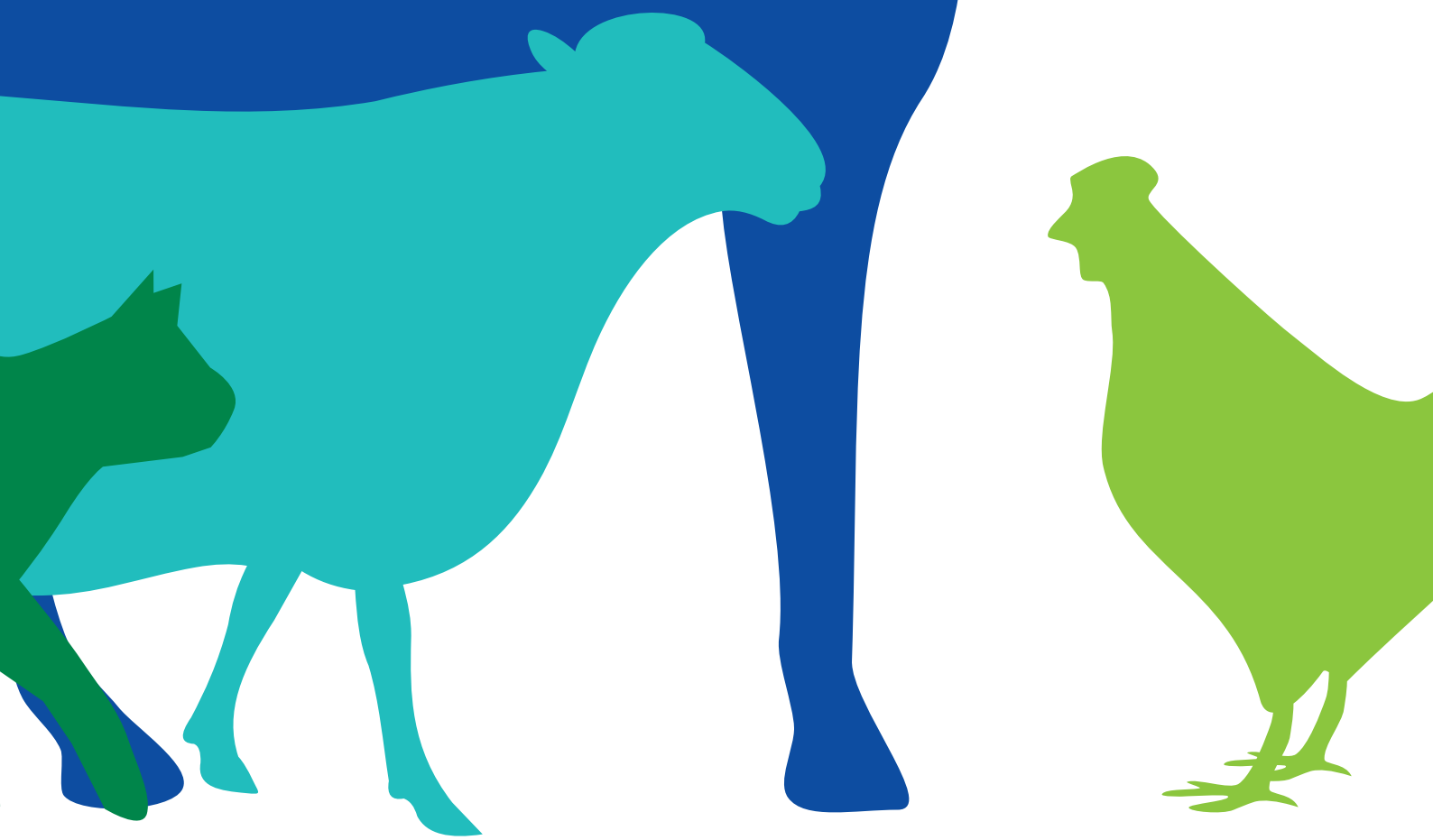
MULE AND DONKEY MEDICINE

EDITED BY: Micaela Sgorbini, Fulvio Laus and Amy Katherine McLean
PUBLISHED IN: Frontiers in Veterinary Science



frontiers Research Topics

ISSN 1664-8714
ISBN 978-2-88976-846-2
DOI 10.3389/978-2-88976-846-2

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

MULE AND DONKEY MEDICINE

Topic Editors:
Micaela Sgorbini, University of Pisa, Italy
Fulvio Laus, University of Camerino, Italy
Amy Katherine McLean, University of California, Davis, United States

Citation: Sgorbini, M., Laus, F., McLean, A. K., eds. (2022). Mule and Donkey Medicine. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88976-846-2

Table of Contents

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 10 September 2020
doi: 10.3389/fvets.2020.00640

Blood Storage Conditions Affect Hematological Analysis in Samples From Healthy Donkeys and Donkeys With Experimentally-Induced Endotoxemia

Alejandro Perez-Ecija[1], *Antonio Buzon-Cuevas*[1], *Raul Aguilera-Aguilera*[1], *Carlos A. Gonzalez-De Cara*[2] *and Francisco J. Mendoza*[1]*

[1] *Department of Animal Medicine and Surgery, University of Cordoba, Cordoba, Spain,* [2] *Department of Pathology and Infectious Diseases, University of Surrey, Guildford, United Kingdom*

OPEN ACCESS

Edited by:
Amy Katherine McLean, University of California, Davis, United States

Reviewed by:
Ignacio Lizarraga, Ross University School of Veterinary Medicine, Saint Kitts and Nevis
Erin L. Goodrich, Cornell University, United States

***Correspondence:**
Francisco J. Mendoza
fjmendoza@uco.es

Specialty section:
This article was submitted to Comparative and Clinical Medicine, a section of the journal Frontiers in Veterinary Science

Received: *14 April 2020*
Accepted: *05 August 2020*
Published: *10 September 2020*

Citation:
Perez-Ecija A, Buzon-Cuevas A, Aguilera-Aguilera R, Gonzalez-De Cara CA and Mendoza FJ (2020) Blood Storage Conditions Affect Hematological Analysis in Samples From Healthy Donkeys and Donkeys With Experimentally-Induced Endotoxemia. Front. Vet. Sci. 7:640. doi: 10.3389/fvets.2020.00640

Preanalytical factors such as storage time and temperature are proved to induce marked artifactual changes in hematological parameters in horses, small animals and humans. These errors can mirror findings typical of endotoxemia, leading to dangerous misdiagnosis. Since donkeys are common in warm climates and remote regions, blood samples from this species can be subjected to long lasting travels from the farm to the nearest laboratory, frequently under suboptimal conditions. Moreover, as other equids, donkeys are prone to suffer endotoxemia. Nonetheless, stability has not been evaluated in samples for hematology in this species. The aim of this study was to characterize the effect of temperature and storage time in hematological parameters from healthy donkeys and donkeys with induced endotoxemia. Blood samples were collected from six healthy female Andalusian donkeys and stored for 6, 12, 24, and 48 h at several temperatures (4, 24, and 35°C). Endotoxemia was induced in the same animals by an intravenous LPS infusion and samples obtained 30 min post-infusion were handled similarly. Hematological analysis was performed using a laser-based analyzer and blood smear examination. Storage at 24°C caused significant neutropenia after 48 h as well as morphological changes typical of endotoxemia in blood from healthy donkeys as soon as 24 h post-storage. Samples kept at 35°C displayed more profound and earlier artifactual variations. Conservation at 4°C did not cause any significant change in blood parameters. Prolonged (48 h) storage of samples from animals with induced endotoxemia at 24 and 35°C accentuated pre-existing leukopenia and neutropenia. These findings highlight that donkey samples should be stored at 4°C, instead of 24°C as recommended for horses. Moreover, blood smear interpretation should be cautious in samples stored for longer than 24 h and could be misleading when blood is kept at 35°C.

Keywords: donkey blood, storage temperature, storage time, sepsis, hematologic analysis

INTRODUCTION

There is a growing interest worldwide in the use of donkeys as pet companion, in assisted therapy, and in high-quality alimentary by-products production, along with their traditional roles in agriculture and transport in developing countries (1–3). Accordingly, the caseload of donkeys referred to veterinary hospitals and the number of donkey blood samples delivered to laboratories have increased in the last years.

Numerous differences have been demonstrated between donkeys and horses (4–6), including marked discrepancies on reference ranges of hematological parameters using either impedance or laser-based analyzers (7–11). Thus, the extrapolation of reference ranges and laboratory guidelines between both species must be avoided.

Preanalytical factors such as storage time and temperature have been related to significant artifacts in hematological parameters in several species (12–17), leading to species-specific recommendations on sample handling. Since donkeys are commonly located in warm and remote regions (18), their blood samples are frequently subjected to long duration travels from the farm to the nearest laboratory, usually under inadequate cold-chain maintenance. Moreover, delays in hematological analysis can also occur due to sample collection on weekends or holidays, overwork in the laboratory, equipment breakdown, etc.

In addition, some of the artifactual changes caused by deficient storage in humans and dogs, such as pseudoleukocytopenia or pseudotoxic changes, can mimic typical features of the systemic inflammatory response syndrome (SIRS) or endotoxemia, leading to erroneous diagnosis (19, 20). Endotoxemia is assumed to be as common in donkeys as in horses (21), but little is known about the hematological response to this disturbance in this species (21, 22).

At the moment, data on the stability of donkeys' hematological parameters during storage are not available. Moreover, whether findings in blood from endotoxemic donkeys could be mimicked by storage artifacts is also unknown. Therefore, the aim of this work was to study the effect of temperature and storage duration on hematological parameters from healthy donkeys and donkeys with experimentally-induced endotoxemia. Our hypothesis was that conservation of donkey samples during large periods and at warm temperatures could cause abnormal findings similar to those observed during induced endotoxemia.

MATERIALS AND METHODS

Animals

Six healthy adult (7.6 ± 0.8 years old) Andalusian non-pregnant jennies (348.3 ± 38.9 kg) housed in the facilities of the Veterinary Teaching Hospital of the University of Cordoba were included in this study. Animals had free access to drinking water and alfalfa hay and oat straw. Jennies were dewormed and had not received any treatment for at least 2 months prior to the study. Animals had no previous history of SIRS or endotoxemia-inducing diseases (e.g., colic, pleuropneumonia, diarrhea). Donkeys were healthy based on clinical history, physical examination and blood work profile (complete blood count, total protein, fibrinogen, albumin, aspartate transaminase -AST-, gamma-glutamyl transferase -γGT-, total bilirubin, creatinine and urea concentrations).

This study was carried out in accordance with the principles of the Basel Declaration and recommendations of the Royal Decree 1386/2018, which establishes the basic rules applicable for the protection of animals used in experimentation and other scientific purposes, Spanish Government. The study was conducted under permits 19-03-15-212 (Welfare Committee of the Conserjeria de Salud y Familias, Junta de Andalucia) and 2015PI/05 (Welfare Committee of the University of Cordoba).

Experimental Design

Thirteen ml of blood was aseptically collected from the left jugular vein of each animal and carefully transferred to 1 ml K_3EDTA tubes (Idexx VetCollect tubes, Idexx Europe, Hoofddorp, the Netherlands) (experiment A). One week later, endotoxemia was induced following previous protocols described in donkeys and horses (21, 23, 24). Briefly, a polyurethane catheter (Milacath, Mila International Inc., KY, USA) was aseptically placed in the left jugular vein, and 20 ng/kg of LPS (Escherichia coli O55:B5, Sigma-Aldrich Quimica, Madrid, Spain) diluted in 500 ml of sterile saline was administered over 30 min. Blood samples (13 ml) were collected 30 min post-LPS infusion into K_3EDTA tubes (experiment B).

Sample Handling and Measurements

One blood sample from experiments A and B was analyzed immediately after the collection (time 0 h). The rest of the tubes were divided in three groups (four tubes of 1 ml each one): one group was stored at 4°C in a refrigerator with an external thermostat controller (TSG505 refrigerator, ThermoFisher Scientist, Waltham, MA, United States), another at controlled room temperature (24°C, using the room air conditioning, a thermostat and a thermometer) and the last one was maintained in an incubator with an external thermostat controller at 35°C (Incubat, JP Selecta, Barcelona, Spain). One tube from each group was retrieved and, after homogenization, analyzed at the following times: 6, 12, 24, and 48 h post-collection using a laser-based hematological analyzer (LaserCyte, Idexx Europe, Hoofddorp, the Netherlands) with a previously validated donkey-specific setup (10). Samples stored at 4°C were raised to room temperature prior to analysis. All tubes were handled in a similar manner and aseptically in order to avoid bacterial contamination, mechanical damage, sample deterioration and any type of bias.

The following parameters were obtained: white blood cell (WBC) count, WBC differential counts, red blood cell (RBC) count, platelet (PLT) count, hematocrit (HCT), hemoglobin concentration, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red cell distribution width (RDW), mean platelet volume (MPV) and platelet distribution width (PDW). Flags displayed by the analyzer were also recorded: VRL (value out of range), RD (RBC and/or platelets abnormal distribution), WD (WBC abnormal distribution) and PA (platelet aggregates).

Additionally, blood smears were prepared by triplicate from each sample. Blood films were air-dried and stained with a modified Wright–Giemsa method (Sigma-Aldrich, St. Louis, MO, United States). Two veterinarians with experience in clinical pathology blindly evaluated every blood smear using a light microscopy (CX31 microscope, Olympus CO., Tokyo, Japan) and ×40 and ×100 objectives. The following morphological abnormalities were recorded: % crenocytes, % ruptured WBC, platelet clumps, toxic neutrophils, pyknotic leukocytes and degranulated eosinophils. Percentages were calculated over a 400 WBC or RBC count. Grading of toxic neutrophils was adapted from a previous report in horses using a scale of 0–9 (25). Briefly, this scale is based on the severity of cytoplasmic vacuolation, cytoplasmic basophilia and Döhle bodies observed and the percentage of neutrophils affected. The rest of the abnormalities were classified as 0 = absent, 1 = scattered, 2 = moderate, 3 = frequent and 4 = highly frequent.

Statistical Analysis

Normality was assessed by a Kolmogorov-Smirnov test. Every hematological analyte, % crenocytes and % ruptured WBC were normally distributed and these results were expressed as mean ± standard deviation of the mean (SD). The rest of morphological parameters (ordinal variables) were non-normally distributed and expressed as median and interquartile range (IQR: 25th percentile−75th percentile); with percentiles calculated using the Tukey's-Hinges test.

Collection time results were compared with post-storage data using an analysis of variance (ANOVA) of repeated measures followed by a Dunnett test. The effect of storage on ordinal variables was analyzed using a Friedman test followed by a Dunn's *post hoc* test. A p-value < 0.05 was considered significant. Inter-observer agreement for categorical variables in blood smears was adapted from a previous report (26). Agreement was calculated using the weighted Fleiss's kappa method with the following interpretation: < 0.20 no agreement; 0.21–0.39 minimal agreement; 0.40–0.59 weak agreement; 0.60–0.79 moderate agreement; 0.80–0.90 strong agreement; >0.90 almost perfect agreement. Statistical analysis was performed using a commercial statistical software (SPSS Statistics 24, IBM, Chicago, IL, USA).

RESULTS

Experiment A: Effect of Storage Time and Temperature on Blood From Healthy Donkeys

Results are shown in **Table 1**. Storage at 35°C caused a significant ($p < 0.05$) reduction in neutrophils compared to basal, both at 24 and 48 h. When stored during 48 h, this temperature also caused significant ($p < 0.05$) decreased WBC, RBC, HCT and MCV. Conservation at 24°C also led to a significant ($p < 0.05$) neutropenia at 48 h. There were not significant changes in hematology parameters when blood was stored at 4°C.

Fourteen samples (14 out of 78, 17.9%) were flagged as VRL (13 samples stored at 35°C and 1 at 24°C). The flags WD (6/78, 7.7%) and RD (5/78, 6.4%) were displayed only in samples at 35°C. Only samples stored for 48 h were flagged.

Morphological changes caused by storage in blood from healthy donkeys are displayed in **Table 2A**. Toxic neutrophils were increased ($p < 0.05$) in blood kept at 24 and 35°C for 24 or 48 h, but no changes were observed at 4°C. Crenocytes were significantly more common in every sample kept at 24 and 35°C, and samples stored at 4°C during 12, 24, and 48 h. Ruptured WBC were significantly more common in every sample kept at 35°C, and samples stored at 4 or 24°C during 12, 24, and 48 h. Pyknotic leukocytes significantly increased beginning at 12 h (35°C) and 24 h (4 and 24°C). Degranulated eosinophils significantly increased at 48 h at every temperature, as well as in samples kept during 24 h at 35°C.

Experiment B: Effect of Storage Time and Temperature on Blood From Donkeys With Experimentally-Induced Endotoxemia

All donkeys safely completed the study and developed typical features of SIRS such as tachycardia, fever and leukopenia. In addition, neutropenia, lymphopenia, eosinopenia and monocytopenia were also observed. Clinical data are reported in a previous study (21).

Results are shown in **Table 3**. Storage of blood from endotoxemic donkeys during 48 h either at 24 or 35°C caused significant decreases ($p < 0.05$) in WBC and neutrophil counts compared to basal results. Samples at 35°C also showed significant differences ($p < 0.05$) in lymphocytes, HCT, MCV, MCH, MCHC and PDW at 48 h. No significant changes were found between basal blood parameters from endotoxemic donkeys and results of samples stored at 4°C.

Sixteen samples (16/78, 20.5%) were flagged as VRL (14 kept at 35°C and 2 at 24°C); 8 (10.2%) as WD (seven stored at 35°C and 1 at 24°C) and three samples stored at 35°C (3.8%) as RD. Only samples stored for 48 h were flagged.

Morphological abnormalities are shown in **Table 2B**. Both toxic changes and platelet clumps were already present at collection time and neither of them were significantly affected by storage. % crenocytes and % ruptured WBC significantly increased ($p < 0.05$) during storage at every temperature. Pyknotic leukocytes only were significantly increased when blood was kept at 35°C during 12, 24, and 48 h as well as in samples kept at 4 and 24°C during 48 h. Degranulated eosinophils were also increased in blood stored during 24 h (24°C) and 48 h (4 and 35°C).

Inter-observer Agreement in Blood Smears

Agreement for toxic neutrophils and platelet clumps was strong (0.88 and 0.81, respectively); while was moderate for degranulated eosinophils (0.71). Slightly lower agreements were detected in samples stored at higher temperatures during 48 h.

DISCUSSION

This study evaluated the stability of EDTA whole-blood samples from healthy donkeys and donkeys with experimentally-induced

TABLE 1 | Effect of temperature and storage time on blood samples from healthy donkeys.

Parameter	Collection	4°C				24°C				35°C			
	0 h	6 h	12 h	24 h	48 h	6 h	12 h	24 h	48 h	6 h	12 h	24 h	48 h
WBC ($\times10^3/\mu l$)	8.9 ± 2.1	9.5 ± 1.9	9.2 ± 2.8	9.3 ± 1.7	8.4 ± 2.1	9 ± 1.8	9.1 ± 2.2	9.5 ± 2.9	5.9 ± 2.1	9.1 ± 2.3	9.1 ± 2.0	6.0 ± 0.8	4.2 ± 1.4[a]
RBC ($\times10^6/\mu l$)	6.3 ± 0.3	6.5 ± 0.4	6.2 ± 0.8	6.3 ± 0.2	6.1 ± 0.4	5.8 ± 0.4	5.2 ± 1.0	6.1 ± 0.4	4.9 ± 1.5	5.8 ± 0.3	5.6 ± 0.2	4.5 ± 1.7	2.3 ± 1.1[a]
Hb (g/dl)	12.5 ± 0.9	11.6 ± 0.9	13.0 ± 0.9	12.3 ± 0.8	13.0 ± 1.1	12.2 ± 0.8	12.1 ± 0.9	12.1 ± 0.9	12.2 ± 1.0	12.2 ± 0.8	12.7 ± 0.9	11.8 ± 1.0	13.0 ± 1.2
HCT (%)	35 ± 2.5	31 ± 3.1	34 ± 5.0	35 ± 2.4	34 ± 3.1	32 ± 3.4	31 ± 3.1	34 ± 2.3	28 ± 9.0	33 ± 2.1	33 ± 1.6	26 ± 10	17 ± 9.7[a]
MCV (fl)	56 ± 1.5	56 ± 1.0	56 ± 1.0	56.2 ± 1.6	56.2 ± 1.4	56 ± 1.2	55 ± 1.4	56.6 ± 1.4	56.8 ± 1.7	56 ± 0.9	56 ± 1.1	56.6 ± 2.0	49.6 ± 3.6[a]
MCH (pg)	20 ± 0.8	21 ± 0.3	21 ± 1.9	19.6 ± 0.7	21.2 ± 0.8	20 ± 0.8	23 ± 4.2	19.8 ± 1.3	28.4 ± 15	20 ± 0.9	21 ± 2.3	30.5 ± 16	75.2 ± 50
MCHC (g/dl)	35 ± 1.4	37 ± 8.4	37 ± 3.9	34 ± 6.3	37 ± 1.3	37 ± 2.0	40 ± 7.6	35 ± 1.4	50 ± 2.7	36 ± 9.0	37 ± 4.1	54 ± 2.9	95 ± 47
RDW (%)	19 ± 0.7	19 ± 0.4	19 ± 0.6	19 ± 0.5	19 ± 0.6	19 ± 0.6	19 ± 0.4	19 ± 0.6	18 ± 0.9	19 ± 0.7	19 ± 0.7	19 ± 1.0	20 ± 0.8
PLT ($\times10^3/\mu l$)	398 ± 78	379 ± 62	316 ± 51	277 ± 61	272 ± 66	374 ± 87	311 ± 71	329 ± 67	372 ± 213	344 ± 88	307 ± 76	259 ± 90	496 ± 268
MPV (fl)	4.5 ± 0.4	5.0 ± 0.3	5.0 ± 0.5	5.1 ± 1.0	5.1 ± 0.8	3.9 ± 0.6	3.7 ± 0.5	4.5 ± 0.4	4.1 ± 0.4	4.4 ± 0.7	4.5 ± 0.6	4.0 ± 0.8	4.5 ± 0.3
PDW (%)	18 ± 0.4	19 ± 0.6	19 ± 0.4	20.0 ± 1.5	20.6 ± 1.0	18 ± 0.2	18 ± 0.4	18.8 ± 0.5	19.8 ± 0.8	19 ± 1.1	18 ± 0.3	20.0 ± 1.0	22.0 ± 1.2
Neu ($\times10^3/\mu l$)	5.3 ± 1.9	5.3 ± 2.1	5.5 ± 2.3	5.3 ± 1.8	4.4 ± 2.1	4.9 ± 1.9	5.0 ± 1.9	5.0 ± 2.5	2.2 ± 1.2[a]	5.3 ± 2.0	4.7 ± 1.7	2.0 ± 1.2[a]	0.6 ± 0.5[a]
Lym ($\times10^3/\mu l$)	2.5 ± 0.9	3.0 ± 1.1	3.0 ± 1.3	2.5 ± 0.4	2.3 ± 0.8	2.9 ± 1.1	2.8 ± 1.0	2.9 ± 0.8	2.4 ± 0.8	3.3 ± 0.9	2.9 ± 1.0	2.2 ± 0.3	2.0 ± 0.9
Mono ($\times10^3/\mu l$)	0.4 ± 0.1	0.5 ± 0.1	0.4 ± 0.1	0.5 ± 0.1	0.6 ± 0.1	0.4 ± 0.1	0.5 ± 0.1	0.5 ± 0.2	0.5 ± 0.2	0.5 ± 0.1	0.6 ± 0.2	1.1 ± 0.8	0.9 ± 1.0
Eos ($\times10^3/\mu l$)	0.5 ± 0.1	0.6 ± 0.1	0.7 ± 0.1	0.8 ± 0.1	0.8 ± 0.1	0.6 ± 0.1	0.7 ± 0.1	0.8 ± 0.1	0.6 ± 0.3	0.7 ± 0.1	0.6 ± 0.1	0.5 ± 0.2	0.6 ± 0.2
Baso ($\times10^3/\mu l$)	0.04 ± 0.0	0.06 ± 0.0	0.06 ± 0.0	0.06 ± 0.0	0.06 ± 0.0	0.05 ± 0.0	0.04 ± 0.0	0.05 ± 0.0	0.04 ± 0.0	0.05 ± 0.0	0.06 ± 0.0	0.04 ± 0.0	0.03 ± 0.0

Results are expressed as mean ± SD. WBC, white blood cell count; RBC, red blood cell count; Hb, hemoglobin concentration; HCT, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; RDW, red cell distribution width; PLT, platelet count; MPV, mean platelet volume; PDW, platelet distribution width; Neu, neutrophil count; Lym, lymphocyte count; Mono, monocyte count; Eos, eosinophil count; Baso, basophil count. [a]$p < 0.05$ compared to Time 0 h (collection time) by a repeated-measures one-way ANOVA. $n = 6$.

TABLE 2 | Effect of temperature and time storage on morphological findings in blood smears from healthy donkeys **(A)** and donkeys with experimentally-induced endotoxemia **(B)**.

Artifact	4°C					24°C				35°C			
	0 h	6 h	12 h	24 h	48 h	6 h	12 h	24 h	48 h	6 h	12 h	24 h	48 h
(A)													
% crenocytes	1 ± 0	3 ± 1	7 ± 1[a]	13 ± 3[a]	16 ± 2[a]	10 ± 1[a]	10 ± 3[a]	11 ± 3[a]	15 ± 3[a]	10 ± 2[a]	15 ± 4[a]	21 ± 4[a]	25 ± 3[a]
% ruptured WBC	1 ± 1	4 ± 1	9 ± 1[a]	17 ± 2[a]	24 ± 4[a]	3 ± 1	10 ± 1[a]	19 ± 3[a]	27 ± 5[a]	20 ± 5[a]	21 ± 6[a]	33 ± 9[a]	44 ± 9[a]
Toxic neutrophils	0 (0–0)	0 (0–0)	0 (0–1)	1 (0–1)	1 (0.25–1)	1 (0.25–1)	1 (0.25–1)	2.5 (2–3)[a]	3.5 (3–4)[a]	1 (0.25–1)	1 (0.25–1)	3.5 (3–4)[a]	4.5 (4–5)[a]
Pyknotic leukocytes	0 (0–0)	0 (0–0)	1 (0.25–1)	2 (2–2)[a]	2.5 (2–3)[a]	1 (0.25–1)	1 (0.25–1)	2.5 (2–3)[a]	2.5 (2–3)[a]	1 (0.25–1)	2.5 (2–3)[a]	3 (3–3)[a]	3.5 (3–4)[a]
Degranulated eosinophils	0 (0–0)	0 (0–0)	0 (0–0.75)	1.5 (1–2)	2.5 (2–3)[a]	0 (0–0)	0 (0–0.75)	1.5 (1–2)	2.5 (2–3)[a]	0 (0–0.75)	0 (0–0.75)	2.5 (2–3)[a]	3.5 (3–4)[a]
Platelet clumps	0 (0–0)	0 (0–0.75)	1 (1–1)	1 (1–2)	1 (1–2)	0 (0–0)	0 (0–0.75)	1 (1–1)	1 (1–2)	0 (0–0.75)	0 (0–0.75)	1 (1–1)	1 (1–2)
(B)													
% crenocytes	5 ± 1	10 ± 2	15 ± 3[a]	14 ± 3[a]	20 ± 3[a]	10 ± 3	12 ± 2[a]	12 ± 4[a]	19 ± 4[a]	20 ± 3[a]	16 ± 5[a]	17 ± 5[a]	16 ± 5[a]
% ruptured WBC	2 ± 1	26 ± 6[a]	35 ± 7[a]	39 ± 8[a]	49 ± 10[a]	29 ± 8[a]	35 ± 8[a]	48 ± 11[a]	51 ± 10[a]	35 ± 6[a]	46 ± 9[a]	48 ± 9[a]	55 ± 8[a]
Toxic neutrophils	3.5 (3–4)	3.5 (3–4)	3.5 (3–4)	3.5 (3–4)	3.5 (3–4)	3.5 (3–4)	3.5 (3–4)	3.5 (3–4)	3.5 (3–4)	3.5 (3–4)	3.5 (3–4)	4 (3–4)	4 (3–4)
Pyknotic leukocytes	0 (0–0)	0 (0–0)	0.5 (0–1)	1.5 (1–2)	2 (2–2)[a]	0 (0–0)	1 (1–1)	1.5 (1–2)	2 (2–2)[a]	1.5 (1–2)	2 (2–2)[a]	2.5 (2–3)[a]	3.5 (3–4)[a]
Degranulated eosinophils	0 (0–0)	1.5 (1–2)	1.5 (1–2)	1.5 (1–2)	2 (2–2)[a]	1.5 (1–2)	1.5 (1–2)	2 (2–2)[a]	1.5 (1–2)	2 (2–2)	1.5 (1–2)	1.5 (1–2)	2 (2–2)[a]
Platelet clumps	1 (1–1)	1 (1–2)	1 (1–2)	1 (1–1)	1.5 (1–2)	1 (1–1)	1 (1–1)	1 (1–1)	1 (1–2)	1 (1–1)	1 (1–1)	1 (1–1)	1 (1–2)

Results are expressed as mean ± SD (% crenocytes and ruptured WBC) or median (25th percentile−75th percentile) for the rest of artifacts. Toxic neutrophils were categorized in a scale 0–9. Pyknotic leukocytes, degranulated eosinophils and platelet clumps were classified in a scale 0–4. WBC, white blood cell counts; C, Celsius degrees. [a]$p < 0.05$ compared to Time 0 h (collection time) by a repeated-measures one-way ANOVA (% crenocytes and ruptured WBC) or $p < 0.05$ compared to collection time using a Friedman test (rest of artifacts). $n = 6$.

TABLE 3 | Effect of temperature and storage time on blood samples from donkeys with experimentally-induced endotoxemia.

Parameter	Collection 0 h	4°C				24°C				35°C			
		6 h	12 h	24 h	48 h	6 h	12 h	24 h	48 h	6 h	12 h	24 h	48 h
WBC ($\times10^3/\mu l$)	1.9 ± 0.4	1.6 ± 0.4	1.4 ± 0.2	1.4 ± 0.4	1.4 ± 0.4	1.6 ± 0.3	1.6 ± 0.4	1.5 ± 0.4	1.0 ± 0.1[a]	1.6 ± 0.3	1.4 ± 0.2	1.4 ± 0.1	0.7 ± 0.0[a]
RBC ($\times10^6/\mu l$)	6.7 ± 1.9	7.2 ± 1.2	7.4 ± 1.3	7.9 ± 1.5	7.1 ± 2.8	6.8 ± 1.7	6.6 ± 0.8	7.4 ± 1.5	5.9 ± 2.8	7.6 ± 0.6	7 ± 1.5	6.6 ± 1.8	4.2 ± 0.7
Hb (g/dl)	14.9 ± 1.5	14.6 ± 1.8	14.8 ± 2.0	15.4 ± 2.3	14.9 ± 2.6	14.6 ± 1.8	14.0 ± 1.5	14.8 ± 1.9	14.6 ± 2.3	14.7 ± 1.0	15.3 ± 0.8	14.4 ± 2.4	15.5 ± 1.0
HCT (%)	37 ± 11	40 ± 6.1	40 ± 5.2	43 ± 8.2	39 ± 15	37 ± 8.1	36 ± 4.2	40 ± 6.6	32 ± 14	41 ± 5.5	38 ± 8.1	37 ± 10	22 ± 4.6[a]
MCV (fl)	54 ± 2.3	55 ± 2.8	54 ± 2.8	55 ± 1.9	55 ± 2.0	55 ± 3.4	54 ± 2	54 ± 2.2	55 ± 2.0	54 ± 3	54 ± 2.2	56 ± 2.0	51 ± 2.7[a]
MCH (pg)	23 ± 7.4	19 ± 1.2	20 ± 1.3	19 ± 1.4	23 ± 10	22 ± 1.6	21 ± 1.1	20 ± 2.5	29 ± 13	19 ± 1.5	24 ± 2.2	22 ± 4.0	36 ± 6.5[a]
MCHC(g/dl)	43 ± 4.2	38 ± 1.0	38 ± 1.0	38 ± 1.5	42 ± 1.8	41 ± 1.1	38 ± 1.9	37 ± 3.5	52 ± 9.1	35 ± 2.6	43 ± 8.1	40 ± 7.6	71 ± 9.5[a]
RDW (%)	19 ± 0.1	19 ± 0.2	19 ± 0.3	19 ± 0.1	19 ± 0.3	19 ± 0.3	19 ± 0.2	19 ± 0.3	19 ± 0.4	19 ± 0.3	19 ± 0.4	20 ± 0.5	20 ± 0.7
PLT($\times10^3/\mu l$)	145 ± 31	133 ± 31	112 ± 22	103 ± 23	104 ± 30	160 ± 31	168 ± 31	139 ± 42	102 ± 48	126 ± 14	97 ± 22	125 ± 58	148 ± 71
MPV (fl)	6.0 ± 0.7	6.1 ± 0.9	5.9 ± 0.4	5.8 ± 0.8	5.5 ± 0.7	4.9 ± 0.4	4.7 ± 0.5	5.2 ± 1.0	5.6 ± 1.3	4.9 ± 0.5	5.3 ± 0.5	4.7 ± 0.5	4.7 ± 1.1
PDW (%)	19 ± 0.4	20 ± 0.7	20 ± 0.4	21 ± 1.6	21 ± 0.7	20 ± 0.5	20 ± 0.5	21 ± 1.3	22 ± 2.0	19 ± 0.5	21 ± 1.4	20 ± 1.1	24 ± 1.7[a]
Neu ($\times10^3/\mu l$)	0.6 ± 0.2	0.7 ± 0.3	0.6 ± 0.2	0.6 ± 0.2	0.4 ± 0.2	0.7 ± 0.2	0.7 ± 0.2	0.6 ± 0.2	0.1 ± 0.0[a]	0.7 ± 0.2	0.5 ± 0.1	0.4 ± 0.1	0.1 ± 0.0[a]
Lym ($\times10^3/\mu l$)	1.1 ± 0.5	0.6 ± 0.2	0.6 ± 0.1	0.6 ± 0.2	0.7 ± 0.5	0.6 ± 0.3	0.7 ± 0.2	0.7 ± 0.3	0.7 ± 0.1	0.7 ± 0.2	0.6 ± 0.2	0.7 ± 0.2	0.3 ± 0.0[a]
Mono ($\times10^3/\mu l$)	0.05 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.0 ± 0.0
Eos ($\times10^3/\mu l$)	0.0 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Baso ($\times10^3/\mu l$)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

Results are expressed as mean ± SD. WBC, white blood cell count; RBC, red blood cell count; Hb, hemoglobin concentration; HCT, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; RDW, red cell distribution width; PLT, platelet count; MPV, mean platelet volume; PDW, platelet distribution width; Neu, neutrophil count; Lym, lymphocyte count; Mono, monocyte count; Eos, eosinophil count; Baso, basophil count. [a]$p < 0.05$ compared to Time 0 h (collection time) by a repeated-measures one-way ANOVA. $n = 6$.

endotoxemia under different storage conditions. Main artifactual changes, both in healthy and endotoxemic donkeys, were observed when blood was kept at 24 and 35°C during 24 and 48 h, while results from blood samples at 4°C were closer to the analysis performed at collection time (0 h).

A previous systematical study on storage of hematological samples in horses recommended conservation at 24°C (13). In our study, blood from healthy donkeys stored at room temperature developed toxic changes in neutrophils as soon as 24 h and neutropenia 48 h post-storage. Since neutropenia and the presence of toxic neutrophils are key features for the diagnosis of sepsis/SIRS in adults and neonates (25, 27), these artifacts could lead to misdiagnosis and unnecessary treatments and economic losses. These discrepancies between donkeys and horses in the effect of temperature on blood parameters could be due to different viability of neutrophils, although variations amongst analyzers used in each study should also be considered (12, 15). The pseudotoxic changes observed on blood smears kept at 24°C also has been reported in dogs (20). Lysed WBCs significantly increased (compared to basal) at 12 h in samples kept at 4 and 24°C. Since this artifactual change can interfere with the impedance and laser-based techniques used by most hematology analyzers to classify leukocytes, a cautious interpretation of differential counts should be recommended on aged samples from donkeys under these conditions.

Storage of blood samples at 4°C is considered more suitable in humans, rodents and dogs (14, 15, 17, 28). This has also been occasionally advised for horses by some authors (29), although published data contradicts this statement (13). In our study, no changes were observed using the analyzer in samples kept at this temperature. However, morphological artifacts (crenocytes and ruptured leukocytes) significantly increased in blood stored for 12 or more hours, which underlines the importance of a rapid blood smear evaluation, even when the sample is kept in the refrigerator. Platelet clumping has been reported to appear earlier in samples kept at 4°C in several species (12, 17). In our study, clumps were observed in samples kept at every tested temperature without significant differences. Nonetheless, these samples were not flagged with PA by the analyzer. While the manufacturer provides no information in this subject, the requirement of a certain amount of aggregates or markedly large clumps in order to display this flag could be a feasible explanation. Newer analyzers, able to perform both impedance and optical-based platelet counts, have been proved to partially solve this problem (17).

Samples stored at 4°C showed no increase in toxic indicators for at least 48 h, which is markedly longer than reported in dogs, where pseudo-Döhle bodies and other toxic changes were apparent at 24 h (20).

Little information is available about the effect of keeping blood in warm environments on hematological parameters. Storage at 35°C caused important changes on both red and white cell counts as soon as 24 h post-sampling, likely secondary to cell lysis and leukoagglutination (15, 19). In contrast, these parameters were not altered in a previous report on pigs, cattle and goats blood stored at 30°C during 48 h (30). Whether these differences are due to species-specific idiosyncrasies or variations in analyzer technologies should be studied. Morphological artifacts on blood smears at 24 and 35°C resembled those from endotoxemic donkeys, which could have prompted a misdiagnosis in these samples. Since lysed WBCs were significantly increased as soon

as 12 h post-sampling, the impact of these abnormalities on the results of the analyzer should also be considered.

LPS administration caused quantitative hematological findings and morphological toxic changes similar to those reported in horses with endotoxemia and SIRS (25, 27). Leukocytopenic samples are proved to be more prone to artifactual changes during storage compared to normal samples in humans (31). Alterations related to storage in endotoxemic blood were similar to those observed in healthy samples, with refrigeration avoiding any significant difference compared to basal results. Prolonged (48 h) storage of endotoxemic samples at 24 and 35°C accentuated pre-existing leukopenia and neutropenia. Although this finding could not affect the diagnosis, it could influence on the prognosis and the evaluation of the response to the treatment (25). Interestingly, toxic neutrophils did not significantly vary during the storage at 24 and 35°C, which could be due to lower percentage of viable neutrophils.

Artifactual changes also depends on the analyzer used for hematological evaluation (12, 15). As previously reported in healthy donkeys (10), proper cell type recognition can be problematic in LaserCyte and blood smears are recommended to verify the results. No sample from this study was flagged as IQA (Internal Quality Assurance Error), which secure that every determination met the analyzer's internal quality assurance checks. Flagging was more prevalent in samples stored for 48 h at higher temperatures; as well as in endotoxemic blood, where cell viability was already altered. Cell lysis, cell swelling and artifactual cytoplasmic changes could be responsible for most of these warnings. The most common observed flag was related to high MCHC, indicating most probably artifactual hemolysis. As stated, platelet aggregates were consistently observed even in unflagged samples, indicating the necessity of a proper blood smear examination in donkey samples, even in the absence of flags.

One limitation of this study is the low number of animal included. Similarly, our results depend on the hematological analyzer used and further studies should be needed in order to describe storage effect on donkey blood using more advanced hematological analyzers. A more delayed sampling post-LPS infusion could have allowed more pronounced abnormalities in blood smear. Finally, it could be of interest to study storage artifacts in blood from donkeys with naturally occurring endotoxemia or SIRS.

To the author's knowledge, this is the first study evaluating the effects of storage temperature and time on donkey hematological parameters both in healthy and those with experimentally-induced endotoxemia Our findings underline the importance of storing donkey blood samples at 4°C, instead of 24°C as recommended for horses, and perform the analysis within 12 h of collection. Moreover, due to the appearance of pseudotoxic changes, blood smear interpretation should be cautious in samples stored longer than 24 h at 24°C or warmer environments.

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 study was conducted under permits 19-03-15-212 (Welfare Committee of the Conserjeria de Salud y Familias, Junta de Andalucia) and 2015PI/05 (Welfare Committee of the University of Cordoba).

AUTHOR CONTRIBUTIONS

AP-E, AB-C, and FM contributed conception and design of the study. AB-C, RA-A, CG-D, and FM performed the experiments concerning induced endotoxemia. AP-E, FM, and CG-D performed hematological determinations. FM organized the database and performed the statistical analysis. AP-E wrote the first draft of the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This study was supported by the Plan Propio de Investigacion from the University of Cordoba (Spain), by the Programa Operativo de fondos FEDER Andalucía (Spain), and Plan Andaluz de Investigacion (AGR-227 group) from the Consejeria de Salud de la Junta de Andalucia (Spain).

ACKNOWLEDGMENTS

The author would like to thank Dr. Juan Antonio Caballero Molina (Dpto. Estadística, Investigación Operativa, Org. de Empresas y Economía Aplicada from the University of Cordoba) for his helpful advice on statistical methods.

REFERENCES

1. Burden F, Thiemann A. Donkeys are different. *J Equine Vet Sci.* (2015) 35:376–82. doi: 10.1016/j.jevs.2015.03.005
2. Camillo F, Rota A, Biagini L, Tesi M, Fanelli D, Panzani D. The current situation and trend of donkey industry in Europe. *J Equine Vet Sci.* (2018) 65:44–9. doi: 10.1016/j.jevs.2017.11.008
3. Costa LR, Aleman M, Davis E. Comprehensive report of the caseload of donkeys and mules presented to a veterinary medical teaching hospital over a ten-year period. *Animals.* (2019) 9:413–26. doi: 10.3390/ani9070413
4. Bazzano M, McLean A, Tesei B, Gallina E, Laus F. Selenium and vitamin E concentrations in a healthy donkey population in central Italy. *J Equine Vet Sci.* (2019) 78:112–6. doi: 10.1016/j.jevs.2019.04.003
5. Mendoza FJ, Perez-Ecija RA, Monreal L, Estepa JC. Coagulation profiles of healthy Andalusian donkeys are different than those of healthy horses. *J Vet Intern Med.* (2011) 25:967–70. doi: 10.1111/j.1939-1676.2011.0748.x
6. Mendoza FJ, Perez-Ecija RA, Toribio RE, Estepa JC. Thyroid hormone concentrations differ between donkeys and horses. *Equine Vet J.* (2013) 45:214–8. doi: 10.1111/j.2042-3306.2012.00622.x

7. Dinev D, Khubenov KD. Normal values of the haematological, biochemical and enzymological indices of the donkey. *Vet Med Nauki.* (1986) 23:69–75.
8. Zinkl JG, Mae D, Merida PG, Farver TB, Humble JA. Reference ranges and the influence of age and sex on hematologic and serum biochemical values in donkeys (*Equus asinus*). *Am J Vet Res.* (1990) 51:408–13.
9. Bonelli F, Rota A, Corazza M, Serio D, Sgorbini M. Hematological and biochemical findings in pregnant, postfoaling, and lactating jennies. *Theriogenology.* (2016) 85:1233–8. doi: 10.1016/j.theriogenology.2015.12.004
10. Perez-Ecija A, Gonzalez-De Cara CA, Aguilera-Aguilera R, Estepa JC, Rubio MD, Mendoza FJ. Comparison of donkey hemogram using the LaserCyte hematology analyzer, an impedance system, and a manual method. *Vet Clin Pathol.* (2014) 43:525–37. doi: 10.1111/vcp.12179
11. Dezzutto D, Barbero R, Valle E, Giribaldi M, Raspa F, Biasato I, et al. Observations of the hematological, hematochemical, and electrophoretic parameters in lactating donkeys (Equus asinus). *J Equine Vet Sci.* (2018) 65:1–5. doi: 10.1016/j.jevs.2018.02.013
12. Ameri M, Schnaars HA, Sibley JR, Honor DJ. Stability of hematologic analytes in monkey, rabbit, rat, and mouse blood stored at 4°C in EDTA using the ADVIA 120 hematology analyzer. *Vet Clin Pathol.* (2011) 40:188–93. doi: 10.1111/j.1939-165X.2011.00304.x
13. Clark P, Mogg TD, Tvedten HW, Korcal D. Artifactual changes in equine blood following storage, detected using the Advia 120 hematology analyzer. *Vet Clin Pathol.* (2002) 31:90–4. doi: 10.1111/j.1939-165X.2002.tb00286.x
14. Cora MC, King D, Betz LJ, Wilson R, Traylos GS. Artifactual changes in Sprague-Dawley rat hematologic parameters after storage of samples at 3 °C and 21 °C. *JALAS.* (2012) 51:616–21.
15. Furlanello T, Tasca S, Caldin M, Carli E, Patron C, Tranquillo M, et al. Artifactual changes in canine blood following storage, detected using the ADVIA 120 hematology analyzer. *Vet Clin Pathol.* (2006) 35:42–6. doi: 10.1111/j.1939-165X.2006.tb00087.x
16. Medaille C, Briend-Marchal A, Braun JP. Stability of selected hematology variables in canine blood kept at room temperature in EDTA for 24 and 48 hours. *Vet Clin Pathol.* (2006) 35:18–23. doi: 10.1111/j.1939-165X.2006.tb00083.x
17. Layssol-Lamour C, Lavabre T, Braun JP, Trumel C, Bourges-Abella N. The effects of storage at 4°C and 20°C on the hemograms of C57BL/6 mice and Wistar rats using the IDEXX ProCyte Dx and blood smear evaluations. *Vet Clin Pathol.* (2019) 48:652–67. doi: 10.1111/vcp.12784
18. Starkey P, Starkey M. Regional and world trends in donkey populations. In: Fielding D, Starkey P, editors. *Donkeys, People and Development. Animal Traction Network for Eastern and Southern Africa (ATNESA).* Wageningen: ATNESA (2004). p. 10–21.
19. Dalal BI, Brigden ML. Artifacts that may be present on a blood film. *Clin Lab Med.* (2002) 22:81–100. doi: 10.1016/S0272-2712(03)00068-4
20. Bau-Gaudreault L, Grimes CN. Effect of time and storage on toxic or pseudo-toxic change in canine neutrophils. *Vet Clin Pathol.* (2019) 48:400–5. doi: 10.1111/vcp.12755
21. Mendoza FJ, Gonzalez-De Cara C, Aguilera-Aguilera R, Buzon-Cuevas A, Perez-Ecija A. Meloxicam ameliorates the systemic inflammatory response syndrome associated with experimentally induced endotoxemia in adult donkeys. *J Vet Intern Med.* (2020) 34:1631–41. doi: 10.1111/jvim.15783
22. El-Ashker MR, El-Sebaei MG, Aamer HG. The influence of experimentally-induced endotoxaemia on clinical variables and markers of systemic inflammation in donkeys (*Equus asinus*). *Vet Med-Czech.* (2017) 62:117–24. doi: 10.17221/133/2016-VETMED
23. Alcott CJ, Sponseller BA, Wong DM, Davis JL, Soliman AM, Wang C, et al. Clinical and immunomodulating effects of ketamine in horses with experimental endotoxemia. *J Vet Intern Med.* (2011) 25:934–43. doi: 10.1111/j.1939-1676.2011.0749.x
24. Barton MH, Parviainen A, Norton N. Polymyxin B protects horses against induced endotoxaemia in vivo. *Equine Vet J.* (2004) 36:397–401. doi: 10.2746/0425164044868350
25. Lambert JL, Fernandez NJ, Roy MF. Association of presence of band cells and toxic neutrophils with systemic inflammatory response syndrome and outcome in horses with acute disease. *J Vet Intern Med.* (2016) 30:1284–92. doi: 10.1111/jvim.13968
26. Fernandez NJ, Gilroy CV, Wagg CR, Kwong GPS, Roy MF. Intra- and inter-rater agreement for the detection of band neutrophils and toxic change in horses. *Vet Clin Pathol.* (2019) 48:668–76. doi: 10.1111/vcp.12811
27. Corley KTT, Furr MO. Evaluation of a score designed to predict sepsis in foals. *J Vet Emerg Crit Car.* (2003) 13:149–55. doi: 10.1046/j.1435-6935.2003.00098.x
28. Lippi G, Salvagno GL, Solero GP, Franchini M, Guidi GC. Stability of blood cell counts, hematologic parameters and reticulocytes indexes on the Advia A120 hematologic analyzer. *J Lab Clin Med.* (2005) 146:333–40. doi: 10.1016/j.lab.2005.08.004
29. Newman AW. Practical tips on sample handling for hematology, chemistry, and cytology testing for equine patients: getting more bang for your buck. *Vet Clin N Am Equine.* (2020) 36:1–14. doi: 10.1016/j.cveq.2019.12.002
30. Ihedioha JI, Onwubuche RC. Artifactual changes in PCV, hemoglobin concentration, and cell counts in bovine, caprine, and porcine blood stored at room and refrigerator temperatures. *Vet Clin Pathol.* (2007) 36:60–3. doi: 10.1111/j.1939-165X.2007.tb00183.x
31. Gulati GL, Hyland LJ, Kocher W, Schwarting R. Changes in automated complete blood cell count and differential leukocyte count results induced by storage of blood at room temperature. *Arch Pathol Lab Med.* (2002) 126:336–42. doi: 10.1043/0003-9985(2002)126<0336:ciacbc>2.0.co;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: 10 September 2020
doi: 10.3389/fvets.2020.00631

Collagen and Eosinophils in Jenny's Endometrium: Do They Differ With Endometrial Classification?

Jordi Miró[1], Miguel Gutiérrez-Reinoso[1], Joana Aguiar da Silva[2], Carina Fernandes[2], Maria Rosa Rebordão[2,3], Graça Alexandre-Pires[2], Jaime Catalán[1] and Graça Ferreira-Dias[2]*

[1] Equine Reproduction Service, Department of Animal Medicine and Surgery, Faculty of Veterinary Medicine, Autonomous University of Barcelona, Cerdanyola del Vallès, Spain, [2] Faculdade de Medicina Veterinária, CIISA - Centro de Investigação Interdisciplinar em Sanidade Animal, Universidade de Lisboa, Lisbon, Portugal, [3] Coimbra College of Agriculture, Polytechnic Institute of Coimbra, Coimbra, Portugal

Collagen fibers and inflammatory cells are the basis for jenny endometrium Kenney and Doig's classification developed for the mare. The infiltration of a large number of eosinophils in the jenny endometrium is intriguing. Eosinophil and fibroblast produced IL33, which has been related to fibrosis development and chronicity. This work on the endometrium consisted of (i) quantification of collagen type I (COL1A2), type III (COL3A1), and IL33 transcripts; (ii) histological localization and quantification of COL1 and COL3 proteins; and (iii) eosinophil and neutrophil count and correlation with collagen area and IL33 transcripts. Localization of COL protein in the jenny endometrium was also compared to the mare endometrium. As fibrosis increased, eosinophil and neutrophil count decreased ($P < 0.05$). A 5-fold increase in IL33 transcripts was noted from categories IIA to III. There was a tendency toward a positive correlation between eosinophil count and IL33 transcripts in category IIA endometrium ($P = 0.055$). Neither transcripts of COL1A2 nor COL3A1 nor the areas of COL1 or COL3 differed with endometrial categories. Unlike for the mare, and regardless of the jenny endometrium classification, COL3 was always found to different extents in the stratum compactum, while COL1 was mainly present in deep stroma. As fibrosis progressed in the mare, an extensive increase in COL1 fibers was notorious under the surface epithelium. Correlations between neutrophil count and COL1 and COL3 areas were observed in the jenny endometrium, although no correlation was found for eosinophil count. Neutrophil count positive correlation with the COL1 area and negative correlation with the COL3 area in endometria with mild lesions suggest that neutrophils in the jenny endometrium may be involved in fibrogenesis. In addition, when eosinophilia subsides, the endometrium reacts with fibrosis establishment, which could be stimulated by the pro-fibrotic cytokine IL33, whose release might then be ascribed to fibroblasts. Further studies are needed to analyze the effect of the presence of COL3 next to the surface epithelium in the stratum compactum, or around the endometrial glands on jenny's endometrial function and fertility.

