjected to aggressive treatments preserve future fertility after transplant (39, 40).

Maca (*Lepidium meyenii* Walp), a plant of the Brassicaceae family from the high Andean region of Peru, grows at 4,000 m.a.s.l., whose traditional use has been for 2,000 years as food and also has a medicinal role (41). Its consumption has been shown to improve fatigue (42), spermatogenesis (32, 41), and erectile dysfunction in animals (43) and in humans (44). Different biological responses of maca ecotypes (32) provide energizing anti-stress and antioxidant effects (33, 34, 45), natural cytoprotection (46), and cytoprotection in oxidative stress conditions (35). Through nuclear magnetic resonance (NMR) and biochemical analysis, Zhao et al. identified that in maca, there are proteins, lipids, carbohydrates, and unsaturated fatty acids and minerals (47). Maca shows properties as a natural potential cryoprotection agent (CPA) to alpaca SSC. On the other hand, it has been observed that maca reduces sperm DNA fragmentation in animals with chemically induced subfertility (31); moreover, long-term treatment with black maca has been reported to enhance daily sperm production and increase sperm motility. These findings indicate differences in the biological response to various maca ecotypes (32), such as anti-stress energizing effects, free radical scavenging, and cytoprotection under oxidative stress conditions (33–35).

For this purpose, we developed cryopreservation media with permeable and non-permeable cryoprotective agents and the addition of a natural supplement, black maca, whose components exert antioxidant effects in mice (31). It was never possible to freeze testicular biopsies or testicular fragments of alpaca with traditional cryopreservation media; however, in some species, such as mice, pigs, cattle, and humans, freezing of testicular material has been successfully performed.

We propose that our freezing media would help tissue and cell cryogenics for promising applications in prepubertal patients and even adult men, as well as for elite animals of high value and genetic lineage, and economic impact, such as alpacas, valued for their quality of fiber and meat, and the conservation of wildlife animals.

Here, we aimed to evaluate the cytoprotective effect of black maca during cryopreservation in both testicular biopsies and isolated SSC before and after *in vitro* proliferation by

analyzing mitochondrial activity, early apoptosis, and post-thawing necrosis.

MATERIALS AND METHODS

Animals

Fifty-one adult male alpacas (4–6 years old), coming from family breeding facilities of the populated centers of the Lacchoc, Cachimayo, Huaracco, and Carhuancho areas (Huancavelica, Peru), were slaughtered in the Municipal Camal of Chuñuranra town, at 3,680 m.a.s.l. The environmental temperature of the area fluctuates between 6 and 8°C, with an average annual rainfall between 400 and 700 mm and an altitude between 3,000 and 4,600 m.a.s.l. Testicles with epididymis were recovered immediately after slaughter in 0.9% NaCl and transported in isothermal boxes provided with gel ice to maintain the cold chain during transport (24 h) to the Laboratory of Reproductive Physiology, Faculty of Biological Sciences, Universidad Nacional Mayor de San Marcos (UNMSM), Lima, Peru.

Samples

Testicles and epididymis were washed in phosphate-buffered saline with 0.1 mg/ml penicillin, 0.1 mg/ml streptomycin, and 0.5 mg/ml gentamicin soon after arrival at the laboratory (UNMSM) in Lima, Peru. The tunica albuginea tissue was removed in sterile conditions. Testes that weighed ≥8 g and exhibited progressive epididymal sperm motility of ≥30% were selected (26).

Epididymal sperm parameters were evaluated according to Organization WH (48). Sperm motility was evaluated and classified as follows: P, progressive motility; NP, non-progressive motility; and I, immotile. Several microscopic fields were evaluated using a Scientific i4 Series light microscope (LW Scientific, Lawrenceville, GA, USA) and a video recorder with an OmniVID camera, and the MicroCap v3.0 software (LW) values were expressed as percentages, based on the observation of 100 spermatozoa in each sample. Samples were classified as good (≥30% of progressive motility of epididymal spermatozoa), regular (20–29% of progressive motility of epididymal spermatozoa), and poor (from 0 to 19% of progressive motility of epididymal spermatozoa), as described by Valdivia et al. (18). Only samples exhibiting good quality were used for the thawing protocol of fragments and isolated spermatogonial cells. Sperm concentration was determined using a hemocytometer and expressed in millions of spermatozoa/ml.

Forty-four samples were frozen as fragments and 32 samples as isolated spermatogonial cells. Eight samples frozen as fragments and isolated spermatogonial cells were analyzed by FC. Fifteen additional samples were used for the *in vitro* proliferation experiment.

Freezing media for fragments and isolated spermatogonial cells were supplemented with atomized black maca (Juvens®; Cayenatur, Lima, Peru) as described by the Research Circle of Plants with Effect on Health (grant no. 010-2014-FONDECYT). Botanical samples were deposited in the HEPLAME MG-2015 (Herbarium of Medicinal Plants, Section of Pharmaceutical Sciences, Faculty of Sciences and Philosophy, Universidad Peruana Cayetano Heredia). Maca components have been previously characterized by NMR (49).

Recovery of Testicular Tissue Fragments and Cryopreservation (Protocol LFR-UNMSM-2)

Longitudinal cuts of testicles were made, and three 25-mg fragments of tissue (8 × 2 × 2 mm) were obtained from each sample and immediately transferred to 80 µl of four media at room temperature: MTDB (HAM-F10 modified, 0.2 M of trehalose, 10% DMSO, and 20 mg/ml atomized black maca Juvens®), MTD (HAM-F10 modified, 0.2 M of trehalose, and 10% DMSO), MSDB (HAM-F10 modified, 0.2 M of sucrose, 10% DMSO, and 20 mg/ml atomized black maca Juvens®), or MSD (HAM-F10 modified, 0.2 M of sucrose, and 10% DMSO).

Samples ($n = 44$) were placed for stabilization for 1 h in a water bath at 4°C. Subsequently, cryopreservation was performed in the Freeze Control CL 3300 heat-controlled system (CryoLogic, Victoria, Australia) using the Cryogenesis IV software. Our in-house designed freezing protocol of testicular fragments is called protocol LFR-UNMSM-2 (30). Freezing begins at 4°C and ends at −35°C. After the process, the samples were suspended in nitrogen vapors (−80°C) for 12 h. Then, the samples were immersed in liquid nitrogen at −196°C. Samples were slowly thawed from −196°C, placing them in a 12 × 8 cm polystyrene box uncovered in room temperature at 19°C for 5 min then to 37°C until it thaws completely (5 min more approximately).

Isolation of SSC

Testicular tissue (0.8 g), except rete testis, was cut into small pieces in Minimum Essential Medium (MEM) supplemented with 100 µg/ml penicillin and 100 µg/ml streptomycin; then, fragments were filtered through a metal mesh in order to disaggregate the tissue into spermatogonial cell suspensions. Subsequently, the samples were processed according to Izadyar et al. (50), by two steps of enzymatic digestion (ED): ED1 and ED2. ED1 was performed in MEM supplemented with 1 mg/ml collagenase, 26.3 µl/ml DNase, and 0.5 mg/ml hyaluronidase for 30 min at 32°C in continuous motion, and then the suspension was washed with MEM by centrifugation and resuspension. The cells were then suspended in ED2 medium, which was supplemented with 1 mg/ml collagenase and 0.5 mg/ml hyaluronidase according to the previously described methodology (50). The cell suspension was centrifuged at 1,800 rpm (300 × *g*) for 5 min, the supernatant was discarded, and the cell pellet was washed three times in MEM supplemented with penicillin and streptomycin. Finally, cell viability was analyzed *via* trypan blue exclusion assay. In this assay, non-stained cells were considered alive, whereas cells staining blue were dead. Samples ($n = 32$) showing ≥80% of cell viability by trypan blue assay were used for cryopreservation and *in vitro* culture experiments.