Keywords: donkey, endometrium, collagen, fibrosis, endometrosis, eosinophils, IL-33

OPEN ACCESS

Edited by:
Amy Katherine McLean, University of California, Davis, United States

Reviewed by:
Marco Antonio Alvarenga, São Paulo State University, Brazil
Miguel Quaresma, Universidade de Trás os Montes e Alto Douro, Portugal

****Correspondence:***
Jordi Miró
jordi.miro@uab.cat

Specialty section:
This article was submitted to Comparative and Clinical Medicine, a section of the journal Frontiers in Veterinary Science

Received: *01 May 2020*
Accepted: *03 August 2020*
Published: *10 September 2020*

Citation:
Miró J, Gutiérrez-Reinoso M, Silva JAd, Fernandes C, Rebordão MR, Alexandre-Pires G, Catalán J and Ferreira-Dias G (2020) Collagen and Eosinophils in Jenny's Endometrium: Do They Differ With Endometrial Classification? Front. Vet. Sci. 7:631. doi: 10.3389/fvets.2020.00631

INTRODUCTION

In jenny's endometrium, the infiltration of eosinophils and the presence of fibrosis to different extents might influence endometrial function and gestation in a dissimilar fashion to the mare. The frequent infiltration of a large number of eosinophils in jenny's endometrium, unlike for the mare, appears to be a physiological feature of the jenny endometrium (1). The eosinophils appear to regulate tissue homeostasis and contribute for inflammatory reactions in tissue remodeling and innate immunity (2). In general, eosinophils are considered as the major effector cells in type two inflammatory diseases, including asthma, helminthic infection, and allergy (2). In fact, eosinophils result from the differentiation of CD117+ progenitor cells, directly stimulated by interleukin 33 (IL33) (3). In airway inflammation, IL33 besides exacerbating eosinophil infiltration in an autocrine and paracrine manner also attracts macrophages, lymphocytes, IL-13, and TGF-β1 (3). A relationship between eosinophil infiltration and the expression of IL33 has also been referred in chronicity and development of fibrosis in liver, skin, and intestine (4–6).

In the mare endometrium, the excessive deposition of collagen in the extracellular matrix, predominantly around the endometrial glands and stroma, which is associated with a chronic degenerative condition, destruction of tissue architecture, and impairment of endometrial function, is named endometriosis (7, 8). In tissue repair, collagen type III (COL3) is the first one to be expressed, being replaced by collagen type I (COL1) in later stages of fibrogenesis (9). Nevertheless, in the mare endometrium at the transcript level, age appeared not to influence COL1 or COL3 mRNA (10). In contrast, quantification of the areas in the endometrium occupied by COL1 and COL3, stained with histochemical picrosirius red staining and visualized by polarized light microscopy (11, 12), showed an increase in COL1 in the tissues with severe endometriosis, in comparison to healthy endometria or with mild fibrotic changes, where COL3 was the most predominant type (10). A previous morphometric analysis of periglandular fibrosis in equine endometria using picrosirius red also referred a predominance of COL1 in severe endometriosis (13).

The main purpose of this work was to contribute to the understanding of the inflammatory processes and fibrosis in jenny's endometrium, classified according to Kenney and Doig's mare grading system (14). Therefore, the main goals of the present study consisted of (i) quantification of transcripts of collagen type I (COL1A2), type III (COL3A1), and IL33 in the endometrium; (ii) localization and quantification of COL1 and COL3 protein in histological sections; and (iii) assessment of eosinophil and neutrophil count and their correlation with collagen and IL33 transcripts. In addition, localization of the COL protein in jenny's endometrium was compared to mare endometrium histological sections.

MATERIALS AND METHODS

Animals and Sample Collection

A total of 80 Catalonian jennies, between 3 and 22 years of age, were divided in eight jenny groups, which were brought together for 75 days (April 1st/June 15th, 2017) with ten Catalan jackasses, aged 5–9 years and of proven fertility, for natural breeding. Jennies were assigned to different groups according to selection criteria. All animals were included in the Catalonian donkey Studbook and under a selection program. One month after jackass removal, a pregnancy diagnosis was done by transrectal ultrasonography using a MyLab™ Gamma (Esaote, Genova, Italy) device.

All animals were owned by a commercial donkey farm, FUIVES (Berga, Barcelona, Spain). Donkeys were kept in paddocks and fed grain forage, straw, hay, and water *ad libitum*.

A total of 29 jennies did not get pregnant (63.75% fertility rate), and 23 of them were included in the present study. Six jennies were discarded for different reasons: three of them presented cervical stenosis, one died, one showed a severe skin disease, and the other one had never foaled before. Endometrial biopsies were obtained during estrous from 23 non-pregnant jennies through the cervix, with an alligator jaw biopsy punch (Kevorkian's uterine biopsy forceps, Hauptner and Herberholz, Solingen, Germany), at estrus. All 23 jennies used in this study had foaled at least once before. Each biopsy was divided into two pieces. One portion was immediately immersed in buffered formaldehyde, for paraffin blocks, and further processed for histological examination. The other piece of the endometrium was placed in cryotubes with RNAlater® (AM7020; Ambion, Applied Biosystems, Foster, CA, USA) and kept at −80°C, for gene transcription studies, by real-time PCR.

Histopathological Analysis

For histopathological analysis, 5-μm-thick sections of formaldehyde-fixed endometrium were stained with hematoxylin (05-06014E; Bio-Optica) and eosin (HT1103128; Sigma-Aldrich). Every jenny endometrial biopsy was evaluated by light microscopy (Leica DM500) regarding the histopathology of endometrial glands, presence of inflammatory cells (neutrophils, eosinophils), and fibrosis in the stroma. Neutrophil and eosinophil count was performed at random in 10 fields/biopsy, at the magnification of × 400. Due to the inexistence of a classification method for jenny's endometrium, the Kenney and Doig (14) grading system for the mare endometrium was used. Thus, a healthy endometrium was considered as category I; an endometrium with mild fibrotic lesions and inflammation as category IIA; when these lesions become moderate as category IIB; or when they are severe as category III (14).

Real-Time PCR for COL1A2, COL3A1, and IL33

Quantification of COL1A2, COL3A1, and IL33 transcripts was accomplished by reverse transcriptase, followed by real-time polymerase chain reaction (qPCR), as previously described (15). Briefly, after endometrial total RNA extraction with TRI Reagent® (T9424; Sigma) according to the manufacturer's instructions, a NanoDrop system (ND200C; Fisher Scientific, Hampton, PA, USA) was used for RNA quantification. Visualization of 28S and 18S rRNA bands on a 1.5% agarose gel confirmed RNA quality. Reverse transcription was performed using M-MLV Reverse Transcriptase (M170B, Promega®) from

TABLE 1 | Primer sequences used in real-time PCR analysis.

Gene (Accession number)	Sequence 5′-3′	Amplicon (base pairs)
COL1A2 (NM_001323780.1)	Forward: CAAGGGCATTAGGGGACACA Reverse: ACCCACACTTCCATCGCTTC	196
COL3A1 (XM_014852914.1)	Forward: CAAAGGAGAGCCAGGAGCAC Reverse: CTCCAGGCGAACCATCTTTG	98
GAPDH (XM_014834961.1)	Forward: CACCCACTCTTCCACCTTCG Reverse: CTTGCTGGGTGATTGGTGGT	173
IL33 (XM_014860749.1)	Forward: CCCCAGCAAAGATGAACAGC Reverse: TGGTACATGCCTTCTGGTGGT	179

COL1A2—Equus asinus collagen type I alpha 2 chain; COL3A1—Equus asinus collagen, type III, alpha 1; GAPDH—Equus asinus glyceraldehyde-3-phosphate dehydrogenase; IL33—Equus asinus interleukin 33.

1 μg of total RNA in a 20-μL reaction volume using an oligo (dT) primer (C1101, Promega®).

Real-time PCR was used for the assessment of mRNA transcription of COL1A2, COL3A1, and IL-33. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was chosen as the reference gene [<2-fold changes between different biological conditions; (16)] from four potential validated genes (ribosomal protein L32, succinate dehydrogenase A complex, subunit A, flavoprotein, and beta-2-microglobulin). Internet-based program Primer-3 (17) and Primer Premier softwares (Premier Biosoft Interpairs, Palo Alto, CA, USA) were used to design the primers listed in **Table 1**.

Target and reference gene amplification was performed on StepOnePlus™ Real-Time PCR System (Applied Biosystems®, California, USA) using Power SYBR Green PCR Master Mix (Applied Biosystems®), after primer concentration optimization. Polymerase chain reaction (PCR) products were run on a 2.5% agarose gel to confirm specificity. Results were evaluated using Real-time PCR Miner 4.0 software. For gene quantification, the average efficiency of each gene (E) and the average cyclic threshold (CT) of each sample were used, in the formula $r_0 = [1/(1 + E)\ CT]$ (18). Relative expression values were calculated by normalizing the expression level of the target genes against that of the reference gene.

Picrosirius Red Staining

In order to assess the extent and localization of COL1 and COL3 in jenny's endometrium, a histological section, other than the one stained with hematoxylin and eosin for histopathological evaluation, was stained with Picrosirius Red Stain (ab#150681, Abcam plc, Cambridge, UK), following the manufacturer's instructions. Thus, a picrosirius red solution was placed onto deparaffinized endometrium sections and incubated for 1 h. Afterward, slides were rinsed with acetic acid solution and absolute alcohol. The slides were observed with an upright widefield microscope (Olympus BX51, Olympus Corporation, Tokyo, Japan) at 200× magnification (20×/0.75NA objective) under polarized light, and ten random images from each stained endometrium section were acquired with a camera (TIS 2MP RGB), with a pixel size of 0.441 μm/pixel (12). Collagen type one fibers appeared in red, while COL3 fibers stained green. A qualitative evaluation of the endometrium sections was performed to enable the identification of the areas where each type of collagen was present. Moreover, a quantitative analysis was performed with ImageJ program (Version 1.52a, National Institutes of Health, USA).

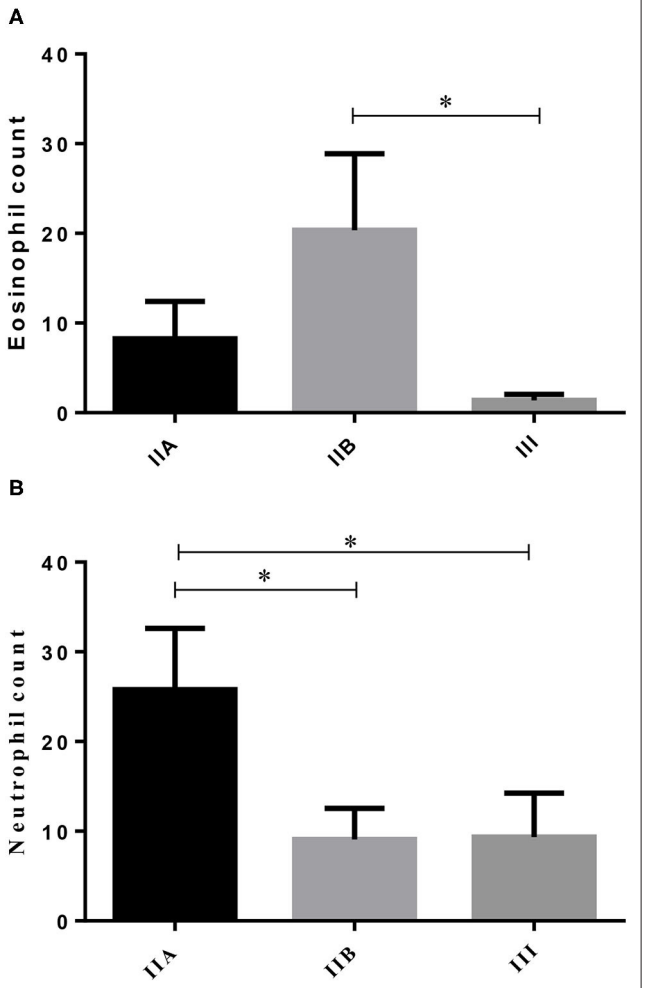


FIGURE 1 | Eosinophil **(A)** and neutrophil **(B)** count in different endometrial categories (IIA, IIB, or III) of the jenny. Bars represent mean ± SEM. Asterisk indicates significant differences between endometrial categories (*$P < 0.05$).

In order to better understand if the localization of collagen fibers, namely, COL1 and COL3, in jenny's endometrium was specific to this species and could be the possible explanation for their longevity in reproduction as compared to the mare, endometrial histological sections from mares, graded according to Kenney and Doig (14), were also stained with PSR and observed for a qualitative assessment.

Statistical Analysis

Data regarding the area in the endometrial biopsy occupied by COL1 or COL3, and jenny's age in relation to endometrial

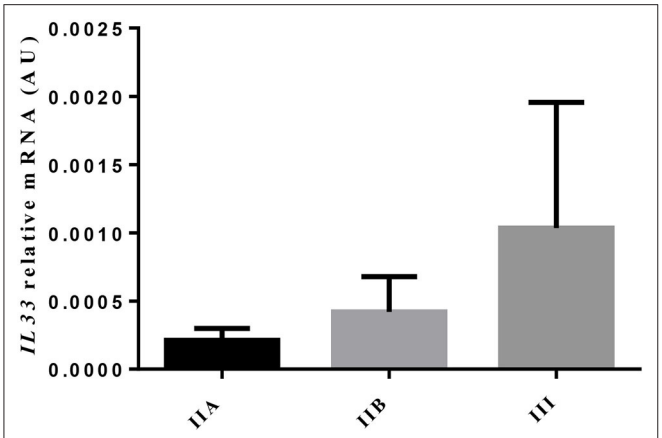


FIGURE 2 | Relative IL33 mRNA levels in different endometrial categories (IIA, IIB, or III) of the jenny. Bars represent mean ± SEM.

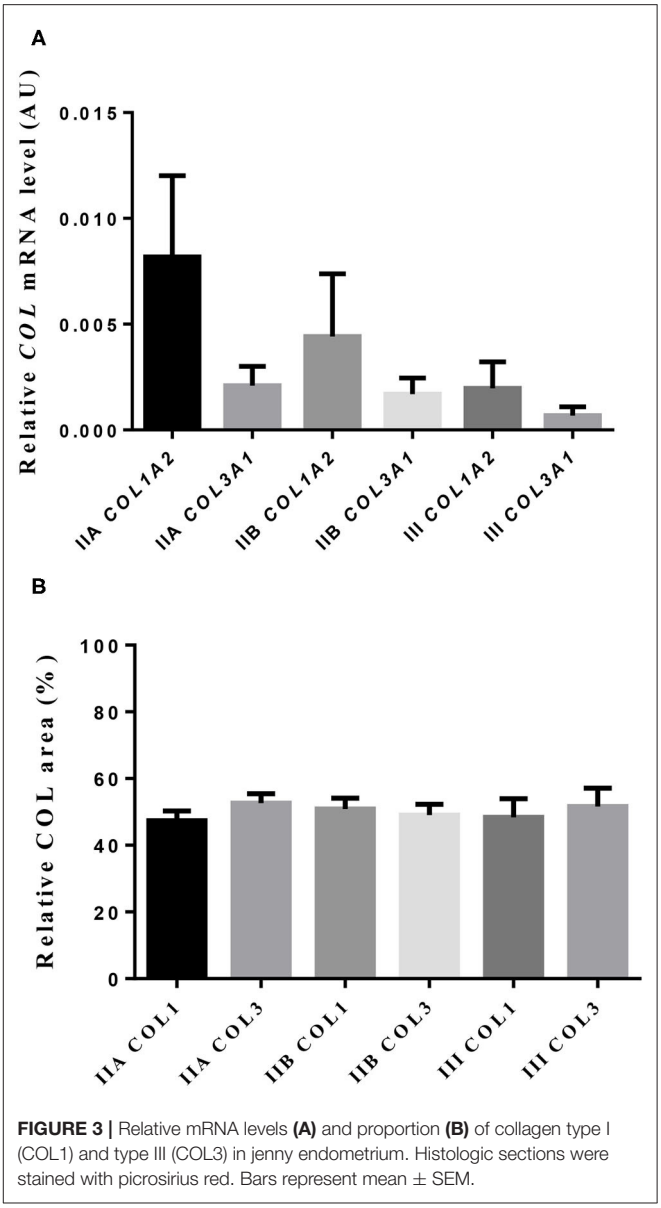


FIGURE 3 | Relative mRNA levels **(A)** and proportion **(B)** of collagen type I (COL1) and type III (COL3) in jenny endometrium. Histologic sections were stained with picrosirius red. Bars represent mean ± SEM.

category were statistically analyzed by one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test. In order to compare the number of eosinophils or neutrophils with IL33, COL1, or COL3 transcripts between endometrial categories, a Kruskal–Wallis analysis followed by Dunn's multiple-comparison test was used. Spearman rank correlation analysis between eosinophil or neutrophil count and IL33 mRNA levels and area occupied by COL1 or COL3 within each endometrial category was also performed. The software GraphPad Prism, Version 6.0, San Diego, CA, USA, was used to analyze these data. Significance was defined with the value of $P < 0.05$. Unless otherwise specified, the presented results are expressed as mean ± SEM.

RESULTS

Histological Classification

From the 23 endometrial biopsies obtained from the jennies, six endometria were graded as category IIA (12.5 ± 3.9 years old), 11 as category IIB (13.8 ± 4.2 y), and six as category III (13.6 ± 2.2 y), according to Kenney and Doig's mare endometrial classification (14). No age differences were found among endometrial categories ($P > 0.05$). As fibrosis increased, from categories IIB to III, a significant 14-fold decrease in the number of eosinophils was depicted (**Figure 1A**; $P < 0.05$). Regarding neutrophil count, there was a fall from the category IIA endometrium to category IIB, and from categories IIA to III. No correlation was found between age and any of the studied factors (PMN, eosinophils, COL1, COL3, COL1, COL3, and IL33 transcripts) (**Figure 1B**; $P > 0.05$).

Real-Time PCR

The transcripts of IL-33, a cytokine involved in eosinophilia and in chronicity and development of fibrosis (6), showed no difference, as endometrial fibrosis advanced, from jenny's endometria with little collagen fibers, such as category IIA, to category III endometria, where fibrosis is visibly established (data not shown). Nevertheless, a 4.9-fold increase in IL33 mRNA levels was noted from category IIA to category III endometrium, even though not significant (**Figure 2**). When all endometrial categories were considered, no correlation was found between eosinophil count and IL33 transcripts (data not shown). However, there was a tendency toward a positive correlation between eosinophil count and IL33 transcripts in category IIA endometrium ($\rho = 0.7714$; $P = 0.055$), mainly due to the effect of a single sample (one out of six) that clearly tipped the line toward significance (data not shown).

Neither transcripts of COL1A2 nor COL3A1 differed between jenny's endometrial categories (**Figure 3A**). However, a 4-fold drop in COL1A2 and a 3-fold fall in COL3A1 in the mRNA levels between category IIA and category III were noted.

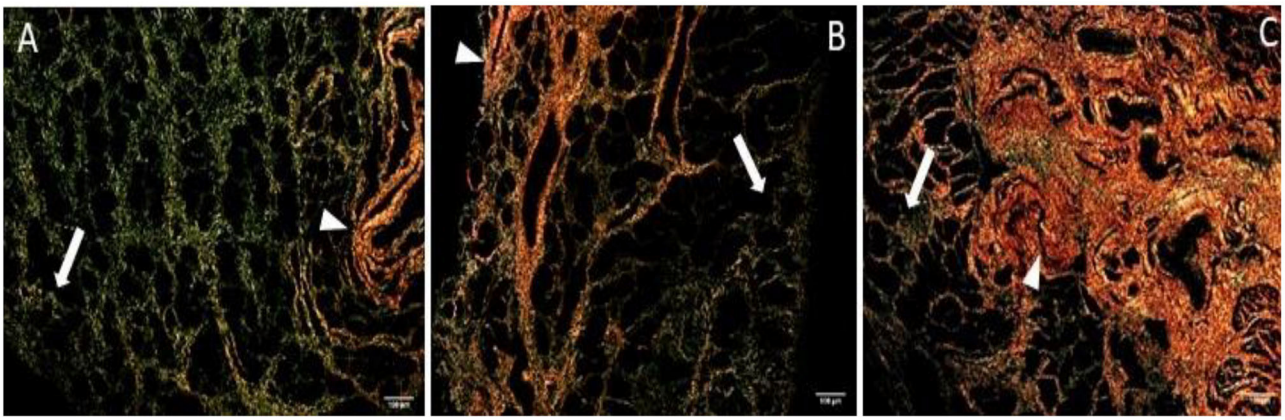


FIGURE 4 | Jenny endometrium sections stained with picrosirius red and observed under polarized light microscopy. Collagen type I (COL1) stains red (arrow head), while collagen type III (COL3) stains green (white arrow). **(A)** Category IIA endometrium—a larger extent of COL3 is present in the stratum compactum under the surface epithelium and periglandular. Some COL1 fibers are present in the deeper stroma. **(B)** Category IIB endometrium—COL3 fibers are depicted under the basement membrane in the stratum compactum and periglandular, but COL3 fibers are also intertwined with some COL1 fibers. **(C)** Category III endometrium—COL3 fibers are still found under the basement membrane and periglandular, but more COL1 is present in the deep stroma, surrounding endometrial glands.

Picrosirius Red Staining

The quantitative assessment of the different types of COL in the jenny endometrium has shown no differences in the area occupied by either COL1 or COL3 (**Figure 3B**). When considering the qualitative evaluation of collagen fibers in jenny's category IIA endometrium, green-stained COL3 was mostly found under the basement membrane and in the stroma, surrounding endometrial glands. However, COL1 fibers, which stained red, were sparse and mainly present in the deeper stroma (**Figure 4**, **Table 2**). In category IIB donkey endometrium, the location of COL3 fibers was also under the basement membrane in the stratum compactum and periglandular, but COL3 fibers were also intertwined with some COL1 fibers. In the stroma, both COL types were found. When fibrosis was strongly established, such as in category III endometrium, COL3 fibers were still found under the basement membrane and periglandular, but more COL1 was seen in the deep stroma, surrounding endometrial glands, lymphatic lacunae, and blood vessels, in comparison to the other Kenney and Doig's endometrial categories (**Figure 4**, **Table 2**). When these data were matched to mare's endometrial localization of COL fibers, visible differences were found between species. In mare endometria graded as category I (not found in the jennies in the present study), COL3 fibers were seen under the basement membrane and in the stratum compactum. As endometriosis progressed, an increase in COL1 fibers was notorious under the basement membrane, which was not seen in the jenny. Actually, in some category III endometria, only COL1 fibers were present (**Figure 5**, **Table 3**).

The correlation between eosinophil or neutrophil count and the area occupied by COL1 or COL3 was also evaluated. Although no correlation was found for eosinophil count (data not shown), correlations between neutrophil count and both COL1 and COL3 areas were observed. Positive correlations were seen for the endometrial area occupied by COL1 when all endometrial categories were considered ($\rho = 0.520$; $P = 0.01$; **Figure 6A**) and for category IIB ($\rho = 0.6770$; $P = 0.03$; **Figure 6B**) but not significantly in category III (**Figure 6C**). Between neutrophil count and COL3 area, a negative correlation was detected when all endometrial categories were considered ($\rho = -0.4716$; $P = 0.03$; **Figure 6D**) and in category IIB ($\rho = -0.6102$; $P = 0.03$; **Figure 6E**). A tendency toward a negative correlation between neutrophil count and COL3 area was also noticed in category III endometrium ($\rho = -0.7537$; $P = 0.07$; **Figure 6F**).

TABLE 2 | Qualitative evaluation of the presence of collagen type I (COL1) and collagen type III (COL3) in jenny endometrium, stained with picrosirius red.

	II A	II B	III
Under basement membrane	COL3	COL3	COL3
Periglandular	COL3	COL3/COL1	COL3/COL1
Stroma	COL3	COL3/COL1	COL1 ↑
Deep stroma	COL1	COL1	COL1 ↑
Around lymphatic			COL1
Around blood vessels			COL1

DISCUSSION

To the best of our knowledge, this is the first study that has evaluated IL33 expression in donkey endometrium and related its transcript levels with eosinophil count and collagen transcripts and deposition. Since IL33 is a critical regulator of a number of processes including inflammation, vascularization, and fibrosis (19, 20), its role on the progression of endometrial inflammation and fibrosis in the jenny is worth evaluating. In fact, IL33 can exacerbate inflammation in collagen-induced arthritis, and in allergic processes (21, 22). Based on studies on peritoneal fluid, serum, or samples from endometriotic lesions in women, it has been suggested that IL33 might play an important role toward inflammation present in advanced-stage endometriosis

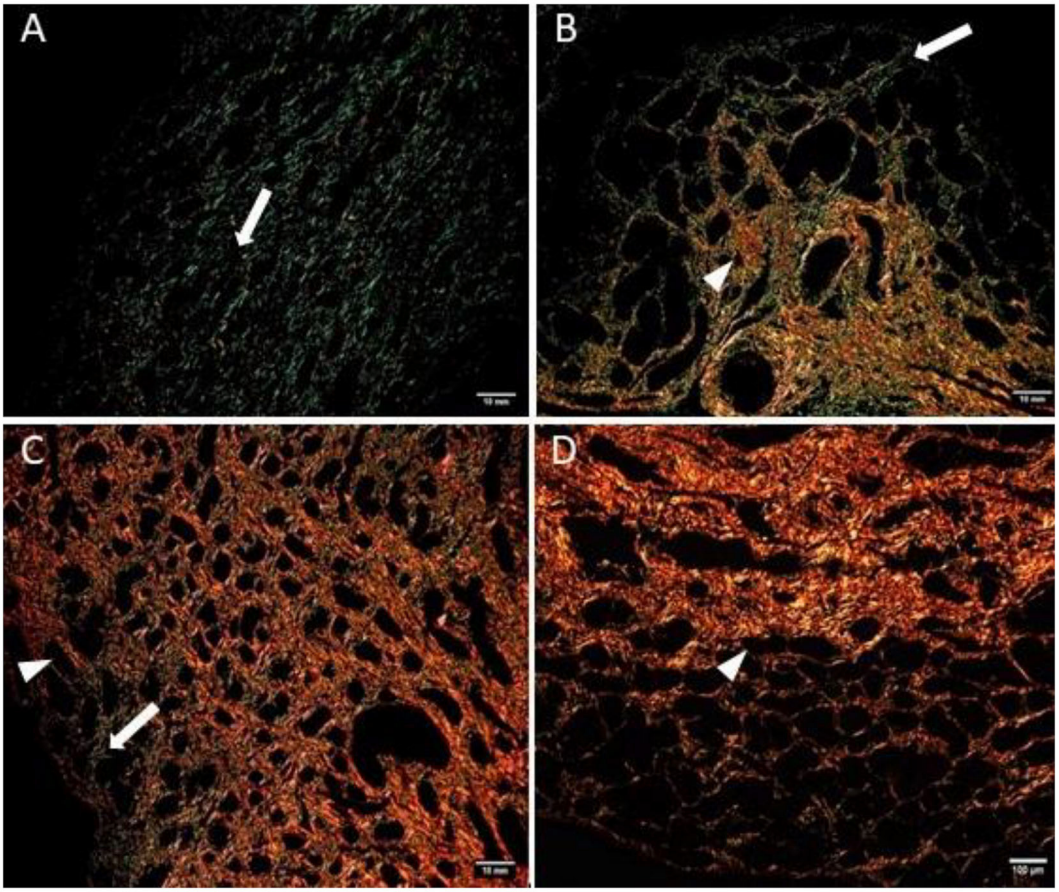


FIGURE 5 | Mare endometrium sections stained with picrosirius red and observed under polarized light microscopy. Collagen type I (COL1) stains red (arrowhead), while collagen type III (COL3) stains green (white arrow). **(A)** Category I endometrium—a larger extent of COL3 is present in the stratum compactum under the surface epithelium and periglandular. **(B)** Category IIA endometrium—COL3 fibers are depicted under the basement membrane in the stratum compactum and periglandular, but COL3 fibers are also intertwined with some COL1 fibers. **(C)** Category IIB endometrium—COL3 fibers are sparsely found under the basement membrane, while COL1 is predominant and present in the deep stroma, surrounding endometrial glands. **(D)** Category III endometrium—COL1 is found almost exclusively in the stratum compactum, periglandular, and under the basement membrane.

TABLE 3 | Qualitative evaluation of the presence of collagen type I (COL1) and collagen type III (COL3) in mare endometrium, stained with picrosirius red.

	II A	II B	III
Under basement membrane	COL3	COL3/COL1	COL1
Periglandular	COL3	COL3/COL1	COL3/COL1
Stroma	COL3	COL3/COL1	COL1 ↑
Deep stroma	COL1	COL1	COL1 ↑
Around lymphatic vessels			COL1
Around blood vessels			COL1

patients and on the progression of the disease (23, 24). Even though endometriosis in women, which consists of the abnormal growth of endometrial tissue outside the uterus (24, 25), and jenny's endometrial fibrosis are different conditions, at a certain stage of their development they both undergo an inflammatory process that ultimately may lead to fibrosis, as an end-point (25–29). We have shown that IL33 transcripts were detected in all jenny endometrial samples, regardless of their histopathological classification. Even though not reaching significance, IL33 mRNA levels in category III jenny endometrium were 5-fold increased, with respect to category IIA, while eosinophil count was about 14-fold lower in severe fibrosis than in healthier tissue (category IIA). The development of fibrosis and its chronicity in liver, skin, and intestine has been related to eosinophil infiltration and the expression of IL33 (4–6). Since a direct relationship between tissue eosinophil infiltration and the expression of IL33 has been referred, the present data on jenny endometria appears somewhat unexpected and cumbersome. In other species and tissues, accumulated observations have improved the understanding of eosinophil stimulation and ability to infiltrate tissues and participate in disease pathogenesis (30, 31). Even though rarely described, endometritis eosinophilica in the mare is a separate endometrial disease responsible for infertility (31). Nevertheless, deciphering the role of eosinophils in jenny's endometrium is still a challenge. It is plausible that jenny's endometrium with mild to moderate fibrosis (categories IIA and IIB) can

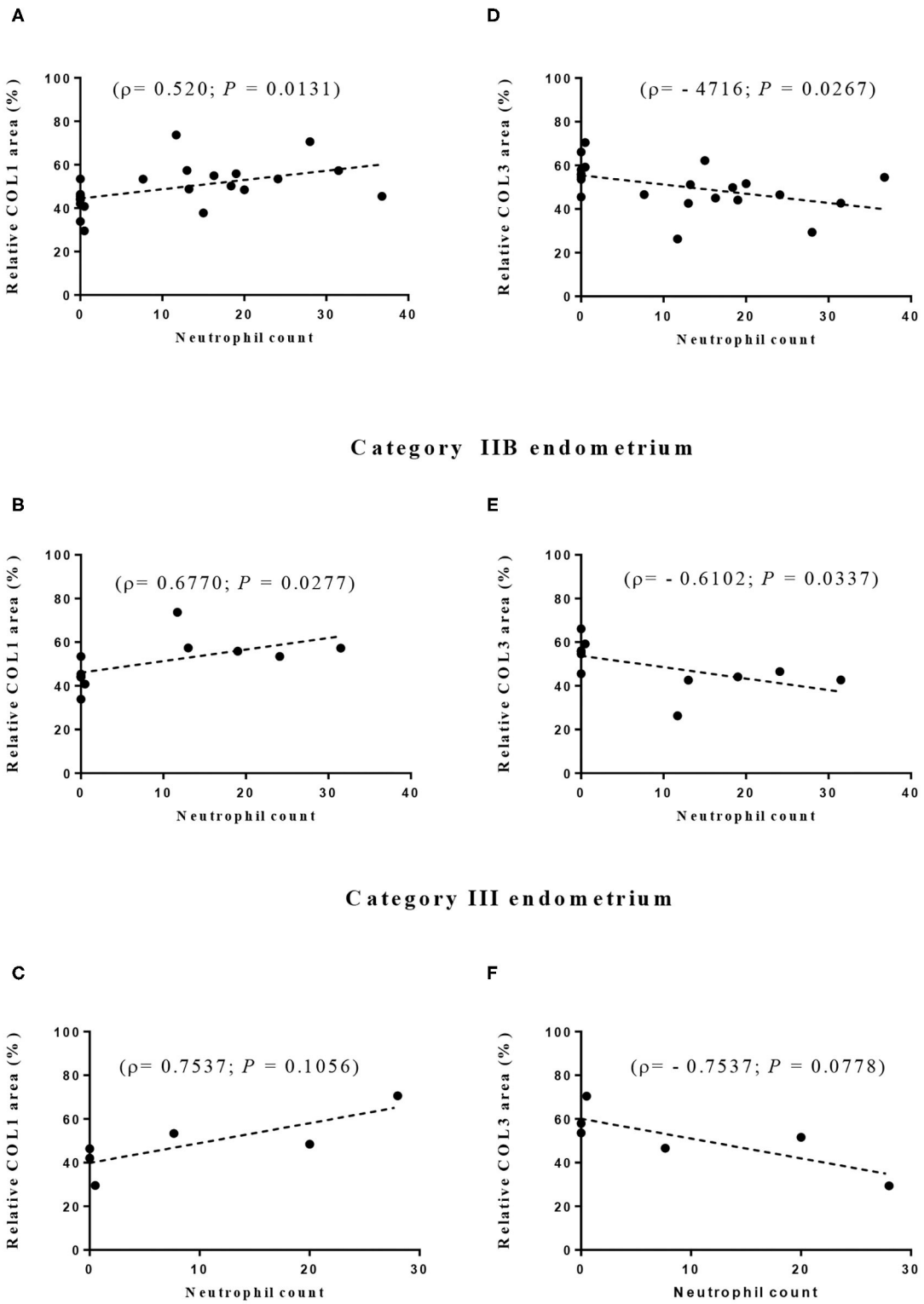


FIGURE 6 | Correlation between neutrophil count and relative proportion of collagen type I (COL1—**A–C**) and type III (COL3—**D–F**) in all **(A,D)** and in IIB **(B,E)**, and III **(C,F)** jenny endometrial categories. ρ—Spearman rank correlation.

mount an inflammatory reaction characterized by the presence of eosinophils and neutrophils. This eosinophil infiltration might be mediated by IL33. In fact, our results appear to indicate a trend toward a positive correlation between eosinophil count and IL33 transcripts in category IIA endometrium. Unlike other cytokines, when epithelial or endothelial cell damage or cell necrosis occurs, IL33 is passively released, suggesting that it may function as an alarmin that stimulates the immune system [reviewed by Chan et al. (22)]. However, in severe fibrosis (category III), as in other tissues where fibrosis is linked to impairment of organ function (32), the endometrial cells may no longer be capable of mounting an inflammatory reaction. Alternatively, eosinophil infiltration subsides, and the tissue reacts with fibrosis establishment, which could be stimulated by the still present pro-fibrotic cytokine IL33 (19), or by other pro-fibrotic stimuli (33). In spite of a decline in eosinophil infiltration in category III endometrium, the release of IL33 might be ascribed to other cells rather than eosinophils, such as fibroblasts [reviewed by Chan et al. (22)]. Nevertheless, this should be further investigated in jenny's endometrium.

In the present study, as fibrosis progressed from categories IIA to III, a 4-fold decrease in COL1A2 and a 3-fold decrease in COL3A1 in the mRNA levels were noted, although not significant. In addition, the percentage of areas occupied by COL1 and COL3 fibers in jenny endometria was similar, despite endometrial histopathological characteristics, in contrast to what we have previously shown for the mare endometrium. A preliminary work on Masson's trichrome-stained histological sections of jenny endometrium classified as I, IIA, or IIB was not conclusive about putative differences in the total area occupied by COL fibers (34). In contrast, in the mare, COL3 was the most predominant type of collagen in healthy endometrium, in comparison to severe endometriosis where COL1 was mostly found (10).

Interestingly, when evaluating the topographic histological distribution of COL1 and COL3 in jenny's endometrium, a different pattern was consistent, despite the histopathological classification and the progress of fibrosis. Even in category III endometrium, there was an area occupied by COL3 fibers under the basement membrane adjacent to the luminal epithelium, in the stratum compactum. In contrast, in the mare endometrium, COL3 fibers under the luminal epithelium were replaced by COL1, as fibrogenesis progressed. In fact, the arrangement of collagen fibers in the mare endometrium and their characteristics were related to the evolution of the process (35, 36). Mare foaling rates do not overcome 11% in category III endometrium (37). However, this correlation remains unknown in donkeys. The analysis of the Catalonian donkey stud book showed that only 3.3% of foaled jennies are older than 15 years of age[1]. Moreover, the generation interval index (average age of one jenny and its selected offspring) of this donkey breed is 7.75 ± 0.25 years[1]. However, based on these data, it is impossible to calculate jenny's reproductive longevity or overall fertility rate, let alone for each endometrial category. In addition, it has been referred that in mare endometriotic tissue, the incidence of periglandular fibrosis should not be based on the increased presence of collagen fibers (38). Instead, the arrangement of fibroblasts around endometrial glands, in one or more layers, which produce the extracellular matrix proteins such as collagen IV, laminin, and fibronectin, should be taken into consideration (38).

The connection between inflammation and fibrosis is well-recognized (31). One putative link between neutrophil and fibrogenesis has been provided by neutrophil extracellular traps (NETs, released by neutrophils) triggering the differentiation of myofibroblasts, cells responsible for COL deposition (17). In the mare, NET components induced an *in vitro* increase in COL1 in the endometrium (39). Likewise, in mares with closed cervix, a marked endometritis and intense neutrophil infiltration, and permanent pathological endometrial changes, including fibrosis were observed (40). In a recent study on jenny's endometrial cytology, an increase in neutrophil count in older and multiparous jennies was referred (41). In the present study, the neutrophil count positive correlations with the area occupied by COL1, and the negative correlation with the area of COL3 observed in endometria with mild lesions, suggest that the presence of neutrophils in jenny's uterus may be involved in endometrial fibrogenesis.

In conclusion, there are important differences in the collagen distribution between jenny and mare endometria. The presence of COL3 next to the surface epithelium, in the stratum compactum, and around endometrial glands might not have a deleterious effect on implantation and endometrial function, as it appears to occur in mares with category III endometrium. Further studies are needed to correlate these findings with jenny's fertility and age Therefore, donkey endometrium classification should be reconsidered. In addition, further studies are needed to unravel the unknown role of neutrophils and eosinophils present in jenny's endometrium on the reproductive performance of 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

Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

JM and GF-D conceived the study, participated in the sample acquisition and analysis, helped conduct the data analysis, contributed to the writing of the original draft, and reviewing and editing the manuscript. MG-R and JC participated in the jenny control, sample acquisition, and process and data curation. JS, CF, and GA-P performed the sample formal analysis, data curation, and analysis. MR participated in sample formal analysis, data curation, and analysis and writing of the original draft. All authors contributed to the article and approved the submitted version.

[1]Rac.uab.cat/enllacos/racenlla.htm

FUNDING

This work was supported by a competitive grant from the Fundação para a Ciência e Tecnologia (PTDC-CVT-REP-4202-2014) and by Project UIDP/CVT/00276/2020 (funded by FCT).

ACKNOWLEDGMENTS

We would like to thank Mrs. Maria do Rosário Luís, from the Laboratory of Pathology (FMV) and Dr. Telmo Nunes from the Laboratory of Microscopy, for the valuable assistance on biopsies histological preparation and observation, respectively.

REFERENCES

1. Vilés K, Rabanal R, Rodríguez-Prado M, Miró J. Effect of ketoprofen treatment on the uterine inflammatory response after AI of jennies with frozen semen. *Theriogenology*. (2013) 79:1019–26. doi: 10.1016/j.theriogenology.2013.01.006
2. Rothenberg ME, Hogan SP. The eosinophil. *Annu Rev Immunol*. (2006) 24:147–74. doi: 10.1146/annurev.immunol.24.021605.090720
3. Gervasi MG, Osycka-Salut C, Caballero J, Vazquez-Levin M, Pereyra E, Billi S, et al. Anandamide capacitates bull spermatozoa through CB1 and TRPV1 activation. *PLoS ONE*. (2011) 6:e16993. doi: 10.1371/journal.pone.0016993
4. Mchedlidze T, Waldner M, Zopf S, Walker J, Rankin AL, Schuchmann M, et al. Interleukin-33-dependent innate lymphoid cells mediate hepatic fibrosis. *Immunity*. (2013) 39:357–71. doi: 10.1016/j.immuni.2013.07.018
5. Rankin AL, Mumm JB, Murphy E, Turner S, Yu N, McClanahan TK, et al. IL-33 induces IL-13–dependent cutaneous fibrosis. *J Immunol*. (2010) 184:1526–35. doi: 10.4049/jimmunol.0903306
6. Masterson JC, Capocelli KE, Hosford L, Biette K, McNamee EN, De Zoeten EF, et al. Eosinophils and IL-33 perpetuate chronic inflammation and fibrosis in a pediatric population with stricturing crohn's ileitis. *Inflamm Bowel Dis*. (2015) 21:2429–40. doi: 10.1097/MIB.0000000000000512
7. Kenney RM. The aetiology, diagnosis and classification of chronic degenerative endometritis (CDE) (endometriosis). In: Allen WR, editor. *Equine Endometritis Worship. Proceedings of the John P. Hughes International Workshop on Equine Endometritis; 1992 Aug 19-20*. Newmarket (1993). doi: 10.1111/j.2042-3306.1993.tb02940.x
8. Hoffmann C, Ellenberger C, Mattos RC, Aupperle H, Dhein S, Stief B, et al. The equine endometrosis: new insights into the pathogenesis. *Anim Reprod Sci*. (2009) 111:261–78. doi: 10.1016/j.anireprosci.2008.03.019
9. Bochsler PN, Slauson DO. Inflammation and repair of tissue. In: Slauson DO, Cooper BJ, editors. *Mechanisms of Disease: A Textbook of Comparative General Pathology*. St. Louis, MO: Mosby (2002). p. 140–245.
10. Pinto-Bravo P, Reborddo MR, Amoral A, Fernandes C, Cuello C, Parrilla I, et al. Is mare endometrosis linked to oviduct fibrosis? *Pferdeheilkunde*. (2018) 34:43–6. doi: 10.21836/PEM20180107
11. Junqueira LCU, Cossermelli W, Brentani R. Differential staining of collagens type I, II and III by sirius red and polarization microscopy. *Arch Histol Jap*. (1978) 41:267–74. doi: 10.1679/aohc1950.41.267
12. Podico G, Canisso IF, Roady PJ, Austin SM, Carossino M, Balasuriya U, et al. Uterine responses and equine chorionic gonadotropin concentrations after two intrauterine infusions with kerosene post early fetal loss in mares. *Theriogenology*. (2020) 147:202–10. doi: 10.1016/j.theriogenology.2019.11.014
13. Porto CD. *Caracterização histoquímica do colágeno e expressão de MMP-2, MMP-9 e TIMP-1 nas endometrites crônicas das éguas* (Dissertation/Master's thesis). Botucatu: University Estadual Paulista "Júlio de Mesquita Filho" (2006).
14. Kenney R, Doig PA. Equine endometrial biopsy. In: Morrow DA, editor. *Current Therapy in Theriogenology*. Philadelphia, PA: WB Saunders (1986). p. 723–9.
15. Rebordão MR, Amaral A, Lukasik K, Szóstek-Mioduchowska A, Pinto-Bravo P, Galvão A, et al. Impairment of the antifibrotic prostaglandin E 2 pathway may influence neutrophil extracellular traps–induced fibrosis in the mare endometrium. *Domest Anim Endocrinol*. (2019) 67:1–10. doi: 10.1016/j.domaniend.2018.10.004
16. Dheda K, Huggett JF, Bustin SA, Johnson MA, Rook G, Zumla A. Validation of housekeeping genes for normalizing RNA expression in real-time PCR. *Biotechniques*. (2004) 37:112–9. doi: 10.2144/04371RR03
17. Chrysanthopoulou A, Mitroulis I, Apostolidou E, Arelaki S, Mikroulis D, Konstantinidis T, et al. Neutrophil extracellular traps promote differentiation and function of fibroblasts. *J Pathol*. (2014) 233:294–307. doi: 10.1002/path.4359
18. Zhao S, Fernald RD. Comprehensive algorithm for quantitative real-time polymerase chain reaction. *J Comput Biol*. (2005) 12:1047–64. doi: 10.1089/cmb.2005.12.1047
19. Gao Q, Li Y, Li M. The potential role of IL-33/ST2 signaling in fibrotic diseases. *J Leukoc Biol*. (2015) 98:15–22. doi: 10.1189/jlb.3RU0115-012R
20. Millar NL, O'Donnell C, McInnes IB, Brint E. Wounds that heal and wounds that don't – the role of the IL-33/ST2 pathway in tissue repair and tumorigenesis. *Semin Cell Dev Biol*. (2017) 61:41–50. doi: 10.1016/j.semcdb.2016.08.007
21. Liew FY. IL-33: a janus cytokine. *Ann Rheum Dis*. (2012) 71:101–4. doi: 10.1136/annrheumdis-2011-200589
22. Chan BCL, Lam CWK, Tam LS, Wong CK. IL33: roles in allergic inflammation and therapeutic perspectives. *Front Immunol*. (2019)10:364. doi: 10.3389/fimmu.2019.00364
23. Santulli P, Borghese B, Chouzenoux S, Vaiman D, Borderie D, Streuli I, et al. Serum and peritoneal interleukin-33 levels are elevated in deeply infiltrating endometriosis. *Hum Reprod*. (2012) 27:2001–9. doi: 10.1093/humrep/des154
24. Miller JE, Monsanto SP, Ahn SH, Khalaj K, Fazleabas AT, Young SL, et al. Interleukin-33 modulates inflammation in endometriosis. *Sci Rep*. (2017) 7:1–11. doi: 10.1038/s41598-017-18224-x
25. Bulun SE. Endometriosis. *N Engl J Med*. (2009) 360:268–79. doi: 10.1056/NEJMra0804690
26. Itoga T, Matsumoto T, Takeuchi H, Yamasaki S, Sasahara N, Hoshi T, et al. Fibrosis and smooth muscle metaplasia in rectovaginal endometriosis. *Pathol Int*. (2003) 53:371–5. doi: 10.1046/j.1440-1827.2003.01483.x
27. Vicino M, Scioscia M, Resta L, Marzullo A, Ceci O, Selvaggi LE. Fibrotic tissue in the endometrioma capsule: surgical and physiopathologic considerations from histologic findings. *Fertil Steril*. (2009) 91:1326–8. doi: 10.1016/j.fertnstert.2008.02.157
28. Sokkar SM, Hamouda MA, El-Rahman SM. Endometritis in she donkeys in Egypt. *J Vet Med B Infect Dis Vet Public Health*. (2001) 48:529–36. doi: 10.1046/j.1439-0450.2001.00457.x
29. Canisso IF, Panzani D, Miró J, Ellerbrock RE. Key aspects of donkey and mule reproduction. *Vet Clin North Am Equine Pract*. (2019) 35:607–42. doi: 10.1016/j.cveq.2019.08.014
30. Akuthota P, Weller PF. Eosinophils and disease pathogenesis. *Semin Hematol*. (2012) 49:113–9. doi: 10.1053/j.seminhematol.2012.01.005
31. Grimm AL, Schoon H-A, Schöniger S. Histopathological features of endometritis eosinophilica in mares. *Histol Histopathol*. (2017) 32:1161–73. doi: 10.14670/HH-11-872
32. Zeisberg M, Kalluri R. Cellular mechanisms of tissue fibrosis. 1. Common and organ-specific mechanisms associated with tissue fibrosis. *J Physiol Cell Physiol*. (2013) 304:216–25. doi: 10.1152/ajpcell.00328.2012
33. Sziksz E, Pap D, Lippai R, Béres NJ, Fekete A, Szabó AJ, et al. Fibrosis related inflammatory mediators: role of the IL-10 cytokine family. *Mediators Inflamm*. (2015) 2015:764641. doi: 10.1155/2015/764641
34. Pires MA, Ferreira-Dias G, Catarino J, Bastos-de-Carvalho C, Silvestre M, Nóvoa M, et al. Correlation between morphological characterization of jenny (*Equus asinus*) endometrial biopsy and quantification of collagen deposition by image analysis. *J Comp Pathol*. (2019) 166:139. doi: 10.1016/j.jcpa.2018.10.125

35. Masseno, APB. *Assessment of endometrial fibrosis and myofibroblasts in endometriosis active and inactive mares* (Dissertation/Ph.D. thesis). Botucatu: University Estadual Paulista "Júlio de Mesquita Filho" (2012).
36. Costa, LD. *Histochemical and immunohistochemical characterization of fibrotic changes in mares endometrosis* (Dissertation/Master's thesis). Botucatu: University Estadual Paulista "Júlio de Mesquita Filho" (2015).
37. Kenney RM. Cyclic and pathologic changes of the mare endometrium as detected by biopsy, with a note on early embryonic death. *J Am Vet Med Assoc*. (1978) 172:241–62.
38. Walter I, Handler J, Reifinger M, Aurich, C. Association of endometriosis in horses with differentiation of periglandular myofibroblasts and changes of extracellular matrix proteins. *Reproduction*. (2001) 121:581–6. doi: 10.1530/reprod/121.4.581
39. Rebordão MR, Amaral A, Lukasik K, Szóstek-Mioduchowska A, Pinto-Bravo P, Galvão A, et al. Constituents of neutrophil extracellular traps induce *in vitro* collagen formation in mare endometrium. *Theriogenology*. (2018) 113:8–18. doi: 10.1016/j.theriogenology.2018.02.001
40. Reilas T, Rivera del Alamo MM, Liepina E, Yeste M, Katila T. Effects on the equine endometrium of cervical occlusion after insemination. *Theriogenology*. (2016) 85:617–24. doi: 10.1016/j.theriogenology.2015.09.053
41. Quartuccio M, Cristarella S, Medica P, Fazio E, Mazzullo G, Rifici C, et al. Endometrial cytology during the different phases of the estrous cycle in jennies: new evidences. *Animals*. (2020) 10:1062. doi: 10.3390/ani10061062

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 October 2020
doi: 10.3389/fvets.2020.569587

Label-Free Mass Spectrometry-Based Quantitative Proteomics Analysis of Serum Proteins During Early Pregnancy in Jennies (*Equus asinus*)

Liang Deng, Yuwei Han, Chi Tang, Qingchao Liao and Zheng Li*

Department of Animal Genetics, Breeding and Reproduction, College of Animal Science and Veterinary Medicine, Shenyang Agricultural University, Shenyang, China

Early pregnancy in jennies is routinely determined by palpation per rectum or ultrasonography and also by detecting steroid hormone and chorionic gonadotropin levels in the blood, plasma, and serum. Herein we applied label-free mass spectrometry-based quantitative proteomics to identify serum proteins that were differentially expressed between early pregnant (day 45 after ovulation) and non-pregnant jennies. Bioinformatics analysis allowed illustration of pathways potentially involved in early pregnancy. We identified 295 proteins from a total of 2,569 peptides. Twenty-five proteins (22 upregulated and three downregulated) were significantly differentially expressed between the early pregnant and non-pregnant groups. The majority of the differentially expressed proteins were involved in defense response, early embryonic development, and hormone signaling pathways. Furthermore, functional protein analyses suggested that proteins were involved in binding, enzyme inhibitor activity, and enzyme regulator activity. Five serum proteins—granulin precursor/acrogranin, transgelin-2, fibronectin, fibrinogen-like 1, and thrombospondin 1—can be considered as novel, reliable candidates to detect pregnancy in jennies. To the best of our knowledge, this is the first study to use label-free mass spectrometry-based quantitative proteomics to analyze serum proteins during early pregnancy in jennies. Our results should facilitate the identification of valuable pregnancy diagnostic markers in early pregnant jennies.

Keywords: proteomics, jennies, early pregnancy, biomarkers, serum proteins

OPEN ACCESS

Edited by:
Micaela Sgorbini,
University of Pisa, Italy

Reviewed by:
Jose Antonio Tapia,
University of Extremadura, Spain
Matteo Tesi,
University of Pisa, Italy

****Correspondence:***
Liang Deng
ldeng@syau.edu.cn

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

Received: *04 June 2020*
Accepted: *18 September 2020*
Published: *22 October 2020*

Citation:
Deng L, Han Y, Tang C, Liao Q and Li Z (2020) Label-Free Mass Spectrometry-Based Quantitative Proteomics Analysis of Serum Proteins During Early Pregnancy in Jennies (Equus asinus). Front. Vet. Sci. 7:569587. doi: 10.3389/fvets.2020.569587

INTRODUCTION

Donkey (*Equus asinus*), a member of the equids, provides substantial societal benefits to humans (1). Considering its novel and evolving role in milk, meat, and skin production and in animal-assisted therapy, there has been a renewed interest in this species. A decline in donkey population and genetic diversity has become a global trend; thus, successful pregnancies are essential for maintaining effective population size and genetic diversity (2). Pregnancy in jennies (female donkeys) can be determined by palpation per rectum at around 40 days after ovulation (3) or by ultrasonography at around 10 days after ovulation (4).

Steroid hormone variations in the blood of pregnant jennies have been previously investigated. Detecting progesterone and estradiol concentrations in the blood, plasma, and serum has become a method for pregnancy diagnosis in jennies (5).

There is a relatively high concentration of proteins present in the blood, plasma, and serum. The serum concentration of proteins in donkeys is 58–82 g/L (6). Molecular events are regulated by the expression of proteins in the blood. Thus, blood proteome could be used to understand biological mechanisms at a molecular level. The blood, plasma, and serum are widely utilized for diagnostic purposes in clinical practice (7) and for the discovery of novel biomarkers (8).

With the development of proteomics, different fractions of the blood proteome can now be analyzed in detail. Thus far, several proteomics approaches have been widely and effectively applied to explore the blood proteome of equids. Two-dimensional (2D) gel electrophoresis is routinely used for assessing quantitative changes in serum and/or plasma proteins of horses, such as for establishing the serum protein map (9, 10), evaluating changes in plasma proteins after prolonged physical exercise (11), and comparing differences in serum protein expression profiles between distinct breeds (12). Henze et al. (13) evaluated genetic differences in the serum proteome of horses, donkeys, and mules using surface-enhanced laser desorption–ionization time-of-flight mass spectrometry (MS), and they found considerable differences in the proteome of horses and donkeys and that the proteome of mules showed a higher similarity to donkeys than to that of horses.

It is well-known that there is an increase in blood protein synthesis during pregnancy (14). In several species, factors that help diagnose pregnancy have been subjected to in-depth research using proteomics. Some previous studies used high-resolution 2D gel electrophoresis in bovine (15, 16) and buffaloes (17) to identify potential pregnancy-specific markers in the serum and plasma. Furthermore, in pregnant women who delivered at term, Scholl et al. (18) used two-dimensional difference in-gel electrophoresis as well as isotope tagging for relative and absolute quantitation to assess changes in relative protein abundance between paired serum samples collected in the first and third trimesters.

A remarkable feature of equine pregnancy is the development of the invasive trophoblast of the chorionic girdle and its formation of gonadotrophin-secreting endometrial cup cells in early pregnancy. Equine chorionic gonadotropin, the main secretory product of the endometrial cups, is secreted during days 40–150 of pregnancy (19). During this period, numerous proteins are involved in the process of fetomaternal interactions. To the best of our knowledge, there are no published data on serum proteins during early pregnancy in jennies. Thus, using label-free MS-based quantitative proteomics, we herein aimed to identify specific serum proteins during early pregnancy in jennies.

MATERIALS AND METHODS

Animals and Sampling

We used Liaoxi donkeys in this study (20). Nine pregnant jennies at day 45 after ovulation and nine non-pregnant jennies (aged 4–8 years) were randomly chosen at a breeding farm in Liaoning Province, China. The animals were considered healthy based on veterinarian records, physical examination, and reproductive tract examination performed by transrectal palpation and ultrasonography. A "B mode" ultrasound scanner equipped with a 7.0 MHz linear array multi-frequency transducer (WED-3000, Shenzhen WELLD, China) was used to monitor ovarian and uterine activity during estrus and pregnancy. The presence of a preovulatory follicle ($\geq$3.5 cm) indicated impending ovulation. Artificial insemination was performed on the day of ovulation, using fresh semen from the same fertile jack donkey. The animals were first examined for pregnancy 12–15 days after ovulation, and re-examined at 40 and 45 days after ovulation to confirm the pregnancy status. Nine jennies that continued pregnancy to 45 days after ovulation were included into the "pregnant group" (P group). Further, nine jennies that repeated their cyclic estrous activity 21–22 days after the last estrus were included into the "non-pregnant group" (NP group). All jennies were maintained in a stable and outdoor paddock with *ad libitum* access to a mixture of cereal straw and grass hay, maize, bran, peas, minerals, vitamins, and water.

Blood samples from the P and NP groups were collected at day 45 after ovulation, and obtained immediately after confirming the non-pregnant state, respectively. Blood samples were collected at 9–10 am. The blood was drawn from the jugular vein and dispensed into 10-mL disposable vacuum tubes (BD Vacutainer, USA) without an anticoagulant. Within 1 h of collection, the samples were centrifuged at 3,000 *g* for 10 min at 4°C, and the serum thus obtained was stored at −80°C until needed. This study was conducted with the approval of Shenyang Agricultural University Animal Care and Use Committee (Permit no.: 201906025).

Protein Extraction and Digestion

To avoid the influence of individual differences on serum proteins, the nine serum samples obtained from each group were randomly divided into three subgroups. The three serum samples (10 μL/sample) within each subgroup were equally pooled to obtain three biological replicates from each group. Proteins were extracted using SDT lysis buffer (4% SDS, 100 mM DTT, 100 mM Tris-HCl, pH 8.0), and protein concentration was determined using the BCA protein assay kit (Bio-Rad, USA). The protein samples were digested with trypsin, in accordance with the filter-aided sample preparation method (21). The digested peptides of each sample were desalted on C18 cartridges [Empore™ SPE C18 Cartridges (standard density), 7 mm bed I.D., 3 mL volume, Sigma], concentrated by vacuum centrifugation, and reconstituted in 40 μL of 0.1% (v/v) formic acid.

Liquid Chromatography (LC)–Electrospray Ionization (ESI)–Tandem MS (MS/MS)

LC–ESI–MS/MS was performed on a Q-Exactive Plus mass spectrometer coupled with an EASY 1200 nano-LC System (Thermo Fisher Scientific, Bremen, Germany). LC–ESI–MS/MS settings were the same as those stated in a previous study (22). Briefly, 3 μg of the peptide mixture was first loaded onto a trap column (100 μm inner diameter, 20 mm long, 5 μm resin,

C18, Dr. Maisch GmbH, Ammerbuch, Germany) in buffer A (0.1% formic acid in water). Reversed-phase high-performance LC was then performed with the EASY 1200 nano LC System (Thermo Fisher Scientific, Bremen, Germany) using a self-packed column (75 μm inner diameter, 150 mm long, 3 μm ReproSil-Pur C18 beads, 120 Å, Dr. Maisch GmbH, Ammerbuch, Germany), and the peptide mixture was separated with a linear gradient of buffer B (0.1% formic acid in 85% acetonitrile) at a flow rate of 300 nL/min controlled by IntelliFlow for 120 min. MS/MS data were acquired using a data-dependent top 20 method by dynamically choosing the most abundant precursor ions. The survey scans were selected with an isolation window of 1.6 Thomson and fragmented by higher energy collisional dissociation with a normalized collision energy of 27 eV. The maximum ion injection times for the survey and MS/MS scans were 50 ms, and the ion target values were set to 1e6 and 1e5, respectively. Selected sequenced ions were dynamically excluded for 60 s.

Sequence Database Searching and Protein Quantification

Raw MS data were analyzed using MaxQuant v1.6.0.16 and searched against UniProtKB *Equus* (28,987 total entries, downloaded on 15/09/2019 from http://www.uniprot.org). The initial search peptide tolerance and mass tolerance were set at 20 ppm for fragment ions. The main search peptide tolerance was 4.5 ppm. The search was performed based on an enzymatic cleavage rule of trypsin/P, and a maximum of two missed cleavage sites were allowed (21). The carbamidomethylation of cysteine residues was defined as a fixed modification, while protein N-terminal acetylation and methionine oxidation were specified as variable modifications for database searching. The false discovery rate for both peptide and protein identification was set to be <0.01 (23). Protein identification was supported by at least one unique peptide identification.

Statistical and Bioinformatics Analysis

Differential proteins were analyzed for significant up- or downregulation, which was assessed using the R statistical computing software (v 3.2.1). Protein abundance information was collected to have at least two valid expression values in each group. Normality was assessed using the Shapiro–Wilk test. This exploratory analysis of the dataset showed that the variables were normally distributed; thus, the independent samples *t*-test was used to determine statistical significance for comparison between the P and NP groups. The significantly differentially expressed proteins (DEPs) were further inspected and the ones with a differential expression ratio of ≥1.5-fold or ≤0.66-fold ($P < 0.05$) were retained.

Analyses of bioinformatics data were performed with Perseus (24), Microsoft Excel, and R statistical computing software. The protein sequences of selected DEPs were extracted from UniProtKB/Swiss-Prot (25), gene ontology (GO) terms, and Kyoto Encyclopedia of Genes and Genomes (KEGG) database (http://geneontology.org/). GO and KEGG enrichment analyses were applied on the basis of Fisher's exact test in which whole quantified protein annotations were considered as background dataset, and only functional categories and pathways with $P < 0.05$ were recognized as significant. The protein–protein interaction (PPI) information of DEPs was retrieved using the STRING (http://string-db.org/) database and Cytoscape software (26).

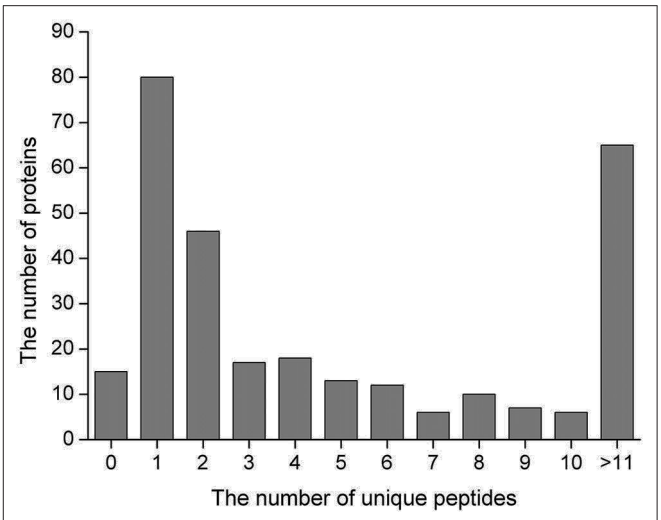


FIGURE 1 | Unique peptides for the identified proteins.

RESULTS

Identification and Quantification of the Serum Proteome

We herein used the label-free MS-based quantitative proteomics to investigate serum proteins in jennies, which led to the identification of 295 proteins (**Supplementary Table 1**) in the serum of early pregnant and non-pregnant jennies with a high confidence level (≥1 unique peptides with false discovery rate of <1%). Overall, 275 proteins were common between the P and NP groups. Of the 295 proteins that were quantified, 27.1% (80/295) were inferred from one peptide, and 22.0% (65/295) were proteins with >11 unique peptides (**Figure 1**). The molecular weight of the identified proteins ranged from 1.53 to 881.23 kDa. Albumin, α-2-macroglobulin, serotransferrin, apolipoprotein A1, and haptoglobin were the major serum proteins. Detailed information pertaining to the identified 2,569 peptides is shown in **Supplementary Table 2**.

Differentially Expressed Serum Protein Profiles

Twenty-five proteins (22 upregulated and three downregulated) were found to be significantly differentially expressed between the P and NP groups (≥1.5-fold, Student's *t*-test, $P < 0.05$) (**Table 1**, **Supplementary Table 3**). Among the 25 DEPs, 12 (10 upregulated and two downregulated) were common between the P and NP groups. According to the fold change and *P*-value between the groups, a volcano plot was drawn to illustrate significant differences (**Figure 2**). In addition, among the 25

TABLE 1 | Differentially expressed proteins in the serum of early pregnant (P group) and non-pregnant (NP group) jennies.

UniProt accession	Protein names	Gene names	Coverage (%)[b]	Molecular weight (kDa)	FC[a]	*P*-value	Change[e]
F6YR34	Thrombospondin 1	THBS1	21.1	129.56	14.43	0.0432	↑
F7CN05	Fibronectin	FN1	39.7	252.55	3.32	0.0045	↑
F6RMK1	C-X-C motif chemokine	LOC100630489	31.5	11.835	2.92	0.0122	↑
F6ZRF6	Serpin family A member 7	SERPINA7	18.6	46.64	2.25	0.0346	↑
A0A0A1E971	Immunoglobulin lambda light chain variable region (Fragment)	IGL	30.4	23.267	1.91	0.0363	↑
F7ATS5	Golgi membrane protein 1	GOLM1	12.5	41.218	1.87	0.0276	↑
Q5IF07	Insulin-like growth factor binding protein-2 (Fragment)	IGFBP-2	17.7	16.042	1.82	0.0014	↑
F6WA57	Lymphocyte cytosolic protein 1	LCP1	35.8	70.347	1.74	0.0409	↑
F6Y950	Chromosome 2 C4orf33 homolog	C2H4orf33	6.5	23.394	1.64	0.0486	↑
F6Y2H3	Peptidase D	PEPD	12.2	54.827	1.53	0.0061	↑
A0A0A1E3W4	Immunoglobulin lambda light chain variable region (Fragment)	IGL	25.4	23.511	P[c]		↑
F6VP61	Serpin family B member 10	SERPINB10	5.8	45.412	P[c]		↑
F6QXN5	Transgelin2	TAGLN2	9.3	23	P[c]		↑
F6RCZ8	Triggering receptor expressed on myeloid cells like 1	TREML1	5	33.597	P[c]		↑
F6RLT8	Calpain small subunit 1	CAPNS1	4.9	28.099	P[c]		↑
F6RMQ1	GLI pathogenesis related 2	GLIPR2	17.2	16.905	P[c]		↑
F6T962	EGF containing fibulin extracellular matrix protein 2	EFEMP2	6.5	49.439	P[c]		↑
F6ZEJ2	HGF activator	HGFAC	7.2	55.76	P[c]		↑
F7AB03	Granulin precursor	GRN	6.6	63.195	P[c]		↑
F7DIN1	Fibrinogen like 1	FGL1	4.4	36.475	P[c]		↑
F7E2K1	Uncharacterized protein	N/A	2.1	72.262	P[c]		↑
P19794	Lutropin/choriogonadotropin subunit beta	LHB	10.1	17.943	P[c]		↑
F7BSN5	Uncharacterized protein	N/A	7.6	59.264	0.51	0.0190	↓
F7DU87	Uncharacterized protein	BPIFA2	39.8	26.915	0.28	0.0012	↓
F7ATL5	Keratin 14	KRT14	8.2	41.036	NP[d]		↓

[a] *FC, fold change, mean value of peak area obtained from the P group/mean value of peak area obtained from the NP group. If the fold-change value was >1.5, the relative content of serum proteins in the P group was higher that than in the NP group, and if the fold-change value was < 0.67, the relative content of serum proteins in the P group was less than that in the NP group.*

[b] *coverage (%) = percentage of the protein sequence covered by identified peptides.*

[c] *uniquely expressed serum proteins in the P group.*

[d] *uniquely expressed serum proteins in the NP group.*

[e] *"↑" = upregulated serum proteins; "↓" = downregulated serum proteins.*

DEPs, there were 12 and one proteins that were uniquely expressed in the P and NP groups, respectively.

Bioinformatics Analysis of DEPs

The 25 DEPs were classified into three distinctive functional sets through GO enrichment analysis, namely biological process (BP), cellular component (CC), and molecular function (MF) (**Figure 3**), with a corrected statistically significant level ($P < 0.05$) based on Fisher's exact test. The most prevalent BPs were response to stimulus, regulation of cellular process, protein metabolic process, and proteolysis. The most enriched CCs were extracellular region, vesicle, extracellular organelle, and extracellular space, and in the MF category, DEPs were mainly involved in binding, enzyme inhibitor activity, and enzyme regulator activity.

The 25 DEPs were related to 17 KEGG pathways; the first 10 pathways are shown in **Figure 4**. The prevalent pathways related to DEPs were involved in bladder cancer, ovarian steroidogenesis, malaria, p53 signaling, prolactin signaling, TGF-beta signaling, and GnRH signaling. Detailed information on the functional enrichment of DEPs is present in **Supplementary Table 4**.

PPI Analysis

Color-coded networks were generated to represent different types of evidence for the biological crosslink between DEPs. The networks contained nine DEPs, and the core factors were fibronectin (FN1), fibrinogen-like 1 (FGL1), and thrombospondin 1 (THBS1). These three proteins were connected by 2–5 nodes (**Figure 5**).

DISCUSSION

Serum proteins during early pregnancy in jennies have not been previously investigated. In this study, we generated proteome profiles of the serum obtained from jennies using label-free MS-based quantitative proteomics, which led to the identification of 25 DEPs. This is the first comprehensive study to explore the potential biological significance of DEPs between

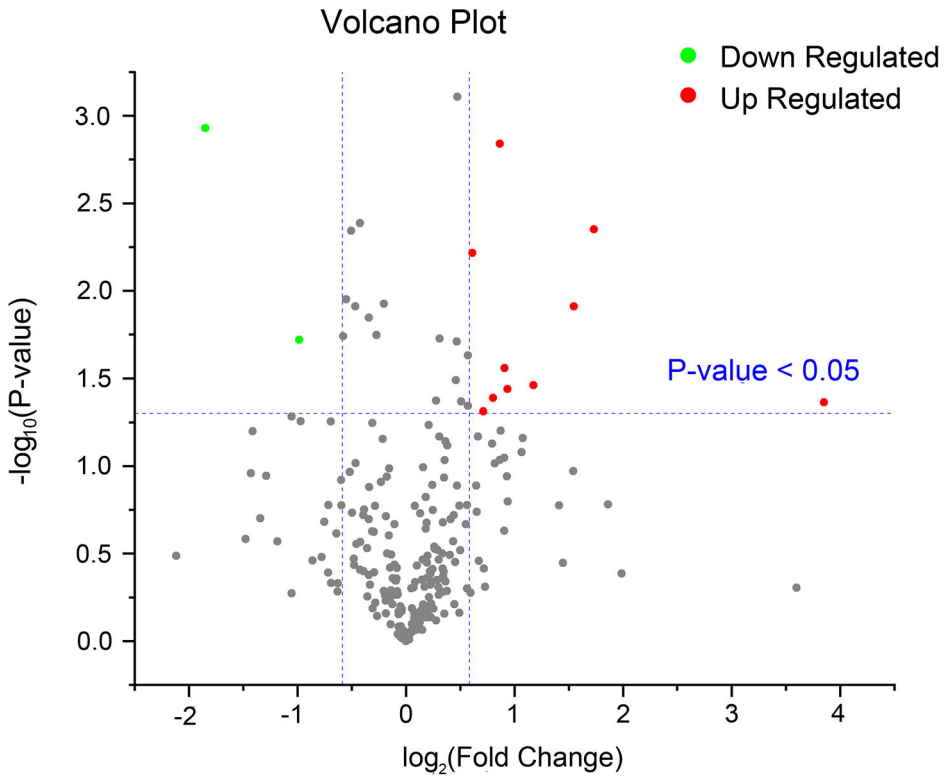


FIGURE 2 | A volcano plot of common proteins identified in the serum of early pregnant and non-pregnant jennies. This plot was drawn on the basis of the fold change and *P*-value between the P and NP groups. Red and green dots represent upregulated and downregulated common proteins, respectively (fold change $\geq$ 1.5 and $P < 0.05$). Gray dots represent proteins without any difference.

early pregnant and non-pregnant jennies, providing valuable insights into pregnancy-specific serum proteins that could be applied for developing pregnancy diagnostic markers in early pregnant jennies.

The label-free MS-based quantitative proteomics method, i.e., spectral counting, compares the number of mass spectra assigned to each protein (27). It offers several advantages such as high sensitivity, real-time measurements, rapid and multiplexed detection, larger quantification dynamic range, and improved reproducibility (28), as compared with the low identification rate of the 2D gel electrophoresis method (27). The label-free MS-based quantitative proteomics method has thus emerged as a promising technique for quantitative protein profiling of complex biological samples, such as serum (29).

Several proteomics-based studies involving various animal species have reported the identification of potential pregnancy-specific serum proteins. These studies have mostly used high-resolution 2D gel electrophoresis and MS for this purpose (15–17). Four differentially expressed serum proteins, namely transferrin, albumin, IgG2a heavy chain constant region, and immunoglobulin gamma heavy chain variable region, were identified as pregnancy-specific proteins in Holstein dairy cattle by 2D gel electrophoresis plus matrix-assisted laser desorption/ionization–time of flight (MALDI–TOF) MS (15). In another study, 2D fluorescence difference gel electrophoresis plus MALDI-TOF MS were used to analyze serum proteins obtained from Holstein dairy cattle, which led to the identification of 16 DEPs spots; among them, upregulated proteins, such as conglutinin precursor, modified bovine fibrinogen, and IgG1, and downregulated proteins, such as hemoglobin, complement component 3, bovine fibrinogen, and IgG2a heavy chain constant region, were considered as pregnancy-specific proteins (16). Further, upon exploration of serum pregnancy-specific proteins in Murrah buffaloes, synaptojanin-1, apolipoprotein a-1, apolipoprotein b, keratin 10, and von Willebrand factors were identified by 2D gel electrophoresis plus MALDI-TOF MS (17). In this study, none of the DEPs identified by us using label-free MS-based quantitative proteomics have been reported in previous studies, indicating interspecific differences.

So far, some pregnancy-specific proteins, such as pregnancy-specific glycoproteins, have been found in trophoblast cells of pregnant mares (30); however, to the best of our knowledge, the identification of pregnancy-specific serum proteins based on proteomics has not been performed in horses. Further studies need to be conducted to reveal more biomarkers of early pregnancy and to compare differences among different *Equus* species.

Herein many DEPs, such as FN1, FGL1, THBS1, granulin precursor/acrogranin (GRN), C-X-C motif chemokine,

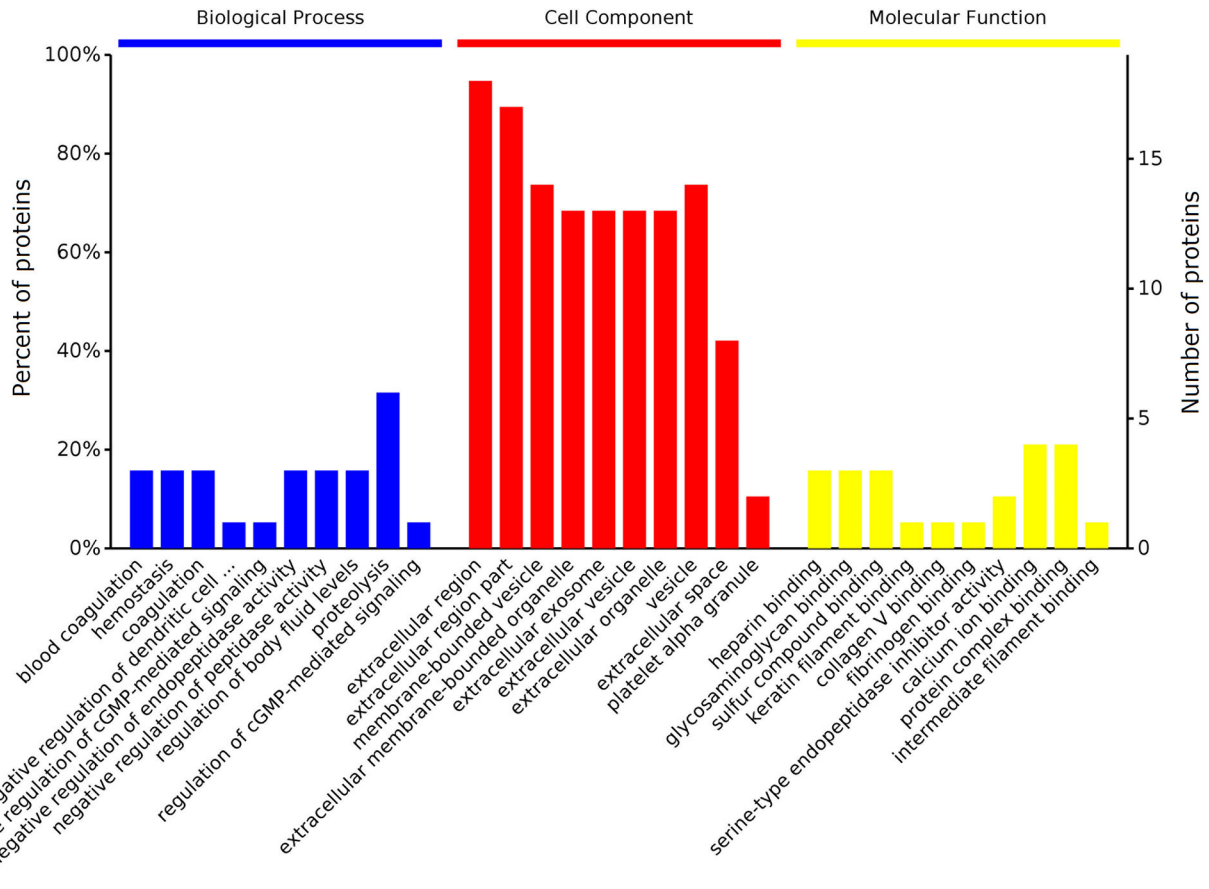


FIGURE 3 | GO enrichment analyses of DEPs identified in the serum of early pregnant and non-pregnant jennies. Most proteins could be divided into three major categories: biological process, cellular component, and molecular function.

triggering receptor expressed on myeloid cells-1 (TREML1), and keratin 14 (KRT14), were identified in the serum obtained from early pregnant jennies, and these were mainly associated with defense response. FN1 usually exists as a dimeric glycoprotein and is a ubiquitous and abundant soluble constituent of the plasma and other body fluids; it is also a part of the insoluble extracellular matrix (31). This extracellular fibronectin niche plays essential roles in wound healing, malignant transformation, inflammation, infection, hemostasis, and thrombosis (32). FGL1 belongs to the fibrinogen family and is primarily secreted from hepatocytes. It is involved in the process of blood clotting (33), and a study reported that recombinant human IL-6 could induce FGL1 expression in Hep G2 cells and that serum FGL1 levels were also enhanced after induction of acute inflammation in rats by subcutaneous injection of turpentine oil (34). THBS1 interacts with a cohort of target molecules through its multifunctional domains and participates in normal development and homeostasis maintenance (35). It also plays an important role in the regulation of coagulation, antiangiogenesis, wound healing, and immune response (36). The 6-kDa form of GRN has been invoked in wound repair (37). GRN is also a potent inhibitor of the inflammatory cytokine tumor necrosis factor-α and regulate inflammation (38). Both C-X-C motif chemokine and TREML1 are involved in platelet activation. KRT14, encoding a basal cell keratin, is expressed during the wound healing process and participates in cell morphogenesis and epidermis development (39). Changes in the serum level of these proteins indicates the involvement of a regulatory mechanism of defense response during early pregnancy.

The presence of many pregnancy-specific proteins associated with early embryonic development have been reported in the serum, other body fluids, and tissues of animals. We found that the expression of GRN and transgelin-2 (TAGLN2) was unique. GRN is an embryo-derived growth factor, which possesses growth regulatory activities principally toward epithelial cells. GRN has been reported to regulate the appearance of the epithelium in developing mouse blastocysts, growth of the trophectoderm, and/or function of the embryonic epithelium (40). As a member of the transgelin family, TAGLN2 plays a significant role in the regulation of F-actin remodeling, which is important for trophoblast cell adhesion and invasion. TAGLN2 may be a potential target to improve embryo implantation rates in *in vitro* fertilization (41). Furthermore, the significantly

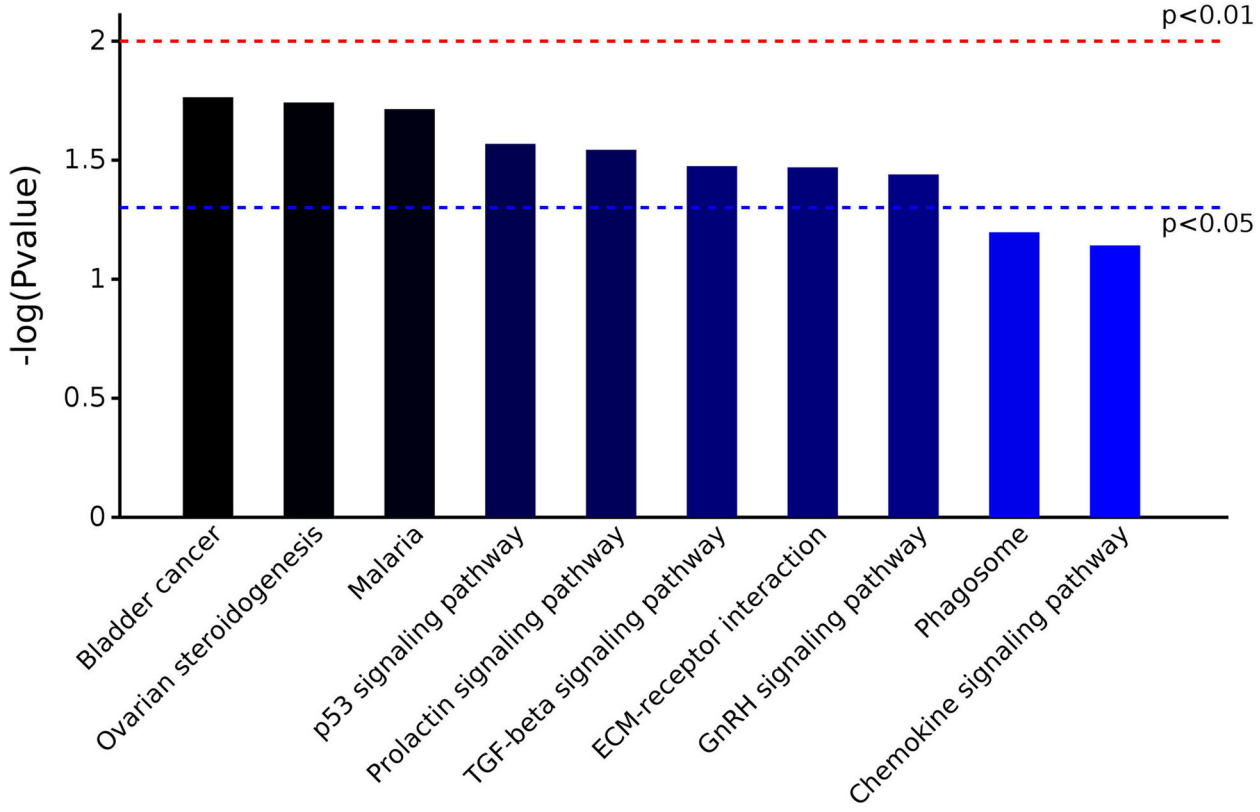


FIGURE 4 | KEGG pathway enrichment analysis of DEPs identified in the serum of early pregnant and non-pregnant jennies.

upregulated proteins FN1, FGL1, and THBS1 are also seemingly involved in the establishment and maintenance of pregnancy. FN1 plays major roles in cell adhesion, migration, growth, and differentiation. It also binds to a number of biologically important molecules (42). Moreover, it is indispensable for embryogenesis; George et al. (43) reported that FN1-deficient mice died around embryonic day 10.5 due to severe defects in mesoderm. FGL1 is also expressed in the placenta and increases the proliferation of trophoblasts through an ERK-dependent pathway (44). THBS1 is reportedly involved in the organization of the cytoskeleton and in the process of adhesion (45). It has also been reported to participate in the adhesiveness of the embryo during the peri-implantation period in pigs (46). Considering our findings pertaining to the differential expression of GRN, TAGLN2, FN1, FGL1, and THBS1 in the serum of early pregnant jennies, which have been proved to play an important role in early embryonic development in other species, these five proteins seem to be novel, reliable candidates to detect pregnancy in jennies.

Equine chorionic gonadotropin is a pregnancy-specific glycoprotein hormone produced by the placenta between day 40 and 150 of pregnancy, and it shows FSH- and LH-like biological activities (19). Equids possess a single lutropin/choriogonadotropin subunit-β (LHB) gene, which confers specificity on the intact hormone (47). In our study, LHB was noted to be uniquely expressed in the serum of pregnant jennies and was probably involved in ovarian steroidogenesis and prolactin signaling and GnRH signaling pathways, according to the KEGG pathway-based enrichment analysis. LHB has been identified as a marker of pregnancy in jennies, although it shows considerably lower FSH-like activity than horse LHB (47).

CONCLUSION

To summarize, this is the first study to report a comprehensive analysis of DEPs in the serum of early pregnant and non-pregnant jennies using label-free MS-based quantitative proteomics. Twenty-two upregulated and three downregulated proteins were significantly differentially expressed in the P group, and these were mainly associated with the defense response, early embryonic development, and hormone signaling pathways. The differences in the expression profiles helped identify candidate proteins to explore pregnancy diagnostic markers related to early pregnancy in jennies. Five serum proteins—GRN, TAGLN2, FN1, FGL1, and THBS1—appear to be novel, reliable candidates to detect pregnancy in jennies. Further studies should be conducted to elucidate their functions in jennies.

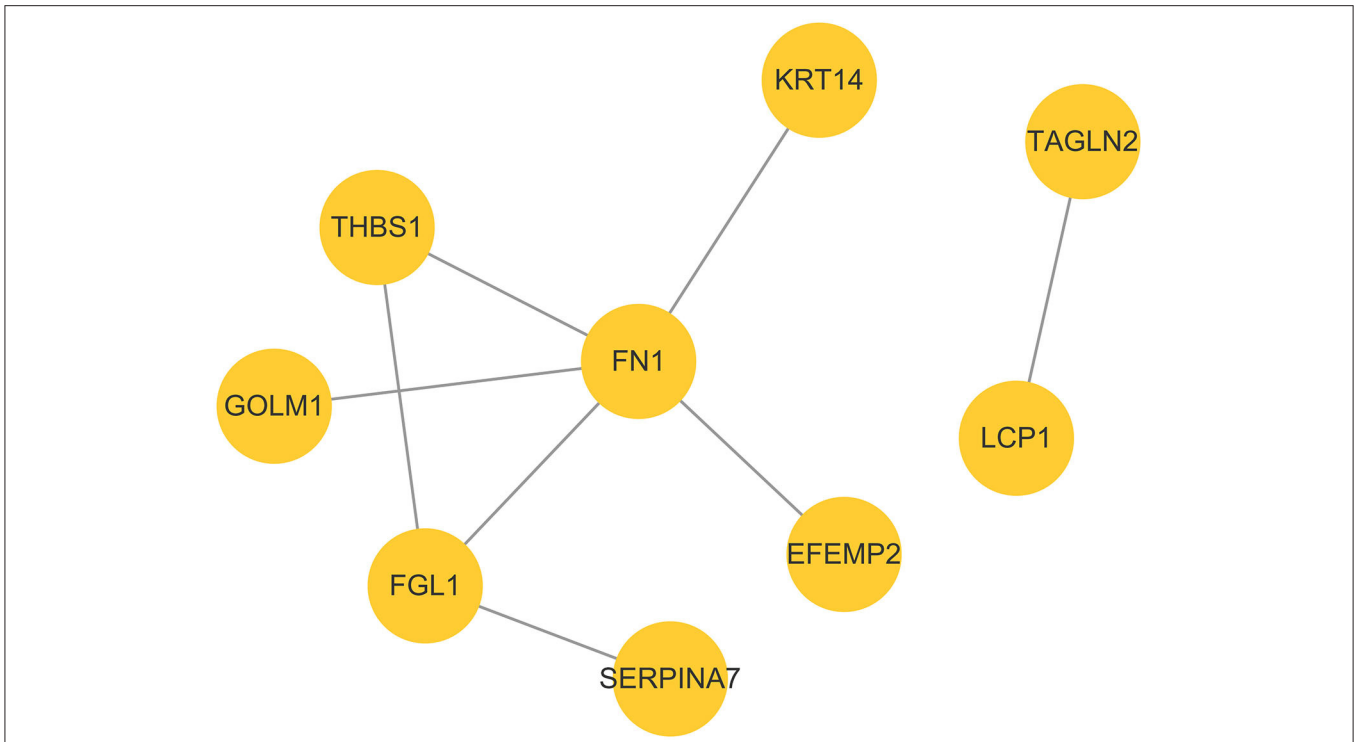


FIGURE 5 | Protein–protein interaction network analysis based on DEPs in the P group vs. the NP group.

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 Shenyang Agricultural University Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

LD performed the experiments. YH and CT participated in data analysis. CT, QL, and ZL helped with sampling. LD and YH edited the manuscript. All authors read and approved the final manuscript.

FUNDING

This study was supported by the National Natural Science Foundation, China (Grant no. 31501952) and the Foundation from the Department of Education of Liaoning Province, China (Grant no. LSNFW201904).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2020.569587/full#supplementary-material

REFERENCES

1. Ali M, Baber M, Hussain T, Awan F, Nadeem A. The contribution of donkeys to human health. *Equine Vet J.* (2014) 46:766–7. doi: 10.1111/evj.12337
2. Tibary A, Sghiri A, Bakkoury M. Reproduction. In: Duncan J, Hadrill D, editors. *The Professional Handbook of the Donkey.* 4th ed. London: Whittet Books (2008). p. 314–41.
3. The Donkey Sanctuary. Reproductive system. In: Evans L, Crane M, editors. *The Clinical Companion of the Donkey*. Leicester: Troubador (2018). p. 67–79.
4. Meira C, Ferreira JC, Papa FO, Henry M. Ultrasonographic evaluation of the conceptus from days 10 to 60 of pregnancy in jennies. *Theriogenology.* (1998) 49:1475–82. doi: 10.1016/S0093-691X(98)00093-4
5. Meira C, Ferreira JC, Papa FO, Henry M. Ovarian activity and plasma concentrations of progesterone and estradiol during pregnancy in jennies. *Theriogenology.* (1998) 49:1465–73. doi: 10.1016/S0093-691X(98)00092-2
6. Svendsen MBE. Biochemical parameters. In: Duncan J, Hadrill D, editors. *The Professional Handbook of the Donkey.* 4th ed. London: Whittet Books (2008). p. 381.
7. Ve′gva′ri Á, Welinder C, Lindberg H, Fehniger TE, Marko-Varga G. Biobank resources for future patient care: developments, principles and concepts. *J Clin Bioinforma.* (2011) 1:24. doi: 10.1186/2043-9113-1-24
8. Beck HC, Overgaard M, Rasmussen LM. Plasma proteomics to identify biomarkers – application to cardiovascular diseases. *Transl Proteomics.* (2015) 7:40–8. doi: 10.1016/j.trprot.2015.01.001

9. Miller I, Friendlein A, Tsangaris G, Maris A, Fountoulakis M, Gemeiner M. The serum proteome of *Equus caballus*. *Proteomics.* (2004) 4:3227–34. doi: 10.1002/pmic.200400846
10. Lepczyński A, Ozgo M, Dratwa-Chałupnik A, Robak P, Pyć A, Zaborski D, et al. An update on medium-and low-abundant blood plasma proteome of horse. *Animal.* (2018) 12:76–87. doi: 10.1017/S1751731117001409
11. Scoppetta F, Tartagila M, Renzone G, Avellini L, Gaiti A, Scaloni A, et al. Plasma protein changes in horse after prolonged physical exercise: a proteomic study. *J Proteomics.* (2012) 75:4494–504. doi: 10.1016/j.jprot.2012.04.014
12. Barsuren E, Namkhai B, Kong HS. Differences in serum protein 2D gel electrophoresis patterns of Przewalski's (Mongolian wild horse) and thoroughbred horses. *Anim Sci J.* (2015) 86:443–8. doi: 10.1111/asj.12303
13. Henze A, Aumer F, Grabner A, Raila J, Schweigert FJ. Genetic differences in the serum proteome of horses, donkeys and mules are detectable by protein profiling. *Brit J Nutr.* (2011) 106:S170–3. doi: 10.1017/S0007114511000845
14. Larsson A, Palm M., Hansson LO, Axelsson O. Reference values for clinical chemistry test during normal pregnancy. *BJOG Int J Obstet Gynaecol.* (2008) 115:874–81. doi: 10.1111/j.1471-0528.2008.01709.x
15. Jin DI, Lee HR, Kim HR, Lee HJ, Yoon JT, Park CS. 151 Proteomics analysis of pregnancy-specific serum proteins in bovine. *Reprod Fertil Dev.* (2005) 18:183. doi: 10.1071/RDv18n2Ab151
16. Lee JE, Lee JY, Kim HR, Shin HY, Lin T, Jin DI. Proteomic analysis of bovine pregnancy-specific serum proteins by 2D fluorescence difference gel electrophoresis. *Asian Australas J Anim Sci.* (2015) 28:788–95. doi: 10.5713/ajas.14.0790
17. Balhara AK, Gupta M, Mohanty AK, Phulia SK, Sharma RK, Singh S, et al. Changes in sera proteome in relation to day of pregnancy in early pregnant buffaloes. *Indian J Anim Sci.* (2014) 84:400–9. doi: 10.2478/aoas-2014-0018
18. Scholl PF, Cole RN, Ruczinski I, Gucek M, Diez R, Rennie A, et al. Maternal serum proteome changes between the first and third trimester of pregnancy in rural Southern Nepal. *Placenta.* (2012) 33:424–32. doi: 10.1016/j.placenta.2012.02.009
19. Antczak DF, de Mestre AM, Wilsher S, Allen WR. The equine endometrial cup reaction: a fetomaternal signal of significance. *Annu Rev Anim Biosci.* (2013) 1:419–42. doi: 10.1146/annurev-animal-031412-103703
20. Deng L, Shi S, Li J, Tang C, Liao Q, Xie P. A cross-sectional survey of foaling-related parameters of Jennies (*Equus asinus*) under smallholder farm conditions in Northeast China. *J Equine Vet Sci.* (2020) 87:102928. doi: 10.1016/j.jevs.2020.102928
21. Wiśniewski JR, Zougman A, Nagaraj N, Mann M. Universal sample preparation method for proteome analysis. *Nat Methods.* (2009) 6:359–62. doi: 10.1038/nmeth.1322
22. Cao X, Song D, Yang M, Yang N, Ye Q, Tao D, et al. Comparative analysis of whey N-glycoproteins in human colostrum and mature milk using quantitative glycoproteomics. *J Agric Food Chem.* (2017) 65:10360–7. doi: 10.1021/acs.jafc.7b04381
23. Luber CA, Cox J, Lauterbach H, Fancke B, Selbach M, Tschopp J, et al. Quantitative proteomics reveals subset-specific viral recognition in dendritic cells. *Immunity.* (2010) 32:279–89. doi: 10.1016/j.immuni.2010.01.013
24. Tyanova S, Temu T, Sinitcyn P, Carlson A, Hein MY, Geiger T, et al. The Perseus computational platform for comprehensive analysis of (prote)omics data. *Nat Methods.* (2016) 13:731–40. doi: 10.1038/nmeth.3901
25. Boutet E, Lieberherr D, Tognolli M, Schneider M, Bansal P, Bridge AJ, et al. UniProtKB/Swiss-Prot, the manually annotated section of the UniProt KnowledgeBase: How to use the entry view. *Methods Mol Biol.* (2016) 1374:23–54. doi: 10.1007/978-1-4939-3167-5_2
26. Shannon P, Markiel A, Ozier O, Baliga NS, Wang JT, Ramage D, et al. Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome Res.* (2003) 13:2498–504. doi: 10.1101/gr.1239303
27. Zhu W, Smith JW, Huang C. Mass spectrometry-based label-free quantitative proteomics. *J Biomed Biotechnol.* (2010) 2010:840518. doi: 10.1155/2010/840518
28. Ray S, Reddy PJ, Jain R, Gollapalli K, Moiyadi A, Srivastava S. Proteomic technologies for the identification of disease biomarkers in serum: advances and challenges ahead. *Proteomics.* (2011) 11:2139–61. doi: 10.1002/pmic.201000460
29. Wang W, Zhou H, Lin H, Roy S, Shaler TA, Hill LR, et al. Quantification of proteins and metabolites by mass spectrometry without isotopic labeling or spiked standards. *Anal Chem.* (2003) 75:4818–26. doi: 10.1021/ac026468x
30. Aleksic D, Blaschke L, Mißbach S, Hänske J, Weiß W, Handler J, et al. Convergent evolution of pregnancy-specific glycoproteins in human and horse. *Reproduction.* (2016) 152:171–84. doi: 10.1530/REP-16-0236
31. Wierzbicka-Patynowski I, Schwarzbauer JE. The ins and outs of fibronectin matrix assembly. *J Cell Sci.* (2003) 116:3269–76. doi: 10.1242/jcs.00670
32. Ni H. Unveiling the new face of fibronectin in thrombosis and hemostasis. *J Thromb Haemost.* (2006) 4:940–2. doi: 10.1111/j.1538-7836.2006.01899.x
33. Rijken DC, Dirkx SP, Luider TM, Leebeek FW. Hepatocyte-derived fibrinogen-related protein-1 is associated with the fibrin matrix of a plasma clot. *Biochem Biophys Res Commun.* (2006) 350:191–4. doi: 10.1016/j.bbrc.2006.09.018
34. Liu Z, Ukomadu C. Fibrinogen-like protein 1, a hepatocyte derived protein is an acute phase reactant. *Biochem Biophys Res Commun.* (2008) 365:729–34. doi: 10.1016/j.bbrc.2007.11.069
35. Lawler J, Sunday M, Thibert V, Duquette M, George EL, Rayburn H, et al. Thrombospondin-1 is required for normal murine pulmonary homeostasis and its absence causes pneumonia. *J Clin Invest.* (1998) 101:982–92. doi: 10.1172/JCI1684
36. Adams JC, Lawler J. The thrombospondins. *Cold Spring Harb Perspect Biol.* (2011) 3:a009712. doi: 10.1101/cshperspect.a009712
37. He Z, Ong CH, Halper J, Bateman A. Progranulin is a mediator of the wound response. *Nat Med.* (2003) 9:225–9. doi: 10.1038/nm816
38. Kessenbrock K, Fröhlich L, Sixt M, Lämmermann T, Pfister H, Bateman A, et al. Proteinase 3 and neutrophil elastase enhance inflammation in mice by inactivating antiinflammatory progranulin. *J Clin Invest.* (2008) 118:2438–47. doi: 10.1172/JCI34694
39. Founds SA, Conley YP, Lyons-Weiler JF, Jeyabalan A, Hogge WA, Conrad KP. Altered global gene expression in first trimester placentas of women destined to develop preeclampsia. *Placenta.* (2009) 30:15–24. doi: 10.1016/j.placenta.2008.09.015
40. Di'az-Cueto L, Stein P, Jacobs A, Schultz RM, Gerton GL. Modulation of mouse preimplantation embryo development by acrogranin (epithelin/granulin precursor). *Dev Biol.* (2000) 217:406–18. doi: 10.1006/dbio.1999.9564
41. Liang X, Jin Y, Wang H, Meng X, Tan Z., Huang T, et al. Transgelin 2 is required for embryo implantation by promoting actin polymerization. *FASEB J.* (2019) 33:5667–75. doi: 10.1096/fj.201802158RRR
42. Pankov R, Yamada KM. Fibronectin at a glance. *J Cell Sci.* (2002) 115:3861–3. doi: 10.1242/jcs.00059
43. George EL, Georges-Labouesse EN, Patel-King RS, Rayburn H, Hynes RO. Defects in mesoderm, neural tube and vascular development in mouse embryos lacking fibronectin. *Development.* (1993) 119:1079–91.
44. Kang L, Li HY, Ou HY, Wu P, Wang SH, Chang CJ, et al. Role of placental fibrinogen-like protein 1 in gestational diabetes. *Transl Res.* (2020) 218:73–80. doi: 10.1016/j.trsl.2020.01.001
45. Adams JC. Thrombospondins: multifunctional regulators of cell interactions. *Annu Rev Cell Dev Biol.* (2001) 17:25–51. doi: 10.1146/annurev.cellbio.17.1.25
46. Kolakowska J, Souchelnytskyi S, Saini RKR, Franczak A. Proteomic analysis of the endometrium during early pregnancy in the domestic pig. *Reprod Fertil Dev.* (2017) 29:2255–68. doi: 10.1071/RD16435
47. Chopineau M, Stewart F, Allen WR. Cloning and analysis of the cDNA encoding the horse and donkey luteinizing hormone β-subunits. *Gene.* (1995) 160:253–6. doi: 10.1016/0378-1119(95)00150-5

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: 06 November 2020
doi: 10.3389/fvets.2020.569916

Incidental Detection of *Onchocerca* Microfilariae in Donkeys (*Equus asinus*) in Italy: Report of Four Cases

Roberto Amerigo Papini, George Lubas and Micaela Sgorbini*

Department of Veterinary Sciences, Veterinary Teaching Hospital "Mario Modenato", University of Pisa, Pisa, Italy

This paper reports the occurrence for the first time in Italy of autochthonous *Onchocerca* infection in donkeys. Four jennies, bred on the same farm, were referred to the Veterinary Teaching Hospital of Pisa for a check-up on ovarian activity ($n = 3$) or for veterinary support during the delivery ($n = 1$). Microfilariae were incidentally detected during the blood smear examination of one jenny. Peripheral blood samples were then collected from the other three jennies and the presence of microfilariae was investigated by Knott's test. Circulating unsheathed microfilariae were identified in all the animals. The level of microfilaraemia was between 1 and 31 microfilariae in 2 mL of blood. Hematological changes showed moderate eosinophilia in one case or both remarkable eosinophilia and basophilia in another case. Based on molecular findings by PCR and sequencing, the microfilariae showed 98% sequence similarity with *Onchocerca* sp. in the NCBI GenBank database (Accession No.: MK541848.1). The present report provides evidence that *Onchocerca* is an etiological agent of parasitic infection in donkeys in Italy. Our findings highlight the importance of screening in donkeys for *Onchocerca* even in the absence of clinical indications.

Keywords: microfilariae, *Onchocerca*, onchocerciasis, donkey, *Equus asinus*, Italy

OPEN ACCESS

Edited by:
Donato Traversa,
University of Teramo, Italy

Reviewed by:
Charles D. Mackenzie,
Task Force for Global Health, United States
Susan Catherine Cork,
University of Calgary, Canada

****Correspondence:***
Roberto Amerigo Papini
roberto.amerigo.papini@unipi.it
orcid.org/0000-0002-5928-0663

Specialty section:
This article was submitted to Comparative and Clinical Medicine, a section of the journal Frontiers in Veterinary Science

Received: *05 June 2020*
Accepted: *13 October 2020*
Published: *06 November 2020*

Citation:
Papini RA, Lubas G and Sgorbini M (2020) Incidental Detection of Onchocerca Microfilariae in Donkeys (Equus asinus) in Italy: Report of Four Cases. Front. Vet. Sci. 7:569916. doi: 10.3389/fvets.2020.569916

INTRODUCTION

Onchocerca cervicalis, *Onchocerca reticulata*, and *Onchocerca raillieti* (Onchocercidae, Spirurida) are filarial thread-like nematodes. *O. cervicalis* and *O. reticulata* infect equines worldwide (1). *O. cervicalis* adult females (50–70 cm long) and males (7–10 cm long) live in the ligamentum nuchae. *O. reticulata* adult worms can measure up to 75 cm (females) or 15–20 cm (males) and inhabit the flexor tendons and suspensory ligament of the fetlock (2). *O. raillietti* has only been reported in donkeys in Africa (1) and adult worms live in the ligamentum nuchae (3). They are all viviparous. Adult females release microfilariae (L1) measuring 190–310 μm for *O. cervicalis*, 330–370 μm for *O. reticulata* (4), and 180–217 μm for *O. raillieti* (3). When microfilariae are released by the females, they migrate through connective tissues and accumulate in the lymphatics of the upper dermis (1, 2). The highest concentrations can be found along the linea alba in the ventral midline, especially near the umbilicus (1, 3, 4). Microfilariae are also commonly found in the dermis of the face, neck, withers, thorax, and eyes (1). *O. raillietti* microfilariae have also occasionally been found in the dermis of the head, back, hips, forelimbs, hind limbs, and perineum, though to a limited extent (3). Information is only available for the life cycle of *O. cervicalis* (4), however the transmission pathways of *O. cervicalis*, *O. reticulata*, and *O. raillieti* are likely to be similar. These parasites are all transmitted by blood-sucking midges of *Culicoides*

species (1, 2, 4, 5). Mosquitoes (*Aedes aegypti*) also act as intermediate hosts for *O. cervicalis* (6). Microfilariae are ingested by insect vectors while feeding on an infected animal. After development within the intermediate host to reach the infective third-stage, L3 larvae are transmitted to other susceptible definitive hosts at the subsequent blood meal (4).