Cryopreservation of Isolated Spermatogonial Cells (Protocol LFR-UNMSM-3)

Similar to testicular fragments, 2×10^6/ml isolated spermatogonial cells ($n = 32$) were mixed with MTDB, MTD, MSDB, or MSD medium at 1:1 ratio in a total volume of 80 µl. Next, 40 µl of the cell suspension was added to 40 µl of four cryopreservation media: MTDB (HAM-F10 modified, 0.2 M of trehalose, 10% DMSO, and 20 mg/ml atomized black maca Juvens®), MTD (HAM-F10 modified, 0.2 M of trehalose, and 10% DMSO), MSDB (HAM-F10 modified, 0.2 M of sucrose, 10% DMSO, and 20 mg/ml atomized black maca Juvens®), or MSD (HAM-F10 modified, 0.2 M of sucrose, and 10% DMSO) at room temperature. After stabilization (for 1 h at 4°C), the samples were frozen in the heat-controlled system using the Cryogenesis IV software and our in-house designed protocol for cell suspensions (LFR-UNMSM-3) (30). The temperature decrease continued at a rate of 2°C/min until reaching a temperature of −7°C, set to last 11 min. At this temperature, ice sowing was induced by seeding. Subsequently, at a rate of 0.3°C/min, the temperature reached −30°C and was then immediately brought down to −84°C at a rate of 6°C/min. At the end of the freezing process, the samples were withdrawn from the cryochamber, suspended in liquid nitrogen vapors for 12 h, and then immersed in liquid nitrogen at a temperature of −196°C. Samples were thawed from −196 to 18°C for 5 min and incubated at 37°C.

In vitro Culture of Isolated Spermatogonial Cells

Other aliquots of the spermatogonial cell suspensions (1×10^6 cells/ml) were cultured *in vitro* according to Valdivia et al. (26) in polystyrene culture plates (SPL Life Science) with Dulbecco's Modified Eagle Medium (DMEM; Gibco) supplemented with 0.29 mg/ml L-glutamine (Sigma-Aldrich), 10 µl/ml non-essential amino acids (Sigma-Aldrich), 100 µg/ml penicillin (Sigma-Aldrich), 100 µg/ml streptomycin (Sigma-Aldrich), 1 µl/ml insulin–selenite–transferrin sodium (Sigma-Aldrich), 30 mg/ml pyruvic acid (Sigma-Aldrich), 1 µl/ml lactic acid (Sigma-Aldrich), 0.5% bovine serum albumin (Sigma-Aldrich), 1% fetal bovine serum (Sigma-Aldrich), and 1% human milk (donated by an anonymous mother). Cells were cultured at 37°C and 5% CO_2 for 15 days. After that, the cells were cryopreserved and thawed following the protocol LFR-UNMSM-03 as described above.

Quantification of SSC and Cells Displaying Normal Mitochondrial Membrane Potential After Cryopreservation by FC

Similarly, the amount of SSC was evaluated in 1×10^6 cells/ml per sample, from fragments ($n = 8$) and isolated cells ($n = 8$) post-thawing, after labeling with 100 µg/ml of the fluorescein isothiocyanate-conjugated DBA. Through this method, we identified SSC as sDBA+ cells, early differentiating cells as weakly marked (wDBA+) cells, and differentiated round cells as DBA-negative (DBA–) cells, according to Valdivia et al. (26). The mitochondrial state of the post-thaw cells was determined with the FlowCellect MitoPotential Red Kit (Merck), which is a dual parameter assay kit for FC including MitoSense Red, a fluorescent cationic dye, and the DNA intercalator 7-Aminoact (7AAD). We classified cells as follows: (1) strongly MitoSense-positive (sMitoSense+) cells with intact mitochondrial activity; weakly MitoSense-positive (wMitoSense+) cells in early apoptosis; and (2) MitoSense-negative (MitoSense–) cells, not labeled by MitoSense Red but showing an intense orange fluorescence due to 7AAD. Cell population analysis was performed with the Amnis imaging flow cytometer (Merck). Histograms produced for each fluorochrome permit with bin tool to calculate the amount of the cell population according to the intensity of the fluorochromes selected. A total of 10,000 events were recorded for analysis using the IDEAS software (EMD Millipore, Burlington, MA, USA). Unfrozen spermatogonial cells, proliferated cells, and fresh cells exposed to UV light were used as control for MitoSense, DBA, and 7AAD staining, respectively.

TABLE 1 | Samples characterization by parameter ranges.

	Range
Number of samples (*n*)	55
Testicular weight (g)	8–19.94
Round spermatogonial cell concentration (millions)	20–171.5
Round spermatogonial viability (%)	80–97.4
Sperm concentration/epididyme (millions/mL)	9–1,029
Sperm viability (%)	30–93.9

Statistical Analysis

Differences between media with or without black maca Juvens® were tested with Kruskal–Wallis tests ($p < 0.05$). Likewise, comparative tests were performed to highlight significant differences between frozen sample types (fragments vs. cells) using the Mann–Whitney *U* test. Statistical analysis was performed with the SPSS statistical package. Tests of homogeneity (Levene's test) and normality (Shapiro–Wilk test) were also carried out.

Ethical Aspects

This study was evaluated, registered, and approved by the UPCH Institutional Ethics Committee for the Use of Animals (CODE SIDISI No. 0000066755). There is no special interest; there is no bias or particular interest in the formulation of the media, the natural supplements used, or the protocols.

RESULTS

To develop an effective alpaca SSC freezing protocol, we collected testicles from 51 animals after 24 h from death. We selected only samples scoring above a minimal threshold for the following parameters on testicular weight, round spermatogonial cell concentration and viability, and sperm viability and motility (**Table 1**). These samples were included in the study to optimize the freezing/thawing process of fragments and isolated SSC. Our results were highly variable, grouped according to spermatogonial cell viability with trypan blue

TABLE 2 | Mean Percentage of cell Viability calculated by Trypan blue exclusion assay.

	$\bar{x} \pm$ SE (*n*)			
	MTDB	**MTD**	**MSDB**	**MSD**
Fragments (37)				
Group 1: > 50%	78.51 ± 2.91 (23)[a]	72.96 ± 2.80 (23)[a]	72.32 ± 2.39 (23)[a]	74.42 ± 2.46 (23)[a]
Group 2: 30-50%	40.90 ± 2.13 (09)[a]	38.55 ± 2.33 (09)[a]	41.70 ± 1.55 (09)[a]	44.71 ± 1.28 (09)[a]
Group 3: < 30%	19.20 ± 4.66 (05) [a]	17.03 ± 6.72 (05)[a]	19.14 ±4.45 (05)[a]	19.20 ± 4.66 (05)[a]
Isolated cells (32)				
Group 1: > 50%	63.60 ± 3.35 (09)[b]	61.75 ± 1.57 (12)[b]	60.51 ± 1.87(8)[b]	59.31 ±1.84 (12)[b]
Group 2: 30–50%	39.65 ± 1.52 (11)[a]	36.42 ± 1.67 (11)[a]	40.59± 1.63 (15)[a]	39.6 2± 1.93 (11)[a]
Group 3: < 30%	20.88 ± 2.12 (12)[a]	20.33 ± 2.23(09)[a]	12.79 ±2.68 (09)[a]	19.92 ± 2.08 (09)[a]

[a,b]*Different letters show statistical differences.*

dye: Group 1: >50%, Group 2: 30–50%, and Group 3: <30%. Mitochondrial activity status was identified in spermatogonial cells using MitoSense fluorochrome and grouped according to mitochondrial activity as Group 1M (≥30% sMitoSense+) and Group 2M (<30% sMitoSense).

Evaluation of Cell Viability by Trypan Blue Staining After the Freeze/Thaw Process

After performing the thawing process, the viability of single round cells, derived from fragments by ED as described for isolated cells, and of isolated frozen cells was evaluated after storage at −196°C for 1–166 days and 1–148 days, respectively, by trypan blue exclusion assay. Forty-four fragment and 32 isolated spermatogonial cell samples were used (**Table 2**). The 37 fragment samples were classified into three groups according to the percentage of viability in MTDB, MTD, MSDB, and MSD media: Group 1, >50%; Group 2, 30–50%; and Group 3, <30%. Group 1 included samples with the highest percentage of viability. In particular, cell viability from thawed fragments belonging to Group 1 was 78.51 ± 2.91% in 60% of evaluated samples in MTDB medium ($n = 23$), 72.96 ± 2.80% in 60% of evaluated samples in MTD medium ($n = 23$), 72.32 ± 2.39% in 64% of evaluated samples in MSDB medium ($n = 23$), and 74.42 ± 2.46% in 60% of evaluated samples in MSD medium ($n = 23$, **Table 2**). The mean percentage of viability of fragment-derived cells did not significantly differ ($p > 0.05$) among the four freezing media, not even among Groups 1, 2, and 3.