Many studies have been conducted on epidemiological, clinical, diagnostic, pathological, and therapeutic aspects of *Onchocerca* infection in horses as reviewed by Dagnaw et al. (1). However, with the exception of a relatively recent case report in Italy (7), all these studies are dated. Moreover, only a limited number of studies have investigated the parasitosis in donkeys, almost all of the published studies in donkeys were from African countries, and most focus on *O. raillieti* (3, 5, 8–10). Currently, little or nothing is known about the current spread of *Onchocerca* infections in donkeys in Italy. In order to fill this gap, we report a case of autochthonous infection in jennies. We discuss various epidemiological and clinical aspects of the infection in this host species.

MATERIALS AND METHODS

Case Presentation and Blood Collection

In June 2019, four adult, pluriparous jennies of the Amiatina breed with ages ranging from 6 to 14 years (median age 13 years) were presented to the Veterinary Teaching Hospital (VTH) of the University of Pisa (geographical coordinates: 43°40′48″N 10°20′55″E). The jennies were from a donkey farm located in central Italy, where animals are used for milk production and reared outdoor in a free animal housing system. On the farm, water *ad libitum* and food were available in shaded resting spots.

Three out of the four were barren jennies admitted to the VTH for an examination of ovarian activity. The other jenny was pregnant and was diagnosed with rupture of the prepubic tendon before pregnancy, thus this jenny needed veterinary care at the time of delivery.

When admitted to the VTH, jennies were housed in collective paddocks 24 h a day, fed with meadow hay *ad libitum* along with commercial equine feed (Equifioc®, Molitoria Val di Serchio, Italy) according to the NCR energy recommendations (National Research Council, NRC, 2007), as well as having free access to clean and fresh water.

Upon admission to the VTH, all jennies underwent a complete clinical assessment and coprological examinations by means of a flotation technique with saturated solution of NaCl (specific gravity = 1.20).

The pregnant jenny showed signs of colic 2 days after delivery and thus underwent further clinical investigations. A complete blood count and biochemistry analyses were also performed. For this purpose, a venous blood sample was drawn via jugular venipuncture and collected in two sterile 10 mL vacutainer tubes, one with K3-EDTA and one plain tube for serum harvesting. As soon as the blood collection was completed, the sample was labeled (jenny I), immediately transferred to the laboratory of veterinary clinical pathology, and examined. Likewise, a blood work was also carried out in the three barren jennies from the same donkey farm. Blood collection was performed in the same way as previously described. The samples were labeled for animal identification (jennies II–IV).

Blood Analysis

Complete blood counts were performed using a combined laser-impedance cell counter (ProCyte®, Idexx Laboratories, Milan, Italy) including red blood cell count, hematocrit, hemoglobin, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, **r**ed blood cell distribution width, total leukocyte count, absolute values of neutrophils, lymphocytes, monocytes, eosinophils, and basophils, platelet count, and mean platelet volume. In order to complete the information on the morphological aspects of red blood cells, white blood cells, and platelets, and to assess the leukocyte differential counts in comparison to the results provided by the cell counter, fresh blood smears were prepared and stained with May-Grundwald-Giemsa (MGG) using an automatic stainer (Wescor Aerospray 7150®, Delcon, Milan, Italy). Using bright-field light microscopy, blood smears were screened at 20× and then examined at 100× by a trained clinical pathologist.

The serum biochemical assays were performed on a combined spectrophotometric-immunoturbidimetric device (Analyzer SAT 450®, Medical System, Guidonia, Rome, Italy) including: total plasma protein, albumin, gamma glutamyl transferase, aspartate aminotransferase, creatine phosphokinase, blood urea nitrogen, creatinine, calcium, phosphate, total bilirubin, and direct bilirubin. In addition, few electrolytes such as sodium, potassium and chloride were assessed with an ion-selective electrodes device (Electrolyte Analyzer GE200®, Medical System, Guidonia, Rome, Italy).

The blood cell counter was set up to evaluate the parameters as equine species. All the reference intervals (RIs) used were derived from an internal assessment carried out by the veterinary clinical pathology laboratory with multi-year experience in examining donkey blood.

Detection of Circulating Microfilariae

Since microfilariae were detected through the blood smear examination of jenny II (**Figure 1**), the occurrence of peripherally-circulating microfilariae was also evaluated in the other three jennies. Microfilariae detection was performed using a modified Knott's test on peripheral EDTA blood samples collected, as described above. Briefly, 8 mL of distilled water were added to 2 mL of whole EDTA blood and mixed thoroughly. Following centrifugation at 3,000 rpm for 5 min, the supernatant was discarded. The sediment was re-suspended in two drops of distilled water, removed with a 1 mL Pasteur pipette, and examined with a light microscope at 100× magnification. The number of microfilariae was counted in the Knott's test sediment. See the Results section for details on morphometric features. After microscopic examination, another aliquot (2 mL) of EDTA blood sample from each positive jenny was stored at −20°C prior to shipment to a sequencing service provider.

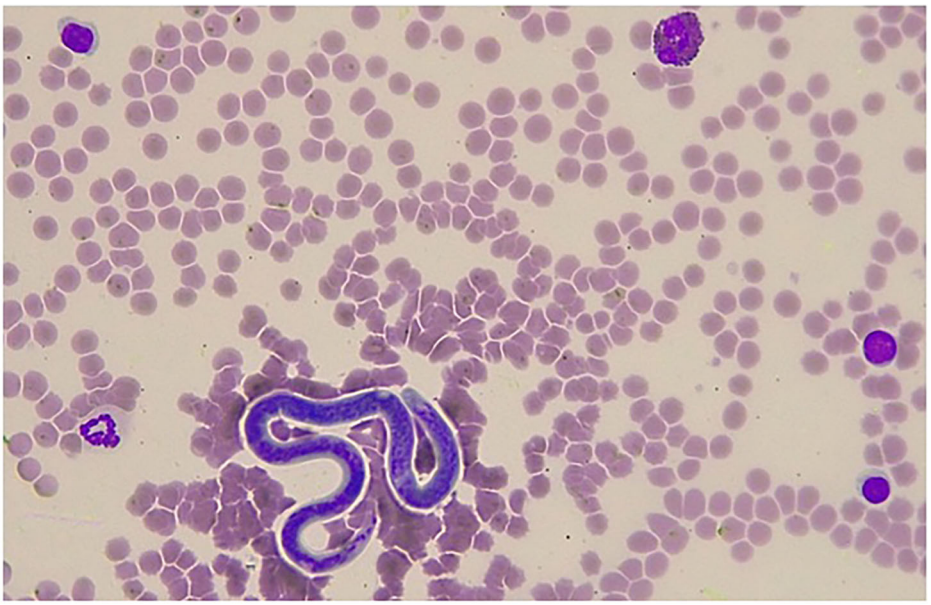


FIGURE 1 | Giemsa stained blood smear showing a single microfilaria of *Onchoerca* sp. detected in a jenny in Italy. In the microscopic field, one eosinophil with characteristic feature of the asinine species, three lymphocytes, and one neutrophil can also be identified (×400 magnification).

Molecular Procedures

Molecular analyses were performed by BMR Genomics (Padua, Italy). After thawing at room temperature, genomic DNA was extracted from positive blood samples using the commercial DNA IQ™ System® (Promega) based on paramagnetic beads, following the manufacturer's instructions. A fragment (about 700 base pairs in length) of the mitochondrial gene for cytochrome c oxidase subunit 1 (COX1) was used as a DNA barcoding system and amplified. The PCR amplification was carried out in a final mixture containing 12.5 μL of Master Mix 2X (GoTaq® Green Master Mix, Promega), 2 μL of genomic DNA extracted from blood, 1 μL (10 μM) of forward primer (5′-TGATTGGTGGTTTTGGTAA-3′), 1 μL (10 μM) of reverse primer (5′-ATAAGTACGAGTATCAATATC-3′), and deionized water, reaching a total reaction volume of 25 μL. The PCR reactions were subjected to the following conditions in thermal cycler (Mastercycler®, Eppendorf): 95°C × 2 min, then 5 cycles (95°C × 40 s, 45°C for 1.5 min, and 72°C for 1.5 min), followed by 35 cycles (95°C for 40 s, 50°C for 1.5 min, and 72°C for 1 min), and finally 72°C for 10 min. The amplification products were visualized after electrophoresis on 1.5% agarose gel. PCR products were purified by AMPure XP Beads (Beckman Coulter), sequenced, and aligned via BLAST analysis to detect their identity by retrieving similar sequences deposited in NCBI's GenBank database (11, 12).

Ethics Approval for the Study

Ethical review was not required as per institutional guidelines/local legislation due to natural infections that occurred spontaneously. Written informed consent was obtained from the owners for the participation of their animals in this study.

RESULTS

All four jennies were apparently healthy on the basis of history and physical examination at admission to the VTH. Three of them (II, III, and IV) remained in this condition throughout hospitalization. However, one jenny (I) was apparently healthy at admission to the VTH but presented signs of colic 2 days after delivering her foal. The results of the complete blood count for all four jennies are reported in **Table 1**. In jenny I, the values evidenced an anemic condition with normocytic hyperchromic red blood cells. Hyperchromia was due the lipemic sample collected, which interferes with the correct spectrophotometric reading of hemoglobin and induces a high value of mean corpuscular hemoglobin concentration. In the leukogram, neutrophilia, and monocytosis were found along with lymphopenia, all three conditions could be related to a long-lasting inflammatory process or simply related to stress. In jennies II and III, the values did not reveal anything particular in the red blood cell compartment. In the leukogram, eosinophilia was found in both animals and in jenny III it was substantial as was the occurrence of basophilia. In jenny IV, the results of the complete blood count were all within the RIs.

The biochemistry analysis in jenny I revealed renal insufficiency highlighted by an increase in both urea (48 mg/dL, RI = 9–31) and creatinine (2.2 mg/dL, RI = 0.59–1.3) as well as a decrease in total proteins (5.6 g/dL, RI = 5.8–7.6) due to a mild reduction in albumin (2.2 g/dL, RI = 2.5–3.2). Both the cholesterol (195 mg/dl, RI = 55–115) and triglycerides

TABLE 1 | Alterations detected in the complete blood count of four jennies found incidentally infected with *Onchocerca* microfilariae.

Hematological values					Reference range*
	I	II	III	IV	
Red blood cells M/μL	*3.40*↓	6.47	5.42	5.25	4.4–7.1
Hematocrit %	18.5	35.2	30.9	29.8	27–42
Hemoglobin g/dL	*7.1*↓	12.1	10.9	10.5	9.1–14.7
Mean corpuscular volume fL	54.4	54.4	57.0	56.8	53–67
Mean corpuscular hemoglobin pg	20.9	18.7	20.1	20.0	17.6–23.1
Mean corpuscular hemoglobin concentration g/dL	*38.4*↑	34.4	35.3	35.2	32–37.5
Red blood cell distribution width %	21.4	*22.9*↑	20.5	22.4	16.1–22.5
Total leukocytes K/μL	14.78	10.95	15.69	6.40	6.2–16
Neutrophils K/μL	*11.82*↑	5.04	5.49	3.07	2.4–6.3
Lymphocytes K/μL	*1.18*↓	4.27	6.12	2.28	1.6–8.5
Monocytes K/μL	*1.77*↑	0.33	0.31	0.32	0.0–0.8
Eosinophils K/μL	0.00	*1.31*↑	*3.45*↑	0.73	0.1–1.0
Basophils K/μL	0.00	0.00	*0.31*↑	0.00	0.0–0.2
Platelets K/μL	296	190	295	190	95–360
Mean platelet volume fL	10.4	10.0	10.4	10.1	8.8–12.0
Appearance of plasma	Lipemia ++	Clear	Clear	Clear	NA
Microfilaraemia at the blood smear	0	2	0	0	NA
Microfilaraemia by Knott's technique	4	31	1	3	NA

**Reference range used internally at the Clinical Pathology Laboratory; the values out-the-range are in italics; ↓, decreased values; ↑, increased values; NA, not applicable.*

(663 mg/dl, RI = 53–248) showed high values. All the other parameters tested, namely calcium, phosphate, gamma glutamyl transferase, aspartate aminotransferase, creatine phosphokinase, total bilirubin, and direct bilirubin were within the RIs. In the electrolytes, sodium (125 mEq/L, RI = 128–138) and potassium (1.6 mEq/L, RI = 3.2–5.1) were low probably due to the interference of the lipemic sample, while chloride was in the normal range. Overall, the trend of these parameters along with the complete blood count results highlighted lipidosis due to a negative energy intake balance related to obstructive colic in a lactating jenny. The clinical symptoms were depression, anorexia, and oliguria (the jenny refused to drink). The mild increase in urea could be related both to anorexia, or along with high levels of creatinine, to an acute pre-renal insufficiency secondary to low ingestion of water. In the other three jennies all parameters on serum biochemistry and electrolytes investigated were within the RIs (values not reported).

In jenny II, microfilaraemia was detected when the blood smear examination by conventional staining and optical microscopy reading was performed by an expert clinical pathologist (**Figure 1**). On the other hand, no microfilariae were detected through the blood smear evaluation in the other three jennies. MGG stained microfilariae had a wider and rounded anterior end, while the posterior end showed a short, thin, and sharply pointed tail. The microfilariae measured ∼214–229 μm in length and 4–5 μm in width. The most prominent feature was the lack of a faintly stained anterior and posterior sheath. This suggested that on the basis of morphological features, they could be differentiated from the sheathed microfilariae of *Setaria* spp and belonged to the genus *Onchocerca*, in which the length of microfilariae between the different species parasitizing donkeys falls within the range of 180–370 μm (3, 4).

Since circulating microfilariae were incidentally detected by microscopic examination of a blood smear from one of the jennies, we thus examined the peripheral blood samples from each jenny by a modified Knott's test, as described above. The Knott's test results showed that all the four jennies presented microfilariae in their peripheral blood samples. Circulating microfilariae appeared to be morphologically identical to each other and from one jenny to another. They were actively motile with serpentine movements, showing the lack of a sac-like hyaline sheath at both ends and a short tail, which matched the characteristics exhibited by the microfilariae in MGG stained blood smears. The characteristic features of the microfilariae appeared to be consistent with *Onchocerca* microfilariae and no further morphometric investigations were carried out, as microfilariae found in Knott's test sediments and in MGG stained smears appeared to be morphologically identical. However, the unusual site of the finding (i.e., peripheral blood stream) precluded any definitive conclusion and only a presumptive diagnosis of asymptomatic onchocerciasis could be made at that time. The levels of microfilaraemia were 31, 4, 3, and 1 microfilariae in 2 mL of blood samples from jennies II, I, IV, and III, respectively. The highest level of microfilaraemia was detected in jenny II which had been found positive from the blood smear. This suggests that positivity at the blood smear was probably related to the higher burden of circulating microfilariae. **Table 1** shows the Knott's test results.

The coprological results were positive for gastrointestinal strongyle eggs.

TABLE 2 | Accession numbers and description of sequences retrieved from GenBank producing significant alignments with COX1 mitochondrial gene of microfilariae incidentally found in peripheral blood samples of four jennies in Italy.

Accession no.	Parasitic organism identification	Bit score	*E*-value	Max identity
MK541848.1	*Onchocerca* sp. JJP-2019	1,138	0.0	98%
KX898458.1	*Onchocerca boehmi* Supperer, 1934	835	0.0	90%
LC318284.1	*Onchocerca flexuosa*	833	0.0	90%
AJ271616.1	*Onchocerca gibsoni*	833	0.0	90%
AM749269.1	*Onchocerca skrjabini*	824	0.0	91%
AP017692.1	*Onchocerca flexuosa*	817	0.0	89%
KX265050.1	*Dirofilaria* sp. "hongkongensis"	817	0.0	89%
AM749270.1	*Onchocerca skrjabini*	813	0.0	91%
AB518693.1	*Onchocerca* sp. wild boar	808	0.0	89%
KX853323.1	*Onchocerca boehmi* Supperer, 1934	806	0.0	90%

The BLAST search results showed that COX1 of microfilariae from the four examined jennies had the closest sequence similarity (i.e., 98%) with that of *Onchocerca* sp. available from GenBank (Accession Number MK541848.1). Sequences producing significant alignments are shown in **Table 2**.

Unfortunately, no follow-up was possible as the jenny with the ruptured prepubic tendon died after delivery, while the others returned to the farm a few days after check-up on ovarian activity. The owner was unwilling either to take them back to the VHT for further examination or to allow us to visit his farm at a later time.

DISCUSSION

To the best of our knowledge, this is the first report of *Onchocerca* infection in donkeys in Italy. The presence of microfilariae in peripheral blood samples was ascertained by laboratory and molecular findings. Unfortunately, the species of microfilariae was not identified. The two *Onchocerca* species infecting donkeys in Europe are *O. cervicalis* and *O. reticulata*, which are both found worldwide (1). The geographical range of *O. raillieti* infection in donkeys known to date is restricted to some countries in Africa (3, 5, 8). In previous studies, when the occurrence of *O. cervicalis* and *O. reticulata* was investigated concurrently, *O. cervicalis* was more prevalent than *O. reticulata*. In fact, prevalence values of 82.98 and 4.26% have been reported for *O. cervicalis* and *O. reticulata* in donkeys, respectively (13). Similarly, prevalence values of 25.42, 5.93, and 2.54% have been reported for *O. cervicalis*, *O. reticulata*, or both infections in horses, respectively (14). Therefore, based on the results of previous surveys, we believe that *O. cervicalis* was most likely the species involved in our study.

All the four *Onchocerca* infected jennies were bred on the same farm and were kept under the same management conditions. They had always lived on the same farm since their birth and there were no movements throughout the country except for their transportation to the VTH. *Onchocerca* infection in horses is considered rare when macrocyclic lactones are used regularly (15), since treatments with ivermectin (16, 17), moxidectin (18, 19) or doramectin (9) are effective in killing microfilariae (but not against adult worms located in the nuchal ligament). However, since our four jennies were milk-producing animals, no treatment for ectoparasite or endoparasite control had never been performed at the donkey farm in order to prevent the risk of contamination with drug residues in milk intended for human consumption. Moreover, the *Culicoides* species, which may act as intermediate hosts for *Onchocerca*, are spread throughout Italy, including central regions (20) where the farm of the infected jennies is located. Therefore, the examined jennies likely acquired the infection locally on the farm and the source of the infection was the bite of infected midges.

There have been few epidemiological studies on *Onchocerca* infection in donkeys. Reported prevalence values are 65.38% (21) and 82.98% (13) for *O. cervicalis* in Egypt, 34% for *O. raillieti* in Sudan (10), and 4.26% for *O. reticulata* in again Egypt (13). In another study, only one donkey was examined and found to be infected with *O. cervicalis* (22). No influence of sex on the infection rate has been observed (5, 10, 13). However, a statistically ($p < 0.05$) higher prevalence has been reported for *O. raillieti* (64.3%) in donkeys aged between 7 and 10 years (10) and for *O. cervicalis* (100%) in donkeys older than 15 years (10). Similarly, *Onchocerca* infection has been reported to be more common in older horses rather younger horses (23–25), particularly over 15 years of age (25, 26), as the prevalence of infection increases with age (25). Our detection of *Onchocerca* infection in jennies aged 6–14 years thus appears to be in agreement with the results of previous prevalence studies on donkeys and horses.

The occurrence, distribution, and population density of microfilariae in tissues of *Onchocerca* infected hosts may vary by season. The peak of distribution of *O. cervicalis* microfilariae in ventral-midline skin of 15 naturally infected pony mares was investigated over a 13-month period and was shown to be highest during the spring and lowest in the winter, disappearing in the surface layers of the dermis during the winter months (27). A distinct pattern of distribution was also reported in blood samples from 284 camels (*Camelus dromedarius*) where the highest monthly prevalence of *Onchocerca* microfilariae throughout a period of 14 months was detected in June, disappearing in July to September and February (28). The authors concluded that this is correlated with an adaptation of the parasite to the

climate, thus affecting the seasonal distribution of the insect vectors (27), and that environmental conditions may arrest the development of microfilariae or influence their distribution in the host's tissues (28). Similar seasonal variations likely occur in the donkey too in terms of the concentration, distribution, and occurrence of microfilariae in tissues, including the bloodstream. Therefore, our finding of *Onchocerca* microfilariae circulating in the peripheral blood of jennies in late spring (June) is in agreement with the results of other studies (27, 28).

Detection of *Onchocerca* microfilariae in the peripheral blood smear of donkeys or other equids is unusual and thus molecular analysis for confirmatory purposes was needed. In our study, donkeys did not show any dermal or ocular sign that might suggest *Onchocerca* infection at the time of admittance to the VTH. Therefore, skin biopsies (see the Section Discussion below) were not included in the initial diagnostic workup. On the other hand, onchocerciasis diagnosis with skin biopsy is laborious and time consuming for equine practitioners and laboratory technicians, as well as being expensive for owners. Even when *Onchocerca* infection was suspected because of microfilariae morphological features, performing skin biopsies of the ventral midline on at least one of the jennies would have required the specific owner's informed consent. Unfortunately, for this veterinary procedure, the jennies need to be sedated to avoid any risk for the operators and also an extended hospitalization time at the VTH, thus leading to economic losses because of the additional costs and lack of income for the owner of a milk production farm. This means that skin biopsies would never have been permitted by the owner given the lack of any clinical evidence and above all with no apparent economic benefit for him. Consequently, we opted to use molecular analysis rather than skin biopsy to confirm a case of microfilaraemia compatible with presumptive laboratory diagnosis of asymptomatic *Onchocerca* infection. This was despite the fact that a skin biopsy is the standard technique for diagnosing equine onchocerciasis. The concentration of microfilariae that we found in the peripheral blood samples (range 1–31 microfilariae in 2 mL) is likely to be much lower than the actual microfilariae concentration in the tissues of the examined jennies because Knott's test is not the most suitable method to detect microfilariae in *Onchocerca* infected equids. *Onchocerca* microfilariae have a very uneven distribution in the dermis of their hosts (29). The most effective method to detect *Onchocerca* microfilariae and accurately estimate their concentration is based on skin biopsies of the midline of the abdomen, preferably near the umbilical region, both in infected donkeys (5, 10) and horses (15, 30–32). For instance, Hussein and El Sammani (5) reported that the density of *O. raillieti* microfilariae in donkeys ranged from 1,200 to 26,200 per gram of skin in the linea alba along the mid-ventral line and from 1,100 to 16,900 per gram of skin in the region of the wither. In donkeys within the age groups of 1–3, 4–6, and 7–10 years, increasing counts per gram of skin were detected with mean values of 1,083 (range 100–2,400), 1,444 (1,000–2,000), and 2,040 (1,330–9,000) microfilariae, respectively (10). The number of *O. cervicalis* microfilariae per 8 or 6 mm of biopsied skin in ponies and horses ranged from 1 to 21,570 and 8 to 55,600, respectively (31). In other surveys on horses, microfilarial counts ranged from 18 to 42,446 microfilariae/skin snip (16) and a concentration of 19,770 microfilariae/mg was detected (26).

All four *Onchocerca* infected jennies appeared to be healthy and clinically normal at the initial clinical examination. This is in agreement with Hussein and El Sammani (5) and Matov et al. (33) in *O. cervicalis* and *O. raillieti* infected donkeys, respectively. Matov et al. (33) reported that 12 out of 41 healthy donkeys harbored adults of *O. cervicalis* and eight had microfilariae in their eyes. Hussein and El Sammani (5) found that at post-mortem examination, fibrous nodules ranging from 5–10 mm to 2–5 cm in diameter, containing degenerated parasites or filled with caseous and calcified necrotic material, were scattered in the ligamentum nuchae. At histological examination, the authors found that live adult worms induced mild inflammation whereas dead worms elicited an intense inflammatory response with the infiltration of large numbers of lymphocytes, macrophages, and fibroblasts intermingled with eosinophils. Moreover, heavy microfilarial skin densities were detected, as mentioned above, and 5 to 15 microfilariae per eye were found, mostly in the anterior chamber. However, despite these findings, none of the 373 infected donkeys had shown clinical signs that could be attributed to the infection, such as poll-evil and fistulous withers, pruritus, blindness or periodic ophthalmia. Likewise, after surveying over 120 horses infected with *O. cervicalis*, Mellor (34) reported that pathological effects of adult worms on the ligamentum nuchae were of little importance and that no pathological conditions were seen, in either the skin or the eyes, that could be definitely attributed to microfilariae. Matov et al. (33) reported that microfilariae of *O. cervicalis* cannot be considered as the primary cause of periodic ophthalmia in horses. However, other authors have associated *O. cervicalis* infection in horses with a variety of ocular lesions of the conjunctiva, cornea, uveal tract, lens and retina (35), fistulous withers (36), and dermatitis of the ventral midline and/or limbs, shoulders, thorax, and withers (17). It is thought that microfilariae migrate through the bloodstream reaching the eyelids, cornea, conjunctiva, and uvea where they concentrate and that a marked inflammatory reaction is triggered by immune responses to antigens released by dead and dying microfilariae (1). However, the pathogenic role of *O. cervicalis* still remains uncertain and controversial (2, 37) as the infection can be seen in large numbers of horses with or without clinical signs (38). Since we observed the absence of dermatological and ocular signs, we concluded that neither adult worms nor microfilariae appear to cause any clinical evidence in donkeys. It is possible that donkeys may have a higher tolerance than horses to *Onchocerca* infection.

To the best of our knowledge, only two studies have previously investigated hematological values in donkeys naturally infected with *Onchocerca*. The results of one of these studies showed that levels of serum total proteins, albumin, and globulins were significantly ($p < 0.05$) higher compared to uninfected donkeys. Other variations included an increase in the level of glucose and a lower concentration of serum cholesterol (10). By contrast, in jenny I we found low values of serum total proteins and a mild reduction in albumin along with increased cholesterol.

However, comparing our findings with those previously reported is not reliable because of the large differences in study design between our report and previous studies. In fact, one study investigated blood biochemical changes following treatment with doramectin against *O. raillietti* microfilariae (9), while another study compared the effects of *O. raillieti* infection on serum total proteins, albumin, glucose, and cholesterol in naturally infected and uninfected donkeys (10). We detected a variety of hematological alterations in one of the jennies (I) with subclinical *Onchocerca* infection. However, all these alterations were probably due to the blood lipemic status secondary to obstructive colic in a lactating jenny and it is very unlikely that *Onchocerca* infection plays a key role in this context. Differential white blood cell counts revealed moderate eosinophilia in jenny II while remarkable eosinophilia and basophilia were present in jenny III. The role of eosinophils in parasitic helminth infections in mammals has been widely demonstrated (39, 40), including in cases of *Onchocerca* infection in humans (41, 42). The relationship between basophilia and helminth infections has also been well established in humans (43) and several animal models (44). However, it cannot be ruled out that these hematological alterations were caused by concurrent infections with other pathogens. Therefore, further investigations are needed to determine whether most donkeys naturally affected by subclinical infection with *Onchocerca* may develop eosinophilia and basophilia.

CONCLUSIONS

Onchocerca microfilariae were incidentally detected in four jennies in Italy. Microfilariae were identified by blood smear, Knott's test, and finally DNA analysis. All the cases were of autochthonous origin because the jennies were born and reared on the same farm located in central Italy and they had never moved outside the farm. The transmission of *Onchocerca* infection to these animals is linked to their exposure to bites of infected *Culicoides*, suggesting that vectors in turn could have acquired the infection locally. The reproductive lifespan of *Onchocerca volvulus*, the etiological agent of river blindness in humans, ranges from 9 to 11 years (45) and adult females can release 700 to 900 microfilariae daily (46). At present, the lifespan of *Onchocerca* species in donkeys is unknown but we can assume that adult females live for years and likewise release hundreds of microfilariae daily during their lifespan. Insect vectors that are suitable for transmitting the infection are widespread in Italy (20). Since the infection can be easily overlooked, a high proportion of donkeys may be infected with minimal or no clinical or hematological evidence, and may act as clinically asymptomatic carriers. Our report demonstrates the occurrence of *Onchocerca* in donkeys in Italy. We thus believe that equine practitioners should be aware of our findings and of their implications for equine health. Our findings highlight: (i) the risk of spreading the infection to other susceptible hosts (donkeys, mules, horses, ponies), (ii) the risk of progression in parasitized equines toward clinical disease, and (iii) the need to control the occurrence of *Onchocerca* infections in the breeding systems of donkeys and horses reared in Italy. The possibility of accidental transmission of *O. cervicalis* to humans has also been reported (47, 48). Our report also provides a basis for further studies to determine infection rates in donkeys and horses, to identify the insect vectors that may act as intermediate hosts, and to implement possible prevention and control measures.

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 was not required as per institutional guidelines/local legislation due to natural infections that occurred spontaneously. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

RP performed parasitological examinations and wrote the first draft of the manuscript. GL performed hematological examinations. MS performed all clinical examinations. All the authors critically edited and reviewed the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This study was supported by the University of Pisa (Fondi di Ateneo 2018).

REFERENCES

1. Dagnaw M, Zemene M, Getaneh G, Tibenu S. A review on equine onchocerchiasis. *Afr J Basic Appl Sci.* (2016) 8:27–33. doi: 10.5829/idosi.ajbas.2016.8.1.22966
2. Melhorn H. *Encyclopedia of Parasitology*. Berlin; Heidelberg; New York, NY: Springer-Verlag (2008).
3. El Sammani SE, Hussein HS. *Onchocerca raillieti*: adult location and skin distribution of the microfilaria in Sudanese donkeys. *J Helminthol.* (1983) 57:355–60. doi: 10.1017/S0022149X0001107X
4. Beveridge I, Gasser R, Sangster N. Horses. In: Beveridge I, Emery D, editors. *Australasian Animal Parasites Inside and Out*. The Australian Society for Parasitology (2015). p. 826–7.
5. Hussein HS, El Sammani SE. *Onchocerca raillieti*: prevalence and pathology in Sudanese donkeys. *J Comp Pathol.* (1985) 95:345–51. doi: 10.1016/0021-9975(85)90038-6
6. Lok JB, Cupp EW, Braide EI, Bernardo MJ. *Aedes aegypti* as a surrogate host for *Onchocerca* spp. *Am J Trop Med Hyg.* (1980) 29:382–8. doi: 10.4269/ajtmh.1980.29.382
7. Lia RP, Mutafchiev Y, Veneziano V, Giannelli A, Abramo F, Santoro M, et al. Filarial infection caused by *Onchocerca boehmi* (Supperer, 1953) in a horse from Italy. *Parasitol Res.* (2017) 116:191–8. doi: 10.1007/s00436-016-5277-x
8. Bain O, Muller R, Khamis Y, Guilhon J, Van Veen TS. *Onchocerca raillieti* sp. (*Filarioidea*) chez l'Ane domestique en Afrique. *J Helminthol.* (1976) 50:287–93. doi: 10.1017/S0022149X00026730

9. Seri HI, ElBashir HM, Elmansoury YHA, Salih MM. Blood biochemical changes in donkeys naturally infected with *Onchocerca raillieti*: the effect of medication with doramectin. *Int J Pharmacol.* (2006) 2:530–5. doi: 10.3923/ijp.2006.530.535
10. Elbasheir HM, Adam DA, Anwar AMA. Epidemiology of *Onchocerca raillietie* in donkeys at Tumbool city and its influence on some serum constituents. *Bull Anim Health Prod Afr.* (2014) 62:339–43.
11. Zhang Z, Schwartz S, Wagner L, Miller W. Greedy algorithm for aligning DNA sequences. *J Comput Biol.* (2000) 7:203–14. doi: 10.1089/106652700500 81478
12. Morgulis A, Coulouri G, Raytselis Y, Madden TL, Agarwala R, Schäffer AA. Database indexing for production MegaBLAST searches. *Bioinformatics.* (2008) 24:1757–64. doi: 10.1093/bioinformatics/btn322
13. Radwan AM, Ahmed NE, Elakabawy LM, Ramadan MY, Elmadawy RS. Prevalence and pathogenesis of some filarial nematodes infecting donkeys in Egypt. *Vet World.* (2016) 9:888–92. doi: 10.14202/vetworld.2016. 888-892
14. Hasanova RI. Distribution of onchocercosis in horses caused by *Onchocerca cervicalis* Raillet et Henry 1910, and *Onchocerca reticulata* Diesing, 1841, in the region of East Caucasus. *Rossiiskii Parazitol Z.* (2014) 3:48–51 (In Russian).
15. Franck MT, Colombet J, Hugnet C, Ducos de Lahitte J, Desmaizières LM, Delverdier M, et al. Research of skin microfilariae on 160 horses from Poland, France and Spain. *Rev Méd Vét.* (2006) 157:323–5.
16. Herd RP, Donham JC. Efficacy of ivermectin against Onchocerca cervicalis microfilarial dermatitis in horses. *Am J Vet Res.* (1983) 49:1102–5.
17. French DD, Klei TM, Foil. CS, Miller RI, Chapman MR, et al. Efficacy of ivermectin in paste and injectable formulations against microfilariae of *Onchocerca cervicalis* and resolution of associated dermatitis in horses. *Am J Vet Res.* (1988) 49:1550–4.
18. Monahan CM, Chapman MR, French DD, Klei TR. Efficacy of moxidectin oral gel against *Onchocerca cervicalis* microfilariae. *J Parasitol.* (1995) 81:117–8. doi: 10.2307/3284021
19. Mancebo OA, Verdi JH, Bulman GM. Comparative efficacy of moxidectin 2% equine oral gel and ivermectin 2% equine oral paste against *Onchocerca cervicalis* (Railliet and Henry, 1910) microfilariae in horses with naturally acquired infections in Formosa (Argentina). *Vet Parasitol.* (1997) 73:243–8. doi: 10.1016/S0304-4017(97)00136-2
20. Goffredo M, Meiswinkel R, Federici V, Di Nicola F, Mancini G, Ippoliti C, et al. The 'Culicoides obsoletus group' in Italy: relative abundance, geographic range, and role as vector for Bluetongue virus. *Vet Ital.* (2016) 52:235–41. doi: 10.12834/VetIt.35.100.1
21. Abou-Rawash AA. Histopathology of nuchal ligament onchocerciasis and microfilarial dermatitis in donkeys in Kafr el-Sheikh governorate. *Minufya Vet J.* (2002) 2:323–32.
22. Loyd S, Soulsby EJ. Survey of infection with *Onchocerca cervicalis* in horses in eastern United States. *Am J Vet Res.* (1978) 39:1962–3.
23. Collins RC. Onchocerciasis of horses in southeastern Louisiana. *J Parasitol.* (1973) 59:1016–20. doi: 10.2307/3278636
24. Stannard AA, Cello RM. *Onchocerca cervicalis* infection in horses from the western United States. *Am J Vet Res.* (1975) 36:1029–31.
25. Schmidt GM, Krehbiel JD, Coley SC, Leid RW. Equine onchocerciasis: lesions in the nuchal ligament of midwestern U.S. horses. *Vet Pathol.* (1982) 19:16–22. doi: 10.1177/030098588201900104
26. Cummings E, James ER. Prevalence of equine onchocerciasis in southeastern and midwestern United States. *J Am Vet Med Assoc.* (1985) 186:1202–3.
27. Foil LD, Klei TR, Miller RI, Church GE, Foil CS, French DD, et al. Seasonal changes in density and tissue distribution of *Onchocerca cervicalis* microfilariae in ponies and related changes in Culicoides variipennis populations in Louisiana. *J Parasitol.* (1987) 73:320–6. doi: 10.2307/328 2085
28. Abdul-Salam J, al-Taqui M. Seasonal prevalence of Onchocerca-like microfilaria in camels in Kuwait. *J Egypt Soc Parasitol.* (1995) 25:19–24.
29. Mellor PS. Studies on *Onchocerca cervicalis* Railliet and Henry 1910: I. *Onchocerca cervicalis* in British horses. *J Helminthol.* (1973) 47:97–110. doi: 10.1017/S0022149X00023774
30. Marcoux M, Fréchette JL, Morin M. Infection par Onchocerca cervicalis au Québec: signes cliniques et méthode de diagnostic. *Can Vet J.* (1977) 18:108–10.
31. Klei TR, Torbert B, Chapman MR, Foil L. Prevalence of *Onchocerca cervicalis* in equids in the Gulf Coast region. *Am J Vet Res.* (1984) 45:1646–7.
32. Marques SM, Scroferneker ML. *Onchocerca cervicalis* in horses from southern Brazil. *Trop Anim Health Prod.* (2004) 36:633–6. doi: 10.1023/B:TROP.0000042863.83233.78
33. Matov K, Vasilev I, Osikpviski E, Yanchev Y. The importance of microfilariae in the aetiology of periodic ophtalmia in horses. *Helminthologia.* (1959) 1:257–66.
34. Mellor PS. Studies on *Onchocerca cervicalis* Railliet and Henry 1910: II Pathology in the horse. *J Helminthol.* (1973) 147:111–8. doi: 10.1017/S0022149X00023804
35. Cello RM. Ocular onchocerciasis in the horse. *Equine Vet J.* (1971) 3:148–54. doi: 10.1111/j.2042-3306.1971.tb04460.x
36. Ottley ML, Dallemagne C, Moorhouse DE. Equine onchocerciasis in Queensland and the Northern Territory of Australia. *Aust Vet J.* (1983) 60:200–3. doi: 10.1111/j.1751-0813.1983.tb09582.x
37. Polley L. Onchocerca in horses from Western Canada and the northwestern United States: an abattoir survey of the prevalence of infection. *Can Vet J.* (1984) 25:128–9.
38. Moran CT, James ER. Equine ocular pathology ascribed to *Onchocerca cervicalis* infection: a re-examination. *Trop Med Parasitol.* (1987) 38:287–8.
39. Behm CA, Ovington KS. The role of eosinophils in parasitic helminth infections: insights from genetically modified mice. *Parasitol Today.* (2000) 16:202–9. doi: 10.1016/S0169-4758(99)01620-8
40. Klion AD, Nutman TB. The role of eosinophils in host defense against helminth infections. *J Allergy Clin Immunol.* (2004) 113:30–7. doi: 10.1016/j.jaci.2003.10.050
41. Pearlman E. Immunopathology of onchocerciasis: a role for eosinophils in onchocercal dermatitis and keratitis. *Chem Immunol.* (1997) 66:26–40. doi: 10.1159/000058664
42. Nfon CK, Makepeace BL, Njongmeta LM, Tanya VN, Bain O, Trees AJ. Eosinophils contribute to killing of adult *Onchocerca ochengi* within onchocercomata following elimination of Wolbachia. *Microb Infect.* (2006) 8:2698–705. doi: 10.1016/j.micinf.2006.07.017
43. Voehringer D. The role of basophils in helminth infection. *Trends Parasitol.* (2009) 25:551–6. doi: 10.1016/j.pt.2009.09.004
44. Mitre E, Nutman TB. Basophils, basophilia, helminth infections. *Chem Immunol Allergy.* (2006) 90:141–56. doi: 10.1159/000088886
45. Plaisier AP, van Oortmarssen GJ, Remme J, Habbema JD. The reproductive lifespan of *Onchocerca volvulus* in West African savanna. *Acta Trop.* (1991) 48:271–84. doi: 10.1016/0001-706X(91)90015-C
46. Schilz-Key H. Observations on the reproductive biology of *Onchocerca volvulus*. *Acta Leidensia.* (1990) 59:27–44.
47. Beaver PC, Yoshimura H, Takayasu S, Hashimoto H, Little MD. Zoonotic Onchocerca in a Japanese child. *Am J Trop Med Hyg.* (1989) 40:298–300. doi: 10.4269/ajtmh.1989.40.298
48. Burr WE Jr, Brown MF, Eberhard ML. Zoonotic Onchocerca (Nematoda: Filarioidea) in the cornea of a Colorado resident. *Ophthalmology.* (1998) 105:1494–7. doi: 10.1016/S0161-6420(98)98035-6

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 November 2020
doi: 10.3389/fvets.2020.579371

Evaluation of Colostral Immunity Against Equine Herpesvirus Type 1 (EHV-1) in Martina Franca's Foals

Cristina E. Di Francesco, Camilla Smoglica, Ippolito De Amicis, Federica Cafini, Augusto Carluccio and Alberto Contri*

Faculty of Veterinary Medicine, University of Teramo, Teramo, Italy

Eight Martina Franca pregnant jennies were selected in order to evaluate the transfer of colostral antibodies against equine herpesvirus type 1 in their relative foals after immunization with a commercial inactivated vaccine, compared with an unvaccinated group. Samples of serum and colostrums/milk were collected from jennies and foals under study starting from 10 min before and up to 21 days after the foaling. Specific anti-EHV-1 antibody titers were evaluated by means of a serum neutralization test, and the results obtained from both groups were analyzed. The serological titers in the vaccinated jennies was significantly higher ($p < 0.01$). No significant differences were found in the specific time-point intervals in both groups examined ($p > 0.05$). The antibody titers in milk at the time of delivery and subsequent withdrawal (T0 and T1) were very high in both groups, but no significant differences were found between the two groups ($p > 0.05$). In the foal sera, a significant difference was found between foals in the vaccinated group compared with those in the unvaccinated group ($p < 0.05$). Finally, a significant correlation ($p < 0.05$) was observed between the antibody titers found in serum and colostrum of jennies and the foal titers in the first time-point sampling (up to 12 h after foaling). The results confirm a substantial homology in the antibody production compared with other most investigated equids, highlighting the efficacy of the vaccination against EHV-1 of the jennies to ensure the protective immunity to their foals during the first weeks after delivery.

Keywords: donkey, Martina Franca breed, serum, colostrum, antibody titer, EHV-1

OPEN ACCESS

Edited by:
Fulvio Laus,
University of Camerino, Italy

Reviewed by:
Abouzar Najafi,
University of Tabriz, Iran
Juan G. Maldonado-Estrada,
University of Antioquia, Colombia

****Correspondence:***
Ippolito De Amicis
ideamicis@unite.it

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

Received: *02 July 2020*
Accepted: *05 October 2020*
Published: *23 November 2020*

Citation:
Di Francesco CE, Smoglica C, De Amicis I, Cafini F, Carluccio A and Contri A (2020) Evaluation of Colostral Immunity Against Equine Herpesvirus Type 1 (EHV-1) in Martina Franca's Foals. Front. Vet. Sci. 7:579371. doi: 10.3389/fvets.2020.579371

INTRODUCTION

The Martina Franca donkey (MFD) is an asinine breed of great value, native from the Murgia dei Trulli area, in the Apulia provinces of Bari, Taranto, and Brindisi, Italy. It is the largest Italian asinine breed, and because of the imposing size, the stallions of this donkey are widely used in the production of mules, in particular the Martinese mule, generated by the crossing with Murgese horse mares. The MFD population includes 850 donkeys with 25 stallions approved for breeding (1). Over the years, as reported by the FAO Domestic Animal Diversity Information System, the asinine breed of Martina Franca has been identified as endangered. Therefore, any conservation strategies aimed to preserve the health status of the animals and to achieve increases in reproductive success should be carefully considered.

For these reasons, in the past the reproductive characteristics of this breed were investigated in order to ensure the reproductive performances of both females and males of MFD (2, 3). More in detail, the immunity transfer from jennies to foals with particular regard to the immunoglobulin (Ig) G and IgA concentrations in the sera, as well as in milk secretions, was characterized showing a substantial similarity with that reported for horses (4). The higher levels of total IgG can be detected in mammary secretions and serum samples of MFD jennies during the first 10 days after birth, while in donkey foals the serum immunoglobulin concentrations did not show statistically significant differences, although high levels of IgG were observed up to 12 h after delivery (5). It is noteworthy that the antibody transfer from the mother to the foal is essential for the acquisition of passive immunity, and it may be achieved if the intake of colostrum occurs within the first 12–24 h of life (6–8) resulting in a protective action of maternal antibodies into possible external infectious agents. On the other hand, any unfavorable event able to compromise the transfer of colostral immunity in the foal could be critical for the onset of infections during the first 4 weeks of life (9).

Among the pathogens responsible for neonatal diseases in equids, equine herpesvirus-1 (EHV-1) is the more prevalent virus associated with respiratory distress in foals, along with stillbirth, neonatal death, and neurological disease (10). In this regard, the prevention plans for EHV-1-associated diseases require the use of a rigorous vaccination protocol, implemented in both mares and jennies, based on three administrations at the 5th, 7th, and 9th months of gestation (11). In horses, despite the use of the vaccine, the EHV-1 antibody-titers in mares and foals do not appear significantly correlated with the protection, and the fluctuations of the serological response observed in mares and foals are probably due to a silent circulation of the virus among the animals (12).

Even if the passive immunity transfer pattern in donkey foals appears to be similar to that reported for the equine foals, the knowledge about the levels of EHV-1-specific antibodies induced by the vaccination in MF mares and the consequent transfer of them to the foals by colostrum are still lacking, and additional studies could be useful to better characterize the physiology of passive immunity in MFD and to identify the more effective strategies to contrast the neonatal mortality in this threatened breed.

The aim of this study was to compare the titers of colostral antibodies against EHV-1 in serum and colostrums/milk samples collected from both vaccinated and unvaccinated MF mares and their foals starting from the day of the birth up to the 21st day after foaling, in order to verify the kinetic of the passive immunity in both groups under field conditions and to obtain information about the effect of the vaccination on the antibody status of the animals.

METHODS

In the period between 2009 and 2014, $n = 13$ pregnant jennies of the Martina Franca breed (MF) and their respective foals, belonging to the same farm within the Faculty of Veterinary Medicine of Teramo, were investigated.

The jennies were older than 4–5 years, and their body weight was between 396 and 420 kg. During the whole observation period, the animals were kept in external paddocks and exposed to natural atmospheric conditions. Daily, the jennies received standard hay *ad libitum* and commercial horse fodder (2 kg). The Body Condition Score (BCS) of all donkeys was between 3/5 and 4/5 and remained constant for the entire duration of the monitoring.

During pregnancy, $n = 8$ jennies were vaccinated against EHV-1 and EHV-4 using the inactivated Duvaxyn TM EHV-1,4 vaccine (Fort Dodge Animal Health SpA, Italy). The vaccine administrations were carried out at the 5th, 7th, and 9th months of gestation, while the remaining $n = 5$ jennies and all relative foals (belonging to both vaccinated and unvaccinated groups) were not subjected to any administration during the observation period. The recruited jennies showed a regular gestation, and the birth took place without the need for obstetric intervention; the foals appeared clinically healthy at foaling, and they began to take the colostrum without any assistance within the first 2 hours (h) after the foaling.

Serum and colostrum/milk samples were collected from each jenny/foal pair 10 min before foaling up to 21 days postpartum (pp) according to the calendar reported in **Table 1** for a total of $n = 143$ colostrum/milk samples and $n = 286$ serum samples.

All samples were frozen within 2 h of collection and stored at −20°C until laboratory investigations were performed. Before testing, the samples were preheated at 56°C for 30 min to inactivate complement and colostrum was centrifuged at 2,000 rpm for 15 min to remove cellular debris and lipid layer and collect only the liquid portion.

All serum and colostrums/milk samples were tested for serum neutralization (SN) against EHV-1 using RK-13 cells for both viral culture and serum testing (13). Briefly, for each test, 96-well SN plates were prepared with 50 μl of each sample, diluted starting from the 1: 2 dilution up to a dilution of 1: 256, and an equal volume of 100 TCID50 of an EHV-1 vaccine strain

TABLE 1 | Temporal intervals for sera and colostrum sampling from mares and foals under study.

ID sampling	Time-point
T0	10 min before foaling
T1	1 h after 1st colostrum suck
T2	1 h after 2nd colostrum suck
T3	12 h from foaling
T4	1 day from foaling
T5	2 days from foaling
T6	3 days from foaling
T7	5 days from foaling
T8	7 days from foaling
T9	14 days from foaling
T10	21 days from foaling

propagated on RK13 cells (1 × 105 ml). After incubation of the plates for 45 min at 37°C and 5% CO2, RK13 cells, generally with passage numbers between 85 and 100, were added in the amount of 100 µl (1 × 105/ml) for each well. Appropriate controls for cell viability, virus infectivity, and serum cytotoxicity were included.

All plates were incubated at 37°C and 5% CO2 for 4 days. Then, all plates were examined to detect the cytopathic effect (CPE) of the virus, characterized by cell rounding, formation of syncytia, and detachment of the cell monolayer. The end-point antibody titer was expressed as the reciprocal of the highest dilution of each sample resulting in a complete neutralization of CPE in the cell monolayer, while a sample was defined negative in the presence of viral replication starting from the first 1: 2 dilution.

Data were reported as mean ± standard error of the mean (SEM). Data distribution was tested using the Shapiro–Wilk test. Since the data were not normally distributed, the statistical evaluation was performed after logarithmic transformation.

The evaluation of the data was performed using a generalized linear model (GLM), based on the univariate ANOVA. The biological matrix (maternal serum, milk, and foal serum), time, and vaccination (vaccinated/unvaccinated) were considered as fixed factors. When necessary, a Scheffè test was performed for the *post-hoc* evaluation.

Any correlations between the SN values in the different biological matrices were tested, at various times, by calculating the Pierson correlation coefficient.

In all cases, differences with $p < 0.05$ were considered statistically significant.

The statistical evaluation was performed using the SPSS software version 17 (SPSS Inc., Chicago, IL, USA).

RESULTS

In the group of unvaccinated jennies, the serum antibody titers against EHV-1 were variable from 0 to 1:16; the latter value was obtained only for a serum sample 3 days after delivery (T6). In contrast, for 6 jennies out of 8 vaccinated and at different times of sampling, the antibody titers in the vaccinated group reached values above 1:16, up to 1:128 (**Figure 1**). The serological titers in the vaccinated jennies was significantly higher ($p < 0.01$). No significant differences were found in the specific time-point intervals in both groups examined ($p > 0.05$).

The antibody titer in milk at the time of delivery and subsequent withdrawal (T0 and T1) were very high in both groups, with titers up to 1:128 in unvaccinated jennies and 1:256 in those vaccinated, even if no significant differences were found between the groups ($p > 0.05$). After T2, the values recorded

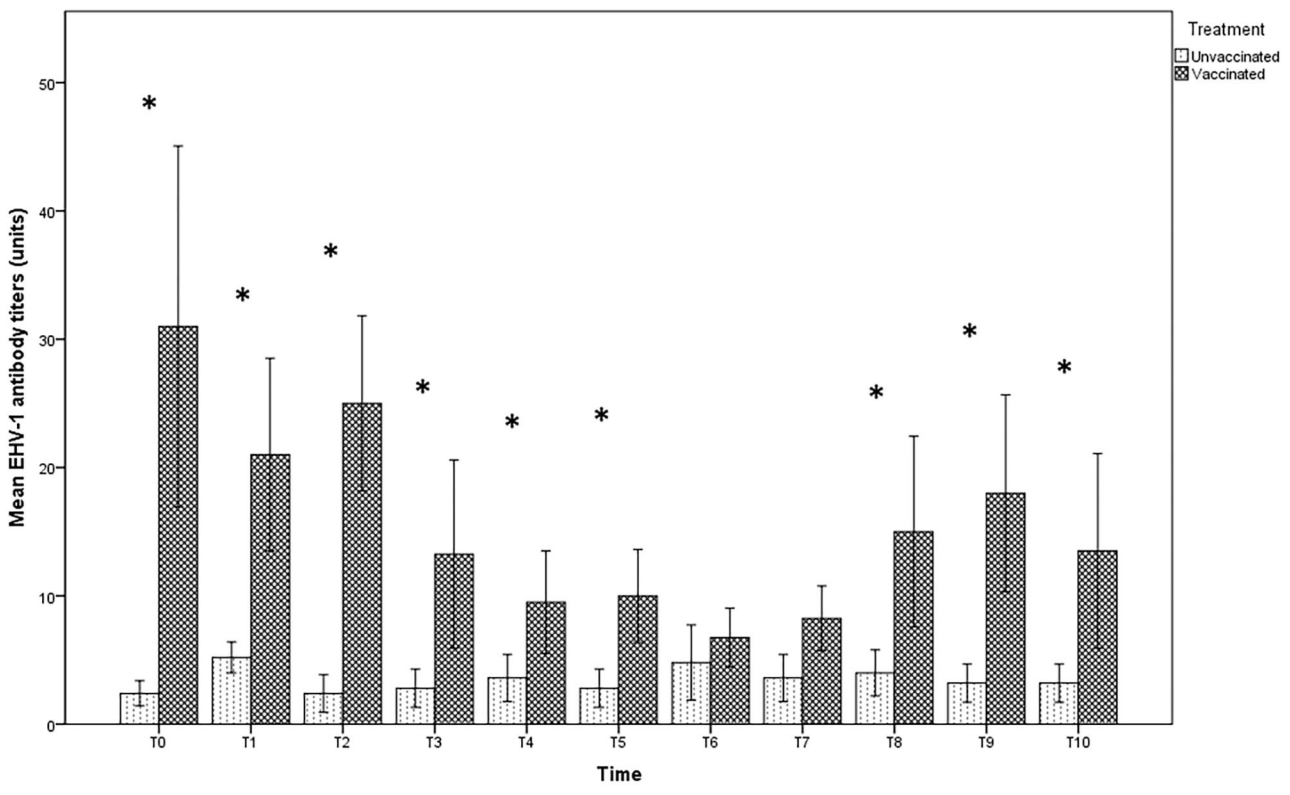


FIGURE 1 | Mean (bar—standard error of the mean) antibody titers against EHV-1 detected by SN test in maternal sera collected during the different time-points of sampling (T0 to T10) from both vaccinated ($n = 8$) and unvaccinated ($n = 5$) jennies under study. At the same time-point, the values of unvaccinated and vaccinated jennies marked with asterisk (*) differ significantly ($p < 0.05$). The titers were expressed as the reciprocal of the highest dilution whit a complete CPE of the cells.

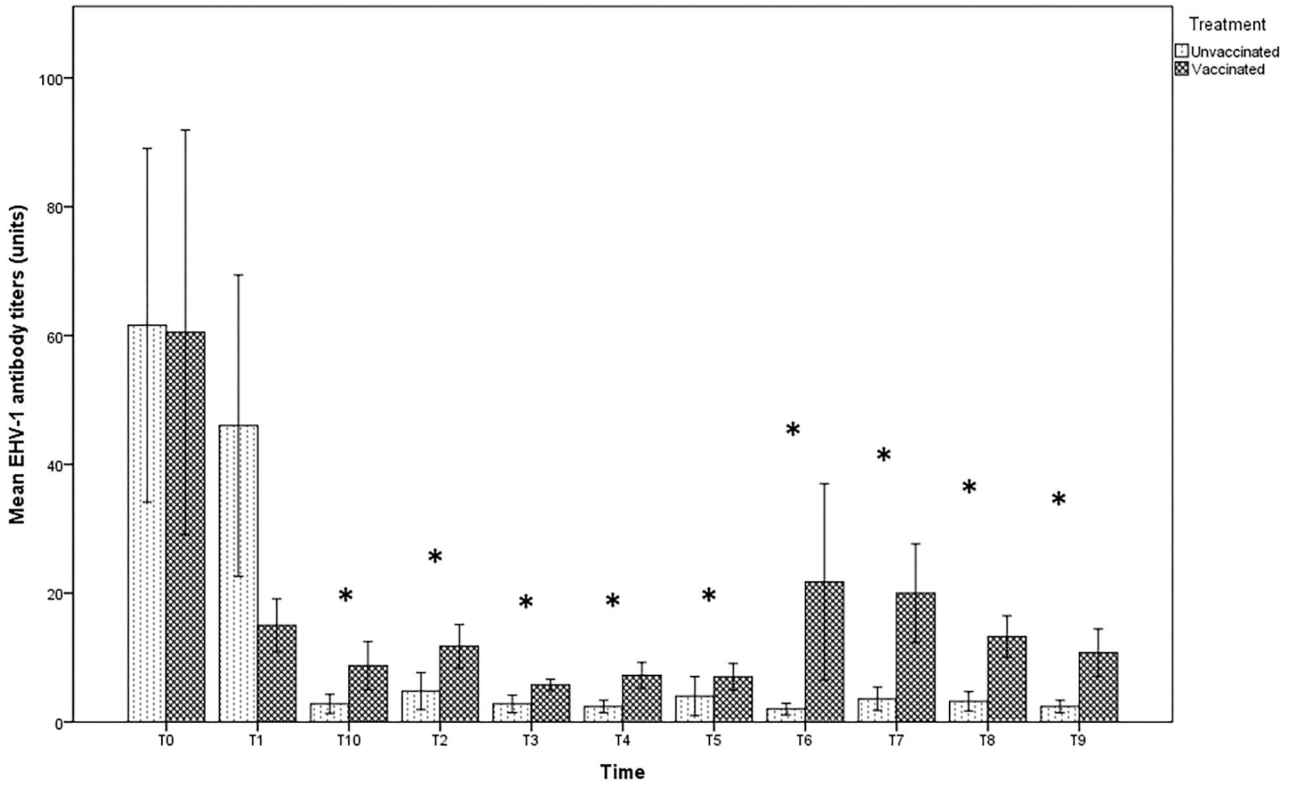


FIGURE 2 | Mean (bar—standard error of the mean) antibody titers against EHV-1 detected by SN test in maternal colostrum/milk samples collected during the different time-points of sampling (T0 to T10) from both vaccinated ($n = 8$) and unvaccinated ($n = 5$) jennies under study. At the same time-point, the values of unvaccinated and vaccinated jennies marked with asterisk (*) differ significantly ($p < 0.05$). The titers were expressed as the reciprocal of the highest dilution with a complete CPE of the cells.

in the milk of vaccinated jennies were significantly higher than those recorded in unvaccinated animals ($p < 0.05$). Indeed, in the group of unvaccinated jennies, the titer decreased reaching the lower values until complete negativity, starting from T2 (after the second sucking). A decrease in antibody concentrations was also found in the group of vaccinated jennies, with antibody titers that returned to increase after T3 until T8 (**Figure 2**).

In the foal serum, antibody titration showed similar values in both vaccinated (1:8) and unvaccinated groups (1:4), until T2. Twelve hours after foaling, however, a significant increase in the serum titer in the vaccinated group, but not in the unvaccinated one, was found (**Figure 3**). Statistically significant differences were found between foals in the vaccinated group compared with those in the unvaccinated group ($p < 0.05$) over T3 until the end of the observational period.

The values of the antibody titer found in the mothers were significantly correlated with the foal titer at T1 ($R = 0.768$, $p < 0.01$), while it was significantly correlated with the milk titer at T2 ($R = 0.729$, $p < 0.01$) and with the foal titer ($R = 0.674$, $p < 0.05$) in the same sampling time.

A further significant correlation was found at T9, in which the titer present in the maternal serum was significantly correlated with the antibody titer in milk ($R = 0.614$, $p < 0.05$) and foal ($R = 0.598$, $p < 0.05$). Furthermore, at the same time, a significant correlation was found between the antibody titer in milk and foal ($R = 0.889$, $p < 0.01$).

DISCUSSION

In this study, the serological investigations were carried out on MF jennies raised in non-experimental field conditions, in open shelters, with the possibility of access to the pasture, and with the presence of other individuals belonging to the same breed and horses. Under these conditions, it is possible to hypothesize a preexisting circulation of HEV-1 among animals, just as it cannot be excluded that other vaccine administrations for the control of the disease have been performed in the previous breeding seasons, considering the age of all jennies subjected to study. Indeed, the infection with HEV-1 is recognized worldwide as endemic among equids, although the vaccination is widely practiced and also thanks to its latency capacity in host cells (14).

In the light of the above information, the presence of anti-EHV-1 antibodies found in the group of jennies that were not subjected to immunization, and persistent throughout the entire study period, even at low levels, is not to be considered unusual. Previous studies have already highlighted that the prevalence

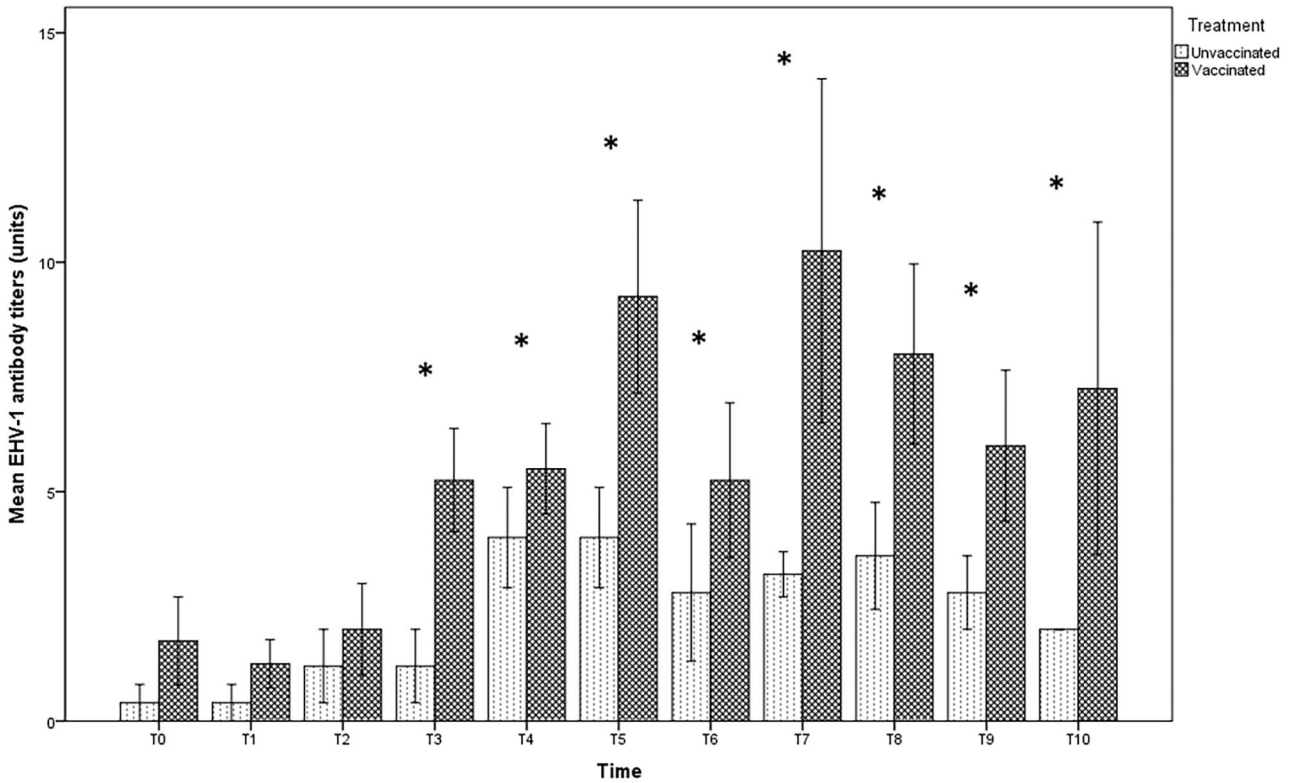


FIGURE 3 | Mean (bar—standard error of the mean) antibody titers against EHV-1 detected by SN test in serum samples collected, at the different time-points (T0 to T10), from foals belonging to both vaccinated ($n = 8$) and unvaccinated ($n = 5$) jennies. At the same time-point, the values of unvaccinated and vaccinated jennies marked with asterisk (*) differ significantly ($p < 0.05$). The titers were expressed as the reciprocal of the highest dilution with a complete CPE of the cells.

of EHV-1 antibody-positive mares and foals did not change significantly when the vaccination is routinely introduced in the horse farms, suggesting a preexisting immunity in the animals consequent to a cycle of infection among the animals (15).

Despite this, the vaccination carried out on a part of the animals has allowed to highlight an overall significant difference in the production of specific antibodies even if this difference is not confirmed by analyzing the individual withdrawal periods. Although a lack of correlation between antibody titer and protection from infection is documented in horses also (16), it is conceivable that the level of antibodies induced by vaccination is able to protect animals from clinical disease and reduce the amount and the duration of viral excretion by the respiratory route. The SN tests carried out starting from milk samples confirmed this difference between vaccinated and unvaccinated subjects, except for T0 and T1 collecting times. In both groups, a significant increase in the concentration of anti-EHV-1 antibodies was observed in the first sampling intervals, confirming the uptake action exerted by the mammary gland in the first hours after delivery, to ensure the massive transfer of immunity to foals through colostrum (17). In the group of vaccinated jennies, moreover, the decrease in antibody levels in the milk appeared to be more gradual and transient, probably favoring a greater intake of specific IgG by the foals in the first 12 h after foaling, when the intestinal absorption of immunoglobulins is greater (18). Similarly, the foal immunity appeared to be influenced by the vaccination of the mothers, with particular emphasis for the samples collected starting from 12 h after foaling. This aspect should be considered when choosing an appropriated vaccination program of the foals, since the presence of high levels of maternal antibody may inhibit the response to the vaccine virus especially for inactivated virus.

The significant correlation observed at T9, 14 days after the delivery, when the transfer of antibodies by the colostrum can be considered completed, appears unusual. Probably, a new introduction of the wild virus strain in both groups under study may have determined this trend, or changes in management and environmental factors resulted in a reactivation of latent infection in stressed mares. Since no movements of new animals or contacts of the foals with other horses were reported during the entire period of the study, the role of stressor factors in the early infection of the foals despite the transfer of passive immunity from vaccinated/seropositive mares should not be ruled out.

In conclusion, this is a first attempt to study the antibody kinetic in MFD after vaccination against EHV-1. The results obtained confirm a substantial homology in the antibody

production compared with other well-characterized equids, highlighting the efficacy of the vaccination of the jennies to ensure the protective immunity of the foals during the first weeks after delivery (11, 19). The control of pathogens potentially able to menace the conservation of threatened species such as MFD remains the main tool to preserve the population, and periodic serological surveys of jennies and foals could be considered a useful diagnostic tool to verify the effective immunity reached by the animals after the vaccine administration.

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 the animal study because In this study the immunological status of donkeys was evaluated after immunization by using commercial vaccine. The antibody titres were obtained from serum samples collected from the animals during the routine clinical investigations in respect of Italian Normative D.Lvo n. 26/2014 (Directive 2010/63/UE).

AUTHOR CONTRIBUTIONS

ACa and CD substantially contributed to conception and design of the research. ACo, CD, CS, and ID contributed to acquisition, analysis, and interpretation of the data. ACo, CD, CS, FC, and ID drafted and critically revised the manuscript. All authors ensured that any part of the work is appropriately investigated and resolved and they gave the final approval of the manuscript.

FUNDING

The present study has been carried out in the framework of the Project Demetra (Dipartimenti di Eccellenza 2018–2022, CUP_C46C18000530001), funded by the Italian Ministry for Education, University and Research.

REFERENCES

1. A.N.A.M.F. *Associazione Nazionale Allevatori del Cavallo delle Murge e dell'Asino di Martina Franca.* (2020). Available online at: https://www.anamf.it/ (accessed March 01, 2020).
2. Carluccio A, Panzani S, Tosi U, Riccaboni P, Contri A, Veronesi MC. Morphological features of the placenta at term in the Martina Franca donkey. *Theriogenology.* (2008) 69:918–24. doi: 10.1016/j.theriogenology.2008.01.004
3. Veronesi MC, Villani M, Wilsher S, Contri A, Carluccio A. A comparative stereological study of the term placenta in the donkey, pony and Thoroughbred. *Theriogenology.* (2010) 74:627–31. doi: 10.1016/j.theriogenology.2010.03.006
4. Carluccio A, Gloria A, Veronesi MC, De Amicis I, Noto F, Contri A. Factors affecting pregnancy length and phases of parturition in Martina Franca jennies. *Theriogenology.* (2015) 84:650–5. doi: 10.1016/j.theriogenology.2015.04.027
5. Veronesi MC, Dall'Ara P, Gloria A, Servida F, Sala E, Robbe D. IgG, IgA, and lysozyme in Martina Franca donkeyjennies and theirfoals. *Theriogenology.* (2014) 81:825–31. doi: 10.1016/j.theriogenology.2013.12.018
6. McGuire TC, Crawford TB. Passive immunity in the foal: measurement of immunoglobulin classes and specific antibody. *Am J Vet Res.* (1973)34:1299–303.
7. Steven DH, Samuel CA. Anatomy of the placental barrier in the mare. *J Reprod Fertil Suppl.* (1975) 23:579–82.
8. Perryman LE, McGuire TC. Evaluation for immune system failures in horses and ponies. *J Am Vet Med Assoc.* (1980) 176:1374–7.
9. Raidal SL. The incidence and consequences of failure of passive transfer of immunity on a Thoroughbred breeding farm. *Aust Vet J.* (1996) 73:201–6. doi: 10.1111/j.1751-0813.1996.tb10035.x
10. Patel JR, Heldens J. Equine Herpes Virus 1 (EHV-1) and 4 (EHV-4), epidemiology, disease and immunoprophylaxis: a brief review. *Vet J.* (2005) 170:14–23. doi: 10.1016/j.tvjl.2004.04.018
11. Kydd JH, Townsend HG, Hannant D. The equine immune response to equine herpesvirus-1: the virus and its vaccines. *Vet Immunol Immunopathol.* (2006) 111:15–30. doi: 10.1016/j.vetimm.2006.01.005
12. Foote CE, Gilkerson JR, Whalley JM, Love DN. Seroprevalence of Equine herpesvirus 1 in mares and foals on a large Hunter Valley stud farm in years pre and postvaccination. *Aust Vet J.* (2003) 81:283–88. doi: 10.1111/j.1751-0813.2003.tb12576.x
13. OIE, Manual of Diagnostic Tests and Vaccines for Terrestrial Animals Terrestrial Manual. *Equine Rhinopneumonitis (infection with equid herpesvirus-1 and−4 (Version adopted in May 2017).* (2019). Available online at https://www.oie.int/standard-setting/terrestrial-manual/access-online/ (accessed March 01, 2020).
14. Nugent J, Paillot R. Equine herpesvirus myeloencephalopathy: unravelling the enigma. *Vet J.* (2009) 180:271–2. doi: 10.1016/j.tvjl.2008.12.002
15. Foote CE, Love DN, Gilkerson JR, Whalley JM. Serological responses of mares and weanlings following vaccination with an inactivated whole virus equine herpesvirus 1 and equine herpesvirus 4 vaccine. *Vet Microbiol.* (2002) 88:13–25. doi: 10.1016/S0378-1135(02)00100-1
16. Hannant D, Jessett DM, O'Neill T, Dolby CA, Cook RF, Mumford JA. Responses of ponies to equid herpesvirus-1 ISCOM vaccination and challenge with virus of the homologous strain. *Res Vet Sci.* (1993) 54:299–305. doi: 10.1016/0034-5288(93)90126-Z
17. Chavatte-Palmer P. Lactation in the mare. *Equine Vet Educ.* (2002) 5:88–93. doi: 10.1111/j.2042-3292.2002.tb01801.x
18. Raidal SL, McTaggart C, Penhale J. Effect of withholding macromolecules on the duration of intestinal permeability to colostral IgG in foals. *Aust Vet J.* (2005) 83:78–81. doi: 10.1111/j.1751-0813.2005.tb12202.x
19. Bresgen C, Lämmer M, Wagner B, Osterrieder N, Damiani AM. Serological responses and clinical outcome after vaccination of mares and foals with equine herpesvirus type 1 and 4 (EHV-1 and EHV-4) vaccines. *Vet Microbiol.* (2012) 160:9–16. doi: 10.1016/j.vetmic.2012.04.042

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.2020.593138

Habituation to Transport Helps Reducing Stress-Related Behavior in Donkeys During Loading

Francesca Dai[1]*, *Silvia Mazzola*[1], *Simona Cannas*[1], *Eugenio Ugo Luigi Heinzl*[2], *Barbara Padalino*[3], *Michela Minero*[1] and *Emanuela Dalla Costa*[1]

[1] Dipartimento di Medicina Veterinaria, Università degli Studi di Milano, Milan, Italy, [2] Direzione Sicurezza, Sostenibilità e Ambiente, Università degli Studi di Milano, Milan, Italy, [3] Department of Agricultural and Food Science, University of Bologna, Bologna, Italy

Adopting proper animal management strategies, including training, might reduce to a substantial extent the adverse effects of transport-related stress in animals. The aim of this study was to evaluate the effect of habituation to transport on stress-related behaviors and physiological indicators during loading and unloading in donkeys. Fourteen donkeys were recruited and divided in two treatment groups: Habituation (H; M = 5, F = 2) and Control (C; M = 5, F = 2). H donkeys were gradually habituated to be transported, traveling together with their mothers and other adult donkeys well-accustomed to transport, while C donkeys had never been transported before. Loading and unloading phases were video recorded and behavior was analyzed. Saliva samples for cortisol concentration determination were collected at rest and after unloading. Latency time to load was significantly shorter for H donkeys than C donkeys (Mann-Whitney; $p = 0.004$). C donkeys also showed significantly more stress-related behaviors (Mann-Whitney; $p = 0.026$) and required a higher but not statistically significant number of human interventions to load. Cortisol concentration increased in both groups, but no differences were found between them (Mann-Whitney; $p > 0.05$). These results suggest that habituation to transport could mitigate stress during loading procedures in donkeys reducing loading time, frequency of stress-related behaviors and diminishing the need of human intervention.

Keywords: habituation, stress, behavior, welfare, transport, donkey

OPEN ACCESS

Edited by:
Amy Katherine McLean, University of California, Davis, United States

Reviewed by:
Jorge Ramón López-Olvera, Autonomous University of Barcelona, Spain
Neil Price Evans, University of Glasgow, United Kingdom

***Correspondence:**
Francesca Dai
francesca.dai@unimi.it

Specialty section:
This article was submitted to Comparative and Clinical Medicine, a section of the journal Frontiers in Veterinary Science

Received: *09 August 2020*
Accepted: *12 November 2020*
Published: *03 December 2020*

Citation:
Dai F, Mazzola S, Cannas S, Heinzl EUL, Padalino B, Minero M and Dalla Costa E (2020) Habituation to Transport Helps Reducing Stress-Related Behavior in Donkeys During Loading. Front. Vet. Sci. 7:593138. doi: 10.3389/fvets.2020.593138

INTRODUCTION

Throughout Europe, a population of about 395,910 donkeys is estimated (1), of which 93,468 are registered in the Equine Italian Database (2). Donkeys in Italy can be kept as pets, or used for leisure activities, therapy programs, or milk and meat production (3).

Transport is part of the management for the majority of pets and farm animals, including donkeys, having different purposes, such as reaching slaughterhouse, moving to a different farm, breeding, competitions and fairs, and medical procedures (4). Transport procedures are known to be stressful for animals, having both short term and prolonged effects on their welfare (4–7). It is also known that transport-related stress could influence meat quality in a variety of species (5, 8–10), thus potentially reducing profits derived from animal farming for meat production. When transported, several potential stressors can impact animal welfare, including interaction with humans, loading, unloading, and penning in a new, unfamiliar environment, and confinement with

and without motion, vibrations, changes in temperature and humidity, inadequate ventilation, and often, deprivation of food, and water (11). In particular, loading is considered to be one of the most stressful components of transport for most animals, including equines (12–17), and it is reported to be the phase with the higher number of transport-related injuries particularly in horses which show transport related behavioral problems or have been trained with inappropriate training methods (18, 19). Several stressors are involved in pre-loading and loading procedures: separation from a familiar environment and social group (6), interactions with humans (14, 20), walking on the ramp (21), entering the trailer (22). Stress and fear during loading are also reinforced by recollections of previous unpleasant traveling experiences (11, 23–25).

The reduction of adverse effects of pre-transport factors decreases the probability of compromising animal welfare during the transport phase itself (7). The pre-transport preparation of animals plays an important role: it is reported that adopting proper management measures might reduce to a substantial extent the adverse effects of loading on stress, improving animal welfare (7). Habituation, in particular, is known to lead to decreased behavioral reactions to a previous novel situation (26), and habituation to loading as a foal, a yearling or an adult horse was reported to make loading behavior become as normal as walking into a stall (23). As highlighted by Padalino and Riley, people involved in equine transport should apply best practices, such as training of animals using evidence-based methodologies (4). In order to evaluate the efficacy of transport training methods, transport-related stress should be evaluated using both behavioral and physiological indicators. In horses, stress-related behaviors during loading include pawing, kicking out, bolting, head-shaking, and avoidance reactions, such as rearing, pulling away sideways, or backwards (13, 16, 17, 26, 27). During transport, reported stress-related behaviors are vocalizing, head tossing, pawing, scrambling, head-turning, kicking at the vehicle, biting and kicking at other horses, and reduced feeding/drinking (27–31). Reported unloading stress-related behaviors are a reluctance to exit the vehicle, prolonged immobility, and running off (21). To the authors' knowledge, no research has been conducted on donkeys to assess stress-related behavior during transport. Several physiological indicators have been proposed to evaluate transport related stress, both in horses and in donkeys, such as cortisol (11, 32–36), ß-endorphin (11, 36, 37), adrenocorticotropic hormone (32) and chromogranin-A (38), and infrared thermography (39).

The aim of this study was to evaluate the effect of habituation to transport procedures on stress related to loading and unloading, using behavioral indicators and salivary cortisol level, in donkeys.

MATERIALS AND METHODS

Ethics Statement

This was an opportunistic study: no animals were transported to record data for the purposes of this study, no farm routine management has been modified for the purpose of the study. To obtain the best from each pasture, donkeys were routinely moved from one pasture to another. Therefore, no extra work was required to the farmer. No animals underwent more than a minimal distress. Transports were conducted in compliance with Council Regulation (EC) No 1/2005 of 22 December 2004 on the protection of animals during transport and related operations. Verbal informed consent was gained from the farmer prior to taking part in this research. Written consent was deemed unnecessary as no personal details of the participants were recorded.

Animals and Facility

All the donkeys kept on farm were health checked and handled daily by the farm manager and/or the groom, so they were used to human contact. Fourteen healthy Romagnolo donkey foals (M = 10; F = 4; 1.2 ± 0.4 years) intended for meat production were included in the study. Only healthy foals born in 2018 were included in the study. Donkeys were born and raised on the same farm located in Northern Italy. Animals were group-housed at pasture with access to clean water *ad libitum* with both automatic drinkers and buckets. Donkeys were free to graze; pastures were managed to guarantee adequate nutrition in terms of quantity and quality of grass. If needed, depending on the season, weather, pasture conditions, and donkey growth rate, hay and mixed feed were provided.

Treatments

Donkeys were randomly divided in two sex-balanced groups of seven subjects each: Control (C; M = 5, F = 2) and Habituation (H; M = 5, F = 2). All the animals were used to be handled and cared for by the same handlers. Foals in the H group were gradually habituated to be transported over short distances (from one pasture to another, about 30 min journeys), traveling together with adult donkeys with travel experience, including their own mothers. This habituation training started when the donkeys aged 6 months and lasted until they were taken to the slaughterhouse (1.2 ± 0.4 years). During the habituation, transport procedures were always performed using the same truck and by the same stockmen people, familiar to the donkeys (farm manager and groom). Foals were left free to load following other donkeys, taking advantage of their gregarious behavior, so they were not led by handlers or pushed by them in anyway. Donkeys in the H group were subjected to a minimum of 5 transports and no injuries were reported in both donkeys and donkey handlers. Donkeys in the C group were naïve to transport since they were housed together in a pasture, different from one of the H group, from birth. All the animals were used to the handlers: while donkeys in the C group were not used to be loaded nor to travel, animals were used to be handled and cared for by the same handlers.

Data Collection

Data were collected during the transport from the pasture (where they were kept) to the main farm. For C donkeys, this was the first transport of their life. All donkeys involved in the study were transported in small groups (two to four donkeys, coming from the same familiar group) with the same truck, for a total of six transports. The transports started at around 4.30 p.m.,

and their durations ranged from 50 to 88 min (mean 64.69 ± 14.57 min). All transport procedures (loading and unloading) were performed by the stockmen according to the usual farm routine. Donkeys were conducted with a lead rope and gently encouraged to move by handlers, also offering food. Animals were loaded in group (two to four donkeys at a time) in order to take advantage of their gregarious behavior. At arrival, the truck door was opened, and the donkeys were left free to unload without leading or encouraging them.

Behavioral Analysis

The loading and unloading phases were video recorded using an HD digital video camera (Canon Legria HFR88) controlled by the researcher. The loading time (from the procedures beginning, with the donkey being in front of the ramp, until the donkey had all four feet on the trailer) and time to unload (from the trailer doors opening until the donkey had all four feet on the ground) was directly recorded using a stopwatch. Donkey behavior during loading and unloading was separately analyzed by a treatment-blind animal scientist, experienced in equine behavior analysis. A focal animal continuous recording method was applied, using the software Solomon Coder beta 17.03.22. The frequency and duration of different behaviors were recorded. Since no literature is available on donkey behavior during loading and unloading, the ethogram was adapted from the one used for horses by Dai et al. (13) (**Table 1**).

Furthermore, each interaction between the handlers and the donkeys was noted from videos. Any interactions to facilitate loading was considered (pulling the rope, pushing the donkey from the back, inciting the animal, offering food).

Salivary Cortisol Evaluation

For cortisol concentration determination, saliva samples were collected using SalivaBio Children's Swab (Salimetrics®, Carlsbad, CA, USA) in the pasture with donkeys at rest immediately before starting loading procedures and immediately after unloading. In order to minimize the impact of the circadian pulsatile cortisol release pattern (40), for each donkey two more samples were taken under control conditions (at the pasture), on the days immediately prior to transport, in the same time slot in which the transport was scheduled (between 4 p.m. and 5 p.m.). The swab was inserted in donkey's mouth, gently restraining the animal with a head collar; the donkey was left free to chew the swab for 1–2 min, then the swab was put in the device tube, closed with a plastic stopper to prevent evaporation, placed in ice and then stored at −20°C immediately after it arrived at the laboratory. The temperature was maintained until analysis. At the time of analysis, the samples were thawed at room temperature and centrifuged (3,500 rpm for 15 min, at 4°C) according to the protocol for salivary samples. Analysis was performed using a commercially available multispecies cortisol enzyme-linked immunosorbent assay (ELISA) kit (Enzo Life Sciences, Farmingdale, NY, USA), following previously validated protocols (41). Samples were aliquoted into wells in duplicate (100 μL), and absorbance measured using a wavelength of 405 nm in a microplate plate reader (Multiskan EX, LabSystem, Thermo Fisher Scientific, Milan, Italy). A recovery test was applied to determine if the value obtained from our samples were accurate (e.g., no interferences with the measurements due to the presence of undesired factors in the sample matrix). The mean recovery was 109.1% ± 8.4, while the average intra- and inter-assay coefficients of variation, respectively, were 3.9 and 7.8%. The assay sensitivity was 56.72 pg/ml (range 156–10,000 pg/ml). The laboratory researcher was blinded to the hypotheses and conditions.

Statistical Analysis

Behaviors of the categories forward locomotion and stress-related behaviors were considered together for the statistical analysis (**Table 1**). Based on the total length of the observation of the video recordings, durations of behaviors were calculated as percentage of total observation time (proportional duration time). Behaviors that were not observed (urinate, paw, sniffing) were not considered for the statistical analysis. Cortisol variations (delta) for each subject of the two groups were calculated. Statistical analysis was performed using SPSS 25 (SPSS Inc., Chicago, IL, USA). Data were tested for normality and homogeneity of variance using the Kolmogorov-Smirnov and Levene test, respectively. Mann-Whitney test was used to investigate differences between groups in behavior during loading and unloading, time to load and unload, human intervention, and cortisol concentration (delta). Statistical significance was accepted at $p \leq 0.05$.

RESULTS AND DISCUSSION

Behavior Analysis

Results of the behavioral analysis showed that latency time to load was significantly shorter in H donkeys (mean 7.97 ± 4.62 s) than in C donkeys (mean 83.23 ± 143.84 s) (Mann-Whitney test; $p = 0.004$) (**Figure 1**). H donkeys showed more forward locomotion toward the truck than C donkeys (87.89 ± 20.48% and 41.71 ± 33.51%, respectively; Mann-Whitney test; $p = 0.026$). Furthermore, C donkeys showed significantly more stress-related behaviors than H donkeys (58.29 ± 33.51% and 12.11 ± 20.48%, respectively; Mann-Whitney test; $p = 0.026$) (**Figure 2**). These results are similar to those reported in trained horses (13, 19). However, positive and negative training reinforcements might require more time than farmers will dedicate (13); for this reason, the proposed and tested habituation training, including the foal following the mother and other known conspecifics, seems instead to be effective in donkeys and may prove to be more feasible when introduced in an on-farm routine as the trailer could be left in the pasture so that the animals can explore it and get habituated to load and unload. Habituation has been strongly recommended for horses (22) and was proven to minimize the incidence of transport related behavioral problems and subsequently injuries (18). In the latter study, self-loading also was found associated with a reduction of loading problem behavior and injuries, however, it is worth to know that self-loading require lots of time, effort, and training skills. Even though, habituation requires some time, Houpt et al. (22) clearly tested that when a foal is habituated to load into a trailer following the mare, loading into a trailer

TABLE 1 | Ethogram for the evaluation of donkey behavior during loading and unloading [modified from (13)].

Behavior	Description	Category
Walk	The donkey walks toward the trailer	Forward locomotion
Trot	The donkey trots toward the trailer	Forward locomotion
Gallop	The donkey gallops toward the trailer	Forward locomotion
Backwards	The donkey moves away from the trailer	Stress-related behavior
Standing	The donkey stands on the four legs	Stress-related behavior
Turn back	The donkey tries to turn all its body in the opposite direction of the trailer	Stress-related behavior
Refuse to walk	The donkey stops moving, digging in its heels, refusing to proceed	Stress-related behavior
Rear	The donkey rears with its front legs	Stress-related behavior
Kick	The donkey kicks, one or two legs is lifted and moved rapidly and forcefully	Stress-related behavior
Mount	The donkey mounts the donkey in front of him/her	Stress-related behavior
Defecate	The donkey drops manure	Stress-related behavior
Urinate*	The donkey drops urines	Stress-related behavior
Paw*	The donkey rises a foreleg and scrapes the floor	Stress-related behavior
Sniffing*	The donkey sniffs the ground	Stress-related behavior

Behaviors that were not observed were not considered for the statistical analysis.

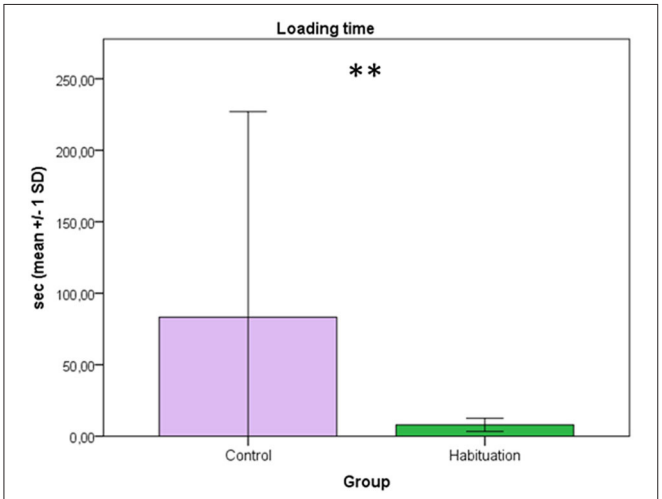


FIGURE 1 | The time (sec) for loading is presented on the y-axis (mean ± 1 SD) with the groups (control vs. habituation) on the x-axis (Mann-Whitney test; $^{**}p = 0.004$).

becomes easy as walking into a box for both the foal and its handler. This is the first study where this training to transport procedures using habituation with the foal following conspecifics was tested in donkeys.

Group C donkeys required a higher but not statistically significant number of human interventions to load compared to H's (H: mean 1.29 ± 0.95; C mean: 7.43 ± 14.03, Mann-Whitney test; $p = 0.32$). The lack of statistically significant difference may probably be due to the high individual variability observed in C subjects and it needs to be ascertained with further studies. However, it is worth noting that this result has interesting practical fallouts considering both animal welfare and human safety. Indeed, interactions with large animals may become dangerous for handlers, especially when animals are stressed and/or frightened: several studies conducted in sport horses with behavioral problems related to transport highlighted the high occurrence of injuries in humans during loading, such as rope burns, lost fingers, broken bones, or bruises, and bleeding (17, 18, 42). Not only loading may be risky for the handlers, but inappropriate animal management in this transport phase has been reported to be a risk factor also for horse injuries (19, 42). The adoption of an adequate training for loading has been deemed useful in increasing human safety by reducing horse-related injuries among handlers (18).

Even if loading represents the most stressful stage of animal transport (7, 12, 43), also unloading may be challenging. Critical factors are steepness and slipperiness of the ramp, and the novel environment the animals are required to enter (6). Consequently to stress and/or anxiety related to unloading, horses have been observed freezing inside the vehicle or performing flight responses (21). In the present study, donkeys did not exhibit abnormal behavior during the unloading phase, and no differences between groups were found in the unloading time (Mann-Whitney test; $p > 0.05$), with C group unloading in 48.9 ± 32.4 s (mean ± 1 SD), while H group unloading in 71.0 ± 31.2 s. Besides, the behavior of the donkeys in the two groups was similar during the unloading procedure (Mann-Whitney test; $p > 0.05$). Having traveled with other members of the social group could have contributed to attenuating the stress at the time of unloading. Taking advantage of the donkeys' gregarious attitude, the animals got out of the truck without showing behaviors attributable to stress.

Salivary Cortisol Evaluation

No differences were found in delta cortisol concentration between groups (Mann-Whitney test; $p > 0.05$) (**Figure 3**). From **Figure 3**, it is evident the great variability of group C data,

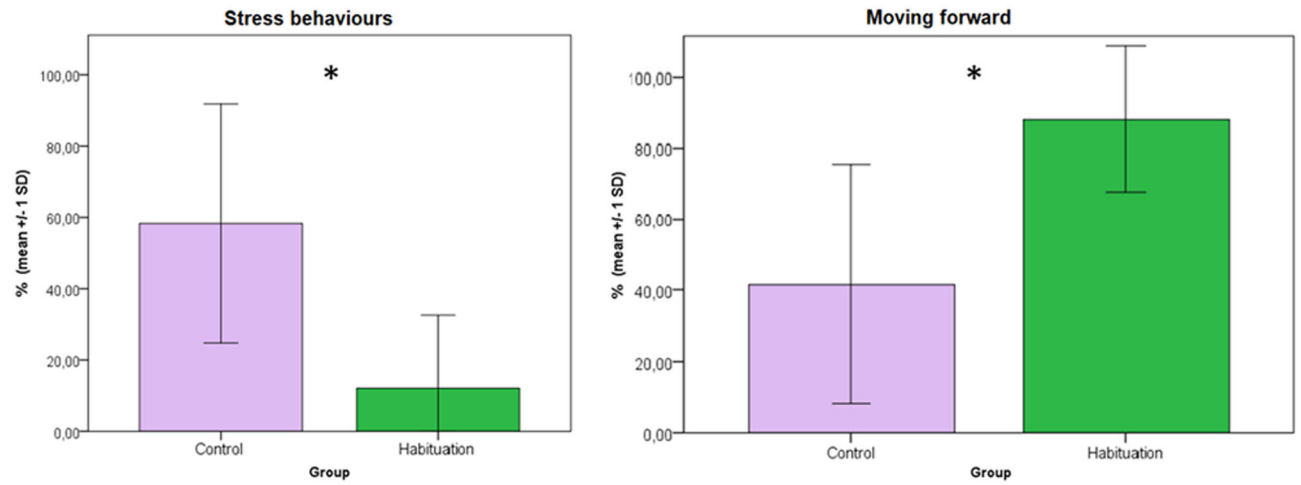


FIGURE 2 | Mean percentages of time (±1 SD) of stress related and moving forward behaviors of donkeys in the two treatment groups during loading procedure. (Mann-Whitney test; $^*p < 0.05$).

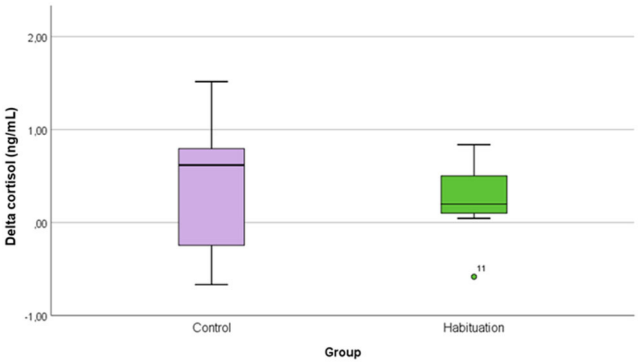


FIGURE 3 | Box plot of the transport-related salivary cortisol variation (delta) in the two groups of donkeys. Outliers are represented with dots.

much greater than those of the group H. In case of acute stress, cortisol secretion increases significantly, with a secretion level that varies from individual to individual, depending on the individual perception of the stressor, but described as correlated with the intensity of the stress (44). These results highlighted the subjectivity of the activation of the hypothalamic-pituitary-adrenal cortex axis induced by the stressogenic stimulus: this variability, associated with the small number of donkeys, may be the basis of the lack of significance of the data. The great variability may be also related to the different space allowance and conditions (group of 2 or 4) during the different journeys tested.

Limitations and Future Perspectives

In this study, no donkeys were transported only for the purpose of data collection: all the transports were part of the farm's management procedures. The limited size of the farm has led to a small number of donkeys available. In a convenient geographical location, there were no donkey farms with the same donkey breed with similar management: therefore, to avoid data bias (especially considering physiological data), the number of subjects observed was not integrated with those of other farms. It clearly appears that the sample size and the unique provenience of the evaluated donkeys represent limitations for the generalization of our results. Further studies applying training to transport procedures through habituation with the foal following conspecifics are foreseen to generalize the results to donkeys kept for other purposes and subjected to different management. As stress related to transport could be affected by several potential stressors (11), future studies should also consider to evaluate different habituation protocols such as load on a trailer without movement and habituation to loading on a trailer and the vehicle movement.

Donkeys were not habituated to saliva sampling. Even if the method is reported to be non-invasive and should not induce a significant stress to the animal, being rapid and permitting animal mobility (38, 41), the procedure represented a novel stimulus for the animal. Although the time required for sampling was not sufficient to allow the presence of free cortisol in the saliva (45), it may not be excluded that the procedure induced a certain degree of stress in donkeys, therefore influencing our result. However, the applied methodology was the same for H and C groups, eliminating any potential bias between treatment groups. Cortisol is released from the adrenal glands in pulses controlled by the hypothalamus's paraventricular nucleus, which receives circadian pulses from the suprachiasmatic nucleus of the hypothalamus and integrates information from cognitive processes and emotional and physical stress reactions (46). The cortisol secretory pulses' variations result from the ultradian rhythm: the secretory episodes occur at a relatively stable frequency, with variable amplitudes, responsible for the typical circadian rhythm. Over 24 h, between 15 and 22 secretory cortisol pulses are expected, with an early morning peak and a nadir by the first half of the night. In the present work, we tried to minimize the impact of this pulsatile circadian release of cortisol:

all transport took place in the afternoon, and, for each animal, salivary sampling was at the transport and also the day before, the same time as transport was planned.

Measuring other physiological indicators, such as heart rate variability (HRV), respiratory parameters, beta-endorphin, catecholamines or glucose levels, would have increased the scientific robustness of results, however, it would have also decreased the study's feasibility increasing the invasiveness of the data collection.

As one of the reasons for breeding donkeys is meat production and numerous studies highlighted how transport stress negatively affects meat quality in several species [see (5) for review]. In future studies, it would be interesting to analyze the effect of transport related stress on donkey meat. As a matter of fact, only few studies described the incidence of transport related stress on equine meat quality (47, 48).

Regardless of the above-mentioned limitations, to the authors' knowledge, this is the first study documenting the effects of habituation to transport procedures in donkeys. In the present study, meat donkeys were taken only as a model, as two groups of animals of the same breed, balanced for sex and age, with the same management and handling from the same stockmen could be subject to the different training procedure. As donkeys are frequently transported for several purposes, including changing ownership, leisure activities, therapy, sport activities, habituation following conspecifics could be helpful in reducing stress related to transport. This result has a practical fallout, since habituation with the foal following the conspecifics could be more feasible, easier and should be recommended for donkeys.

CONCLUSIONS

These results, although preliminary, suggest that habituation to transport following conspecifics could mitigate stress responses during loading in donkeys, reducing loading time, the frequency of stress-related behaviors and the handler's intervention. Further research, conducted on a larger donkey population on several farms, is needed in order to confirm these results.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

FD, ED, and MM: conceptualization. FD and BP: methodology. ED: formal analysis and visualization. FD, EH, and SM: investigation. MM: resources. FD: data curation. FD, SM, and ED: writing—original draft preparation. MM, SC, ED, EH, and BP: writing—review and editing. MM: supervision and project administration. All authors have read and agreed to the published version of the manuscript.

ACKNOWLEDGMENTS

Authors are grateful to Azienda Agricola Il Colmetto for hosting the experiment and for their precious help in data collection. Authors wish to thank Miss Giulia Segati for her contribution in data collection.

REFERENCES

1. FAOSTAT (2018). Available online at: http://www.fao.org/faostat/en/#data/QA (accessed June 2, 2020).
2. Banca Dati Equidi. (2020). Available online at: http://www.anagrafeequidi.it/tabstatistiche_Report_report.php (accessed June 2, 2020).
3. Dai F, Dalla Costa E, Murray LMA, Canali E, Minero M, Anne Murray LM, Canali E, Minero M. Welfare conditions of donkeys in europe: initial outcomes from on-farm assessment. *Animals.* (2016) 6:5. doi: 10.3390/ani6010005
4. Padalino B, Riley CB. Editorial: the implications of transport practices for horse health and welfare. *Front Vet Sci.* (2020) 7:202. doi: 10.3389/fvets.2020.00202
5. Schwartzkopf-Genswein K, Faucitano L, Dadgar S, Shand P, González L, Crowe T. Road transport of cattle, swine and poultry in North America and its impact on animal welfare, carcass and meat quality: a review. *Meat Sci.* (2012) 92:227–43. doi: 10.1016/j.meatsci.2012.04.010
6. Padalino B. Effects of the different transport phases on equine health status, behavior, and welfare: a review. *J Vet Behav Clin Appl Res.* (2015) 10:272–82. doi: 10.1016/j.jveb.2015.02.002
7. Šímová V, Večerek V, Passantino A, Voslárová E. Pre-transport factors affecting the welfare of cattle during road transport for slaughter – a review. *Acta Vet Brno.* (2016) 85:303–18. doi: 10.2754/avb201685030303
8. Petracci M, Bianchi M, Cavani C, Gaspari P, Lavazza A. Preslaughter mortality in broiler chickens, turkeys, and spent hens under commercial slaughtering. *Poult Sci.* (2006) 85:1660–4. doi: 10.1093/ps/85.9.1660
9. Marìa G, Villarroel M, Sanudo C, Olleta J, Gebresenbet G. Effect of transport time and ageing on aspects of beef quality. *Meat Sci.* (2003) 65:1335–40. doi: 10.1016/S0309-1740(03)00054-8
10. Polkinghorne R, Philpott J, Thompson J. Do extended transport times and rest periods impact on eating quality of beef carcasses? *Meat Sci.* (2018) 140:101–11. doi: 10.1016/j.meatsci.2018.02.017
11. Fazio E, Ferlazzo A. Evaluation of stress during transport. *Vet Res Commun.* (2003) 27:519–24. doi: 10.1023/B:VERC.0000014211.87613.d9
12. Trunkfield H, Broom DM. The welfare of calves during handling and transport. *Appl Anim Behav Sci.* (1990) 28:135–52. doi: 10.1016/0168-1591(90)90050-N
13. Dai F, Dalla Costa A, Bonfanti L, Caucci C, Di Martino G, Lucarelli R, et al. Positive reinforcement-based training for self-loading of meat horses reduces loading time and stress-related behavior. *Front Vet Sci.* (2019) 6:350. doi: 10.3389/fvets.2019.00350
14. York A, Matusiewicz J, Padalino B. How to minimise the incidence of transport-related problem behaviours in horses: a review. *J Equine Sci.* (2017) 28:67–75. doi: 10.1294/jes.28.67
15. Padalino B, Rogers C, Guiver D, Bridges J, Riley C. Risk factors for transport-related problem behaviors in horses: a New Zealand survey. *Animals.* (2018) 8:134. doi: 10.3390/ani8080134
16. Shanahan S. Trailer loading stress in horses: behavioral and physiological effects of nonaversive training (TTEAM). *J Appl Anim Welf Sci.* (2003) 6:263–74. doi: 10.1207/s15327604jaws0604_1
17. Ferguson DL, Rosales-Ruiz J. Loading the problem loader: the effects of target training and shaping on trailer-loading behavior of horses. *J Appl Behav Anal.* (2001) 34:409–24. doi: 10.1901/jaba.2001.34-409
18. Padalino B, Henshall C, Raidal SL, Knight P, Celi P, Jeffcott L, et al. Investigations into equine transport-related problem behaviors: survey results. *J Equine Vet Sci.* (2017) 48:166-173.e2. doi: 10.1016/j.jevs.2016.07.001
19. Yngvesson J, de Boussard E, Larsson M, Lundberg A. Loading horses (Equus caballus) onto trailers - behaviour of horses and horse owners

during loading and habituating. *Appl Anim Behav Sci.* (2016) 184:59–65. doi: 10.1016/j.applanim.2016.08.008
20. Casamassima D, Palazzo M, Presutti T, Cinone M. Effects of two tame systems on physiological parameters of Arab horses subjected to load in the trailer. *Ippologia*. (2008) 19:13–9.
21. Siniscalchi M, Padalino B, Lusito R, Quaranta A. Is the left forelimb preference indicative of a stressful situation in horses? *Behav Processes.* (2014) 107:61–7. doi: 10.1016/j.beproc.2014.07.018
22. Houpt KA, Wickens CL. Handling and transport of horses. In: Grandin T, editor. *Livestock Handling and Transport*. Boston, MA: CABI (2014). p. 347–69.
23. Weeks C, McGreevy P, Waran N. Welfare issues related to transport and handling of both trained and unhandled horses and ponies. *Equine Vet Educ.* (2012) 24:423–30. doi: 10.1111/j.2042-3292.2011.00293.x
24. Cregier S. Reducing equine hauling stress: a review. *J Equine Vet Sci Vet Sci.* (1982) 2:187–98. doi: 10.1016/S0737-0806(82)80025-7
25. Houpt KA. Behavioral problems in horses. In: *Proceedings of the Annual Convention of the American Association of Equine Practitioners* (1986).
26. Waran NK, Cuddeford D. Effects of loading and transport on the heart rate and behaviour of horses. *Appl Anim Behav Sci.* (1995) 43:71–81. doi: 10.1016/0168-1591(95)00555-7
27. Lee J, Houpt K, Doherty O. A survey of trailering problems in horses. *J Equine Vet Sci.* (2001) 21:235–8. doi: 10.1016/S0737-0806(01)70042-1
28. Kay R, Hall C. The use of a mirror reduces isolation stress in horses being transported by trailer. *Appl Animal Behav Sci.* (2009) 116:237–43. doi: 10.1016/j.applanim.2008.08.013
29. Houpt KA. Stable vices and trailer problems. *Vet Clin North Am Equine Pract.* (1986) 2:623–33. doi: 10.1016/S0749-0739(17)30710-1
30. Padalino B, Maggiolino A, Boccaccio M, Tateo A. Effects of different positions during transport on physiological and behavioral changes of horses. *J Vet Behav Clin Appl Res.* (2012) 7:135–41. doi: 10.1016/j.jveb.2011.09.003
31. Riley CB, Noble BR, Bridges J, Hazel SJ. Horse injury during non-commercial transport: findings from researcher-assisted intercept surveys at southeastern australian equestrian events. *Animals.* (2016) 6:65. doi: 10.3390/ani6110065
32. Fazio E, Medica P, Cravana C, Ferlazzo A. Pituitary-adrenocortical adjustments to transport stress in horses with previous different handling and transport conditions. *Vet World.* (2016) 9:856–61. doi: 10.14202/vetworld.2016.856-861
33. Strzelec K, Kankofer M, Pietrzak S. Cortisol concentration in the saliva of horses subjected to different kinds of exercise. *Acta Vet Brno.* (2011) 80:101–5. doi: 10.2754/avb201180010101
34. Fazio E, Medica P, Cravana C, Ferlazzo A. Cortisol response to road transport stress in calm and nervous stallions. *J Vet Behav Clin Appl Res.* (2013) 8:231–7. doi: 10.1016/j.jveb.2012.10.010
35. Tajik J, Nazifi S, Eshtraki R. The influence of transportation stress on serum cortisol, thyroid hormones, and some serum biochemical parameters in Iranian cashmere (Raini) goat. *Vet Arh.* (2016) 86:795–804. Available online at: https://hrcak.srce.hr/170145
36. Fazio E, Medica P, Cravana C, Aveni F, Ferlazzo A. Comparative endocrinological responses to short transportation of Equidae (Equus asinus and Equus caballus). *Anim Sci J.* (2013) 84:258–63. doi: 10.1111/j.1740-0929.2012.01065.x
37. Alberghina D, Medica P, Cusumano F, Fazio E, Ferlazzo A. Effects of transportation stress and influence of different distance and age on bendorphin, ACTH and cortisol levels of horses. In: *Proceedings of the 34th International Congress of the International Society for Applied Ethology*. Florianópolis (2000).
38. Dai F, Dalla Costa E, Cannas S, Heinzl EUL, Minero M, Mazzola SM. May salivary chromogranin a act as a physiological index of stress in transported donkeys? A pilot study. *Animals.* (2020) 10:972. doi: 10.3390/ani10060972
39. Butterfield C, Grumpelt B, Kimmel D, Patterson R, Jones K, Scott SL, et al. The Pretransport Management of Stress in Performance Horses. *J Equine Vet Sci.* (2018) 69:145–8. doi: 10.1016/j.jevs.2018.07.006
40. Trifonova ST, Gantenbein M, Turner JD, Muller CP. The use of saliva for assessment of cortisol pulsatile secretion by deconvolution analysis. *Psychoneuroendocrinology.* (2013) 38:1090–101. doi: 10.1016/j.psyneuen.2012.10.016
41. Bonelli F, Rota A, Aurich C, Ille N, Camillo F, Panzani D, et al. Determination of salivary cortisol in donkey stallions. *J Equine Vet Sci.* (2019) 77:68–71. doi: 10.1016/j.jevs.2019.02.027
42. Padalino B, Hall E, Celi P, Knight P, Jeffcott L, Muscatello G. Health problems and risk factors associated with long haul transport of horses in Australia. *Animals.* (2015) 5:1296–310. doi: 10.3390/ani5040412
43. Tateo A, Padalino B, Boccaccio M, Maggiolino A, Centoducati P. Transport stress in horses: effects of two different distances. *J Vet Behav Clin Appl Res.* (2012) 7:33–42. doi: 10.1016/j.jveb.2011.04.007
44. Hellhammer DH, Wüst S, Kudielka BM. Salivary cortisol as a biomarker in stress research. *Psychoneuroendocrinology.* (2009) 34:163–71. doi: 10.1016/j.psyneuen.2008.10.026
45. Kirschbaum C, Hellhammer DH. Salivary cortisol in psychoneuroendocrine research: recent developments and applications. *Psychoneuroendocrinology.* (1994) 19:313–33. doi: 10.1016/0306-4530(94)90013-2
46. Ulrich-Lai YM, Herman JP. Neural regulation of endocrine and autonomic stress responses. *Nat Rev Neurosci.* (2009) 10:397–409. doi: 10.1038/nrn2647
47. Roy R, Cockram M, Dohoo I, Ragnarsson S. Transport of horses for slaughter in Iceland. *Anim Welf.* (2015) 24:485–95. doi: 10.7120/09627286.24.4.485
48. Roy RC, Cockram MS, Dohoo IR, Riley CB. Injuries in horses transported to slaughter in Canada. *Can J Anim Sci.* (2015) 95:523–31. doi: 10.4141/cjas-2015-032

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: 18 December 2020
doi: 10.3389/fvets.2020.602984

Reference Ranges for Hematological and Biochemical Profile of Martina Franca Donkeys

Francesca Trimboli[1], Ippolito De Amicis[2], Antonio Di Loria[3], Carlotta Ceniti[1] and Augusto Carluccio[2]*

[1] *Interdepartmental Services Centre of Veterinary for Human and Animal Health, Department of Health Science, University Magna Græcia of Catanzaro, Catanzaro, Italy,* [2] *Department of Veterinary Medicine, University of Teramo, Teramo, Italy,* [3] *Department of Veterinary Medicine and Animal Productions, University of Napoli Federico II, Naples, Italy*

The Martina Franca donkey (MFd) is one of the largest Italian donkey breeds, considered as endangered breed. To support the conservation strategies, knowledge about the physiologic hematological parameters of MFds is needed. The aims of the study were to determine reference value for hematological and major serum parameters in a population of healthy MFds and to estimate the influence of age on these parameters. Eighty-one clinically healthy MFds (17 males and 64 females) in different ages were enrolled: group A (foals, n° 16, animals < 1 year old) group B (young animals, n° 36, from 1 to 3 years old), and group C (adult animals, n° 29, over 3 years old). Red blood cell count (RBC); hematocrit value (HCT); hemoglobin concentration (HGB); mean corpuscular volume (MCV); mean corpuscular hemoglobin (MCH); hemoglobin concentration distribution width (HDW); RBC distribution width (RDW); total white blood cell (WBC); WBC differential count for neutrophils, lymphocytes, monocytes, eosinophils and basophils, and platelets (PLT); mean platelet volume (MPV); platelet volume distribution width; and plateletcrit (PCT) were analyzed. The biochemistry panel included aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), gamma glutamyl transferase (GGT), total serum protein (TP), albumin (ALB), cholesterol (CHOL), triglyceride (TGL), blood urea nitrogen (BUN), creatinine (CREA), glucose (GLU), Ca^{2+}, phosphorus, Na^{+}, Cl^{-}, K^{+}, and Mg^{2+}. The effect of age on hematological parameters was investigated using one-way ANOVA test. Age of donkeys does not influence total WBC, HGB, HCT, platelet count and MPV, and PCT ($P > 0.05$). Some leukocyte populations such as eosinophils, monocytes, and basophils showed age-linked variations ($P < 0.05$). RBC count, RDW, and HDW decrease with age whereas MCV and MCH increase. Na^{+}, K^{+}, Cl^{-}, Ca^{2+}, phosphorus, ALP, GGT, CREA, GLUC, and CHOL decrease with age ($P < 0.05$), while AST and TP showed an increase with aging ($P < 0.05$). ALB reaches the lowest values in young donkeys and returns to values of foals in older animals ($P < 0.05$).