Similarly, 32 samples of isolated spermatogonial cells were thawed, and cell viability was evaluated by trypan blue staining. Group 1 included samples with the highest percentage of viability. In particular, cell viability from thawed isolated spermatogonial cells was 63.60 ± 3.35% in 28% of evaluated samples in MTDB medium ($n = 9$), 61.75 ± 1.57% in 37.5% of evaluated samples in MTD medium ($n = 12$), 60.5 ± 1.87 in 25% of evaluated samples in MSDB medium ($n = 8$), and 59.31 ± 1.84% in 37.5% of evaluated samples in MSD medium ($n = 12$). There were no significant differences in cell viability between cells frozen in different media nor among Groups 1, 2, and 3 (**Table 2**). However, when compared, the viability in cell derived from fragments vs. isolated cell of Group 1 did significantly differ ($p < 0.05$) in all media (**Table 2**).

Quantification of SSC and Determination of Mitochondrial Membrane Potential After Cryopreservation by FC

The percentage of SSC (sDBA+) and their mitochondrial membrane potential were analyzed in cell extracts from thawed fragments and isolated spermatogonia.

Samples were classified according to the intensity of MitoSense staining into two groups: Group 1M, ≥30% of sMitoSense+ cells (**Table 3**) and Group 2M, <30% of sMitoSense+ cells, for identifying the best freezing medium with good and low samples, respectively (**Table 4**).

Further, cells displaying strong mitochondrial activity were revealed as sMitoSense+ cells, cells undergoing early apoptosis as wMitoSense+ cells, and necrotic cells as 7AAD+ cells (**Figure 1**). In Group 1M, the percentage of SSC (sDBA+ cells) and sMitoSense+ for SSC derived from fragments among MTDB, MTD, MSDB, and MSD freezing media did not significantly differ ($p > 0.05$). On the other hand, we found statistically significant differences ($p < 0.05$) in sDBA+% in MSDB (2.73 ± 2.02%) and MSD (1.09 ± 0.84%) with MTDB (5.95 ± 2.62%) and MTD (5.07 ± 1.87%) freezing media in isolated cryopreserved cell and with cell derived from fragments cryopreserved in MSDB (9.67 ± 2.22%) and MSD (7.41 ± 2.37%) media (**Table 3**).

In particular, the high percentage of sMitoSense+ cells among thawed isolated spermatogonia was 63.68 ± 8.90% (**Figure 2**, **Table 3**) and significantly higher than MTDB, MTD, and MSD media and with cell derived from fragments in MSDB medium ($p < 0.05$). There were no significant differences between apoptosis and necrosis in cells cryopreserved derived from fragments or isolated cells.

On the other hand, among the samples of Group 2M (<30% sMitoSense+), the percentages of sDBA+ with sMitoSense+ or wMitoSense+ derived from fragments did not significantly differ among the cryopreservation media. Conversely, the percentage of necrotic cells (7AAD+) was 27.03 ± 5.80% in sDBA+ derived from frozen fragments in MSDB medium (**Table 4**), significantly higher than in MTDB, MTD, and MSD media ($p < 0.05$).

TABLE 3 | Mean percentage of spermatogonial stem cell with intact mitochondrial activity (sMitoSense+), early apoptosis (wMitoSense) and necrosis (7AAD+) in-group 1M.

Group 1M: ≥30% (sMitoSense+)	**$\bar{x}$ ± S.E(%)**			
TESTICULAR FRAGMENT (n = 8)	**MTDB**	**MTD**	**MSDB**	**MSD**
sDBA+	8.43 ± 3.37[a]	13.52 ± 1.27[a]	9.67 ± 2.22[a]	7.41±2.37[a]
wDBA+	70.3 ± 6.48[a]	68.73 ± 2.32[a]	71.98 ± 1.60[a]	67.58 ± 2.88[a]
DBA-	11.15 ± 5.38[a]	15.40 ± 8.65[a]	15.92 ± 4.15[a]	14.29 ± 9.78[a]
sMitoSense+	57.20 ± 9.57[a]	34.07 ± 12.20[a]	48.53 ± 10.46[a]	41.96 ± 12.92[a]
wMitoSense+	23.7 ± 6.25[a]	29.57 ± 19.07[a]	20.55 ± 5.41[a]	34.18 ± 5.88[a]
7AAD+	11.15 ± 5.38[a]	15.40 ± 8.65[a]	16.00 ± 4.15[a]	14.29 ± 9.78[a]
ISOLATED SPERMATOGONIAL (n = 8)				
sDBA+	5.95 ± 2.62[a]	5.07 ± 1.87[a]	2.73 ± 2.02[b]	1.09 ± 0.84[b]
wDBA+	34.50 ± 7.57[b]	40.92 ± 6.20[a]	39.05 ± 13.56[a]	41.17 ± 6.94[a]
DBA-	53.97 ± 9.95[a]	47.42 ± 3.28[a]	51.45 ± 13.80[a]	52.02 ± 6.07[a]
sMitoSense+	46.90 ± 2.73[a]	43.58 ± 2.95[a]	63.68 ± 8.90[b]	53.00 ± 11.65[a]
wMitoSense+	25.58 ± 4.88[a]	31.65 ± 4.28[a]	32.46 ± 12.05[a]	33.23 ± 5.57[a]
7AAD+	6.71 ± 2.29[a]	8.10 ± 2.41[a]	5.18 ± 2.13[a]	10.03 ± 3.70[a]

[a,b]*Different letters show statistical differences.*

TABLE 4 | Mean percentage of spermatogonial stem cells with intact mitochondrial membranes (sMitoSense+), undergoing, early apoptosis (wMitoSense+) and necrosis (7AAD+) in group 2M.

Group 2M: < 30% (sMitosense+)	**$\bar{x}$ ± EE**			
TESTICULAR FRAGMENT (n = 8)	**MTDB**	**MTD**	**MSDB**	**MSD**
sDBA+	2.96 ± 2.54[a]	2.68 ± 2.68[a]	2.96 ± 2.55[a]	2.68 ± 2.69[a]
wDBA+	54.87 ± 16.43[a]	48.65 ± 22.11[a]	57.6 ± 19.33[a]	65.8 ± 18.15[a]
DBA-	39.69 ± 17.72[a]	47.45 ± 24.20[a]	38.4 ± 19.76[a]	25.80 ± 20.05[a]
sMitoSense+	16.50 ± 11.27[a]	20.92 ± 12.42[a]	12.41 ± 5.19[a]	16.64 ± 8.13[a]
wMitoSense+	57.85 ± 14.28[a]	58.9 ± 12.26[a]	57.23 ± 6.73[a]	50.90 ± 14.77[a]
7AAD+	19.76 ± 7.45[a]	18.52 ± 4.36[a]	27.03 ± 5.80[b]	13.78 ± 0.03[a]
ISOLATED SPERMATOGONIAL (n = 8)				
sDBA+	21.9 ± 11.40[b]	6.8 ± 6.8[a]	3.36 ± 1.52[a]	5.51 ± 1.69[a]
wDBA+	43.18 ± 32.02[a]	29.62 ± 20.49[b]	53.00 ± 10.60[a]	66.75 ± 12.25[a]
DBA-	28.7 ± 17.90[a]	61.1 ± 28.30[b]	39.15 ± 7.15[a]	23.9 ± 9.90[a]
sMitoSense+	2.05 ± 1.51[a]	9.6 ± 5.90[a]	34.40 ± 23[b]	27.7 ± 8.70[b]
wMitoSense+	65.1 ± 16.8[a]	52.2 ± 6.40[a]	39.45 ± 16.95[a]	43.80 ± 0.10[a]
7AAD+	23.04 ± 13.36[a]	24.05 ± 12.55[a]	14.51 ± 9.29[a]	16.65 ± 11.35[a]

[a,b]*Different letters show statistical differences.*

Moreover, after thawing of the isolated spermatogonial cells, the amounts of SSC with intact mitochondrial membranes in MSDB (34.40 ± 23%) and MSD (27.7 ± 8.70%) media were significantly different from those in MTDB and MTD freezing media ($p < 0.05$). Finally, the comparison of the percentage of sMitoSense+ cells between fragments and isolated cells belonging to Group 2M (**Table 4**), frozen in MSDB (12.41 ± 5.19 and 34.40 ± 23%, respectively) or MSD medium (16.64 ± 8.13 and 27.7 ± 8.70%, respectively), showed significant differences ($p < 0.05$).