OPEN ACCESS

Edited by:
Micaela Sgorbini, University of Pisa, Italy

Reviewed by:
Giuseppe Piccione, University of Messina, Italy
Pasquale De Palo, University of Bari Aldo Moro, Italy

****Correspondence:***
Antonio Di Loria
adiloria@unina.it

Specialty section:
This article was submitted to Comparative and Clinical Medicine, a section of the journal Frontiers in Veterinary Science

Received: *04 September 2020*
Accepted: *16 November 2020*
Published: *18 December 2020*

Citation:
Trimboli F, De Amicis I, Di Loria A, Ceniti C and Carluccio A (2020) Reference Ranges for Hematological and Biochemical Profile of Martina Franca Donkeys. Front. Vet. Sci. 7:602984. doi: 10.3389/fvets.2020.602984

Finally, a difference among groups for BUN and TGL was not found ($P < 0.05$). The results suggest how even for the MFd breed, age is a variable that affects different hematological and biochemical parameters. Compared to other donkey and horses, the MFd breed showed some differences that clinicians involved during conservation strategies need to be consider.

Keywords: donkey, reference ranges, hematobiochemical profile, healthy, Martina Franca breed

INTRODUCTION

Donkeys (*Equus asinus*) were traditionally used as working animals for transport, riding, and farm activities. In Italy, at present, data from FAO list a total of eight breeds of donkeys: Amiata, Asinara, Martina Franca, Ragusano, Sardo, Romagnolo, Viterbese, and Pantelleria, of which a very small number of pure specimens exist (1, 2). In recent years, the growing use of donkey milk especially in children allergic to cow's milk proteins (2, 3) has seen an increasing number of donkey farms throughout Italy. The Martina Franca (MF) donkey breed, an ancient native breed of Apulia famous in the past for mule production, is one of the largest Italian donkey breeds. The population of MF donkeys consists of 1,239 specimens including 132 jackasses and 698 jennies approved for breeding (1). Notwithstanding the increasing population trend, MF donkeys are considered an endangered breed (1, 4, 5). Therefore, the exact knowledge about the physiological and pathological conditions of MF donkeys is indispensable in supporting the conservation strategies adopted for this breed. Although the knowledge of species-specific normal values of hematological and biochemical parameters is essential to classifying even in donkey the health or pathological status, in the past donkeys were considered similar to horses, limiting the study on donkey-specific reference ranges of biochemical and hematological parameters (4, 5). Many factors influence blood parameters: the most important of them are age, gender, physiological status, or circadian rhythms that can strongly affect plasma concentrations of melatonin and glucose (6).

In literature, little information is available about donkey hematological and biochemical parameters. Studies that involved donkeys of different breed as Pêga (7), Ragusana (4), Kirgyz (8), or Northwestern donkeys (9) highlighted breed-related differences in some blood parameters as lymphocytes and neutrophils that are higher in Ragusana (4) than in crossbred donkeys (10).

In donkeys of the MFd breed, the reference ranges for main biochemical and hematological parameters are poor and limited to a specific physiological status such as pregnancy (5) or limited to a particular period such as the neonatal one (11). In particular, age represents an important variable that should be considered for the evaluation of the hematological and biochemical reference range; indeed, it can influence the physiological and/or pathological status of the animal.

Sgorbini et al. (10) provided age-related changes in hematological and biochemical parameters of Amiata donkey foals from birth up to the second month of life.

The investigation of hematological and biochemical parameters in endangered Balkan donkey, autochthonous of the Serbian territory, revealed significant differences in some parameters (white blood cell, mid cell, and granulocyte counts and alkaline phosphatase) related to age (12).

Another factor influencing biochemical parameters is gender. Girardi et al. (7) reported a highest total protein value in females attributing them to the physiological responses of females in puerperium and lactation. More recently, de Palo et al. (13) showed that a different breeding technique in the early life affects biochemical profiles and lipid peroxidation patterns in donkey foals. Taking into account what has been said so far, the construction of a reference range must necessarily include the effect of influenced factors as age or breed, for the appropriate interpretation of serum biochemical results.

Finally, the instrument–instrument variation of analysis methods represents a significant influencing factor for the generation of a reference range; therefore, it is also important that each laboratory develops its own normal range. However, the determination of a normal range for the principal biochemistry and hematological parameters in an endangered donkey breed population such as those of Martina Franca is not easy due to the limited number of animals, which makes it difficult to assess the effects of age, but also of sex on them. Therefore, the aim of this study was to contribute to increasing the knowledge of the physiology of the Martina Franca donkey breed using high-quality methods and taking into account different age groups, proposing reference ranges for the main hematological and biochemical parameters in a general population of Martina Franca donkeys.

MATERIALS AND METHODS

Animals

After a complete clinical exam, 81 clinically healthy Martina Franca donkeys (17 males and 64 females) from the same breeding farm located in Puglia (Italy) were enrolled. Donkeys were divided into three groups according to their age [4]: group A (foals, n° 16, animals < 1 year old), group B (young animals, n° 36, from 1 to 3 years old), and group C (adult animals, n° 29, over 3 years old). In details, group A included 16 donkeys (five males, 11 females, mean ± SD age: 5.6 ± 3.2 months) with age between 1 and 11 months. Group B included 36 animals (12 males and 24 females, mean ± SD age: 19.7 ± 7.2 months) from 12 to 36 months of age. Group C included 29 jennies from 4 to 22 years (mean ± SD age: 8.5 ± 4.0 years). Regarding reproductive status and lactation number of donkeys, 16 were pregnant and pluripara

and 48 were non-pregnant. Seven out of the 48 were pluripara and the remaining ones were nullipara.

Blood Sample Collection and Complete Blood Count Analysis

Blood samples came from leftover samples of the previous routine clinical investigation and were collected from the jugular vein, through an 18-gauge needle (Vacutest Kima srl, Arzergrande, Italy) into 9-ml blood vacuum tubes (Vacuette, Austria) containing K_3EDTA for the hematological exam and into 10-ml blood collection tubes containing activation clot for the biochemical profile. Blood collection was taken during the spring season in the same day from 7.00 to 10.00 a.m. to avoid the possible influence of different photoperiods on the studied parameters and then promptly transported in a cooler to the veterinary laboratory at the University of Magna Græcia (Catanzaro, Italy). Hematological exam and serum separation were promptly performed, whereas the biochemical profile and serum protein electrophoresis were performed within 24 h after blood sampling. The serum was separated by centrifugation at 1,700 × g for 10 min at room temperature and was stored at +4°C until use.

The hematological parameters analyzed were red blood cell count (RBC); hematocrit value (HCT); hemoglobin concentration (HGB); mean corpuscular volume (MCV); mean corpuscular hemoglobin (MCH); hemoglobin concentration distribution width (HDW); RBC distribution width (RDW); total white blood cell (WBC); and WBC differential count for neutrophils (NEUT), lymphocytes (LYMPH), monocytes (MONO), eosinophils (EOS), and basophils (BASO) as percentage as absolute count (n° cell × $10^3/\mu$L). Platelets and their indices were also analyzed and included platelet count (PLT), mean platelet volume (MPV), platelet counts (PLT), PLT volume distribution width (PDW), and plateletcrit (PCT). All hematological analyses were performed with an automatic cell counter equipped with software dedicated for veterinary blood analysis (ADVIA 2120, Siemens Healthcare Diagnostic, Germany).

The biochemistry profile was performed on an automated biochemistry analyzer (Dimension EXL, Siemens Healthcare Diagnostic, Germany) using commercial reagents (Siemens Healthcare Diagnostics, Germany) for each parameter and dedicated standards (Siemens Healthcare Diagnostics, Germany) to set the analyzer. In particular, the biochemistry panel included the determination of the following parameters: aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), gamma glutamyl transferase (GGT), total serum protein (TP), albumin (ALB), cholesterol (CHOL), triglyceride (TGL), blood urea nitrogen (BUN), creatinine (CREA), glucose (GLU), calcium (Ca^{2+}), phosphorus (Phos), sodium (Na^+), chloride (Cl^-), potassium (K^+), and magnesium (Mg^{2+}).

Statistical Analysis

Data analysis was performed using a statistical software (GraphPad InStat version 3.10, GraphPad Software Inc., San Diego, CA). Each parameter was tested for normality applying the Kolmogorov and Smirnov method. The following statistical parameters were calculated for each group of donkeys: mean, standard deviation, median, and 2.5th and 97.5th percentiles. The reference intervals were given as mean ± 1.96 SD for parameters normally distributed while non-normally distributed parameters were expressed as median and 2.5th and 97.5th percentiles (14).

One-way ANOVA test followed by Tukey–Kramer multiple-comparison posttest was used to determine the effect of age on biochemical and hematological parameters for normally distributed data, and if data did not have Gaussian distribution, one-way ANOVA on rank test followed by Dunn's multiple-comparison posttest was used. The level of statistical significance was set at $P < 0.05$.

RESULTS

Donkeys included in this study have a mean age of 4 years (± 4 years) and a range of age between 1 month and 22 years.

Reference ranges for hematological parameters were described in **Table 2**. Excluding lymphocytes (% and n° cell × $10^3/\mu$L) and MCH, all other cell populations were not normally distributed.

Age of donkeys did not influence the number of total WBC ($P > 0.05$; **Table 1**), but some leukocyte populations such as EOS, MONO, and BASO showed age-linked variations. EOS was the leukocyte population mainly affected by age ($P < 0.05$): it showed a strong increment over the years (group A vs. group C) as percentage as absolute value (n° cell × $10^3/\mu$L) (**Table 1**). Variations of MONO (n° cell × $10^3/\mu$L) and BASO (n° cell × $10^3/\mu$L) were less pronounced: both tend to decrease in older (group C) than in young animals (group B).

Overall, RBC count decreased with age ($P < 0.05$) with simultaneous increases of MCV and MCH ($P < 0.05$) and decreases of RDW ($P < 0.05$) and HDW ($P < 0.01$). Age did not show any effect on HGB and HCT ($P > 0.05$). Platelet count and their associated parameters (MPV and PCT) were not influenced by age ($P < 0.05$) excepting PDW which results lower in older with respect to younger donkeys ($P < 0.05$) (**Table 1**).

Reference ranges of main biochemical parameters are listed in **Table 2**. Most of the variables were not normally distributed with exception of ALP and AST enzymes. The serum Na^+, K^+, and Cl^- electrolytes were found different among foals (group A) and young (group B) and adult donkeys (group C), showing a decrement with age ($P < 0.05$) (**Table 2**). Significant differences ($P < 0.05$) were observed between groups A and B for Ca^{2+} and Phos: both have higher values in foals, and in particular, Phos concentration tends to halve in older donkeys (**Table 2**). In older donkeys, Mg^{2+} showed a weak increase with respect to young donkeys ($P < 0.05$).

Donkeys under 1 year of age (group A) showed the highest ALP activity ($P < 0.05$) while ALT was not influenced by age. Moreover, AST activity was higher in older (group C) than in young (group B) donkeys ($P < 0.05$), and GGT activity was lower in older animals than in foals ($P < 0.05$).

The serum TP showed an increase with aging ($P < 0.01$) while ALB reached the lowest values in young donkeys (group B) (P

TABLE 1 | Reference range of Martina Franca donkeys were calculated by 2.5th−97.5th percentiles when values were not normally distributed or by mean ± 1.96 SD (§) when values were normally distributed.

Hematological parameters	All donkeys ($n^\circ = 78$)	Group A ($n^\circ = 15$)	Group B ($n^\circ = 36$)	Group C ($n^\circ = 29$)
White cell line				
WBC (× 10^3 cells/μL)	9.5–22.1	12.8 (9.8–17.5)	14.4 (9.3–24.5)	12.4 (10.2–18.5)
NEUT (%)	29.4–69.3	42.9 (39.5–64.7)	44.9 (29.6–81.0)	42.3 (29.4–60.1)
LYM (%)	18.9–57.8§	43.6 (23.9–43.9)	39.5 (14.9–56.8)	38.5 (25.4–50.4)
MONO (%)	2.6–8.5	4.5 (3.5–6.4)	4.0 (2.6–8.7)	3.9 (2.6–7.3)
EOS (%)	2.7–19.8	7.0 (5.4–8.5)[a]	6.7 (2.3–13.8)[a,b]	13.6 (4.9–20.2)[c]
BASO (%)	0.2–0.6	0.3 (0.2–0.4)	0.40 (0.2–0.5)	0.3 (0.2–0.6)
NEUT (× 10^3 cells/μL)	3.5–13.7	5.6 (4.4–10.6)	6.5 (3.3–15.8)	5.2 (3.6–10.9)
LYM (× 10^3 cells/μL)	2.3–8.5§	5.7 ± 1.6	5.6 ± 1.9	4.9 ± 1.0
MONO (× 10^3 cells/μL)	0.3–1.1	0.5 (0.3–0.8)	0.6 (0.4–1.2)[a]	0.5 (0.3–1.0)[b]
EOS (× 10^3 cells/μL)	0.3–2.8	0.7 (0.3–1.1)[a]	1.0 (0.3–2.0)[a,b]	1.8 (0.7–2.9)[c]
BASO (× 10^3 cells/μL)	0.02–0.1	0.04 (0.02–0.09)	0.05 (0.02–0.10)[a]	0.03 (0.02–0.10)[b]
Red cell line				
RBC (× 10^6 cells/μL)	4.2–8.6	6.6 (6.1–7.0)[a]	5.6 (3.9–9.3)[a,b]	5.1 (4.4–6.7)[b]
HGB (g/dL)	7.5–13.1	10.2 (9.2–10.3)	9.6 (7.0–14.3)	10.0 (8.8–11.8)
HCT (%)	24.3–39.6	32.1 (30.8–32.9)	31.2 (22.3–46.8)	32.3 (28.6–38.2)
MCV (fL)	40.5–66.7	48.5 (43.9–50.2)[a]	56.0 (45.8–63.6)[a,b]	62.3 (57.1–67.6)[c]
MCH (pg)	13.6–21.3§	14.8 (13.5–15.7)[a]	17.3 (13.9–20.0)[a,b]	19.3 (17.7–20.7)[c]
MCHC (g/dL)	29.7–39.4	30.9 (29.7–31.8)	30.8 (29.7–31.7)	30.8 (29.9–31.6)
RDW (%)	15.6–20.5	18.5 (17.4–18.7)[a]	16.8 (16.2–19.4)[b]	16.3 (15.4–17.4)[c]
HDW (g/dL)	1.6–2.1	2.0 ± 0.15[a]	1.8 ± 0.2[a,b]	1.7 ± 0.1[c]
Platelets				
PLT (× 10^3 cells/μL)	74–469	159 (77–325.7)	157.5 (59–478)	220 (48–450)
MPV (fL)	6.0–11.4	8.9 (7.5–10.2)	7.7 (6.3–10.7)	7.5 (6.0–11.4)
PCT (%)	0.06–0.33	0.12 (0.08–0.15)	0.13 (0.05–0.40)	0.16 (0.05–0.30)
PDW (%)	18.45–73.6	58.8 (51.1–74.7)[a]	42.7 (18.2–73.6)[b,c]	47.5 (20.2–59.3)[c]

§*mean ± 1.96 SD.* [a,b,c]*Letters identify differences among groups for P-values < 0.05.*

Donkeys were divided in three groups according to age: group A (foals, animals below 1 year of age), group B (young animals, from 1 to 3 years of age), and group C (adult animals, over 3 years old), and hematological parameters were indicated by median (2.5th−97.5th percentiles) or by mean ± 1.96 SD.

< 0.05) and returned to the values of foals (group A) in older animals (group C) ($P < 0.05$).

Finally, the difference among groups for BUN and TGL ($P > 0.05$) was not found, as opposed to CREA, GLUC, and CHOL whose concentrations drastically decrease with aging ($P < 0.05$) (**Table 2**).

DISCUSSION

Donkeys as horses belong to the Equidae family with which they share some physiological similarities but show also some species-specific differences. Some studies were carried out to establish in donkeys reference ranges of hematological and biochemical parameters, enrolling donkeys of different breeds as Pêga (7), Ragusana (4), Kirgyz (8), or Northwestern donkeys (9). The present study investigates for the first time the hematological and biochemical reference range of Martina Franca donkeys in a general population and then evaluates the eventual influence of age on these parameters.

In our study, Martina Franca donkey's total with cells, neutrophils, and lymphocytes is constant throughout their life and did not differ between the age groups considered according to those observed in crossbred donkeys (15) but not in Ragusana where an increased absolute value of lymphocytes and neutrophils in older animals was reported (4). According to other authors (15, 16), with respect to young and old animals MF foals show the lowest concentration of eosinophils. The number of eosinophils tends to increment with age probably due to the progressive exposure of animals to parasites during their life (17). We observed a slight decrease in basophil number between young and adult animals, not observed in the only other study, to our knowledge, present in literature which provides information about basophil count (15). Finally, the monocyte population remains substantially constant throughout life in disagreement with that reported for Ragusana (4) and crossbreed (15).

RBC and RBC indexes are dynamic parameters throughout the life of donkeys and specially during the neonatal period (11, 16, 18). Unlike horses (19), in the present study we observed that foals and young donkeys do not show signs of physiological

TABLE 2 | Reference range of Martina Franca donkeys were calculated by 2.5th−97.5th percentiles when values were not normally distributed or by mean ± 1.96 SD (§) when values were normally distributed.

Biochemical parameters	All donkeys ($n^\circ = 78$)	Group A ($n^\circ = 15$)	Group B ($n^\circ = 36$)	Group C ($n^\circ = 29$)
Electrolytes				
Na^+ (mmol/L)	128.0–152.0	145 (132.4–166.3)[a]	139 (125.4–149.4)[b,c]	136 (130.0–148.6)[c]
K^+ (mmol/L)	4.2–5.9	5.0 (4.2–5.9)[a]	4.8 (4.3–5.9)	4.6 (4.3–5.3)[b]
Cl^- (mmol/L)	96.0–120.0	107.0 (98.4–122.9)[a]	103.0 (93.6–111.1)[b]	101.0 (96.0–109.3)[b]
Ca^{2+} (mg/dL)	12.6–17.0	15.7 (13.0–20.0)[a]	14.1 (12.0–15.7)[b]	14.6 (13.1–16.5)
Phos (mg/dL)	2.0–7.7	6.1 (4.2–9.5)[a]	3.5 (2.6–5.1)[b]	2.7 (1.9–4.0)[c]
Mg^{2+} (mg/dL)	1.6–2.6	2.0 (1.6–3.1)	1.9 (1.6–2.3)[a]	2.1 (1.8–2.2)[b]
Enzymes				
ALP (UI/L)	85.8–392.4§	285.9 ± 61.2[a]	216.7 ± 74.5[b]	247.6 ± 77.7
ALT (UI/L)	14.0–23.0	17.0 (15.0–21.3)	17.0 (13.9–21.1)	17.0 (14.7–24.8)
AST (UI/L)	158.9–371.1§	272 (194.9–370.9)	247.5 (155.6–351.6)[a]	288 (179.0–369.4)[b]
GGT (UI/L)	26.0–69.0	49 (29.7–65.8)[a]	37.5 (25.7–69.7)	32.0 (26.0–53.7)[b]
Protein metabolism				
TP (g/dL)	5.5–9.3§	6.8 ± 0.8[a]	7.3 ± 1.1[a,b]	8.0 ± 0.6[c]
ALB (g/dL)	1.5–3.8	2.6 (1.8–4.4)[a]	2.1 (1.3–2.7)[b]	2.4 (1.9–3.0)[ac]
BUN (mg/dL)	7.0–36.0	24.0 (5.1–58.5)	13.5 (4.9–25.7)	16.0 (5.0–22.3)
CREA (mg/dL)	0.1–0.7	0.40 (0.10–0.60)[a]	0.35 (0.09–0.74)[a,b]	0.2 (0.10–0.40)[c]
Energy metabolism				
GLUC (mg/dL)	28–107	84.0 (60.8–118.8)[a]	58.5 (27.5–88.6)[b]	59.0 (27.4–73.5)[b,c]
CHOL (mg/dL)	42–131	102.0 (72.1–158.7)[a]	61.0 (40.5–99.0)[b]	57.0 (48.4–79.9)[b,c]
TGL (mg/dL)	10–83	37 (17.3–78.0)	28 (2.0–62.5)	32.0 (8.0–85.7)

§*mean ± 1.96 SD.* [a,b,c]*Letters identify differences among groups for P-values < 0.05.*

Donkeys were divided in three groups according to age: group A (foals, animals below 1 year of age), group B (young animals, from 1 to 3 years of age), and group C (adult animals, over 3 years of age), and biochemical parameters were indicated by median (2.5th−97.5th percentile) or by mean ± SD.

anemia: the HGB concentration remains constant throughout life despite that MCV values are below the adult range while hemoglobinization (HDW) and anisocytosis (RDW) indexes are higher than adult animals. In agreement with previous reports (4, 20), we observed greater RBC counts in under 1-year-old than in oldest donkeys. Since the HGB concentration values are constant over the life, the contemporary reduction of RBC with aging explains the increment of MCH observed in older MF donkeys.

Platelet count was not influenced by age showing reference ranges comparable to reports of other studies (16, 20). The reference range of mean platelet volume (MPV), a PLT index of heterogeneity of platelet volume, overlaps with those reported in Northeastern donkeys (21). Decrement of platelet distribution width (PDW), a measurement of heterogenicity in platelet morphology (22), was recorded in MF similarly to that reported for Ragusana donkeys (4); this reduction was related with age progression but reached a value higher than the Ragusana breed probably due to physiological reasons.

About the biochemical profile, our study showed that many analytes are age-influenced, warning about the importance in these cases to establish the appropriate reference ranges. The only two studies published on MF breed were aimed to provide biochemical blood analysis in foals immediately after parturition, from 12 h to 11 days (11), or in jennies during pregnancy (5). Therefore, our biochemicals data are the first provided for a general population of MF donkeys.

According to Bature et al. (9) with the exception of Cl^- not influenced by age, electrolytes and in particular Na^+, K^+, and Cl^- showed a significant decrease with advancement of age (9). Conversely, in Pêga breed donkeys these analytes showed an opposite trend (7) similar to that reported only for Cl^- in Ragusana breed where Na^+ and K^+ are not influenced by age (4).

In our study, the marker of bone metabolism tends to decrease with aging as a probably natural consequence of age-related bone remodeling decline. In particular, in Kyrgyz donkeys the highest value of serum Ca^{2+} was found in foals and the increment of serum Phos concentration gradually decays with aging (8), corroborating the observations of Caldin et al. (4), Zinkl et al. (23), and Jordana et al. (24). Generally, a high serum Phos concentration is indicative of a fast bone growth as happens during the neonatal and youth periods. Then, the Phos concentration gradually decays with aging (25), probably due to decline of bone metabolism (23). Serum Mg^{2+} concentration observed was on average higher than that reported by other authors (4, 8). Although also influenced by age, the higher ALP activity observed in MF foals under 1 year of age seems to follow the evolution bone metabolism characterized by an initial rapid growth that decreases with aging (19, 26).

Transaminase enzyme (ALT, AST) and GGT activity showed an age-related trend different to reports on donkeys by other authors (4, 7, 15). In particular, ALT activity is not influenced by age according to Caldin et al. (4) and to that observed in

horses (27) and in very young foals (11), but in disagreement with that reported by Girardi et al. (7) which observed the highest value in old donkeys. In our work, AST activity reaches its lowest values in young donkeys, confirming the observations of other authors (7, 15). As reported for Pêga (7), also in MF donkeys GGT activity is higher in foals than in older donkeys differently to those reported by observed in Ragusana and crossbred donkeys (4).

Total proteins, albumin, and BUN are biomarkers of protein metabolism which provide information about the nutritional status of animals. The range for TP and ALB values in the MF donkey population was slightly higher than that described in the Kyrgyz breed (27). Moreover, MF donkey's TP concentration resulted to be age-related with higher values in older donkeys, contrary to those reported by Kisadere et al. (27) but in agreement with Girardi et al. (7). The ALB concentration showed the same trend observed in Ragusana donkeys (4): presumably, the age-related ALB concentration increment is caused by a tendency to establish a dehydration status in old animals. Finally, BUN concentrations remain constant throughout the MF donkey's life with values comparable to those reported for very young MF foals (11) while CREA drastically decreases with aging probably due to physiological reduction of muscular mass in old animals. These results partially concord to those of Caldin et al. (4) which were observed as CREA as urea levels reach the lowest values in older animals.

As also described by other authors, the MF donkeys under 1 year of age showed the highest mean values of CHOL (4, 7) and GLUC (7) concentrations and a possible explanation could be in the kind of feeding of foals that primary is milk-based. The TGL concentration in MF donkeys is not influenced by age as reported by Caldin et al. (4), but the mean values in the correspondent age group were lower than those described in Ragusana donkeys (4) probably because they were affected by different diets.

The MF donkey population examined belongs to a unique farm and so makes it impossible to estimate the effects of the feeding management or environment. The lack of evaluation of the effects of sex on biochemical and hematological parameters in this study certainly represents a limit, especially for some hematological parameters such as RBC, HGB, and HCT which in other species, including humans, notoriously are higher in males than in females (17).

Moreover, biochemical parameters as glucose or creatinine increase during the first weeks of pregnancy (5), and also RBC and HCT are higher in late pregnancy than at foaling (28). Unfortunately, the number of pregnant and nonpregnant jennies was too different to correctly analyze the effects of pregnancy on biochemical and hematological parameters representing another limitation of this study.

CONCLUSIONS

The MF donkey is a breed considered endangered, so knowledge about the normal values of hematological and biochemical parameters is essential to classifying animal as healthy or affected by a pathological condition. This knowledge plays an important role in the strategies aimed at the conservation of endangered breeds as Martina Franca donkeys and more in general in the panorama of the biodiversity of equidae. Further studies are needed to have more data including a wider population of MF donkeys to set a valid breed-based reference range.

In conclusion, the results suggest that for the Martina Franca donkey breed, age is a variable that, influencing different hematological and biochemical parameters, needs to be better considered in order to obtain a correct clinical evaluation of the animals.

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 the animal study because all analyses were performed in leftover blood samples from routinary investigations performed for clinical purposes and for the benefit of the patient.

AUTHOR CONTRIBUTIONS

FT, ID, AD, and AC designed the study. FT, ID, and CC performed the experiments. FT analyzed the data. AD, FT, ID, and AD wrote the paper with input from all authors. AC supervised this work. All authors contributed to the article and approved the submitted version.

FUNDING

The present study has been carried out in the framework of the Project Demetra (Dipartimenti di Eccellenza 2018 – 2022, CUP_C46C18000530001), funded by the Italian Ministry for Education, University and Research.

REFERENCES

1. Domestic FAO. *Animal Diversity Information System*. (2019) Available online at: http://www.fao.org/dad-is / (accessed January 31, 2020).
2. Monti G, Bertino E, Muratore MC, Coscia A, Cresi F, Silvestro L, et al. Efficacy of donkey's milk in treating highly problematic cow's milk allergic children: an *in vivo* and *in vitro* study. *Pediatr Allergy Immunol*. (2007) 18:258e64. doi: 10.1111/j.1399-3038.2007.00521x
3. Cavallarin L, Giribaldi M, Soto-Del Rio MD, Valle E, Barbarino G, Gennero MS. A survey on the milk chemical and microbiological quality in dairy donkey farms located in Northwestern Italy. *Food Control*. (2015) 50:230e5. doi: 10.1016/j.foodcont.2014.08019
4. Caldin M, Furlanello T, Solano-Gallego L, de Lorenzi D, Carli E, Tasca S, et al. Reference ranges for haematology, biochemical profile and electrophoresis in a single herd of Ragusana donkeys from Sicily (Italy). *Comp Clin Pathol*. (2005) 14:5–12. doi: 10.1007/s00580-005-0544-8

5. Gloria A, Veronesi MC, Carluccio R, Parrillo S, de Amicis I, Contri A. Biochemical blood analysis along pregnancy in Martina Franca jennies. *Theriogenology*. (2018) 115:84–9. doi: 10.1016/j.theriogenology.2018.04026
6. Piccione G, Caola G, Mortola JP. Scaling the daily oscillations of breathing frequency and skin temperature in mammals. *Comp Biochem Physiol Part A Mol Integr Physiol*. (2005) 140:477–86. doi: 10.1016/j.cbpb.2005.02010
7. Girardi AM, Marques LC, de Toledo CZP, Barbosa JC, Maldonado W, Jorge RLN, et al. Biochemical profile of the Pêga donkey (*Equus asinus*) breed: influence of age and sex. *Comp Clin Pathol*. (2014) 23:941–7. doi: 10.1007/s00580-013-1718-4
8. Kisadere I, Donmez N, Omurzakova N. Serum biochemical reference values of Kyrgyz donkeys (*Equus asinus*). *Comp Clin Pathol*. (2018) 28:817–23. doi: 10.1007/s00580-018-2864-5
9. Bature I, Shehu BM, Barje PP. Serum biochemical parameters of donkeys (*Equus asinus*) as affected by age, location and sex in northwestern Nigeria. *J Anim Prod Res*. (2018) 30:123–33.
10. Sgorbini M, Bonelli F, Rota A, Baragli P, Marchetti V, Corazza M. Hematology and clinical chemistry in amiata donkey foals from birth to 2 months of age. *J Equine Vet Sci*. (2013) 33:35–9. doi: 10.1016/j.jevs.2012.04010
11. D'Alessandro AG, Casamassima D, Palazzo M, Jirillo F, Jirillo E, Martemucci G. Values of energetic, proteic and hepatic serum profiles in neonatal foals of the martina franca donkey breed. *Macedonian J Anim Sci*. (2012) 2:213–7.
12. Ljubodrag S, Vladimir D, Predrag S, Nada L, Ivica R, Ante I, et al. Morphological, biochemical and hematological characterization of endangered balkan donkey breed. *Acta Vet*. (2015) 65:125–36. doi: 10.1515/acve-2015-0010
13. de Palo P, Maggiolino A, Albenzio M, Casalino E, Neglia G, Centoducati G, et al. Survey of biochemical and oxidative profile in donkey foals suckled with one natural and one semi-artificial technique. *PLoS ONE*. (2018) 13:e0198774. doi: 10.1371/journal.pone0198774
14. CLSI and IFCC. *C28-A3 Document; Defining, Establishing and Verifying Reference Intervals in the Clinical Laboratory: Approved Guideline*. Wayne, PA: 3rd ed (2008).
15. Dezzutto D, Barbero R, Valle E, Giribaldi M, Raspa F, Biasato I, et al. Observations of the hematological, hematochemical, and electrophoretic parameters in lactating donkeys (*Equus asinus*). *J Equine Vet Sci*. (2018) 65:1–5. doi: 10.1016/j.jevs.2018.02013
16. Girardi AM, Marques LC, de Toledo CZP, de Campos Filho E. Hematological variables of the Pêga donkey (*Equus asinus*) breed: influence of age and sex. *Comp Clin Pathol*. (2015) 24:335–42. doi: 10.1007/s00580-014-1905-y
17. Weiss DJ, Wardrop KJ. *Schalm'S Veterinary Hematology*. Philadelphia, PA: John Wiley & Sons (2010).
18. Veronesi MC, Gloria A, Panzani S, Sfirro MP, Carluccio A, Contri A. Blood analysis in newborn donkeys: hematology, biochemistry, and blood gases analysis. *Theriogenology*. (2014) 82:294–303. doi: 10.1016/j.theriogenology.2014.04004
19. Barton MH. How to interpret common hematologic and serum biochemistry differences between neonatal foals and mature horses. *AAEP Proc*. (2015) 61:125–9.
20. Folch P, Jordana J, Cuenca R. Reference ranges and the influence of age and sex on haematological values of the endangered Catalonian donkey. *Vet J*. (1997) 154:163–8. doi: 10.1016/S1090-0233(97)80054-8
21. da Silva GB, da Silva CJ, de Souza LA, Hunka MM, Ferreira L, CC HE. Hematological and blood chemistry values of donkeys (Equus africanus osinus) in different management systems. *Pferdeheilkunde*. (2018) 34:253–9. doi: 10.21836/PEM20180306
22. Osselaer JC, Jamart J, Scheiff JM. Platelet distribution width for differential diagnosis of thrombocytosis. *Clin Chem*. (1997) 43:1072–6. doi: 10.1093/clinchem/43.61072
23. Zinkl JG, Mae D, Guzman PM, Farver TB, Humble JA. Reference ranges and the influence of age and sex on hematologic and serum biochemical values in donkeys (*Equus asinus*). *Am J Vet Res*. (1990) 51:408–13.
24. Jordana J, Folch P, Cuenca R. Clinical biochemical parameters of the endangered Catalonian donkey breed: normal values and the influence of sex, age, and management practices effect. *Res Vet Sci*. (1998) 64:7–10. doi: 10.1016/S0034-5288(98)90107-2
25. Evans GO. *Animal Clinical Chemistry—A Practical Guide for Toxicologists and Biomedical Researchers*. 2nd ed. Boca Raton, FL: CRC Press (2009).
26. Pitel P, Moulin M, Valette JP, Dumontier S, Petit L, Fortier G, et al. Approche des valeurs hématologiques et biochimiques chez deux races asines. *Prat Vet Equine*. (2006) 38:19–25.
27. Kisadere I, Bayraktar M, Salykov R. Some hematological and biochemical reference values of the thoroughbred Appaloosa horse breeds reared in Kyrgyzstan. *Comp Clin Pathol*. (2018) 28:1651–60. doi: 10.1007/s00580-019-02991-2
28. Bonelli F, Rota A, Corazza M, Serio D, Sgorbini M. Hematological and biochemical findings in pregnant, postfoaling, and lactating jennies. *Theriogenology*. (2016) 85:1233–8. doi: 10.1016/j.theriogenology.2015.12004

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 December 2020
doi: 10.3389/fvets.2020.601665

Radiographic and Venographic Appearance of Healthy and Laminitic Feet in Amiata Donkeys

Irene Nocera[1], Benedetta Aliboni[1], Liri Ben David[2], Luis Alfonso Gracia-Calvo[3], Micaela Sgorbini[1] and Simonetta Citi[1]*

[1] Department of Veterinary Science, Veterinary Teaching Hospital, University of Pisa, Pisa, Italy, [2] Private Equine Practitioner, Tel Aviv, Israel, [3] Faculty of Veterinary Science, Veterinary Teaching Hospital, University of Helsinki, Helsinki, Finland

Introduction: Laminitis is a debilitating disorder resulting in irreversible anatomical changes in the feet of equids. Assessing specific anatomical features through radiography and venography provides diagnostic and prognostic information. The reference ranges are well-established in horses, but not in donkeys. It is also uncertain as to whether these ranges can be applied to every donkey breed. The present study characterizes the radiological and venographic hoof anatomy of healthy feet of Amiata donkeys and defines the changes associated with severe and mild laminitis.

Materials and Methods: A total of 16 forefeet were evaluated in 8 Amiata jennies. The animals underwent musculoskeletal examination, Obel grading assessment and radiological evaluation. Based on clinical examination and radiographic findings, the forefeet were grouped as healthy, mild or severe laminitic feet, thus the digital venograms were performed according to the group definition.

Results: Radiology revealed 7/16 healthy, 4/16 mild laminitic, and 5/16 severe laminitic forefeet. Statistical analysis showed differences between the healthy and laminitic forefeet for the dorsal angle ($p < 0.0001$) and angle of solar aspect ($p < 0.0001$) of the distal phalanx, for deviation between dorsal aspect of distal phalanx and the hoof wall ($p < 0.0001$) and phalangeal rotation angle ($p = 0.0032$). Venography was abnormal in mild and severe laminitic forefeet. In particular, the vascularization was reduced or absent at the lamellar-circumflex junction dorsally, at the sub-lamellar vascular bed and at the circumflex veins. Coronary plexus vascularization was absent in severe laminitic forefeet.

Discussion and Conclusions: This study provides the radiological parameters for the assessment of healthy and laminitic forefeet of Amiata donkeys. The mild laminitic foot venogram showed decreased vascularization mainly on lamellar-circumflex junction and sub-lamellar vascular bed, in latero-medial views. The severe laminitic foot showed very poor or absent vascularization in multiple areas. The technique is easily applicable and provides diagnostic support in laminitis.

Keywords: Amiata donkey, laminitis, radiology, venography, foot

OPEN ACCESS

Edited by:
Ellen Roelfsema, Utrecht University, Netherlands

Reviewed by:
Annamaria Grandis, University of Bologna, Italy
Domenico Caivano, University of Perugia, Italy

****Correspondence:***
Irene Nocera
irene.nocera@vet.unipi.it

Specialty section:
This article was submitted to Comparative and Clinical Medicine, a section of the journal Frontiers in Veterinary Science

Received: *01 September 2020*
Accepted: *27 November 2020*
Published: *21 December 2020*

Citation:
Nocera I, Aliboni B, Ben David L, Gracia-Calvo LA, Sgorbini M and Citi S (2020) Radiographic and Venographic Appearance of Healthy and Laminitic Feet in Amiata Donkeys. Front. Vet. Sci. 7:601665. doi: 10.3389/fvets.2020.601665

INTRODUCTION

Laminitis is an extremely painful disease that compromises the integrity of the digital dermis and the normal biomechanics of the equine foot (1). It can result in lameness and alterations in hoof horn production and in digit positioning, leading to chronic and acute foot pain (2). Chronic and irreversible cases, where animal euthanasia is the only humane option, are not rare (1).

The pathophysiology of laminitis is still not completely understood. It has been defined as the failure of the attachment of the distal phalanx and the inner hoof wall (1). The most commonly accepted causes are inflammatory and septic response, insulin resistance, mechanical overloading, and failure of the normal vascular perfusion within the foot (3).

These mechanisms lead to significant anatomical changes (4). This is of clinical importance because the diagnostic assessment can be aimed at detecting and quantifying of the anatomical changes (4–7). Today, radiological assessments are considered of primary diagnostic importance and represent the "gold standard" for the diagnosis of laminitis (8).

Digital venogram assessments have been shown to be very important for the assessment of vascular perfusion and integrity in the horse (9–12). This diagnostic method has the potential to provide information on the status of the blood supply within the foot capsule, to assess disease severity, to develop treatment strategies, and to monitor the response to treatment (10). Digital venograms can also be performed in very painful cases, even if the horse is not able to move at all, thus preventing further mechanical damage to the integrity of the digit dermis (10).

There is little information on the radiological anatomy of donkey feet (13–15), and therefore there has been a tendency to apply an equine model to help in the diagnostic interpretation (4, 7).

Laminitis is currently an underestimated pathology in donkeys, despite being a common disease in this species, due to the lack of physiological data on the donkey digit (16).

Few studies have been conducted on evaluating the radiographic appearance of the donkey digit, either in healthy or laminitic animals (4, 14), and few studies have been carried out on the normal aspect of the venogram in healthy donkeys (13, 17) and with evidence of laminitis (18).

The aim of this study was to assess the radiographic and venographic parameters of healthy Amiata donkey feet, to define the changes associated with mild and severe laminitis and to compare the results with other donkey breeds and horses.

MATERIALS AND METHODS

Study Group

A cohort of 8 Amiata donkeys belonging to the Regional Stud Farm of Tuscany and housed at the Veterinary Teaching Hospital, Department of Veterinary Sciences, University of Pisa were enrolled in this study. Approval to conduct this study was obtained from the ethical committee of the University of Pisa, according to the D. Lgs. 26/14 (Number 23/19).

TABLE 1 | Grading of the wall deformity (20, 21).

	Wall deformity scale
Grade 0	No visible changes
Grade 1	Converging hooves growth rings involving 1/3 of the hoof wall
Grade 2	Converging hooves growth rings involving 2/3 of the hoof wall
Grade 3	Wall changes involving the entire hoof wall

All the animals enrolled were barren jennies used for reproduction purposes and were considered non-athlete. The animals were aged between 9 and 19 years (median age 13 years), the body weight (BW) ranged between 283 and 393 kg (median BW 342 kg), and the body condition score (BCS) ranged between 5 and 6.5/9 (median BCS 5.75/9). Jennies were housed in collective paddocks 24 h a day, fed with meadow hay *ad libitum* along with commercial equine feed in line with the NCR energy recommendations (19). All the subjects were barefoot and underwent periodical hoof trimming every 50/60 days.

None of the jennies had a previous history of foot-related problems. An orthopedic evaluation was performed on all the animals to assess the following clinical signs related to laminitis: stance and gait irregularities, soundness according to the Obel score (2), digital pulse amplitude, increased hoof temperature, presence of supra-coronary depression, and increased sensitivity to hoof testers.

Hooves were inspected visually to assess the presence of the following signs of laminitis: presence of converging hoof growth rings, deformity of hoof capsule shape, slipper foot conformation, flattening of the sole, and widening of the white line. Since no previous studies are available in the literature, the presence of converging rings was assessed according to a grading scale specifically designed for this study. The wall deformity was thus scored from 0 to 3 (**Table 1**).

Radiographic Technique

For all the jennies enrolled in the present study, x-ray views were obtained for both forelimbs, for a total of 16 forefoot radiographs. Prior to radiography, the feet were thoroughly cleaned. All subjects were sedated with detomidine chloride (10 μg/Kg, IV) (Detogesic®, Zoetis Italia, Italy) and buthorphanol 0.025 mg/kg, IV (Nargesic®, ACME, Italy) (22). Baseline radiographs, dorso-palmar (DP) and latero-medial (LM) views, were taken. The jennies were then placed on wooden blocks for the feet (8 cm in height) (4), positioning both forelimbs in a way that the metacarpi were perpendicular to the ground and in close contact with the radiograph cassette to prevent any image magnification (23). Radiopaque barium paste was applied on the dorsal hoof wall at the midline, up to the palpable proximal coronary band (23).

All radiographs were obtained at a focal distance of 80 cm, with the beam focused midway between the dorsal and palmar aspect of the foot, and midway between the coronary band and the weight bearing border, in accordance

TABLE 2 | Direct and derived radiological parameters relevant in laminitis, for the forefoot and their definitions, in latero-medial and dorso-palmar radiographic views [modified from (4)].

Parameter	Definition	Method of determination
ANGULAR PARAMETERS OF THE FOOT		
Latero-medial radiographic view		
S	Dorsal hoof wall angle	Angle subtended between the dorsal aspect of the hoof wall and the ground line direct parameter
Ts	Dorsal angle of the distal phalanx	Angle subtended between the dorsal aspect of the DP and the ground line Direct parameter
U	Angle of proximal phalanx	Angle subtended between the long axis of the PP and the ground line Direct parameter
C	Angle of middle phalanx	Angle subtended between the long axis of the MP and the ground line Direct parameter
SA	Angle of solar aspect of the distal phalanx	Angle subtended between the solear aspect of the DP and the ground line Direct parameter
PA (U-C)	Angle of pastern axis	Angular difference between long axis of the PP and MP Derived parameter.
HPA (U-S)	Angle of hoof pastern axis	Angular difference between the dorsal hoof wall angle and the long axis of the PP
H Ang (Ts-S)	Angular deviation between the dorsal aspect of the DP and dorsum of the hoof wall	Angular difference between dorsal aspect of the DP and the dorsal hoof wall angle Derived parameter
F Ang (C-Ts)	DIP rotation Angle	Angular difference between dorsal aspect of the DP and the long axis of the MP Derived parameter
R Ang (U-Ts)	Phalangeal rotation angle	Angular difference between dorsal aspect of the DP and the long axis of the PP Derived parameter
LINEAR PARAMETERS		
Latero-medial radiographic view		
D	Distal displacement of the distal phalanx	Perpendicular linear distance between the proximal limit of the hoof wall and the extensor process of the DP
MPL	Middle phalanx length	Liner measurement of long axis of the middle phalanx
IDA	Proximal integument depth of the dorsal aspect of the foot	Perpendicular linear distance between the dorsal aspect of the hoof wall and the dorsal surface of the DP, immediately distal to the distal limit of the extensor process
IDB	Distal integument depth of the dorsal aspect of the foot	Perpendicular linear distance between the dorsal aspect of the hoof wall and the dorsal surface of the DP proximal to the apex of the DP
IDM	Mid integument depth of the dorsal aspect of the foot	Perpendicular linear distance between the dorsal aspect of the hoof wall and the dorsal surface of the DP at the midpoint between the IDA and IDB measurement sites
IDR	IDA/IDB ratio	
Dorso-palmar radiographic view		
SL	Lateral sole thickness	Perpendicular linear distance between the lateral solar aspect of the DP and the ground
SM	Medial sole thickness	Perpendicular linear distance between the medial solar aspect of the DP and the ground
LHW	Lateral hoof wall thickness	Perpendicular linear distance between the distal lateral aspect of the DP and the lateral hoof wall
MHW	Medial hoof wall thickness	Perpendicular linear distance between the distal medial aspect of the DP and the medial hoof wall
MORPHOMETRIC PARAMETERS		
Latero-medial radiographic view		
PPCA	Proximal palmar cortex angle	Angle subtended between the proximal palmar cortex of the DP and the ground line
PPCL	Proximal palmar cortex length	Linear distance between the point of insertion of DDFT and the articular process of the navicular joint
PCL	Palmar cortex length	Linear distance between the apex of the DP and the articular process of the navicular joint
RA	Reflex angle of palmar cortex	Internal angle subtended between the proximal and distal palmar cortex of the DP
AA	Apex angle	Internal angle subtended between the distal palmar cortex and the dorsal aspect of the DP
Surface Convexity	Dorsal surface of the coffin bone convexity	Quality evaluation of the convexity of the parietal surface of the distal phalanx
Erosion	Osteolysis of Distal margin of the coffin bone	Quality evaluation of resorption and remodeling of bone at the dorsal solear margin of the distal phalanx
Distal Margin Lip	Remodeling of the distal margin of the coffin bone	Quality evaluation of new bone formation on the dorsal aspect of the toe of the distal phalanx

with the literature (4). All radiographic procedures were performed using a portable machine (GIERTH HF100 M), with the following settings: exposure factors of 59 kV at 1.2 mAs, for a 100 mm wide hoof (adjusting 1kV according to 5 mm change in width) (4). One single experienced operator performed all the radiographs (IN). Radiographs were scanned and the digitized images were analyzed using commercial software (Horos^{TM}–DICOM).

Radiological Parameters

All the forefoot radiographs obtained were assessed in terms of the radiological parameters relevant to laminitis. These radiometric parameters and associated definition are shown in **Table 2** (4, 14).

Subgroup Definition and Inclusion Criteria for Venography Protocol

Based on the results obtained from clinical examination and radiographic evaluation, the feet were retrospectively divided into three groups, as previously reported (15, 24): (A) healthy foot (7/16), which is normal at clinical examination and radiographic parameters where within normal limits, (B) foot showing mild laminitic changes (4/16), namely Obel grade <1

TABLE 3 | Venographic parameters relevant in laminitis, for the forefoot and their definitions, in LM and DP radiographic views [modified from (24)].

Parameter	Definition	Quality evaluation of contrast distribution
VENOGRAPHIC PARAMETERS		
Latero-medial radiographic view		
PDV	Palmar Digital Vein	Present—Altered—Absent
TA	Terminal Arch	Present—Altered—Absent
CV	Circumflex Vessels	Location of the Circumflex Vessels distal to the palmar rim of the distal phalanx
LCJ	Lamellar-Circumflex Junction	Normal—Mild—Folded—Void of Contrast
SLVB	Sublamellar Vascular Bed	Uniform Line—Rectangular Shape—Triangular Shape—Void of Contrast
CP	Coronary Plexus	Normal—Abnormal—Void of Contrast
Parameter	**Definition**	**Method of determination**
Dorso-palmar radiographic view		
PDV	Palmar Digital Vein	Present—Altered—Absent
TA	Terminal Arch	Present—Altered—Absent
CVM	Circumflex Vessels Medial	Location of the Medial Circumflex Vessels distal to the palmar rim of the distal phalanx
CVL	Circumflex Vessels Lateral	Location of the Lateral Circumflex Vessels distal to the palmar rim of the distal phalanx
LCJ	Lamellar-Circumflex Junction	Normal—Mild—Folded—Void of Contrast
SLVB	Sublamellar Vascular Bed	Uniform Line—Rectangular Shape—Triangular Shape—Void of Contrast
CPL	Coronary Plexus Lateral	Normal—Abnormal—Void of Contrast
CPM	Coronary Plexus Medial	Normal—Abnormal—Void of Contrast

TABLE 4 | Average values obtained from clinical evaluation, reported according to the subgroups.

	Lameness	Altered stance at rest	Obel grade	Digital pulse amplitude	Hoof temperature	Response to hoof tester	BCS	Weight (kg)
PHYSICAL EXAMINATION								
Normal	0	No	0	No	Normal	Negative	5	289
Mild	0	No	0	No	Normal	±	5.5	322
Severe	0-3	No	1	No	Warm	Positive	6	316
	Palpable depression at coronary band	**Convergent hoof rings**	**Shape deformity**	**Dropped sole**	**Widening white line**	**Slipper foot conformation**		
EXTERNAL HOOF CAPSULE								
Normal	No	1.1	Yes	No	±	No		
Mild	No	0.5	Yes	±	Yes	No		
Severe	Yes	1.2	Yes	Yes	Yes	No		

BCS, Body Condition Score.

and no hoof capsule changes and radiographic findings of distal phalanx rotation, and (C) foot showing severe laminitic changes (5/16), which is characterized by Obel grade >1, hoof capsule deformation, and radiographic findings of distal phalanx displacement and remodeling. The venography exams were performed on 3/7 healthy feet, 4/4 mild laminitic feet, and 5/5 severe laminitic feet.

Venographic Technique

All the venographic exams were performed under sedation with detomidine chloride (10 μg/Kg, IV) (Detogesic®, Zoetis Italia, Italy) and buthorphanol 0.025 mg/kg, IV (Nargesic®, ACME, Italy) (22). The hair was clipped from the distal third of the metacarpus up to the coronary band. The low four-point nerve block was performed by injecting 3 ml of lidocaine perineural at each site (lidocaine 2%, Zoetis Italia, Italy) and a tourniquet was tightly wrapped slightly above the fetlock, using constant tension. The area above the later palmar digital vein was scrubbed and a 21G butterfly IV catheter (Terumo Italia Srl, Italy) with an extension tube line was placed.

A total of 20 ml of contrast agent (Iopamiro 300®, Bracco Imaging, Italia) was used for an average-sized foot and two different syringes were used to avoid excessive injection pressure, and thus to prevent any perivascular extravasation or wall vein damage (24). The first 10 ml contrast was injected with the foot in weight-bearing position, immediately afterwards the second 10 ml was injected while the limb was gently flexed, thus taking the weight off the foot (24).

After all the contrast has been injected, the butterfly catheter was left in place and the tube line was taped proximally to the limb, until the radiographs were performed (18). The latero-medial and dorso-palmar views were taken with the limb in weight bearing position, within 45 s of the injection (24).

Six areas were evaluated on the venogram image (**Table 3**), as previously reported in the horse (24): palmar digital vein (PDV), terminal arch (TA), circumflex vessels (CV), lamellar–circumflex junction (LCJ), sub-lamellar vascular bed (SLVB), and coronary plexus (CP).

These areas were examined both in LM and DP views for a quality evaluation of the contrast distribution (24).

Statistical Analysis

Data were assessed for distribution. Since some parameters were normally distributed and others were not, we decided to express all the results as not Gaussian distributed. The results were reported as median and standard error, minimum and maximum values.

The Kruskal-Wallis-test for multiple comparisons was applied to verify differences between the three groups concerning the numerical data (angular and linear radiographic parameters, lameness and clinical parameters of hoof convergent rings), BW and BCS. The significance level was set at $p<0.05$. Statistical analysis was performed with GraphPad Prism v. 8.3.1 (GraphPad Software Inc., San Diego, CA, USA).

TABLE 5 | Angular radiographic parameters from latero-medial view, expressed as degree, for the healthy, mild, and laminitic study groups.

	Angular parameters																											
	S			Ts			U			C			SA			PAxis (U-C)			HPAxis (U-S)			H Ang (Ts-S)			F Ang (C-Ts)			R Ang (U-Ts)		
	H	M	S	H	M	S	H	M	S	H	M	S	H	M	S	H	M	S	H	M	S	H	M	S	H	M	S	H	M	S
Me	60.1	59.3	61.9	62.5	66.4	71.3	61.2	60.2	63.8	64.7	58.6	67.9	7.7	11.3	15.7	−0.8	0.0	3.5	2.2	0.1	8.2	2.5	7.6	11.1	2.2	−7.8	−5.0	0.6	−5.9	−7.2
m	59.0	58.2	55.6	60.6	66.1	68.3	58.4	50.0	61.3	53.1	50.3	58.1	6.1	10.1	14.4	−7.7	−0.4	−6.6	−2.1	−9.4	−0.7	0.1	4.1	6.5	−7.4	−17.1	−10.7	−3.5	−17.4	−10.
M	61.1	62.0	63.4	64.1	67.3	78.6	69.1	62.5	73.0	72.1	61.3	76.6	9.2	13.7	22.4	5.3	4.6	5.7	8.0	3.2	9.7	3.1	8.0	16.7	9.2	−4.7	−2.0	5.0	−4.1	−1.2

Me, median; m, minimum values; M, maximum value; H, healthy group; M, mild laminitic group; S, severe laminitic group.

TABLE 6 | Linear radiographic parameters from latero-medial view, expressed in cm, for the healthy, mild, and laminitic study groups.

	Linear parameters																	
	D			MPL			IDA			IDB			IDM			IDR		
	H	M	S	H	M	S	H	M	S	H	M	S	H	M	S	H	M	S
Me	3.3	1.6	1.7	3.4	3.7	3.5	2.5	2.6	2.4	2.7	2.9	3.0	2.6	3.0	2.8	1.0	0.8	0.8
m	1.2	0.8	1.3	3.3	3.4	3.4	1.7	2.0	2.1	2.1	2.4	2.5	2.0	2.2	2.4	0.8	0.8	0.8
M	3.9	2.2	2.2	3.7	4.0	3.7	3.0	3.3	2.9	3.2	3.4	3.2	2.9	3.2	3.2	1.2	1.1	0.9

Me, median; m, minimum values; M, maximum value; H, healthy group; M, mild laminitic group; S, severe laminitic group.

TABLE 7 | Morphometric radiographic parameters from latero-medial view, for the healthy, mild, and laminitic study groups.

	Morphometric parameters														
	PPCA (mm)			PPCL (mm)			PCL (mm)			RA (degree)			AA (degree)		
	H	M	S	H	M	S	H	M	S	H	M	S	H	M	S
Me	49.0	53.5	59.3	2.5	2.7	2.5	4.4	4.7	4.4	140.6	137.4	137.4	52.4	57.2	53.4
m	41.6	50.9	51.7	2.0	2.4	2.0	4.0	4.5	4.1	124.3	135.9	134.0	45.1	54.6	50.6
M	61.5	55.0	61.5	2.9	3.5	2.9	5.2	5.2	5.0	147.4	144.6	145.3	56.8	64.6	60.3

Me, median; m, minimum values; M, maximum value; H, healthy group; M, mild laminitic group; S, severe laminitic group.

TABLE 8 | Linear radiographic parameters from dorso-palmar view for the healthy, mild, and laminitic study groups.

	Linear parameters											
	SL (cm)			SM (cm)			LHW (cm)			MHW (cm)		
	H	M	S	H	M	S	H	M	S	H	M	S
Me	2.5	2.0	2.1	2.4	2.3	1.9	2.0	2.2	2.0	1.8	1.7	1.8
m	1.9	1.5	1.8	1.7	2.1	1.8	1.6	1.2	1.4	1.5	1.5	1.2
M	2.9	2.8	2.7	3.1	2.7	2.9	2.4	2.7	2.8	2.0	1.8	2.0

Me, median; m, minimum values; M, maximum value; H, healthy group; M, mild laminitic group; S, severe laminitic group.

RESULTS

The results obtained from physical and external hoof examinations are reported in **Table 4**, according to the different subgroups. In particular, the severe laminitic feet group presented 4/6 physical parameters and 4/6 altered external hoof parameters, compared to the results obtained for the normal and mild groups.

The results obtained for the angular, linear and morphometric radiological parameters relevant to the laminitis evaluation are shown in **Tables 5**–**8**, according to the different subgroups. Statistical analysis showed differences between the healthy and the severe laminitic donkeys in terms of Ts ($p < 0.0001$), SA ($p < 0.0001$), H ang ($p < 0.0001$) and R ang ($p = 0.0032$) (**Figure 1**). No statistical differences were obtained for the other parameters. According to the evaluation of the angular, linear and morphometric radiological parameters, only 1 out of 8 (12.5%) donkeys presented the left forefoot healthy and the right forefoot mild laminitic.

The results obtained from the evaluation of the venographic parameters are given in **Table 9**. Within the severe laminitic group, the main altered parameters both in the LM and DP were LCJ, SLVB and CP (both in the medial and lateral areas on DP view), compared to the healthy group. These parameters differ from the normal shape, with a total lack of contrast in the severe cases (5/16) (**Figures 2**, **3**).

Finally, statistical differences were found for BCS ($p = 0.0052$), but not for BW ($p = 0.0713$) between severe laminitic vs. healthy group. In particular, the BCS was higher in the laminitic group.

DISCUSSION

The radiographic and venographic appearance of healthy and laminitic feet in donkeys were assessed in order to define the changes associated with mild and severe laminitis and to compare the results with other donkey breeds and horses.

Overall, we found statistical differences between healthy vs. severe laminitic donkeys for Ts, SA, H, and R ang, but not between mild vs. severe laminitic or healthy feet. No differences were found for other radiographic parameters. Thus, our results support the idea that the bone alignment and its relationship with the hoof capsule is relevant in the evaluation of laminitis in donkeys, in line with literature (4).

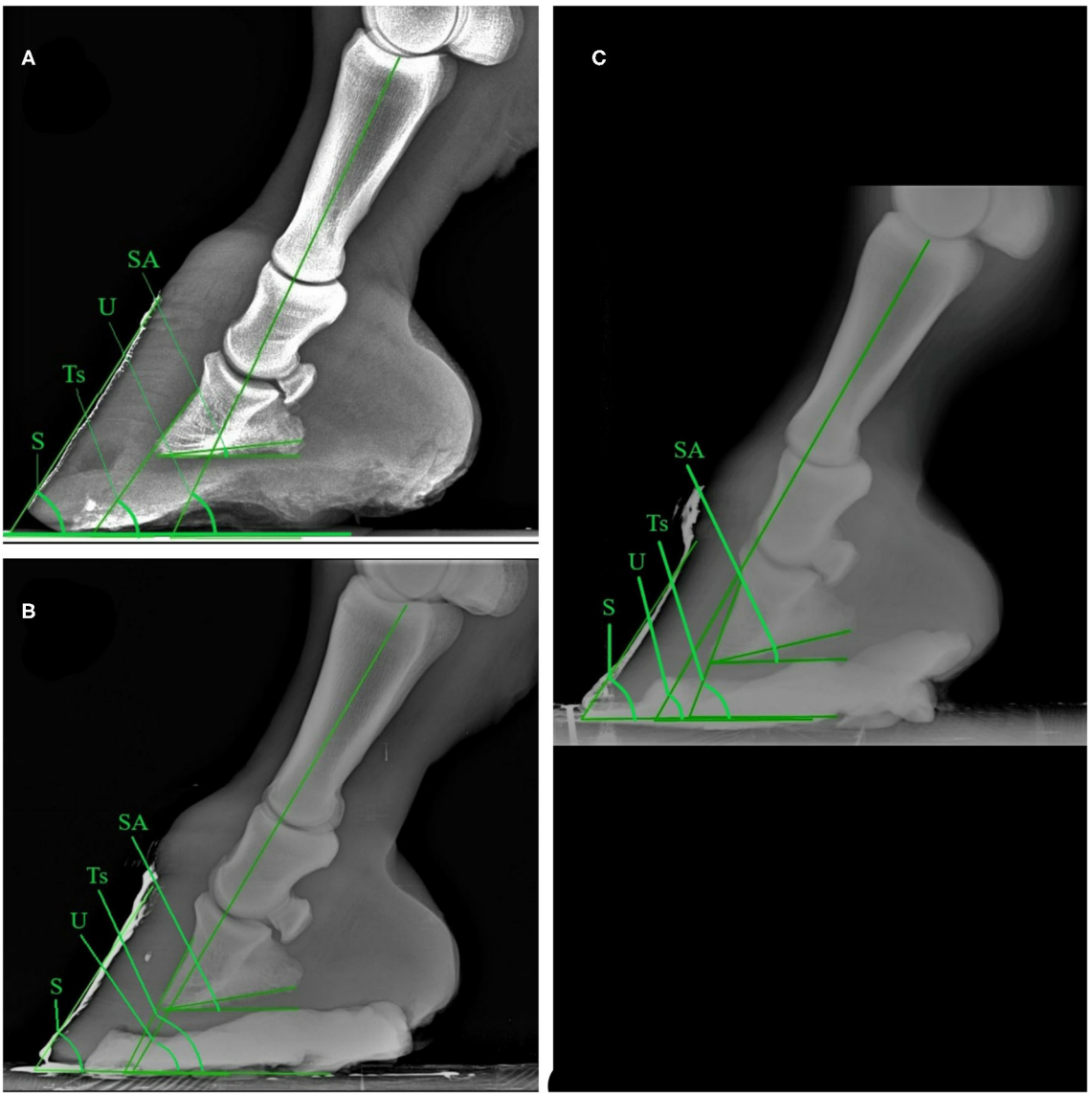


FIGURE 1 | Latero-medial plain radiographic images of healthy **(A)**, mild **(B)**, and severe **(C)** laminitic donkey feet. SA, Angle of solar aspect of the distal phalanx; S, Dorsal hoof wall angle; Ts, Dorsal angle of the distal phalanx; U, Angle of proximal phalanx.

In our study, Ts and SA values differed significantly between severe laminitic and healthy group. In particular, Ts and SA values were higher in the severe laminitic group compared to the healthy group, in agreement with Collins et al. (4). Moreover, the Ts and SA values obtained in the severe laminitic group were comparable with the laminitic values reported by Collins et al. (4) in the horse, even if we found a narrower range of values for Ts (68.30–78.60° vs. 57–94°) (4). This difference might be related to the small population enrolled in this study, which could be considered a limitation to the present work. SA is commonly considered to be useful in the diagnosis and prognosis of laminitis in horses and the degree of rotation has been inversely related to the prognosis (25).

The statistically differences obtained in the H Ang values between healthy and severe laminitic groups were in line with the previous study by Collins et al. (4). Moreover, the laminitic H Ang values obtained in this study were comparable to what found both in donkeys (4) and horses (6) in previous studies. The H Ang value usually indicates the correct presence of the parallelism between the dorsal surface of the distal phalanx and the dorsal wall of the hoof capsule, both in donkeys (4, 14, 15) and horses (23, 26). The divergence in alignment supported by the increased H ang value might be indicative of dorsal distal phalanx rotation (23, 27).

In this work, the R Ang values statistically differ between healthy and severe laminitic groups, as also reported by Collins

TABLE 9 | Values obtained from the evaluation of venographic parameters, shown according to subgroups. Minimum and maximum values are reported for each parameter.

	Normal group	Mild group	Severe group
LATERO-MEDIAL RADIOGRAPHIC VIEW			
PDV	Present	Present	Present
TA	Present	Present	Present
CV (mm)	4.3 – 4.7	4.0 – 5.7	2.9 – 6.2
LCJ	Normal	Mild - Folded	Mild - Void of contrast
SLVB	Uniform Line	Rectangular to Triangular shape	Triangular Shape -Void of contrast
CP	Normal	Normal	Normal - Void of contrast
DORSO-PALMAR RADIOGRAPHIC VIEW			
PDV	Present	Present	Present
TA	Present	Present	Present
CVM (mm)	2.2–3.8	3.0–6.4	2.3–5.7
CVL (mm)	2.6–4.6	2.5–5.1	2.4–4.6
LCJ	Normal	Normal—Folded	Normal—Void of Contrast
SLVB	Uniform Line	Uniform line—Rectangular shape	Rectangular shape—Void of contrast
CPL	Normal	Normal	Normal—Abnormal
CPM	Normal	Normal	Normal—Void of Contrast

et al. (4). In particular, the laminitic R Ang values obtained in this study were lower than results found in previous studies performed both in donkeys (4) and horses (6). This finding might be related to the large variability in digit values reported in different studies performed in donkeys (4, 14, 15, 18). For this reason, even if the R Ang value is considered relevant for diagnosis of laminitis (4), it needs to be evaluated with caution.

The lack of differences between mild vs. severe laminitic feet or healthy feet for Ts, SA, H, and R ang might be related to the small number of animals included in the study and/or by the wide variability in digit values registered in donkey breeds (4, 14, 18) and, in particular, in Amiata donkeys (15).

We found 1/8 donkey presenting one healthy forefoot and the other one showing mild laminitic changes, in line with literature. In fact, laminitis may affect only one foot, if the causes are repeated trauma on the foot, abnormal distribution of the loading force and any other alteration of the normal gait (11, 23).

The PDV and TA values obtained from venographic studies in healthy donkeys were clearly evident, in agreement to literature (18). In this study, the PDV and TA values were evident also in mild and laminitic donkeys. These results are in line with previous studies in which the PDV and TA values were rarely altered in laminitic animals, even in severe cases (such as in DP distal displacement, infarcts, and thickening of the distal aspect of the deep digital flexor tendon) (10, 24).

In our study, the CV values, obtained both in the LM and DP views, showed wide ranges both in the mild and severe laminitic groups compared with the healthy one. To the best of authors' knowledge, the CV range has not been reported in healthy donkey feet yet. The results obtained for CV parameter in healthy donkeys in this study is comparable to what reported in a previous study (24). Sound horses showed a large variation in the normal appearance of the CVs and sole and may be affected by abnormalities others than laminitis (10, 24). Thus, our findings obtained in the mild and severe laminitic groups might be distorted by the coexistence of foot problems other than laminitis. CV should still be evaluated during a venogram laminitis assessment because an increase in solar depth and CV appearance may be related to successful treatment (10, 24).

On the other hand, our results for the LCJ and SLVB revealed strongly altered values in both the mild and severe laminitic groups compared with the healthy group, both in DP and LM views. Donkeys show a well-developed anastomosis in foot circulation compared to horses (18). In horses, LCJ and SLVB parameters were related to displacement of the DP, damage to the vessels secondary to displacement, and sub-lamellar edema (10, 24). It is possible that the particular extensive foot circulation in donkeys may lead these parameters get altered earlier compared to the other venogram parameters. Evaluation of LCJ and SLVB values may therefore be useful to promptly detect a potential anatomical vasculature alteration within the hoof.

Lastly, in our study CP parameters showed comparable results between the healthy and mild laminitic groups. On the other hand, the CP ranged from normal to void of contrast in the severe laminitic group. These findings may be explained considering that occasionally an inadequate volume of contrast creates a technical artifact, reducing the filling of CP, as reported in horses (24). Moreover, in severe laminitic horses, the CPs were found

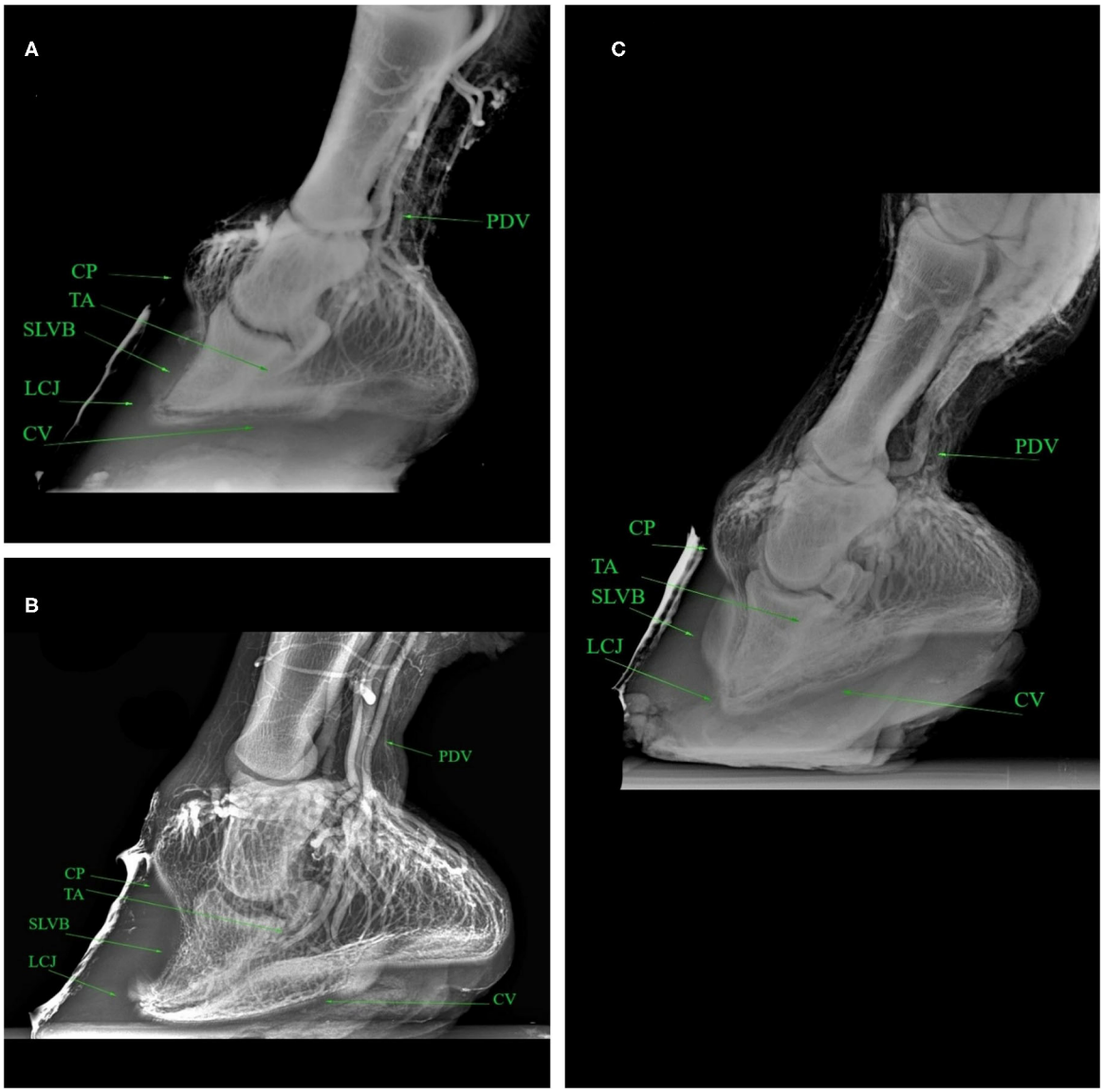


FIGURE 2 | Latero-medial venograms of healthy **(A)**, mild **(B)**, severe **(C)** laminitic donkey feet. PDV, Palmar Digital Vein; CP, Coronary Plexus; TA, Terminal Arch; SLVB, Sublamellar Vascular Bed; LCJ, Lamellar-Circumflex Junction; CV, Circumflex Vessels.

to be permanently distorted and so contrast may be reduced (10, 24).

As reported in literature, the presence of dermal pathology may be overlooked without venograms and the appropriate treatment may be delayed (24). According to this, our results seem to suggest that, also in donkeys as in horses, the venogram could detect mild laminitis change within the foot earlier than radiogram. In our study, the venogram was useful in demonstrate vascular and dermal integrity. It could be an important tool for the assessment of severity of the disease and the development of the treatment strategies also for donkeys.

This study has some limitations. The venogram appearance was evaluated in a small number of healthy feet, thus this might have led to a bias in the interpretation of results. Therefore, more studies needed to correctly establish the normal venogram appearance of healthy donkey feet in order to properly evaluate a pathological foot and to verify the reproducibility of the study.

DATA AVAILABILITY STATEMENT

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

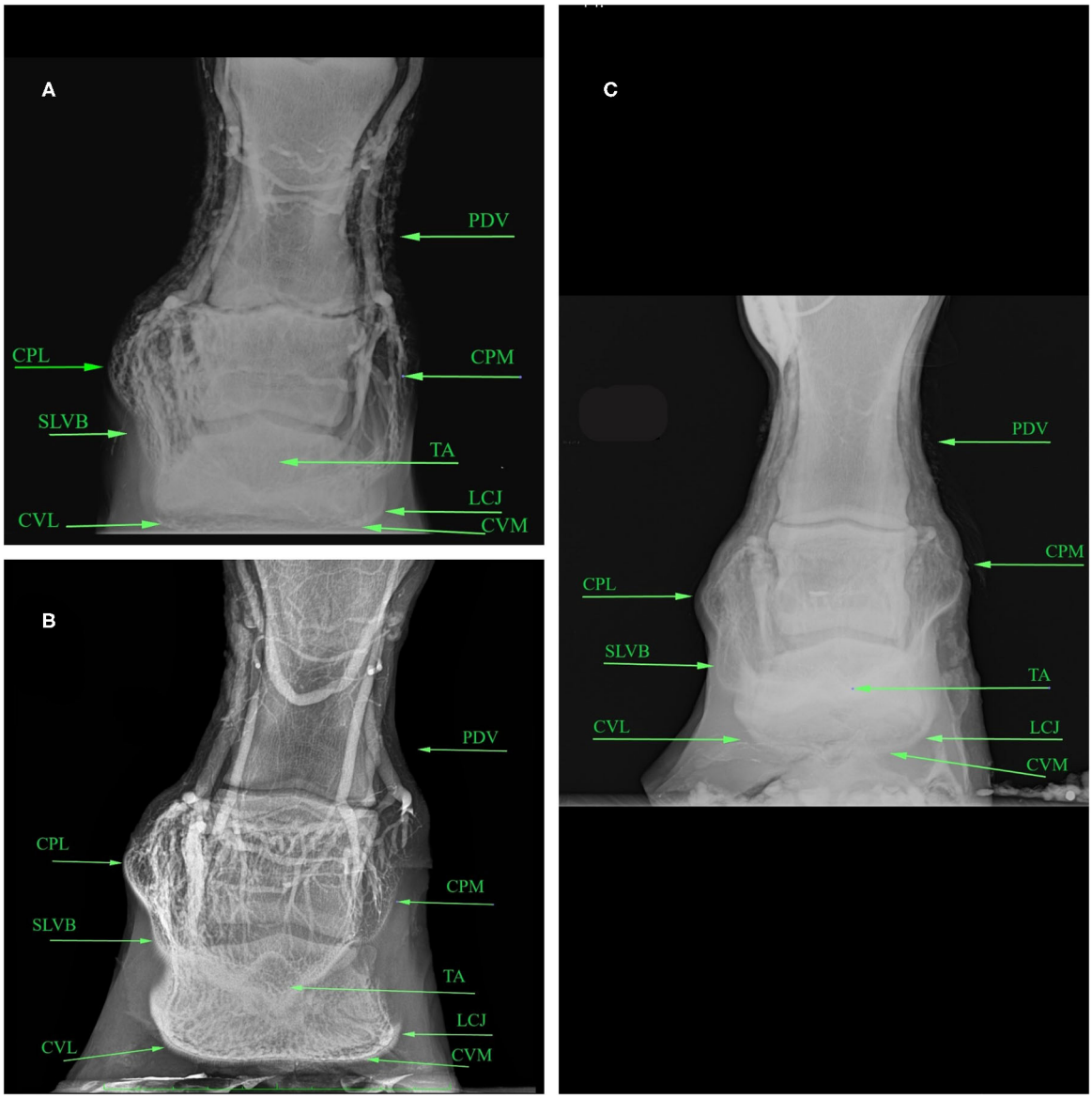


FIGURE 3 | Dorso-palmar venograms of healthy **(A)**, mild **(B)**, laminitic **(C)** donkey feet. PDV, Palmar Digital Vein; CPL, Coronary Plexus Lateral; CPM, Coronary Plexus Medial; TA, Terminal Arch; SLVB, Sublamellar Vascular Bed; LCJ, Lamellar-Circumflex Junction; CVL, Circumflex Vessels Lateral; CVM, Circumflex Vessels Medial.

ETHICS STATEMENT

The animal study was reviewed and approved by the Ethical Committee of the University of Pisa, according to the D. Lgs. 26/14 (Number 23/19). Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

MS and SC conceived, designed, and supervised the project. IN, BA, and LB executed the experiment. IN, BA, and LG-C analyzed the data. MS executed the formal analysis. IN and MS had full access to all the data in the study and take responsibility for the integrity of the data, and the accuracy of the data analysis. All the authors interpreted the data, wrote and critically revised the manuscript for intellectual content, and approved the final version. All authors contributed to the article and approved the submitted version.

ACKNOWLEDGMENTS

We are grateful to the Regional stud farm Ente Terre Regionali Toscane (Tuscany, Italy) for allowing us to use the animals for this study. The authors thank Giuseppe Conte for statistical support.

REFERENCES

1. Patterson-Kane JC, Karikoski NP, McGowan CM. Paradigm shifts in understanding equine laminitis. *Vet J*. (2018) 231:33–40. doi: 10.1016/j.tvjl.2017.11.011
2. Obel N. *Studies on the Histopathology of Acute Laminitis*. Uppsala: Almqvist and Wiksells (1948).
3. Mitchell B. The importance of dietary management in laminitis. *Equine Health*. (2015) 22:10–5. doi: 10.12968/eqhe.2015.22.10
4. Collins SN, Dyson SJ, Murray RC, Burden F, Trawford A. Radiological anatomy of the donkey's foot: Objective characterisation of the normal and laminitic donkey foot. *Equine Vet J*. (2011) 43:478–86. doi: 10.1111/j.2042-3306.2010.00312.x
5. Kameya T. Clinical studies on laminitis in the racehorse. *Exp Rept Eq Health Labor*. (1973) 10:19–40.
6. Stick JA, Jann HW, Scott EA, Robinson NE. Pedal bone rotation as a prognostic sign in laminitis of horses. *J Am Vet Med A*. (1982) 180:251–3.
7. Cripps PJ, Eustace RA. Radiological measurements from the feet of normal horses with relevance to laminitis. *Equine Vet J*. (1999) 31:427–32. doi: 10.1111/j.2042-3306.1999.tb03844.x
8. Tachio G, Davies HMS, Morgate M, Bernardini D. A radiographic technique to assess longitudinal balance in front hooves. *Equine Vet J Suppl*. (2002) 34:368–72. doi: 10.1111/j.2042-3306.2002.tb05450.x
9. Floyd AE. Environmental management of the severe laminitic horse. In: Floyd AE, Mansmann RA, editors. *Equine Podiatry*. St Louis, MO: Saunders Elsevier (2007). p. 359–69. doi: 10.1016/B978-072160383-4.50023-2
10. D'Arpe L, Bernardini D. Digital venography in horses and its clinical application in Europe. *Vet Clin North Am Equine*. (2010) 26:339–59. doi: 10.1016/j.cveq.2010.06.006
11. Hunt RJ, Wharton RE. Clinical presentation, diagnosis, and prognosis of chronic laminitis in North America. *Vet Clin North Am Equine*. (2010) 26:141–53. doi: 10.1016/j.cveq.2009.12.006
12. Eastman S, Redden RF, Williams CA. Venograms for use in laminitis treatment. *J Equine Vet Sci*. (2012) 32:757–9. doi: 10.1016/j.jevs.2012.02.020
13. Alves FR, Guerra PC, Fioretto ET, Júnior AANM, Machado FCF, Aranha RMC, et al. Angiographic aspect of the distal forelimb in donkeys (*Equus asinus*) used for animal traction. *Biotemas*. (2009) 22:163–7. doi: 10.5007/2175-7925.2009v22n4p163
14. El-Shafaey EA, Salem MG, Mosbah E, Zaghloul AE. Morphometric evaluation of relevant radiographic parameters of the forefeet of clinically normal donkeys (Equus αsinus). *J Hell Vet Med Soc*. (2017) 68:467–78. doi: 10.12681/jhvms.15543
15. Nocera I, Aliboni B, Puccinelli C, Pietrini G, Sgorbini M, Citi S, et al. Radiographic parameters of the digit in a cohort population of Amiata donkeys. *Open Vet J*. (2020) 10:354–62. Available online at: https://www.bibliomed.org/?mno=126378
16. Burden F, Thiemann A. Donkeys are different. *J Equine Vet Sci*. (2015) 3:376–82. doi: 10.1016/j.jevs.2015.03.005
17. Said AH, Khamis Y, Mahfouz MF, El-Keiey MT. Angiographic appearance of the metacarpus, phalanges and foot of the donkey. *Zbl Vet Med A*. (1983) 30:788–95. doi: 10.1111/j.1439-0442.1983.tb01905.x
18. Salem M, El-Shafaey ES, Mosbah E, Ei A. Plain and contrast radiographic evaluation of the digit in donkeys (*Equus asinus*). *Mans Vet Med J*. (2016) 18:51–65. Available online at: https://journals.ekb.eg/article_126856_0.html
19. National Research Council. *The Nutrient Requirements of Horses*. 6th ed. Washington, DC: National Academy Press (2007).
20. Herthel D, Hood DM. Clinical presentation, diagnosis, and prognosis of chronic laminitis. *Vet Clin North Am Equine*. (1999) 15:375–94. doi: 10.1016/S0749-0739(17)30151-7
21. Van Eps A. General clinical aspects of the laminitis case. In: Belknap JK, Geor RJ, editors. *Equine Laminitis*. 1st ed. Hoboken, NJ: John Wiley and Sons Inc. (2017). p. 183–90. doi: 10.1002/9781119169239.ch21
22. Evangelista F, Tayari H, Degani M, Nocera I, Briganti A. Sedative and respiratory effects of intramuscular detomidine and butorphanol in donkeys sedated for bronchoalveolar lavage. *J Equine Vet Sci*. (2018) 69:96–101. doi: 10.1016/j.jevs.2018.06.011
23. Parks AH, Belknap JK. Diagnostic imaging. In: Belknap JK, Geor RJ, editors. *Equine Laminitis*. 1st ed. Hoboken, NJ: John Wiley and Sons Inc. (2017). p. 226–39.
24. Rucker A. The digital venogram. In: Belknap JK, Geor RJ, editors. *Equine Laminitis*. 1st ed. Hoboken, NJ: John Wiley and Sons Inc. (2017). p. 240–51.
25. Linford RL, O'Brien TR, Trout DR. Qualitative and morphometric radiographic findings in the distal phalanx and digital soft tissues of sound Thoroughbred racehorses. *Am J Vet Res*. (1993) 54:38–51.
26. Butler JA, Colles CM, Dyson SJ, Kold SE, Poulos PW, Puchalski S. The foot. In: Butler JA, Colles CM, Dyson SJ, Kold SE, Poulos PW, Puchalski S, editors. *Clinical Radiology of the Horse*. 4th ed. The Atrium, Southern Gate, Chichester, West Sussex: John Wiley and Sons. (2017). p. 55–148.
27. Goulet C, Olive J, Rossier Y, Beauchamp G. Radiographic and anatomic characteristics of dorsal layers in non laminitic horses. *Vet Radiol Ultrasound*. (2015) 56:589–94. doi: 10.1111/vru.12280

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 April 2021
doi: 10.3389/fvets.2021.645627

Nasopharyngeal Microbiomes in Donkeys Shedding *Streptococcus equi* Subspecies *equi* in Comparison to Healthy Donkeys

Yiping Zhu[1†], *Shulei Chen*[1†], *Ziwen Yi*[1], *Reed Holyoak*[2], *Tao Wang*[3], *Zhaoliang Ding*[3] *and Jing Li*[1*]

[1] *Equine Clinical Diagnostic Center, College of Veterinary Medicine, China Agricultural University, Beijing, China,* [2] *College of Veterinary Medicine, Oklahoma State University, Stillwater, OK, United States,* [3] *Dong-E-E-Jiao Co., Ltd., Dong-E County, China*

Streptococcus equi subsp. equi (*S. equi*) is the pathogen causing strangles, a highly infectious disease that can affect equids including donkeys of all ages. It can persistently colonize the upper respiratory tract of animals asymptomatically for years, which serves as a source of infection. Several strangles outbreaks have been reported in the donkey industry in China in the last few years and pose a great threat to health, production, and the welfare of donkeys. Nasopharyngeal swab samples for culture and PCR are used widely in strangles diagnosis. Additionally, microbiomes within and on the body are essential to host homoeostasis and health. Therefore, the microbiome of the equid nasopharynx may provide insights into the health of the upper respiratory tract in animals. There has been no study investigating the nasopharyngeal microbiome in healthy donkeys, nor in donkeys shedding *S. equi*. This study aimed to compare nasopharyngeal microbiomes in healthy and carrier donkeys using 16S rRNA gene sequencing. Nasopharyngeal samples were obtained from 16 donkeys recovered from strangles (group S) and 14 healthy donkeys with no history of strangles exposure (group H). Of those sampled, 7 donkeys were determined to be carriers with positive PCR and culture results in group S. In group H, all 14 donkeys were considered free of strangles based on the history of negative exposure, negative results of PCR and culture. Samples from these 21 donkeys were used for microbial analysis. The nasopharyngeal microbiome composition was compared between the two groups. At the phylum level, relative abundance of Proteobacteria was predominantly higher in the *S. equi* carrier donkeys than in healthy donkeys ($P < 0.01$), while Firmicutes and Actinobacteria were significantly less abundant in the *S. equi* carrier donkeys than in healthy donkeys ($P < 0.05$). At the genus level, *Nicoletella* was detected in the upper respiratory tract of donkeys for the first time and dominated in carrier donkeys. It is suspected to suppress other normal flora of URT microbiota including *Streptococcus* spp., *Staphylococcus* spp., and *Corynebacterium* spp. We concluded that the nasopharyngeal microbiome in *S. equi* carrier donkeys still exhibited microbial dysbiosis, which might predispose them to other airway diseases.

Keywords: carrier, donkeys, nasopharyngeal microbiomes, *Streptococcus equi*, 16S rRNA

OPEN ACCESS

Edited by:
Fulvio Laus,
University of Camerino, Italy

Reviewed by:
Valentina Stefanetti,
University of Perugia, Italy
Karen Frances Steward,
Animal Health Trust, United Kingdom

***Correspondence:**
Jing Li
jlivet@cau.edu.cn

[†]These authors have contributed equally to this work

Specialty section:
This article was submitted to Comparative and Clinical Medicine, a section of the journal Frontiers in Veterinary Science

Received: *23 December 2020*
Accepted: *29 March 2021*
Published: *22 April 2021*

Citation:
Zhu Y, Chen S, Yi Z, Holyoak R, Wang T, Ding Z and Li J (2021) Nasopharyngeal Microbiomes in Donkeys Shedding Streptococcus equi Subspecies equi in Comparison to Healthy Donkeys. Front. Vet. Sci. 8:645627. doi: 10.3389/fvets.2021.645627

INTRODUCTION

Streptococcus equi subspecies *equi* (*S. equi*), one of the common upper respiratory tract (URT) pathogens in equids, has been causing the infection referred to as strangles, which still remains an important disease worldwide (1–3). Strangles is highly contagious and pathogenic, resulting in abrupt onset of fever, mucopurulent nasal discharges, coughing and abscesses in submandibular and retropharyngeal lymph nodes (4–6). In cases with severe complications, animals are significantly compromised due to pharynx obstruction, metastatic abscessation (bastard strangles) as well as purpura hemorrhagica (7–9). In uncomplicated cases of high morbidity and low mortality, strangles usually lasts about 25–35 days in most animals (7, 10). However, infection may be extended to chronic or endemic status with constant or intermitted shedding of *S. equi* (7, 11). In most animals, nasal shedding of *S. equi* begins the first few days after onset of pyrexia and persists for 2–3 weeks. However, some animals may keep periodically shedding for a much longer time due to a persistent infection in the guttural pouch (12).

In the last few years, there have been outbreaks of strangles reported in donkeys in China (13, 14). Donkeys <1 year of age were found having much higher morbidity (40.3%) and mortality rates compared to older age groups (13). As the donkey industry has expanded tremendously in China, strangles has emerged as a disease of concern, especially when donkeys are raised at a relatively high stocking density. Additionally, shedding of *S. equi* after the resolution of clinical signs is a potential threat to the industry.

The URT hosts a variety of microorganisms which form a complex microbial community with synergistic and competitive interactions (15). The harmonious coexistence of microbiota is necessary to maintain the health of the URT of animals (16). Dysbiosis of these microbial populations is associated with disruption of respiratory tract health (17) and increased risk of respiratory tract infection (18). The nasopharynx, as part of the URT, frequently harbors both commensal and pathogenic bacteria (19). Investigation of the relationship between nasopharyngeal microbes impacted by respiratory tract diseases is anticipated to increase understanding of the pathogenesis of URT infections (19). Changes of the nasal microbial population before and after long distance transportation in clinically healthy donkeys has recently been reported (17). However, samples obtained using non-guarded swabs might be exposed to contaminants from different parts of the URT including sinuses, nostrils, and oropharynx, each colonized with its own system of microorganisms (20). Therefore, sampling from the deeper nasopharyngeal area may lead to different results.

To our knowledge, there has been no study focusing on the population of donkeys asymptomatically shedding *S. equi*. In this study, we would like to investigate whether donkeys shedding *S. equi* have a perturbed microbial environment in the nasopharynx, which may create a predisposition to other respiratory tract infections (18). Based on this objective, we identified *S. equi* carrier donkeys without signs of clinical disease from a donkey farm that just experienced a strangles outbreak using both culture and PCR techniques. Then nasopharyngeal samples were obtained and the nasopharynx microbiomes in *S. equi* carrier donkeys and healthy donkeys were detected and compared using a 16S rRNA high-throughput sequencing technique. After sequencing, we assessed the differences within the microbial communities between the two groups that were associated with asymptomatic *S. equi* infection.

MATERIALS AND METHODS

Ethics Statement

All procedures involving animals were conducted in compliance and within the license (No.AW11101202-2-0) granted by the Animal Welfare and Ethics Committee of China Agriculture University.

Animals and Sample Collection

The study was conducted in an intensive donkey farm in Shandong Province with about 800 Dezhou donkeys in total. The recently weaned 6-month-old donkeys were arranged in 24 barns aligned in eight rows with three barns per row. The donkeys were provided with hay and commercial concentrates daily with free choice of water. The farm experienced a strangles outbreak 3 months earlier. It was also the first outbreak known to have occurred in that area. As the commercial strangles vaccine was not available in China, the donkey population on the farm had not been vaccinated against *S. equi* before or after the outbreak. Donkeys affected by strangles was clinically healthy previously according to the health records of the farm. Donkeys which had displayed positive clinical signs were first noted in one barn and quickly isolated. All movement of those donkeys was stopped and a quarantine zone was established to minimize the spread of infection. Their manure and waste feed were also composted separately. A specific group of staff members had been and continued to be assigned to take care of these donkeys using a separate set of equipment, which were disinfected daily. Donkeys in surrounding barns were also limited in movement and monitored closely. Throughout the outbreak, three barns in the same row were affected, while donkeys in other barns remained clinically healthy. Only individuals with severe lymphadenopathy were treated with 3% penicillin gel topically, while other isolated donkeys were monitored three times a day and provided with food and water *ad libitum a*ccording to the local veterinarian's instructions. None of the donkeys involved in this study had been treated with antibiotics throughout the outbreak prior to sampling. The brief signalment and history of the animals involved in this study were summarized in **Supplementary Tables 1**, **2**.

A group of 16 male donkeys of ∼6 months of age were randomly selected from the group that had clinically recovered from strangles. Also, none had current clinical signs of disease. These donkeys were designated as group S. Nasopharyngeal swabs were collected using the technique described by Holman (21). Two nasopharynx samples were collected from each donkey. Before sampling, the nostril that was to be used

for sampling was wiped clean with 70% ethanol. A 70 cm double-guarded sterile uterine swab (IMV Technologies, L'Aigle, France) was inserted into the nasal cavity passing through ventral meatus gently to approximate depth of 15 cm (22, 23). The guarded inner swab was then extended from the casing, rotated 360° 2–3 times, maintaining contact for about 20 s and then drawn back into the guarded tube. This sample was then stored in a sterile empty vacutainer tube with two to three drops of saline and kept on ice for no more than 6 h. Upon arrival at the laboratory it was used for aerobic culture and PCR. A second sample from the same nostril was obtained using the exact same technique. This swab sample was stored in −80°C until potential 16S rRNA gene sequencing.

A group of 14 male donkeys of approximately 6 months of age was also randomly selected from a different barn located at the furthest row about 200 m away in the same farm, housing donkeys with no history of being exposed and exhibited no clinical signs of *S. equi* infection during the outbreak. These donkeys were still clinically healthy based on a physical exam and were designated as group H. Two nasopharyngeal swab samples were collected with the exact same technique from each donkey, then stored and processed in the same way described above for group S, with the first swab being submitted for aerobic culture and PCR, and the second swab stored for potential 16S rRNA gene sequencing.

Streptococcus equi Aerobic Culture, Biochemical Identification, and PCR

S. equi swabs submitted from group S for culture of *S. equi* were processed as previously described (24). Namely, Colombia blood agar (Huankai Microbial, Guangzhou, China) was used to streak the collected swabs for bacterial culture (17). Cultures were incubated at 37°C for 24 h. Isolates were sub-cultured two times on blood agar before being identified. Colonies identified via beta-hemolysis and colony appearance were subsequently tested by Gram staining. Biochemical tests, including ferment lactose, sorbitol and trehalose, were conducted using Micro-Biochemical Identification Tube (Hopebio, Qingdao, China).

DNA from each swab sample was extracted using a Hi-Swab DNA kit (Tiangen, Beijing, China) according to manufacturer's instructions. PCR was performed using primer set of ICESE2GC2F (5′-TTACCTCCATTACTTGACAATCCAT-3′) and ICESE2GC2R (5′-GATTTGCAACATGAAACATTTACAG-3′) (25), which was specific for *S. equi*. Successful amplification was confirmed by 1% agarose gel electrophoresis of the PCR products. Samples with both positive culture and PCR results were selected for further 16S rRNA gene sequencing analysis.

Nasopharyngeal swab samples from group H were tested by aerobic culture and PCR in the same way as described above. Individuals with both negative culture and PCR results were selected for microbial composition analysis with 16S rRNA gene sequencing technique.

DNA Preparation for 16S rRNA Gene Sequencing

Samples of selected individuals from each group stored at −80°C were thawed for DNA extraction using E.Z.N.A soil DNA Kit (Omega Bio-tek, Norcross, GA, U.S.) in accordance with manufacturer's instructions. Positive DNA amplification was verified by 1% agarose gel electrophoresis. DNA concentration was determined with NanoDrop 2000 UV-vis spectrophotometer (Thermo Scientific, Wilmington, USA).

High-Throughput Sequencing

The V3–V4 hypervariable regions (26) were amplified by PCR using primer set of 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′). The 16S rRNA gene amplification of each sample was performed in a 20 μL reaction system. Reaction conditions were set as follows: initial denaturation at 95°C for 3 min, followed by 27 thermal cycles of denaturing at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 45 s. The whole process then ended with a single extension at 72°C for 10 min. 2% agarose gel was used to reveal the PCR products. Extraction and purification of these PCR products were performed using AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA). Amplicons were quantified by Quanti-Fluor^{TM}-ST fluorometer (Promega, Madison, WI, USA). Amplicon library pools were gel-sized ahead of sequencing paired-end on Illumina MiSeq platform (Illumina, San Diego, CA, USA).

Bioinformatics Analysis

FASTQ (v0.20.0) (27) was used to quality-filter the raw 16s rRNA gene sequencing. Sequencing reads were merged using FLASH (v1.2.7). USEARCH (v7.0) was applied to process and cluster sequences for operational taxonomic units (OTU) analysis. OTUs derived from clustering 16S rRNA gene rDNA sequences were used as approximations of microbial taxa (28). The most abundant sequence of each OTU was chosen by QIIME (30) (v1.9.1). RDP Classifier (v2.2) was used to assign a taxonomy with a minimum threshold of 0.7 and the representative sequence of OTU with 97% similarity (29).

Statistics Analysis

Alpha diversity analysis including Shannon and Chao indexes was carried out using Mothur (v1.30.1) to analyze evenness and richness of OTUs, respectively. Mann–Whitney test was performed to illustrate the diversity difference at different levels between the two groups in this study. The cut-off for rejecting the null hypothesis was set at $P < 0.05$, which indicated no difference between two groups. The OTUs of different levels in each group were assessed by community barplot. Principal coordinate analysis (PCoA) as part of beta diversity was conducted using QIIME (30) (v1.9.1) to reflect the difference and distance between groups based on the Bray–Curtis algorithms. Liner discriminant analysis (LDA) effect size (LEfSe) with an LDA threshold score of >3 was used to determine which taxa and OTUs were most associated with each group and contributed to the differences.

RESULTS

Aerobic Culture and PCR Results

Seven donkeys from group S were positive for culture, biochemical identification (non-fermented lactose, sorbitol and trehalose) and PCR. Therefore, they were identified as asymptomatic carriers of *S. equi* and selected for 16S rRNA high-throughput sequencing. In group H, all donkeys remained clinically healthy and yielded negative results on both aerobic culture and PCR. Subsequently, all frozen samples from this group were selected for further microbial composition analysis.

Microbiota Overview

Nasopharyngeal swabs taken from healthy donkeys and donkeys shedding *S. equi* were assessed by sequencing the bacterial 16S rRNA V3–V4 region. An average of 468 base pairs were obtained from the PCR products. The average DNA concentration yield was 198 ng/μL in group S and 146 ng/μL in group H. A total of 1,385,649 sequence reads were obtained from 21 samples. After removing low-quality reads, singletons, and triplicates, 401,877 sequences were retained (97% sequence similarity). A total of 2,291 OTUs were identified and classified into 36 phyla, 99 classes, 226 orders, 390 families, 835 genera, and 1,326 species. 2,142 and 809 OTUs have been detected in group H and group S, respectively. Raw sequences reads from the samples were deposited at NCBI Sequence Read Archive (SRA) database (accession number PRJNA695404).