Cryopreservation of Isolated Spermatogonial Cells Before and After *in vitro* Proliferation

Other samples were analyzed by FC, to detect SSC in isolated spermatogonial cells by DBA staining as well as to evaluate mitochondrial activity in these cells. These parameters were estimated for freshly isolated spermatogonial cells, cryopreserved SSC, and cryopreserved SSC after proliferation in MSDB freezing medium. Among samples, the freshly isolated spermatogonial cells showed 7.4 ± 0.33% of SSC, a significantly higher amount than that of cryopreserved SSC (1.46 ± 0.08%; $p < 0.05$),

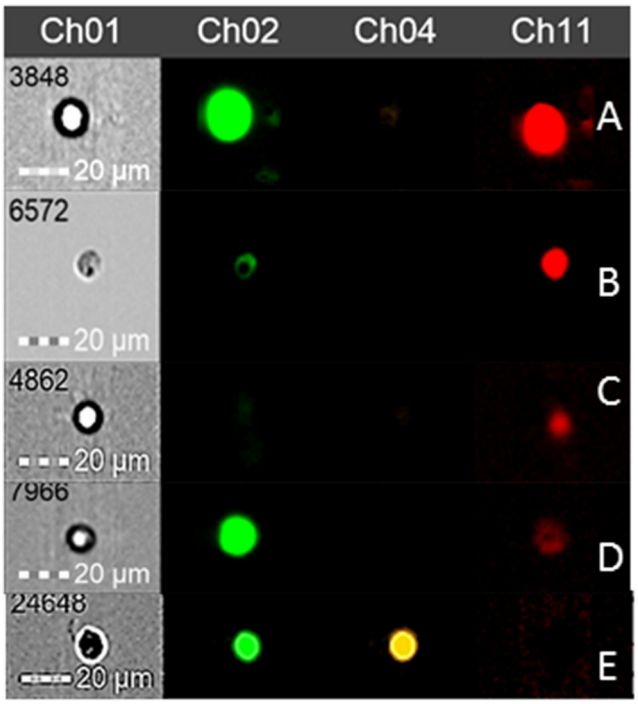


FIGURE 1 | Round spermatogonial cells observed by flow cytometry with DBA (Channel 2), 7AAD (Channel 4), and MitoSense (Channel 11) fluorochromes. **(A)** SSC (sDBA+) with intact mitochondrial membrane (sMitoSense+). **(B)** Early differentiating SSC (wDBA+) with intact mitochondrial membrane (sMitoSense+). **(C)** Differentiated round spermatogonial cell (DBA-) in early apoptosis (wMitoSense+). **(D)** SSC (sDBA+) in early apoptosis (wMitoSense+). **(E)** Necrotic SSC (7AAD+).

but not significantly different than that of cryopreserved SSC after proliferation (6.29 ± 0.04; $p > 0.05$). In this case, wDBA+ cells that correspond to the SSC in early differentiation express significant differences ($p < 0.05$) with wDBA+ cells of freshly isolated spermatogonial and cryopreserved SSC after proliferation. Moreover, the percentages of cells displaying intact mitochondrial membranes, early apoptosis, necrosis, and DBA-negative (differentiated) cells did not show significance (**Table 5**).

DISCUSSION

Alpaca is an important resource for the Peruvian economy due to its high-quality meat and fiber, but its reproductive rate is low. Alpaca testes are relatively small in proportion to the body (51, 52), and high variability exists among adult animals (53). The season does not affect the testes' size but shows age-related size variation abnormalities, from 0.6 g at 6 months to 13.6 g at 36 months (54). In our case, we used the adult's testes collected all year from alpacas slaughtered at 4–6 years, and the variation of testicular weight ranged from 8 to 19.94 g, probably the testes around 8 g showed pathological conditions in alpaca. On the other hand, the male pathological condition has a high incidence in slaughtered animals at about 18.1% with an increased incidence of testicular abnormalities: hypoplasia 10.8%, cryptorchidism 3%, ectopic testes 1.9%, and cysts 14.5% (53). These pathological conditions could explain the high variation among samples (**Tables 1–3**) in the round spermatogonial cell and sperm concentration, low viability with trypan blue dye, and low mitochondrial activity after cryopreservation.

However, SSC isolation, molecular characterization, and cryopreservation can substantially improve the genetic conservation and reproductive fitness of these animals. In this frame, cell viability has often been evaluated by staining with trypan blue, an exclusion dye that marks only living cells (55, 56). However, this method can lead to overestimating cell viability because it cannot discriminate living cells from cells in early apoptosis. For this reason, in the present study, trypan staining was used in combination with the evaluation of mitochondrial activity by FC using the fluorochromes MitoSense and 7AAD; the first allows to determine the integrity of mitochondrial membranes while undergoing early apoptosis, and the second highlights cells necrosis. Likewise, the DBA marker was used to identify SSC.

In our assays, the highest percentage of cell viability upon trypan blue staining was observed in cells recovered from fragments of testes tissue, in medium supplemented with DMSO and sucrose or trehalose; there were statistical differences in good samples of Group 1 (**Table 2**) on cell derived from fragments and isolated cell.

Interestingly, freezing biopsies with DMSO results in low recovery and functionality of spermatogonia in mouse, rabbit, hamster, and monkey allotransplants or xenotransplants (39, 57), whereas in pig and human post-thawed biopsies, spermatogenesis can be completed (11, 58).

Notably, our results have demonstrated the possibility of maintaining frozen alpaca SSC for long periods (1–166 days), similar to mice (59). This opens the possibility of storing frozen alpaca samples for several years, as also observed in mice (60), if collected within 24 h from death of the alpaca.

In our work, isolated cells and testicular biopsies showed different cryopreservation efficiencies and different requirements for CPA. However, previous reports have shown variable results concerning the type of tissue being frozen. For example, Pacchiarotti et al. (61) found no differences between the efficiency of freezing between isolated cell suspensions and tissues, whereas Yango et al. (62) showed that the viability of cell suspensions was greater than that of biopsies in adults, but not in newborns, thus suggesting that age is an important factor for the selection of the right method for the cryopreservation of testicular tissue or isolated cells. In our study, only testicular samples from adult alpacas were used; therefore, the differences in post-thaw cell viability between biopsies and cell suspensions are consistent with the aforementioned studies. However, in mice, freezing of testicular tissue was more effective than freezing of cell suspensions (63). This report contrasts with our results, since we observed better mitochondrial activity in thawed isolated alpaca cells.

In our experiment with FC using MitoSense, the most prominent and significant statistical differences in the cryopreserved isolated cells occurred in the MSDB medium. In

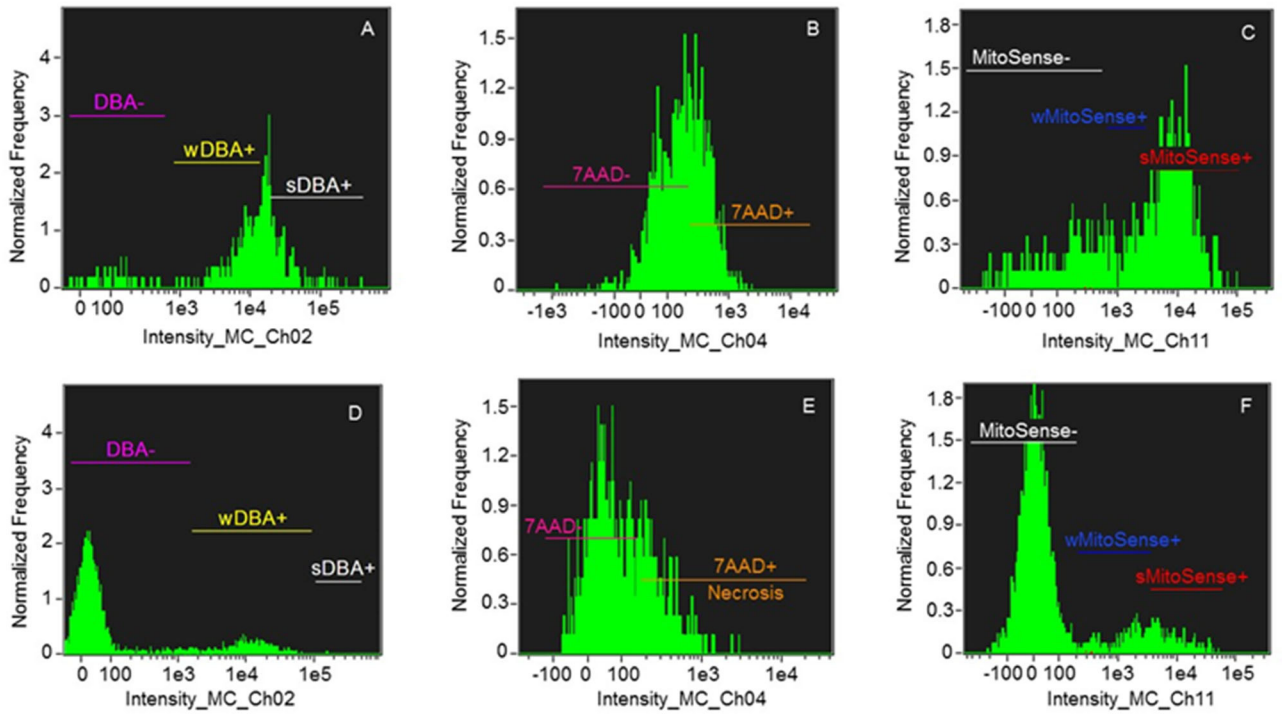


FIGURE 2 | Histograms of samples of Group 1: > 30% sMitoSense+ **(A–C)** and Group 2 < 30% sMitoSense+ **(D–F)**. **(A,D)** DBA histograms showing strongly DBA-positive (sDBA+) cells corresponding to SSC, weakly DBA-positive (wDBA+) cells corresponding to early differentiating SSC, and DBA-negative (DBA-) cells corresponding to differentiated round spermatogonia. **(B,E)** Histograms of necrotic cells (7AAD+). **(C,F)** Histograms of cells with intact mitochondrial membranes (sMitoSense+), weakly MitoSense-positive (wMitoSense+) cells, undergoing early apoptosis, and MitoSense-negative (MitoSense−) cells.

TABLE 5 | Mean percentage of spermatogonial stem cell before and after *in vitro* culture and freeze in MSDB freezing medium.

		$\overline{X} \pm$ SE					
Samples	**n**	**sDBA+**	**wDBA+**	**DBA-**	**sMitoSense**	**wMitoSense**	**7AAD+**
Fresh *in vitro* culture	15	6.4 ± 0.3^{a}	21.4 ± 1.9^{a}	69.3 ± 2.9^{a}	81.3 ± 2.2^{a}	8.3 ± 3.0^{a}	$8,0 \pm 0.7^{a}$
Cryopreserved before *in vitro* culture	15	1.5 ± 0.1^{b}	5.4 ± 0.1^{b}	87.6 ± 2.1^{a}	70.8 ± 2.9^{a}	14.0 ± 1.3^{a}	$13,3 \pm 3,2^{a}$
Cryopreserved after *in vitro* culture	15	6.30 ± 0.4^{a}	11.1 ± 2.6^{a}	75.9 ± 2.8^{a}	77.1 ± 2.8^{a}	8.1 ± 0.7^{a}	$8,0 \pm 2,0^{a}$

^{a,b}*Different letters show statistical differences.*

particular, the highest mitochondrial activity was observed in isolated cells in freezing medium supplemented with DMSO, sucrose, and black maca. Cells from testicular biopsies also showed good results about the conservation of mitochondrial activity when frozen in medium supplemented with DMSO, trehalose, and black maca but not significant. However, it is the way to easy conservation of testes tissue, cheaper, and without complex methods. The detected differences between frozen fragments and isolated cells may be due to the differing physical dimensions of the samples, the distribution of cryoprotectants, and the uniformity of temperature during cooling (63–65). In mice, better results have been observed when freezing whole testes (66), whereas in rats, freezing of small testicular biopsies showed better results than those obtained with fragments twice the size (67). On the other hand, the size of the testicular biopsies did not significantly affect the efficiency of cryopreservation either in pigs (58) or in prepubertal children (9, 38, 68–70). The biopsy size in our previous study was 8 × 2 × 2 mm (26); therefore, in this work, we used the same size and observed an average viability of 57.2% in samples of good quality (>50% viability), suggesting that this size is adequate for freezing of alpaca testicular biopsies. On the other hand, the size of the biopsies used for human SSC cryopreservation was not indicated. However, the freezing of testicular biopsies is recommended (71).

Interestingly, we found that media supplemented with non-permeable CPA, such as sucrose, exerted a greater cryoprotective effect on isolated cells. This sugar has previously been shown to mediate better results than trehalose when freezing bovine spermatozoa (72). In the case of pigs, it has been observed that sucrose at 280 mM allowed better vitality of cryopreserved SSC (29), which is consistent with our results for freezing of isolated alpaca SSC. On the other hand, Jung et al. (73)

found that stabilization of isolated mouse spermatogonia with trehalose reduced the cytotoxicity of the cells and improved post-thaw survival. Similar results were found by Syvyk et al. (74) in isolated rat SSC. Sucrose was not used in these studies; however, the results of these studies differ from ours, since we observed that trehalose did not improve mitochondrial activity in isolated cells. This could indicate that the efficacy of CPA may be species dependent. On the other hand, previous research in tissue freezing indicates that the use of trehalose confers greater cellular vitality (75), consistently with our results, as trehalose was better than sucrose for freezing of testicular tissue.

Moreover, antioxidants can improve the cryopreservation process (13, 76, 77), and maca is considered a natural antioxidant because it can help maintain DNA integrity. In particular, some derivatives of maca can protect cells against oxidative stress (31, 33), due to the presence of phytochemicals capable of scavenging free radicals, and thus can help fight chronic inflammation (33). In the current study, we observed that the presence of black maca helps alpaca isolated SSC to retain efficient mitochondrial activity, suggesting the presence of maca metabolites with antioxidant properties that protect the mitochondria as observed in isolated cell of good (>30% sMitoSense+) and regular (<30% sMitoSense+) quality of samples (**Tables 3**, **4**). In addition to the antioxidant activity of maca, high concentrations of sucrose may play a key role in cryoprotection (45, 47, 49), since, as previously stated, the presence of sucrose as a cryoprotectant improves post-thaw cell viability (MSDB medium). Therefore, antioxidants and cryoprotectant components of maca can be useful for cryopreservation; this phenomenon could be explained by the fact that maca belongs to the Brassicaceae family, which easily adapts to heat and hydric stress conditions at the heights of the Andes (78). The phytochemicals of maca allow its survival in the difficult conditions of its habitat, and this could explain their protective effect in cryopreservation.

In the present study, DBA staining, together with the FlowCellect MitoPotential Red kit, allowed to identify intact mitochondrial membrane activity (sMitoSense+), early apoptosis (wMitoSense+), and necrosis (7AAD+) in SSC. This analysis, carried out by FC, makes it possible to better recognize the events and damages generated by the cryopreservation process and the degree of effective conservation through this process, compensating the insufficient indications provided by trypan blue staining alone.

In conclusion, our study showed that the supplementation of natural atomized black maca in the cryopreservation medium improves survival and preserves intact mitochondrial membrane activity in alpaca SSC. Furthermore, another CPA, trehalose, was found to be a better cryoprotectant for testicular fragments, whereas sucrose would be more effective for cryopreserving isolated SSC. We conclude that it is possible to cryopreserve alpaca SSC collected after 24 h from death in the form of biopsies or isolated cells in the presence of DMSO and natural supplement of black maca in the freezing media for cryopreserved good and regular quality samples. Furthermore, we recommend the use of these MSDB media for the cryopreservation of isolated SSC after *in vitro* proliferation.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Comité Institucional de ética para el uso de animales CIEA, Universidad Peruana Cayetano Heredia Codigo SIDISI N° 0000066755.

AUTHOR CONTRIBUTIONS

MV did cryopreservation protocols for isolated and testis tissue and analysis by CF. JR has done SSC *in vitro* proliferation. ZB did statistical analyses and discussion. GG, MV, and JR did the analysis of the results. All authors contributed to the article and approved the submitted version.

FUNDING

This study was supported by grants PIBA15A106 and B17100101 of the Universidad Nacional Mayor de San Marcos.

ACKNOWLEDGMENTS

This article includes results of Martha Valdivia Doctoral Thesis of Sciences with mention of Physiology, Universidad Peruana Cayetano Heredia. MV thanks Dr. Alexei Santiani for the use of CF (National Council of Science Technology and Innovation No. 313-2011-CONCYTEC-OAJ).

REFERENCES

1. Tagelenbosch RA, de Rooij DG. A quantitative study of spermatogonial multiplication and stem cell renewal in the C3H/101 F1 hybrid mouse. *Mutat Res Fund Mol Mech Mutag.* (1993) 290:193–200. doi: 10.1016/0027-5107(93)90159-D
2. Vansandt LM, Livesay JL, Dickson MJ, Li L, Pukazhenthi BS, Keefer CL. Conservation of spermatogonial stem cell marker expression in undifferentiated felid spermatogonia. *Theriogenology.* (2016) 86:1022–35. e3. doi: 10.1016/j.theriogenology.2016.03.031
3. Ryu BY, Orwig KE, Oatley JM, Avarbock MR, Brinster RL. Effects of aging and niche microenvironment on spermatogonial stem cell self-renewal. *Stem Cells.* (2006) 24:1505–11. doi: 10.1634/stemcells.2005-0580
4. González R, Tang L, Dobrinski I. *Application of Spermatogonial Transplantation in Agricultural Animals. The Biology of Mammalian Spermatogonia.* Springer. (2017). p. 343–77. doi: 10.1007/978-1-4939-7505-1_14
5. Kanatsu-Shinohara M, Takehashi M, Takashima S, Lee J, Morimoto H, Chuma S, et al. Homing of mouse spermatogonial stem cells to germline niche depends on β1-integrin. *Cell Stem Cell.* (2008) 3:533–42. doi: 10.1016/j.stem.2008.08.002

6. Singh SR, Burnicka-Turek O, Chauhan C, Hou SX. Spermatogonial stem cells, infertility and testicular cancer. *J Cell Mol Med.* (2011) 15:468–83. doi: 10.1111/j.1582-4934.2010.01242.x
7. Phillips BT, Gassei K, Orwig KE. Spermatogonial stem cell regulation and spermatogenesis. *Philosoph Trans R Soc B Biol Sci.* (2010) 365:1663–78. doi: 10.1098/rstb.2010.0026
8. De Rooij DG, Griswold MD. Questions about spermatogonia posed and answered since (2000). *J Androl.* (2012) 33:1085–95. doi: 10.2164/jandrol.112.016832
9. Curaba M, Poels J, Van Langendonckt A, Donnez J, Wyns C. Can prepubertal human testicular tissue be cryopreserved by vitrification? *Fertil Steril.* (2011) 95:2123. e9-e12. doi: 10.1016/j.fertnstert.2011.01.014
10. Yokonishi T, Sato T, Komeya M, Katagiri K, Kubota Y, Nakabayashi K, et al. Offspring production with sperm grown in vitro from cryopreserved testis tissues. *Nat Commun.* (2014) 5:1–6. doi: 10.1038/ncomms5320
11. Baert Y, Braye A, Struijk RB, van Pelt AM, Goossens E. Cryopreservation of testicular tissue before long-term testicular cell culture does not alter in vitro cell dynamics. *Fertil Steril.* (2015) 104:1244–52. e4. doi: 10.1016/j.fertnstert.2015.07.1134
12. Baukloh V. Retrospective multicentre study on mechanical and enzymatic preparation of fresh and cryopreserved testicular biopsies. *Hum Reprod.* (2002) 17:1788–94. doi: 10.1093/humrep/17.7.1788
13. Aliakbari F, Gilani MAS, Amidi F, Baazm M, Korouji M, Izadyar F, et al. Improving the efficacy of cryopreservation of spermatogonia stem cells by antioxidant supplements. *Cell Reprogramm.* (2016) 18:87–95. doi: 10.1089/cell.2015.0067
14. Takashima S, Shinohara T. Culture and transplantation of spermatogonial stem cells. *Stem Cell Res.* (2018) 29:46–55. doi: 10.1016/j.scr.2018.03.006
15. Lee Y-A, Kim Y-H, Ha S-J, Kim B-J, Kim K-J, Jung M-S, et al. Effect of sugar molecules on the cryopreservation of mouse spermatogonial stem cells. *Fertility and sterility.* (2014) 101:1165–75. e5. doi: 10.1016/j.fertnstert.2013.12.033
16. Kim K-J, Lee Y-A, Kim B-J, Kim Y-H, Kim B-G, Kang H-G, et al. Cryopreservation of putative pre-pubertal bovine spermatogonial stem cells by slow freezing. *Cryobiology.* (2015) 70:175–83. doi: 10.1016/j.cryobiol.2015.02.007
17. Lee W-Y, Lee K-H, Heo Y-T, Kim N-H, Kim J-H, Kim J-H, et al. Transcriptional coactivator undifferentiated embryonic cell transcription factor 1 expressed in spermatogonial stem cells: a putative marker of boar spermatogonia. *Anim Reprod Sci.* (2014) 150:115–24. doi: 10.1016/j.anireprosci.2014.09.010
18. Valdivia M, Castañeda-Zegarra S, Lévano G, Lazo J, Reyes J, Bravo Z, et al. Spermatogonial stem cells identified by molecular expression of PLZF, integrin β1 and reactivity to Dolichos biflorus agglutinin in alpaca adult testes. *Andrologia.* (2019) 51:e13283. doi: 10.1111/and.13283
19. Kim S-M, Fujihara M, Sahare M, Minami N, Yamada M, Imai H. Effects of extracellular matrices and lectin Dolichos biflorus agglutinin on cell adhesion and self-renewal of bovine gonocytes cultured in vitro. *Reprod Fertil Dev.* (2014) 26:268–81. doi: 10.1071/RD12214
20. La Manna V, La Terza A, Ghezzi S, Saravanaperumal S, Apaza N, Huanca T, et al. Analysis of genetic distance between Peruvian Alpaca (*Vicugna Pacos*) showing two distinct fleece phenotypes, Suri and Huacaya, by means of microsatellite markers. *Ital J Anim Sci.* (2011) 10:e60. doi: 10.4081/ijas.2011.e60
21. Renieri C, Valbonesi A, La Manna V, Antonini M, Asparrin M. Inheritance of Suri and Huacaya type of fleece in Alpaca. *Ital J Anim Sci.* (2009) 8:83–91. doi: 10.4081/ijas.2009.83
22. Elzawam AZ. *Establishment of Spermatogenesis Following Testicular Tissue Ectopic Xenografting in Alpaca*. Pullman, WA: Whashington state university (2013). Available online at: https://research.wsulibs.wsu.edu/xmlui/bitstream/handle/2376/4743/Elzawam_wsu_0251E_10700.pdf?sequence=1
23. Abraham MC. *Reproductive Biotechnologies in Swedish Male Alpacas*. Uppsala: Swedish University of Agricultural Sciences (2016). Available online at: https://pub.epsilon.slu.se/13255/
24. Santiani A, Huanca W, Sapana R, Huanca T, Sepúlveda N, Sánchez R. Effects on the quality of frozen-thawed alpaca (*Lama pacos*) semen using two different cryoprotectants and extenders. *Asian J Androl.* (2005) 7:303–9. doi: 10.1111/j.1745-7262.2005.00021.x
25. Mamani-Mango G, Moina Gonzales M, Ramos Hidalgo M, Mendoza Mallma J, Ruiz Bejar J, Rivas Palma V, et al. Effect of extender and freezing rate on quality parameters and in vitro fertilization capacity of alpaca spermatozoa recovered from cauda epididymis. *Biopreservation Biobank.* (2019) 17:39–45. doi: 10.1089/bio.2018.0021
26. Valdivia M, Reyes J, Bravo Z, Cancho C, Castañeda S, Limaymanta O, et al. In vitro culture of spermatogonial stem cells isolated from adult alpaca (*Vicugna pacos*) testes analysed with Dolichos biflorus by flow cytometry. *Andrologia.* (2019) 51:e13269. doi: 10.1111/and.13269
27. Savvulidi F, Ptacek M, Vargova KS, Stadnik L. Manipulation of spermatogonial stem cells in livestock species. *J Anim Sci Biotechnol.* (2019) 10:46. doi: 10.1186/s40104-019-0355-4
28. Zarandi NP, Galdon G, Kogan S, Atala A, Sadri-Ardekani H. Cryostorage of immature and mature human testis tissue to preserve spermatogonial stem cells (SSCs): a systematic review of current experiences toward clinical applications. *Stem Cells Cloning.* (2018) 11:23. doi: 10.2147/SCCAA.S137873
29. PAN C-Y, Shuai Y, Zhang P-F, Bo W, Zhu Z-D, Liu Y-Y, et al. Effect of sucrose on cryopreservation of pig spermatogonial stem cells. *J Integr Agricult.* (2017) 16:1120–9. doi: 10.1016/S2095-3119(16)61489-2
30. Valdivia M. *Efecto de la Suplementación de Maca Roja o Negra Atomizada Comercial Combinada con un Potencial Crioprotector Para Células Madre Espermatogoniales (SSC) de alpaca Vicugna Pacos Tanto en SSC Aisladas Como En Fragmentos Testiculares.* Lima: Universidad Peruana Cayetano Heredia (2019). Available online at: http://190.116.48.43/handle/upch/7307
31. Valdivia M, Yarasca De La Vega K, Levano Sanchez G, Vasquez Cavero J, Temoche Garcia H, Torres Torres L, et al. Effect of *Lepidium meyenii* (maca) on testicular function of mice with chemically and physically induced subfertility. *Andrologia.* (2016) 48:927–34. doi: 10.1111/and.12682
32. Gonzales C, Rubio J, Gasco M, Nieto J, Yucra S, Gonzales GF. Effect of short-term and long-term treatments with three ecotypes of *Lepidium meyenii* (MACA) on spermatogenesis in rats. *J Ethnopharm.* (2006) 103:448–54. doi: 10.1016/j.jep.2005.08.035
33. Sandoval M, Okuhama NN, Angeles FM, Melchor VV, Condezo LA, Lao J, et al. Antioxidant activity of the cruciferous vegetable Maca (Lepidium meyenii). *Food Chem.* (2002) 79:207–13. doi: 10.1016/S0308-8146(02)00133-4
34. Lee K-J, Dabrowski K, Sandoval M, Miller MJ. Activity-guided fractionation of phytochemicals of maca meal, their antioxidant activities and effects on growth, feed utilization, and survival in rainbow trout (*Oncorhynchus mykiss*) juveniles. *Aquaculture.* (2005) 244:293–301. doi: 10.1016/j.aquaculture.2004.12.006
35. Gonzales-Castañeda C, Rivera V, Chirinos AL, Evelson P, Gonzales GF. Photoprotection against the UVB-induced oxidative stress and epidermal damage in mice using leaves of three different varieties of *Lepidium meyenii* (maca). *Int J Dermatol.* (2011) 50:928–38. doi: 10.1111/j.1365-4632.2010.04793.x
36. Brook PF, Radford JA, Shalet SM, Joyce AD, Gosden RG. Isolation of germ cells from human testicular tissue for low temperature storage and autotransplantation. *Fertil Steril.* (2001) 75:269–74. doi: 10.1016/S0015-0282(00)01721-0
37. Gosden R, Nagano M. Preservation of fertility in nature and ART. *Reprod Cambridge-.* (2002) 123:3–11. doi: 10.1530/reprod/123.1.3
38. Keros V, Hultenby K, Borgström B, Fridström M, Jahnukainen K, Hovatta O. Methods of cryopreservation of testicular tissue with viable spermatogonia in pre-pubertal boys undergoing gonadotoxic cancer treatment. *Hum Reprod.* (2007) 22:1384–95. doi: 10.1093/humrep/del508
39. Shinohara T, Inoue K, Ogonuki N, Kanatsu-Shinohara M, Miki H, Nakata K, et al. Birth of offspring following transplantation of cryopreserved immature testicular pieces and in-vitro microinsemination. *Human reproduction.* (2002) 17:3039–45. doi: 10.1093/humrep/17.12.3039
40. Gies I, Oates R, De Schepper J, Tournaye H. Testicular biopsy and cryopreservation for fertility preservation of prepubertal boys with Klinefelter syndrome: a pro/con debate. *Fert Ster.* (2016) 105:249–55. doi: 10.1016/j.fertnstert.2015.12.011
41. Gonzales GF, Cordova A, Vega K, Chung A, Villena A, Góñez C. Effect of Lepidium meyenii (Maca), a root with aphrodisiac and fertility-enhancing properties, on serum reproductive hormone levels in adult healthy men. *J Endocrinol.* (2003) 176:163–8. doi: 10.1677/joe.0.1760163

42. Gonzales GF, Villaorduña L, Gasco M, Rubio J, Gonzales C. Maca (Lepidium meyenii Walp), una revisión sobre sus propiedades biológicas. *Rev Per Med Exp Y Salud Pública.* (2014) 31:100–10. doi: 10.17843/rpmesp.2014.311.15
43. Zheng BL, He K, Kim CH, Rogers L, Shao Y, Huang ZY, et al. Effect of a lipidic extract from lepidium meyenii on sexual behavior in mice and rats. *Urology.* (2000) 55:598–602. doi: 10.1016/S0090-4295(99)00549-X
44. Zenico T, Cicero AF, Valmorri L, Mercuriali M, Bercovich E. Subjective effects of Lepidium meyenii (Maca) extract on well-being and sexual performances in patients with mild erectile dysfunction: a randomised, double-blind clinical trial. *Andrologia.* (2009) 41:95–9. doi: 10.1111/j.1439-0272.2008.00892.x
45. Zha S, Zhao Q, Chen J, Wang L, Zhang G, Zhang H, et al. Extraction, purification and antioxidant activities of the polysaccharides from maca (Lepidium meyenii). *Carbohydrate polymers.* (2014) 111:584–7. doi: 10.1016/j.carbpol.2014.05.017
46. Rubio J, Riqueros MI, Gasco M, Yucra S, Miranda S, Gonzales GF. *Lepidium meyenii* (Maca) reversed the lead acetate induced—Damage on reproductive function in male rats. *Food Chem Toxicol.* (2006) 44:1114–22. doi: 10.1016/j.fct.2006.01.007
47. Zhao J, Avula B, Chan M, Clément C, Kreuzer M, Khan IA. Metabolomic differentiation of maca (Lepidium meyenii) accessions cultivated under different conditions using NMR and chemometric analysis. *Planta Med Nat Products Med Res.* (2012) 78:90. doi: 10.1055/s-0031-1280117
48. Organization WH. *WHO Laboratory Manual for the Examination and Processing of Human Semen*. Geneva (2010).
49. Gonzales-Arimborgo C, Yupanqui I, Montero E, Alarcón-Yaquetto DE, Zevallos-Concha A, Caballero L, et al. Acceptability, safety, and efficacy of oral administration of extracts of black or red maca (Lepidium meyenii) in adult human subjects: a randomized, double-blind, placebo-controlled study. *Pharmaceuticals.* (2016) 9:49. doi: 10.3390/ph9030049
50. Izadyar F, Spierenberg G, Creemers L, Ouden Kd, De Rooij D. Isolation and purification of type A spermatogonia from the bovine testis. *Reprod Cambridge.* (2002) 124:85–94. doi: 10.1530/rep.0.1240085
51. Fowler ME. *Medicine and Surgery of South American Camelids: Llama, Alpaca, Vicuna, Guanaco.* Ames, IA: Iowa State University Press (1998).
52. Tibary A, Anouassi A. *Male Breeding Soundness Examination*. Theriogenology in Camelidae: Anatomy, Physiology, BSE, Pathology and artificial breeding, Actes Editions, Institut Agronomique et Vétérinaire Hassan II. (1997). p. 79–111.
53. Sumar J. Studies on reproductive pathology in alpacas. (1983).
54. Tibary A, Vaughan J. Reproductive physiology and infertility in male South American camelids: a review and clinical observations. *Small Rum Res.* (2006) 61:283–98. doi: 10.1016/j.smallrumres.2005.07.018
55. Sato K. On the real nature of vital staining. *Folia Anatomica Jap.* (1930) 8:51–61. doi: 10.2535/ofaj1922.8.1_51
56. Altman SA, Randers L, Rao G. Comparison of trypan blue dye exclusion and fluorometric assays for mammalian cell viability determinations. *Biotechnol Progress.* (1993) 9:671–4. doi: 10.1021/bp00024a017
57. Schlatt S, Kim SS, Gosden R. Spermatogenesis and steroidogenesis in mouse, hamster and monkey testicular tissue after cryopreservation and heterotopic grafting to castrated hosts. *Reprod Cambridge.* (2002) 124:339–46. doi: 10.1530/reprod/124.3.339
58. Abrishami M, Anzar M, Yang Y, Honaramooz A. Cryopreservation of immature porcine testis tissue to maintain its developmental potential after xenografting into recipient mice. *Theriogenology.* (2010) 73:86–96. doi: 10.1016/j.theriogenology.2009.08.004
59. Avarbock MR, Brinster CJ, Brinster RL. Reconstitution of spermatogenesis from frozen spermatogonial stem cells. *Nat Med.* (1996) 2:693–6. doi: 10.1038/nm0696-693
60. Wu X, Goodyear SM, Abramowitz LK, Bartolomei MS, Tobias JW, Avarbock MR, et al. Fertile offspring derived from mouse spermatogonial stem cells cryopreserved for more than 14 years. *Hum Reprod.* (2012) 27:1249–59. doi: 10.1093/humrep/des077
61. Pacchiarotti J, Ramos T, Howerton K, Greilach S, Zaragoza K, Olmstead M, et al. Developing a clinical-grade cryopreservation protocol for human testicular tissue and cells. *BioMed Res Int.* (2013) 2013:1–10. doi: 10.1155/2013/930962
62. Yango P, Altman E, Smith JF, Klatsky PC, Tran ND. Optimizing cryopreservation of human spermatogonial stem cells: comparing the effectiveness of testicular tissue and single cell suspension cryopreservation. *Fert Ster.* (2014) 102:1491–8. e1. doi: 10.1016/j.fertnstert.2014.07.1250
63. Onofre J, Baert Y, Faes K, Goossens E. Cryopreservation of testicular tissue or testicular cell suspensions: a pivotal step in fertility preservation. *Hum Reprod Update.* (2016) 22:744–61. doi: 10.1093/humupd/dmw029
64. Karlsson JO, Toner M. Long-term storage of tissues by cryopreservation: critical issues. *Biomaterials.* (1996) 17:243–56. doi: 10.1016/0142-9612(96)85562-1
65. Yavin S, Arav A. Measurement of essential physical properties of vitrification solutions. *Theriogenology.* (2007) 67:81–9. doi: 10.1016/j.theriogenology.2006.09.029
66. Gouk SS, Loh YFJ, Kumar SD, Watson PF, Kuleshova LL. Cryopreservation of mouse testicular tissue: prospect for harvesting spermatogonial stem cells for fertility preservation. *Fert Steril.* (2011) 95:2399–403. doi: 10.1016/j.fertnstert.2011.03.035
67. Travers A, Milazzo J, Perdrix A, Metton C, Bironneau A, Mace B, et al. Assessment of freezing procedures for rat immature testicular tissue. *Theriogenology.* (2011) 76:981–90. doi: 10.1016/j.theriogenology.2011.04.025
68. Wyns C, Curaba M, Vanabelle B, Van Langendonckt A, Donnez J. Options for fertility preservation in prepubertal boys. *Hum Reprod Update.* (2010) 16:312–28. doi: 10.1093/humupd/dmp054
69. Poels J, Van Langendonckt A, Many M-C, Wese F-X, Wyns C. Vitrification preserves proliferation capacity in human spermatogonia. *Hum Reprod.* (2013) 28:578–89. doi: 10.1093/humrep/des455
70. Picton HM, Wyns C, Anderson RA, Goossens E, Jahnukainen K, Kliesch S, et al. A European perspective on testicular tissue cryopreservation for fertility preservation in prepubertal and adolescent boys. *Hum Reprod.* (2015) 30:2463–75. doi: 10.1093/humrep/dev190
71. Moraveji SF, Esfandiari F, Sharbatoghli M, Taleahmad S, Nikeghbalian S, Shahverdi A, et al. Optimizing methods for human testicular tissue cryopreservation and spermatogonial stem cell isolation. *J Cell Biochem.* (2019) 120:613–21. doi: 10.1002/jcb.27419
72. Woelders H, Matthijs A, Engel B. Effects of trehalose and sucrose, osmolality of the freezing medium, and cooling rate on viability and intactness of bull sperm after freezing and thawing. *Cryobiology.* (1997) 35:93–105. doi: 10.1006/cryo.1997.2028
73. Jung S-E, Kim M, Ahn JS, Kim Y-H, Kim B-J, Yun M-H, et al. Effect of equilibration time and temperature on murine spermatogonial stem cell cryopreservation. *Biopreserv Biobank.* (2020) doi: 10.1089/bio.2019.0116
74. Syvyk T, Djachenko L, Syvyk A. Optimization of freezing conditions for cryopreservation of rat spermatogonial stem cell. *J Microb Biotech Food Sci.* (2020) 9:947–50. doi: 10.15414/jmbfs.2018-19.8.3.947-950
75. Lee W-Y, Park H-J, Lee R, Lee K-H, Kim Y-H, Ryu B-Y, et al. Establishment and in vitro culture of porcine spermatogonial germ cells in low temperature culture conditions. *Stem Cell Res.* (2013) 11:1234–49. doi: 10.1016/j.scr.2013.08.008
76. Aliakbari F, Sedighi Gilani MA, Yazdekhasti H, Koruji M, Asgari HR, Baazm M, et al. Effects of antioxidants, catalase and α-tocopherol on cell viability and oxidative stress variables in frozen-thawed mice spermatogonial stem cells. *Artif Cells Nanomed Biotech.* (2017) 45:63–8. doi: 10.3109/21691401.2016.1138491
77. Amidi F, Rashidi Z, Khosravizadeh Z, Khodamoradi K, Talebi A, Navid S, et al. Antioxidant effects of quercetin in freeze-thawing process of mouse spermatogonial stem cells. *Asian Pacific J Reprod.* (2019) 8:7. doi: 10.4103/2305-0500.250417
78. Zhang J, Tian Y, Yan L, Zhang G, Wang X, Zeng Y, et al. Genome of plant maca (*Lepidium meyenii*) illuminates genomic basis for high-altitude adaptation in the central Andes. *Mol Plant.* (2016) 9:1066–77. doi: 10.1016/j.molp.2016.04.016

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Advantages of publishing in Frontiers

OPEN ACCESS
Articles are free to read for greatest visibility and readership

FAST PUBLICATION
Around 90 days from submission to decision

HIGH QUALITY PEER-REVIEW
Rigorous, collaborative, and constructive peer-review

TRANSPARENT PEER-REVIEW
Editors and reviewers acknowledged by name on published articles

Frontiers
Avenue du Tribunal-Fédéral 34
1005 Lausanne | Switzerland

Visit us: www.frontiersin.org
Contact us: frontiersin.org/about/contact

REPRODUCIBILITY OF RESEARCH
Support open data and methods to enhance research reproducibility

DIGITAL PUBLISHING
Articles designed for optimal readership across devices

FOLLOW US
@frontiersin

IMPACT METRICS
Advanced article metrics track visibility across digital media

EXTENSIVE PROMOTION
Marketing and promotion of impactful research

LOOP RESEARCH NETWORK
Our network increases your article's readership