Alpha Diversity Analysis

Five phyla with relative abundance of >1% were identified in the nasopharyngeal microbiota of the donkeys from group H. The microbial communities of healthy donkeys were dominated by Proteobacteria (42.82%), Firmicutes (41.71%), Actinobacteria (11.32%), Chloroflexi (1.41%), and Bacteroidetes (1.38%) (**Figure 1A**). There were four phyla with relative abundance of >1% in the communities of *S. equi* carrier (group S) donkeys and they were Proteobacteria (75.01%), Firmicutes (16.32%), Actinobacteria (4.70%), and Bacteroidetes (3.46%) (**Figure 1A**). In group S donkeys, Proteobacteria were significantly more abundant ($P < 0.01$) than in group H donkeys, while the abundance of Firmicutes and Actinobacteria were notably lower ($P < 0.05$) in group S as compared to group H. At the genus level, 12 genera had a relative abundance of >1% with *Streptococcus* (28.93%) and unclassified *Moraxellaceae* (13.85%) being the most abundant in healthy donkeys. Whereas in group S, 10 genera had a relative abundance of >1% characterized by *Nicoletella* (37.70%) and *Moraxella* (21.48%) (**Figure 1B**). The relative abundance of *Nicoletella* and *Moraxella* in group S were notably higher ($P < 0.01$) than in group H. While *Streptococcus*, *Staphylococcus*, and *Corynebacterium* were much lower in abundance ($P < 0.01$) than in group H. There were also a substantial amount of OTUs of unclassified *Moraxellaceae* inhabiting healthy donkeys vs. carrier donkeys.

The richness (Chao) and diversity (Shannon) of the partitions were calculated. Group S had a microbial composition significantly lower in richness than group H as illustrated by the Chao index (**Figure 2A**; $P = 0.004$). The Shannon indexes did not differ significantly between the healthy and carrier donkeys (**Figure 2B**; $P = 0.057$).

Beta Diversity Analysis

Principal coordinate analysis (PCoA) based on Bray-Curtis algorithms between the two groups was performed and showed differences in microbial communities of the nasopharynx between healthy and carrier donkeys (**Figure 3**). The ANOSIM of Bray-Curtis distances revealed significant differences of the microbiota composition between group H and group S donkeys ($R = 0.531$, $P = 0.002$).

The linear discriminant analysis (LDA) effect size (LEfSe) method was applied to identify high-dimensional biomarkers and assess the differences between the two groups at the genus or higher taxonomic level. The LDA was set to 4.0 as a threshold for identification of biomarkers of different taxa in LEfSe analyses. From all 2,291 OTUs, 257 OTUs were significantly different between the healthy and carrier donkeys ($P < 0.05$). From 835 genera or taxa at higher levels, 379 taxa were significantly different between the two groups ($P < 0.05$); Among them, 70 taxa had LDA scores above 3. Sixteen taxa were associated with group S donkeys and the rest of the taxa (54) were associated with healthy donkeys. The most discriminating taxa in the samples of group S donkeys were Pasteurellales, *Pasteurellaceae*, and *Nicoletella*. *Nicoletella* belongs to the order Pasteurellales and the family *Pasteurellaceae*. In group H donkeys, the most associated taxa were Firmicutes, Bacilli and unclassified *Moraxellaceae* (**Figure 4**). *Bacilli* is at the class level belonging to the phylum Firmicutes, while *Moraxellaceae* is at the family level belonging to the phylum Proteobacteria.

DISCUSSION

The previous "gold standard" for detection of *S. equi* was aerobic culture of samples from the upper respiratory tract, including nasal washes, nasal/nasopharyngeal swabs, nasopharyngeal washes or purulent material aspirated from abscesses (31). However, these samples may result in false negatives, especially in the early febrile state. Hence, culture of these samples is no longer considered as the gold standard for strangles diagnosis, even though they are still commonly used (6, 7, 32). Animals recovered from strangles can be asymptomatic carriers and intermittently shed the pathogen (33, 34). They can be a source of infection leading to new or recurrent diseases even in well-managed groups of animals (1) and are also challenging for accurate diagnosis due to the periodical shedding. There is evidence that PCR is more specific and sensitive for detecting *S. equi* than culture (24, 32). Quantitative PCR (qPCR) also referred as real-time PCR has been developed for more sensitive and rapid diagnosis of strangles (35, 36) and guttural pouch lavage qPCR is recommended to detect carriers (6). However, the PCR technique is not able to differentiate viable from non-viable organisms, and it is currently not widely available in China. As a result, a combination of culture and regular PCR was applied to achieve a more reliable diagnosis for carrier donkeys in this study. Since *S. equi* may periodically shed from the guttural pouch into the nasopharynx, rostral nasal

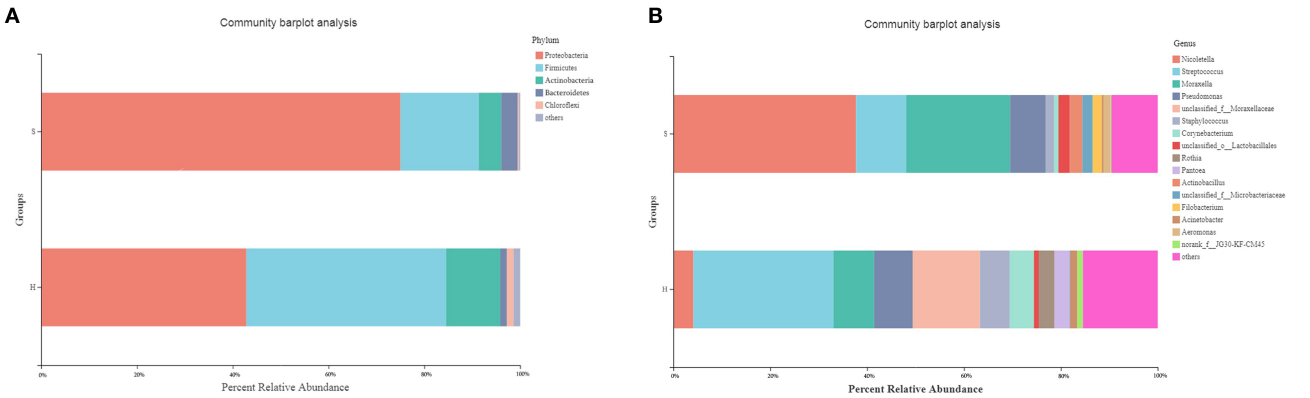


FIGURE 1 | Relative abundance of predominant (>1%) phyla **(A)** and genera **(B)** in the nasopharyngeal microbiota of healthy donkeys and donkeys shedding *S. equi*. H: Healthy donkeys ($n = 14$); S, donkeys shedding *S. equi* ($n = 7$). Other: bacterial taxa with ≤1% abundance, Unclassified, sequences which could not be classified.

swabs may present false negative results (6). However, compared to guttural pouch lavage, swab samples are easier to obtain without the requirement of endoscopic equipment. Hence, we chose to sample the nasopharyngeal area with double-guarded uterine swabs. In this study, 7 out of 16 donkeys from group S were positive on culture and PCR tests. Therefore, they were determined to be asymptomatic carriers. The remaining nine donkeys in group S were not double positive on these tests, therefore, their carrier state was not confirmed at that point and they were not included in this study. Guttural pouch lavage PCR with visual inspection of the guttural pouch guided by endoscopy would be necessary to detect other *S. equi* carriers in this group. The "carriers" defined in the study, along with the other donkeys in the same barn, continued to be isolated, waiting for the future inspection of the local veterinary officials. As for group H, these donkeys had no exposure to *S. equi* and a history free of clinical signs during the outbreak. In addition, they were all under strict biosecurity and negative on both culture and PCR. Thus, they were considered not infected with *S. equi* at that point and were used as negative controls of group S. Ideally, two blood samples for serology test with 2 weeks apart are recommended to help confirm their negative infection status (6).

We hypothesized that asymptomatic carriers of *S. equi* would have dysbiosis of the microbial community of their upper respiratory tract despite absence of clinical signs of disease. The 16S rRNA sequencing results revealed differences between the two study groups and were able to detect microbial changes within carrier donkeys compared to those that remained healthy. Nasopharyngeal samples have been used as diagnostic samples of *S. equi* (32) and representative samples of upper respiratory tract microbiomes (37). To our knowledge the current study is the first to compare the nasopharyngeal microbiota of healthy donkeys with donkeys shedding *S. equi* using high-throughput 16S rRNA gene sequencing.

In this study, the nasopharynx in healthy donkeys hosted richer microbiomes than donkeys shedding *S. equi*. A healthy microbiome is usually characterized by a diverse community of organisms, which are more stable and more resistant to

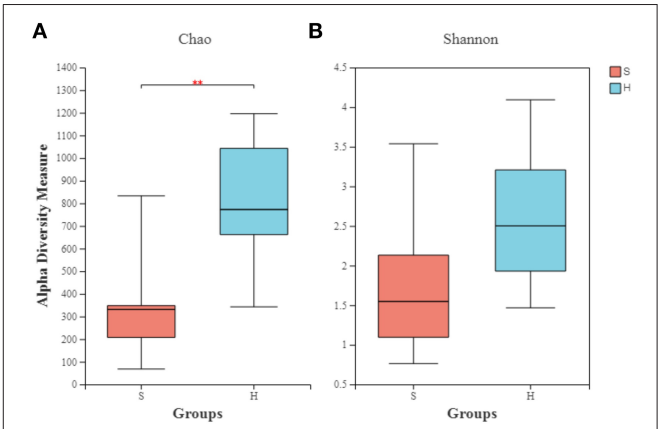


FIGURE 2 | Alpha diversity indexes of the nasopharyngeal microbiota of healthy and carrier donkeys. **(A)** Shannon index of OTU level. **(B)** Chao index of OTU level. S, donkeys shedding *S. equi* ($n = 7$) in orange. H, Healthy donkeys ($n = 14$) in light blue; **significant decrease ($P = 0.004$, Mann–Whitney test) in the richness of the bacterial communities in donkeys shedding *S. euqi* (S) compared to healthy donkeys (H).

overgrowth of pathogenic microbiomes. Whereas a dysbiotic community, associated with chronic disease, usually has a markedly lower diversity and is often dominated by a few pathogenic species (38). There is also strong evidence that dysbiotic microbial communities are more susceptible to inflammation and diseases (39). The significantly decreased diversity of microbial composition and number of OTUs in group S were consistent with dysbiotic communities within the URT. Similar results were also noted in published research of other species. For example, cattle with bovine respiratory disease complex (BRDC) tend to not only have a less rich source of bacteria, but also lower numbers of OTUs in the nasopharynx, as compared to healthy cattle (21). In humans it has been reported that chronic inflammatory diseases have been associated with a decrease in microbial diversity, evenness, as well as richness (20, 40, 41). This change in microbial richness under circumstances characterized with infection or inflammation was probably due

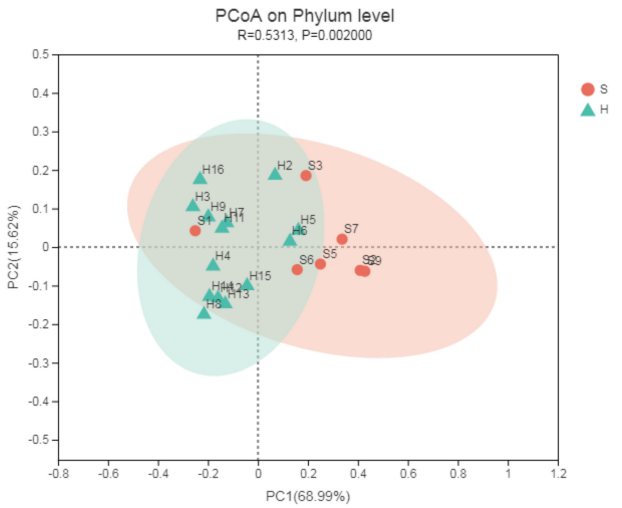


FIGURE 3 | Principal coordinate analysis (PCoA) of the nasal microbiota of healthy and carrier donkeys based on Bray-Curtis distance. S, donkeys shedding *S. equi* ($n = 7$) in orange; H, Healthy donkeys ($n = 14$) in green.

to an increased colonization of anaerobic bacteria, which was facilitated by biofilm formation (42, 43).

According to the microbial composition analysis, the dominant phyla were Proteobacteria, Firmicutes, Actinobacteria and Bacteroidetes in both groups. The results of healthy donkeys in this study are comparable with Zhao's study conducted in clinically healthy donkeys with non-guarded nasal swabs (17). Similar results have also been mentioned in studies of healthy URT microbiomes in horses (37), cattle (21), humans (44), as well as in swine (45). Proteobacteria occupied a notably larger portion of the nasopharyngeal microbiome in donkeys shedding *S. equi* than in healthy donkeys. There is evidence that Proteobacteria is associated with increased severity of inflammation and respiratory tract disease (46). *Moraxella* and *Nicoletella*, the two bacterial genera belonging to Proteobacteria phylum, were predominant in *S. equi* carrier donkeys. *Moraxella spp*. have been considered as pro-inflammatory bacteria associated with asthma in humans and horses (37, 47). It has also been found having a dramatically high relative abundance in cattle with BRDC vs. healthy cattle, which could potentially cause disorders such as pneumonia and otitis (48). In our study, the abundance of *Moraxella* spp. increased significantly in carrier donkeys as opposed to donkeys free of strangles. This is consistent with previous findings in respiratory diseases in other species. Even though donkeys shedding *S. equi* are usually asymptomatic, they are still likely to undergo inflammatory processes in the upper respiratory tract especially guttural pouches (49). In consequence, donkeys shedding S. equi were still having microbial perturbation consistent with the shifted predominance of *Moraxella* in this study.

In donkeys, *Nicoletella spp*. were first identified and reported in our study with a predominant presence in carrier donkeys shedding *S. equi*. *Nicoletella* spp. are rarely reported in other species, except for horses. *Nicoletella semolina* is the species repeatedly isolated from horses with airway disease and first reported in 2004 (50). As a new member in the *Pasteurellaceae* family, it is usually present as part of the normal flora in the equine airway. It has been isolated from URT of healthy horses and horses with airway disease in similar proportions in Europe, however, marked growth of *N. semolina* was observed in tracheal aspirate cultures from horses with respiratory disorders (51). Pulmonary disease potentially associated with *N. semolina* infection has been reported in three young horses in North America (52). All three cases were characterized with chronic airway infection and heavy growth of *N. semolina* was observed in transtracheal wash cultures along with other equine airway flora. The results indicated that *N. semolina* might overgrow and cause airway disease in a dysbiotic community. In 2020, *Nicoletella semolina* was first reported as a sole isolate in a horse with a pulmonary infection in New Zealand. Nonetheless, whether it acted as a primary or opportunistic pathogen was still not determined (53). According to results of LEfSe herein, *Nicoletella* was also the genus most often associated with *S. equi* carrier donkeys, while it was not discriminative in the group of healthy donkeys. Therefore, the overgrowth of *Nicoletella spp*. in carrier donkeys might be associated with URT dysbiosis and possible chronic airway inflammation caused by *S. equi*. Further investigation involving more samples will be required to facilitate the understanding of its role in URT diseases in donkeys.

Streptococcus belonging to the Firmicutes phylum was another genus showing a significant difference between the two groups at the genus level. Its relative abundance was notably lower in group S donkeys compared to healthy donkeys. Since the classifier used in this study was the RDP Classifier, which is only able to classify 16S rRNA genes from phylum to genus (54), the species of *Streptococcus* were not determined definitively. Our hypothesis to explain this finding was that the predominant *Streptococcus spp*. in healthy donkeys could be *S. equi* subsp. *zooepidimicus*, a common flora in the URT, while in carrier donkeys, the presence of *Nicoletella semolina* might inhibit the normal growth of *S. equi* subsp. *zooepidimicus* and contribute to microbial dysbiosis. Besides *Streptococcus* spp., *Staphylococcus* spp., and *Corynebacterium* spp., which are considered part of microbiota of healthy equine airways (55), also demonstrated significantly lower abundance in Group S. Herein, we inferred that suppression from *Nicoletella semolina* might also lead to this finding.

LEfSe analysis was conducted to identify the most discriminating bacterial taxa of each group. Pasteurellales, *Pasteurellaceae* and *Nicoletella* were the top three taxa associated with donkeys shedding *S. equi*. Thereinto *Nicoletella* belongs to the order Pasteurellales and the family *Pasteurellaceae*. As these bacterial groups were shown at multiple taxonomic levels, the association between these bacterial taxa and donkeys shedding *S. equi* was more robust. Based on LDA, majority of taxa with an LDA score of >3 was associated with healthy donkeys. Hence, there seemed to be no single bacterial taxon strongly associated with healthy donkeys, which reflects a more balanced microbial composition.

Even though strangles has been known to produce chronic shedding individuals, the long-term impact of *S. equi* on the URT from the point of view of microbiomes was still unclear. This study demonstrated a characteristic nasopharyngeal microbiota

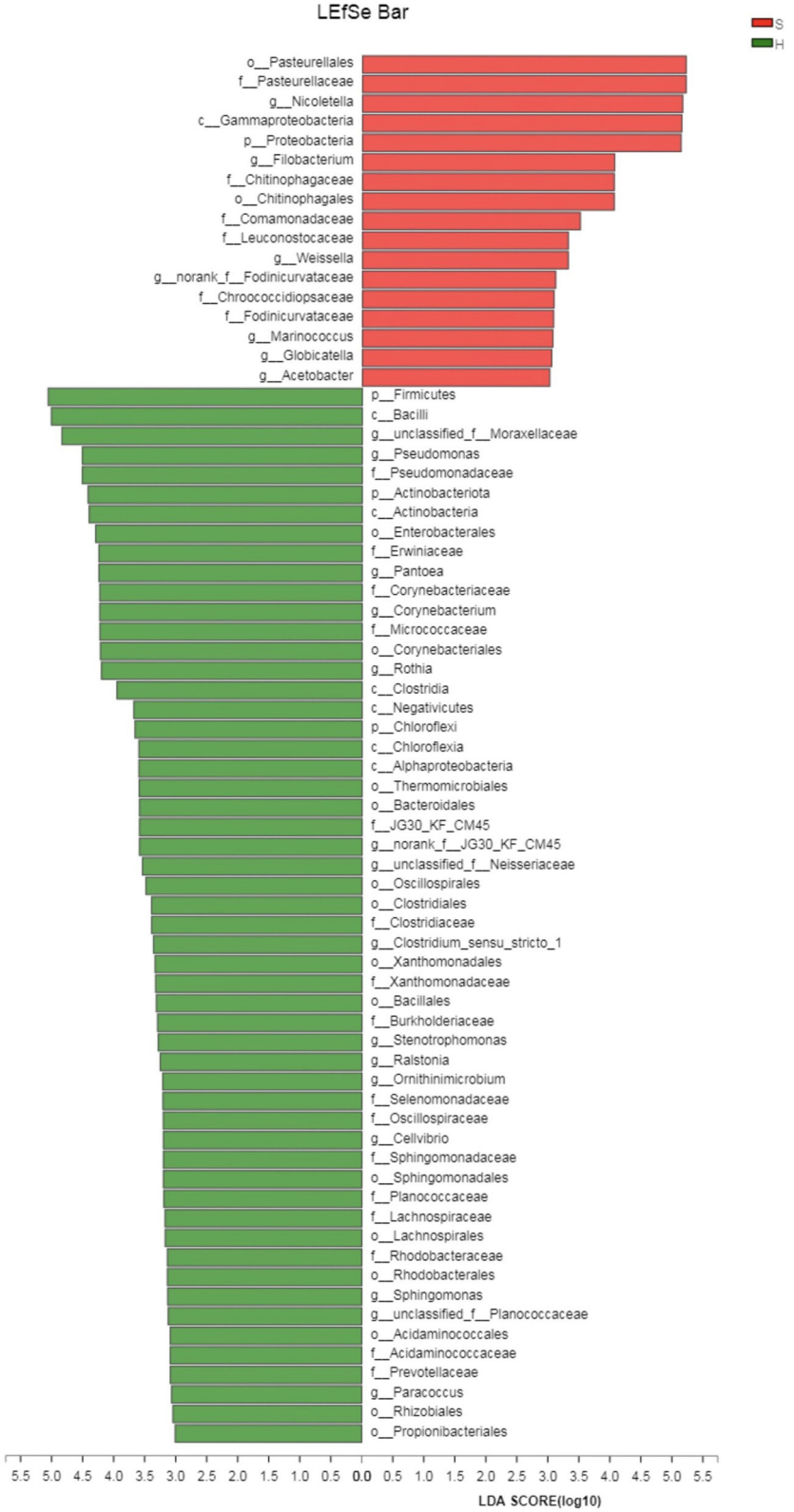


FIGURE 4 | Linear discriminant effect size analysis (LEfSe) of the nasopharygeal microbiota of healthy and donkeys shedding *S. equi*. Bacterial taxa at the genus level and higher in group S (carrier donkeys, in red) and group H (healthy donkeys, in green) were demonstrated by LDA scores >3. Ranking of taxa was based on effect size in LEfSe.

profile of donkeys shedding *S. equi* and of healthy donkeys, which may promote future research on the microbial dysbiosis of URT in donkeys and other susceptible animals. However, the sample size was relatively small and one round of sequencing analysis only represented the nasopharyngeal microbiota of the point when the samples were taken. Although it would be difficult to perform, larger groups of samples acquired at different stages of the disease may facilitate greater understanding of the pathogenesis, as well as the long-term influence of strangles on the URT of infected animals.

CONCLUSIONS

There were significant differences shown in microbiota analysis between the two groups, including OTU richness and microbial composition at different taxonomic levels. The significantly increased abundance of *Nicoletella* spp. and *Moraxella* spp. in *S.equi* carrier donkeys indicated a dysbiotic URT microbial community possibly associated with chronic airway perturbation. Significantly decreased abundance of *Streptococcus* spp., *Staphylococcus* spp., and *Corynebacterium* spp. in group S was probably due to inhibition by *Nicoletella* spp. Therefore, the role of *Nicoletella* in the URT of donkeys requires further investigation.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found here: NCBI PRJNA695404/SRP304294.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Welfare and Ethics Committee of China Agriculture University. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

JL and YZ conceived the study. YZ, SC, ZY, TW, ZD, and JL participated in animal acquisition and sample collection. SC helped conduct data analysis. YZ, RH, and SC contributed to the writing of the original draft. RH and JL helped review and edit the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the Introduction of Talent Research Start-up Fund from China Agricultural University (grant number 31051017). The funder was not involved in the study design, collection, analysis, interpretation of data, the writing of this article, or the decision to submit it for publication.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2021.645627/full#supplementary-material

REFERENCES

1. Newton JR, Wood JL, Dunn KA, DeBrauwere MN, Chanter N. Naturally occurring persistent and asymptomatic infection of the guttural pouches of horses with Streptococcus equi. *Vet Rec.* (1997) 140:84–90. doi: 10.1136/vr.140.4.84
2. Slater JD. Strangles, bastard strangles, vives and glanders: archaeological relics in a genomic age. *Equine Vet J.* (2003) 35:118–20. doi: 10.2746/042516403776114252
3. Robinson C, Waller AS, Frykberg L, Flock M, Zachrisson O, Guss B, et al. Intramuscular vaccination with strangvac is safe and induces protection against equine strangles caused by Streptococcus equi. *Vaccine.* (2020) 38:4861–68. doi: 10.1016/j.vaccine.2020.05.046
4. Sweeney CR, Whitlock RH, Meirs DA, Whitehead SC, Barningham SO. Complications associated with *Streptococcus equi* in a horse farm. *J Am Vet Med Assoc.* (1987) 191:1446–8.
5. Libardoni F, Machado G, Gressler LT, Kowalski AP, Diehl GN, Santos LC, et al. Prevalence of *Streptococcus equi* subsp. *equi* in horses and associated risk factors in the State of Rio Grande do Sul, Brazil. *Res Vet Sci.* (2016) 104:53–7. doi: 10.1016/j.rvsc.2015.11.009
6. Boyle AG, Timoney JF, Newton JR, Hines MT, Waller AS, Buchanan BR. *Streptococcus equi* infections in horses: guidelines for treatment, control, and prevention of strangles- revised consensus statement. *J Vet Intern Med.* (2018) 32:633–47. doi: 10.1111/jvim.15043
7. Sweeney CR, Timoney JF, Newton JR, Hines MT. *Streptococcus equi* infections in horses: guidelines for treatment, control, and prevention of strangles. *J Vet Intern Med.* (2005) 19:123–34. doi: 10.1111/j.1939-1676.2005.tb02671.x
8. Whelchel DD, Arnold CE, Chaffin MK. Subscapular lymph node abscessation as a result of metastatic *Streptococcus equi* subspecies *equi* infection: an atypical presentation of bastard strangles in a mare. *Equine Vet Educ.* (2009) 21:131–4. doi: 10.2746/095777309X386619
9. Boyle AG. Strangles and its complications. *Equine Vet Educ.* (2017) 29:149–57. doi: 10.1111/eve.12568
10. Swerczek TW. Exacerbation of *Streptococcus equi* (strangles) by overly nutritious diets in horses: a model for infectious bacterial diseases of horses and other livestock. *Anim Vet Sci.* (2019) 7:18–23. doi: 10.11648/j.avs.20190701.13
11. George JL, Reif JS, Shideler RK, Small CJ, Ellis RP, Snyder SP, et al. Identification of carriers of *Streptococcus equi* in a naturally infected herd. *J Am Vet Med Assoc.* (1983) 183:80–4.
12. Chanter N, Newton JR, Wood JL, Verheyen K, Hannant D. Detection of strangles carriers. *Vet Rec.* (1998) 142:496.
13. Dong J, Gao N, Waller AS, Cook FR, Yuan D, Du Y, et al. An outbreak of strangles associated with a novel genotype of *Streptococcus equi* subspecies *equi* in donkeys in China during 2018. *Equine Vet J.* (2019) 51:743–8. doi: 10.1111/evj.13114
14. Liu Y, Gao N, He D, Waller A, Gu J, Wang T,Han W. (2019). Identification of a novel genotype of *Streptococcus equi* subspecies *equi* in a donkey suffering from strangles. *Pak Vet J.* 39:609–11. doi: 10.29261/pakvetj/2019.087
15. Bosch AATM, Biesbroek G, Trzcinski K, Sanders EAM, Bogaert D. Viral and bacterial interactions in the upper respiratory tract. *LOS Pathog.* (2013) 9:e1003057. doi: 10.1371/journal.ppat.1003057
16. Man WH, de Steenhuijsen Piters WAA, Bogaert D. The microbiota of the respiratory tract: gatekeeper to respiratory health. *Nat Rev Microbiol.* (2017) 15:259–70. doi: 10.1038/nrmicro.2017.14
17. Zhao F, Jiang G, Ji C, Zhang Z, Gao W, Feng P, et al. Effects of long-distance transportation on blood constituents and composition of the nasal microbiota in healthy donkey. *BMC Vet Res.* (2020) 16:338–48. doi: 10.1186/s12917-020-02563-5
18. Niederwerder MC. Role of the microbiome in swine respiratory disease. *Vet Microbiol.* (2017) 209:97–106. doi: 10.1016/j.vetmic.2017.02.017

19. Allen EK, Koeppel AF, Hendley JO, Turner SD, Winther B, Sale MM. Characterization of the nasopharyngeal microbiota in health and during rhinovirus challenge. *Microbiome.* (2014) 2:22. doi: 10.1186/2049-2618-2-22
20. Schenck LP, Surette MG, Bowdish DME. Composition and immunological significance of the upper respiratory tract microbiota. *Febs Lett.* (2016) 590:3705–20. doi: 10.1002/1873-3468.12455
21. Holman DB, McAllister TA, Topp E, Wright AG, Alexander TW. The nasopharyngeal microbiota of feedlot cattle that develop bovine respiratory disease. *Vet Microbiol.* (2015) 80:90–5. doi: 10.1016/j.vetmic.2015.07.031
22. Artiushin S, Timoney JF. PCR for detection of *Streptococcus equi*. *Adv Exp Med.* (1997) 418:359–61. doi: 10.1007/978-1-4899-1825-3_86
23. Pusterla N, Mapes S, Wilson WD. Diagnostic sensitivity of nasopharyngeal and nasal swabs for the molecular detection of EHV-1. *Vet Rec.* (2008) 162:520–1. doi: 10.1136/vr.162.16.520
24. Newton JR, Verheyen K, Talbot NC, Timoney JF, Wood JL, Lakhani KH, et al. Control of strangles outbreaks by isolation of guttural pouch carriers identified using PCR and culture of *Streptococcus equi*. *Equine Vet J.* (2000) 32:515–26. doi: 10.2746/042516400777584721
25. Cordoni G, Williams A, Durham A, Florio D, Zanoni RG, La Ragione RM. Rapid diagnosis of strangles (*Streptococcus equi* subspecies equi) using PCR. *Res Vet Sci.* (2015) 102:162–66. doi: 10.1016/j.rvsc.2015.08.008
26. Neefs JM, Van de Peer Y, De Rijk P, Chapelle S, De Wachter R. (1993). Compilation of small ribosomal subunit RNA structures. *Nucleic Acids Res.* 21:3025–49. doi: 10.1093/nar/21.13.3025
27. Chen S, Zhou Y, Chen Y, Gu J. fastp: an ultra-fast all-in-one FASTQ preprocessor. *Bioinformatics.* (2018) 34:884–90. doi: 10.1093/bioinformatics/bty560
28. Goodrich JK, Di Rienzi SC, Poole AC, Koren O, Walters WA, Caporaso JG, et al. Conducting a microbiome study. *Cell.* (2014) 158:250–62. doi: 10.1016/j.cell.2014.06.037
29. Edgar RC. UPARSE: highly accurate OTU sequences from microbial amplicon reads. *Nat Methods.* (2013) 10:996–98. doi: 10.1038/nmeth.2604
30. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, et al. QIIME allows analysis of high-throughput community sequencing data. *Nat Methods.* (2010) 7:335–6. doi: 10.1038/nmeth.f.303
31. Taylor SD, Wilson WD. *Streptococcus equi* subsp. *equi* (strangles) infection. *Clin Tech Equine Pract.* (2006) 5:211–17. doi: 10.1053/j.ctep.2006.03.016
32. Lindahl S, Båverud V, Egenvall A, Aspán A, Pringle J. Comparison of sampling sites and laboratory diagnostic tests for S. *equi* subsp. *equi* in horses from confirmed strangles outbreaks. *J Vet Intern Med.* (2013) 27:542–47. doi: 10.1111/jvim.12063
33. Davidson A, Traub-Dargatz JL, Magnuson R, Hill A, Irwin V, Newton R, et al. Lack of correlation between antibody titers to fibrinogen-binding protein of *Streptococcus equi* and persistent carriers of strangles. *J Vet Diagn Investig.* (2008) 20:457–62. doi: 10.1177/104063870802000407
34. Knowles EJ, Mai TS, Butcher N, Waller AS, Wood JL. Use of a novel serological test for exposure to *Streptococcus equi* subspecies *equi* in hospitalised horses. *Vet Rec.* (2010) 166:294–97. doi: 10.1136/vr.166.10.294
35. North SE, Wakeley PR, Mayo N, Mayers J, Sawyer J. Development of a real-time PCR to detect *Streptococcus equi* subspecies *equi*. *Equine Vet J.* (2014) 46:56–9. doi: 10.1111/evj.12088
36. Pusterla N, Leutenegger CM, Barnum SM, Byrne BA. Use of quantitative real-time PCR to determine viability of *Streptococcus equi* subspecies *equi* in respiratory secretions from horses with strangles. *Equine Vet J.* (2018) 50:697–700. doi: 10.1111/evj.12809
37. Bond SL, Timsit E, Workentine M, Alexander T, Léguillette R. Upper and lower respiratory tract microbiota in horses: bacterial communities associated with health and mild asthma (inflammatory airway disease) and effects of dexamethasone. *BMC Microbiol.* (2017) 17:184. doi: 10.1186/s12866-017-1092-5
38. Welp AL, Bomberger JM. Bacterial Community interactions during chronic respiratory disease. *Front. Cell Infect Microbiol.* (2020) 10:213. doi: 10.3389/fcimb.2020.00213
39. Lawley TD, Clare S, Walker AW, Goulding D, Stabler RA, Croucher N, et al. Antibiotic treatment of clostridium difficile carrier mice triggers a supershedder state, spore-mediated transmission, and severe disease in immunocompromised hosts. *Infect Immun.* (2009) 77:3661–69. doi: 10.1128/IAI.00558-09
40. Abreu NA, Nagalingam NA, Song Y, Roediger FC, Pletcher SD, Goldberg AN, et al. Sinus microbiome diversity depletion and *Corynebacterium tuberculostearicum* enrichment mediates rhinosinusitis. *Sci Transl Med.* (2012) 4:151. doi: 10.1126/scitranslmed.3003783
41. Hoggard M, Waldvogel-Thurlow S, Zoing M, Chang K, Radcliff FJ, Mackenzie BW, et al. Inflammatory endotypes and microbial associations in chronic rhinosinusitis. *Front Immunol.* (2018) 9:2065. doi: 10.3389/fimmu.2018.02065
42. Dlugaszewska J, Leszczynska M, Lenkowski M, Tatarska A, Pastusiak T, Szyfter W. The pathophysiological role of bacterial biofilms in chronic sinusitis. *Eur Arch Otorhinolaryngol.* (2016) 273:1989–94. doi: 10.1007/s00405-015-3650-5
43. Stephenson M-F, Mfuna L, Dowd SE, Wolcott RD, Barbeau J, Poisson M, et al. Molecular characterization of the polymicrobial flora in chronic rhinosinusitis. *J Otolaryngol Head Neck Surg.* (2010) 39:182–87. doi: 10.2310/7070.2009.090060
44. Bogaert D, Keijser B, Huse S, Rossen J, Veenhoven R, Van Gils E, et al. Variability and diversity of nasopharyngeal microbiota in children: a metagenomic analysis. *PLoS One.* (2011) 6:e17035. doi: 10.1371/journal.pone.0017035
45. Weese JS, Slifierz M, Jalali M, Friendship R. Evaluation of the nasal microbiotaslaughter-age pigs and the impact on nasal methicillin resistant *Staphylococcus aureus* (MRSA) carriage. *BMC Vet Res.* (2014) 10:69. doi: 10.1186/1746-6148-10-69
46. Kuhar HN, Tajudeen BA, Mahdavinia M, Heilingoetter A,Ganti A, Gattuso P, et al. Relative abundance of nasal microbiota in chronic rhinosinusitis by structured histopathology. *Int Forum Allergy Rhinol.* (2018) 8:1430–37. doi: 10.1002/alr.22192
47. Yang X, Li H, Ma Q, Zhang Q, Wang C. Neutrophilic asthma is associated with increased airway bacterial burden and disordered community composition. *BioMed Res Int.* (2018) 2018:1–11. doi: 10.1155/2018/9230234
48. Lima SF, Teixeira AGV, Higgins CH, Lima FS, Bicalho RC. The upper respiratory tract microbiome and its potential role in bovine respiratory disease and otitis media. *Sci Rep.* (2016) 6:29050. doi: 10.1038/srep29050
49. Verheyen K, Newton JR, Talbot NC, Brauwere MND, Chanter N. Elimination of guttural pouch infection and inflammation in asymptomatic carriers of Streptococcus equi. *Equine Vet J.* (2000) 32:527–32. doi: 10.2746/042516400777584703
50. Kuhnert P, Korczak B, Falsen E, Straub R, Hoops A, Boerlin P, et al. *Nicoletella semolina* gen. nov., sp. nov., a new member of Pasteurellaceae isolated from horses with airway disease. *J Clin Microbiol.* (2004) 42:5542–8. doi: 10.1128/JCM.42.12.5542-5548.2004
51. Hansson I, Persson KE, Riihimäki M. The clinical significance of *Nicoletella semolina* in horses with respiratory disorders and a screening of the bacterial flora in the airways of horses. *Vet Microbiol.* (2013) 162:695–99. doi: 10.1016/j.vetmic.2012.09.016
52. McConachie EL, Hart KA, Welchel EL, Schroeder HC, Schott II, Sanchez S. Pulmonary disease potentially associated with *Nicoletella semolina* in 3 young horses. *J Vet Intern Med.* (2014) 28:939–43. doi: 10.1111/jvim.12349
53. Riley CB, Icon O, Aldrich ED, Pemeberton SL, Mirza AA. First report of pulmonary disease associated with *Nicoletella semolina* in a horse in New Zealand. *N Z Vet J.* (2020) 69:65–9. doi: 10.1080/00480169.2020.1811795
54. Gao X, Lin H, Revanna K, Dong Q. A Bayesian taxonomic classification method for 16S rRNA gene sequences with improved species-level accuracy. *BMC Bioinformatics.* (2017) 18:247. doi: 10.1186/s12859-017-1670-4
55. Manguin E, Pépin E, Bolvin R, Leclere M. Tracheal microbial populations in horses with moderate asthma. *J Vet Intern Med.* (2020) 34:986–95. doi: 10.1111/jvim.15707

Conflict of Interest: TW and ZD were employed by the company Dong-E-E-Jiao Co., Ltd.

The remaining 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 September 2021
doi: 10.3389/fvets.2021.662887

Semen Quality and Freezability Analyses in the Ejaculates of Two Poitou Donkeys in the Southern Hemisphere

Francisca Ebel[1†], *Omar Ulloa*[2], *Pablo Strobel*[1] *and Alfredo Ramírez-Reveco*[1*]

[1] *Instituto de Ciencia Animal, Facultad de Ciencias Veterinarias, Universidad Austral de Chile, Valdivia, Chile,* [2] *Haras Militar Pupunahue, DGFER-Ejército de Chile, Los Lagos, Chile*

OPEN ACCESS

Edited by:
Amy Katherine McLean, University of California, Davis, United States

Reviewed by:
Abouzar Najafi, University of Tehran, Iran
Islam M. Saadeldin, Zagazig University, Egypt
Nucharin Songsasen, Smithsonian Conservation Biology Institute (SI), United States

***Correspondence:**
Alfredo Ramírez-Reveco
alfredoramirez@uach.cl

†Present address:
Francisca Ebel, Instituto de Biotecnología Ambiental y Salud (INIBIAS), Facultad de Ciencias Exactas, Físico-Químicas y Naturales, Universidad Nacional de Río Cuarto, Río Cuarto, Córdoba, Argentina

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

Received: *01 February 2021*
Accepted: *28 July 2021*
Published: *03 September 2021*

Citation:
Ebel F, Ulloa O, Strobel P and Ramírez-Reveco A (2021) Semen Quality and Freezability Analyses in the Ejaculates of Two Poitou Donkeys in the Southern Hemisphere. Front. Vet. Sci. 8:662887. doi: 10.3389/fvets.2021.662887

The Baudet du Poitou is a vanishing donkey breed recognized for engendering robust working mules. In Chile, only two pure breed Poitou males exist, which belong to the Chilean army and are used for mule production. We performed an extensive sperm and seminal analysis of these two jackasses aged 3 and 6 years and investigated the use of a simple hypometabolic extender for sperm cryopreservation. Computer-assisted sperm analysis showed high motility, velocity, and linearity in sperm movement. The seminal plasma analysis revealed that sodium and chloride were the main electrolytes, and globulins were the main metabolites. Active and variable enzymatic activity was observed. New information is reported about gamma-glutamyltransferase, aspartate aminotransferase, zinc, and magnesium concentrations in seminal plasma of Poitou donkeys. Ejaculates among jackasses showed some variability due to individual variability and different stages in sexual maturation according to age. The freezability index analysis based in viability, total motility and progressive motility with Botucrio extender (57.1 ± 11.0%; 56.6 ± 20.0%; and 22.6 ± 10.3%, respectively) were significantly higher ($p < 0.05$, $p < 0.0001$, and $p < 0.0001$, respectively) than with HM-0 extender (42,6 ± 11.4%; 14.9 ± 5.1%; and 1.0 ± 2.5%, respectively). We report new information on Poitou donkey semen and cryopreservation in the Southern Hemisphere that could be useful in donkey breeding and conservation programs to develop strategies that improve the effectiveness of population management of this breed.

Keywords: Baudet du Poitou, donkey, sperm quality, seminal plasma, cryopreservation, Southern hemisphere

INTRODUCTION

Donkeys are an important socio-economic support for human livelihood (1, 2). One of the oldest domestic donkey breeds is the Baudet du Poitou (Studbook since 1884). This French breed is highly recognized and requested for engendering robust mules useful in agricultural and army activities (3). This breed is classified as "endangered-maintained," which indicates a worldwide population of pure breed animals of up to 6–20 males and 100–1,000 females (4). The steady decrease in the population is due to the industrialization of agriculture and the closure of national stud farms in the 20th century (5). There is limited empirical information on the current world population of Poitou donkeys because they are mainly owned by hobby-breeders of small farms (6)

and breeders' organizations (i.e., UPRA and SABAUD[1]), with a small number of animals per farm (4). This situation hinders the census logistics of their population by statistical organizations (7). Thus, information on the Poitou donkey population is mainly provided by breeders' associations or organizations through informal data (CREGENE, 2021[2]) According to recent data from the Domestic Animal Diversity Information System (DAD-IS) for 2018, there were 2,903 Baudet du Poitou donkeys in the world−1,648 females and 486 males. Ninety-nine females of this population are pure breed and only five males participate in the national cryopreservation bank for insemination programs (4). Besides, in France, which has the greatest population of this breed, the French Institute of Horse and Riding reported 117 new births in 2017 (IFCE, 2019)[3] Nonetheless, the Rare Breeds Trust of Australia, which declared two animals of this breed, reported that the international status of the Poitou donkeys is "critical" and estimated that there are fewer than 200 Poitou Donkeys in the world today (RBTA, 2019[4]) Similarly, in the United States, the University of Illinois Veterinary Teaching Hospital, which keeps five Poitou jennies, stated that fewer than 70 and 500 Poitou donkeys remain throughout the United States and the world, respectively (University of Illinois College of Veterinary Medicine, 2020[e]). In South America, only five Poitou donkeys were reported (three females and two males). These specimens belong to the Chilean army and are used to produce mules, whose purpose is the transport of supplies and materials to mountain areas, which are otherwise difficult to reach (8).

To preserve the livestock genetic diversity and populations of Poitou donkeys, it is crucial to know their normal seminal characteristics and develop efficient techniques for semen cryopreservation that will ensure optimal results in conservation programs. Consequently, several studies have reported the importance of seminal plasma for spermatozoa (9–14), semen preservation (15, 16), and reproduction (17, 18). Donkeys differ strikingly in their reproductive process from horses and among donkey breeds, which is why data comparisons between donkeys and horses and between donkeys of different breeds are questionable (9, 12). Research on fresh and cryopreserved semen of Poitou donkeys has been performed (9, 18–24) but a detailed study of semen characterization, including biochemical analysis of semen, has been only partially conducted in this breed.

All investigations on fresh and cryopreserved Poitou ejaculates have been performed only in the Northern Hemisphere. Since fertility in related species such as stallions is higher in the Southern than in the Northern Hemisphere (25), we fulfill the requirements to analyze the ejaculates' behavior of Poitou jackass in the Southern Hemisphere and compare it with that of Northern Hemisphere jackasses. This study aimed (1) to perform an extensive semen analysis, including sperm quality and biochemical profile of seminal plasma, on the ejaculates of the two Poitou jackasses aged 3 and 6 years; and (2) to investigate the effect of a simple hypometabolic extender on sperm cryopreservation.

[1]http://www.racesmulassieresdupoitou.com
[2]http://www.cregene.org/Mamiferes.html
[3]https://www.ifce.fr/ifce/connaissances/statistiques-et-donnees/
[4]https://rarebreedstrust.com.au/

MATERIALS AND METHODS

Animals and Semen Samples

Semen was obtained from two fertile Poitou jackasses belonging to the Chilean army (Haras Militar Pupunahue, Los Lagos, Chile; 39°47′32″S, 72°53′34″O). Jackass 1 was 6 years old and weighed 450 kg. Jackass 2 was 3 years old and weighed 380 kg. Both animals were fed in *ad libitum* pasture and water conditions. As these donkeys are used in labor activities, alfalfa hay and corn supplementation were also provided twice daily. Animals were kept in paddocks and only if the weather conditions were adverse during the winter were they housed in a barn.

Donkey semen without extra-gonadal sperm reserves (with Daily Sperm Output [DSO] stabilized) was collected using an artificial vagina of an estrus female. Fourteen ejaculates (seven from each jackass) were collected during the breeding season (summer). All ejaculates were used for sperm evaluation, and 12 (6 from each donkey) were used for biochemical evaluation.

Semen Evaluation

Sperm Quality

Seminal volume was determined by graduated containers, removing the gel-free fraction, and sperm concentration was estimated with a spectrophotometer (AccuRead, IMV Technologies, France).

For the sperm analysis, 40 μl of each ejaculate was diluted 1:5 in UHT skim milk and kept at 37°C for motility assessment. The remaining sample was diluted 1:1 with skim milk and kept at 37°C for concentration, viability, and morphology evaluation.

Plasma membrane integrity (viability) and morphology were assessed by using an eosin-nigrosin staining dye technique (18, 19, 26). Briefly, the sperm samples were mixed 1:1 with a stain solution (50 g/L eosin; 100 g/L nigrosin) on a tempered microscope slide and analyzed under a bright field microscope (Nikon Eclipse E200). The percentage of sperm viability was obtained according to the permeability of the plasma membrane of the sperm head to the eosin. The sperm morphology was determined as the percentage of normal spermatozoa without morphological abnormalities in the head, midpiece, and tail. At least 500 spermatozoa for each sample were analyzed in triplicate.

Total and progressive motility and sperm kinetic parameters were assessed using computer-assisted sperm analysis (CASA system, SCA, Microptic S.L., Spain) according to Córdova et al. (27). Aliquots of the sperm samples were then briefly placed on a pre-warmed slide at 37°C. The samples were analyzed by using a phase-contrast microscope (Nikon Eclipse E200) with 10× magnification (negative phase), coupled with a high-velocity camera (scA780 54tc). Twenty-five consecutive photographs were taken per second and at least 500 spermatozoa for each sample were analyzed in triplicate in three to six separate fields for each sample. For sperm kinetic analysis, the following parameters were considered: curvilinear velocity (VCL), linear velocity (VSL), mean velocity (VAP), linearity coefficient (LIN), straightness coefficient (STR), wobble coefficient (WOB), mean

amplitude of lateral head displacement (ALH), and frequency of head displacement (BCF). Following these parameters, total motility was defined as the percentage of spermatozoa showing a VCL of above 10 μm/s, and progressive motility was defined as the percentage of spermatozoa showing an STR above 75%. At least six fields and 500 spermatozoa were measured in each evaluation.

Biochemical Evaluation

Fifteen milliliters of each ejaculate (gel-free fraction) was centrifuged twice at 2,700×g for 15 min. The supernatant of each tube was stored at −20°C until pH [PL-600] and osmolarity (Osmomat030) analyses. The enzymes, ions, and metabolites of the seminal plasma were analyzed by Laboratorio de Patología Clínica of Universidad Austral de Chile (Valdivia, Chile). The following parameters were considered in the analysis: (a) enzymes: alkaline phosphatase [[ALP]; alkaline phosphatase liquicolor], aspartate aminotransferase [[AST]; GOT IFCC mod. LiquiUV], and gamma-glutamyltransferase [[GGT]; γ-GT liquicolor]; (b) ions: total calcium [[Ca2+]; calcium liquicolor], inorganic phosphorus [[Pi]; phosphorus liquirapid], magnesium [[Mg2+]; magnesium liquicolor], sodium [[Na+]; atomic absorption spectrophotometry], chloride [[Cl−]; chloride liquicolor], potassium [[K+]; potassium liquirapid], zinc [[Zn2+]; atomic absorption spectrophotometry], and Ca:Pi ratio; (c) metabolites: cholesterol (cholesterol liquicolor), total protein (total protein liquicolor), albumin (albumin liquicolor), globulins (total protein minus albumin), urea (urea liquiUV), and glucose (glucose liquicolor). All tests were supplied by HUMAN (Weisbaden, Germany).

Freezability Test

Freezing Protocol

Fresh semen samples with >70% viability and total motility were diluted in UHT skim milk at 37°C, centrifuged, and resuspended in the following freezing extenders (80 × 106 sperm/ml):

- The commercial extender Botucrio (Botupharma, Brazil). Osmolarity: 1,244 ± 1 mOsm/L. pH: 7.4. Glucose concentration: 99.0 ± 6.1 mM (mean ± SD).
- The hypometabolic extender HM-0 Tris, composed of 300 mM Tris base, 94.7 mM citric acid, 2% (v:v) glycerol, 15% (v:v) egg yolk, 0.5 mg/ml streptomycin sulfate, and 0.05 mg/ml gentamicin. Osmolarity: 592 ± 4 mOsm/L. pH: 7.4. Glucose concentration: 1.9 ± 0.1 mM (mean ± SD) (27).

The freezing–thawing protocol and the freezing extender used were based on Cordova et al. (27). Briefly, ejaculates were collected using an artificial vagina, filtered to remove the gel, diluted 1:1 in pre-warmed UHT skimmed milk (isothermal condition), and centrifuged to 1,000×*g* for 20 min. Post-centrifugation sperm pellets were suspended in the extenders previously tempered at 20°C, packed in 0.5 ml at 50 × 10^6 sperm/ml in straws, and cooled to 5°C for 90 min. Afterward, straws were subsequently exposed to liquid nitrogen vapors for 20 min and finally plunged and stored in liquid nitrogen for at least 2 months before analysis. The temperature of the samples was recorded during the whole process using a temperature probe coupled to a USB data logger (ThermoWorks, Alpine-UT, USA).

Post-thawed Sperm Evaluation

Samples were centrifuged and resuspended in Tris/citrate buffer extender for sperm quality evaluation. Each evaluation was established from a count of at least six different fields and 500 spermatozoa in each assessment.

The plasma membrane integrity (viability) was evaluated by the CASA system using Ethidium Bromide (EB)/Acridine Orange (OA) double staining technique according to Córdova et al. (27). Briefly, sperm samples were mixed (1:1) on a tempered microscope slide with a staining aqueous solution composed of 10 μM EB and 20 μM AO (EB/AO solution). Samples were immediately visualized and analyzed using the viability module from the CASA system (Sperm Class Analyzer, Microptic, Spain) coupled to an epifluorescence microscope (Nikon E200, upright microscope) with a high-velocity camera (Basler AG, Germany, scA780 54tc). Viable spermatozoa were green-stained on the head, whereas non-viable spermatozoa were red-stained on the head. The sperm motility was evaluated by the CASA system as previously described for fresh samples.

The acrosome integrity analysis was evaluated by FITC-PSA dye (Sigma Aldrich, USA), according to Ramirez et al. (28). Briefly, sperm aliquots were fixed and permeabilized for at least 30 min at 4°C in 100% methanol, to allow entry of PSA. Permeabilized spermatozoa, dried onto slides, were then covered with a droplet of 100 mg/ml FITC-PSA PBS for 10 min. Later, the slides were washed with bi-distilled water, and the spermatozoa were analyzed with an inverted epifluorescence microscope (Leica DMI3000 B). The emission fluorescence of PSA-FITC was detected using a 450- to 490-nm UV excitation filter, a 510-nm dichroic mirror, and a 520-nm barrier filter. Data were analyzed in triplicate. Intense acrosome staining was indicative of an intact acrosome. Sperm with structurally altered acrosomes were those displaying a slight fluorescence or no fluorescence at all on the sperm head. To compare the extender freezing ability, a Freezability Index (FI) was calculated for viability and total and progressive motility [FI [%] = AF/BF × 100; sperm values after [VAF] and values before freezing [VBF]].

Statistical Analysis

Data were analyzed using GraphPad Prism 6 statistical software (USA) and they included mean values, standard deviation (mean ± SD). For comparative analysis of post-thawing semen parameters and freezability analysis between freezing extenders, the paired sample *t*-test were used. *p*-values of <0.05 and <0.01 were considered significant and highly significant differences, respectively.

RESULTS

Semen Evaluation

Sperm Quality

The results of sperm quality and motility parameters of both Poitou donkeys are shown in **Tables 1**, **2**, respectively. The mean values for jackass 1 and 2 were 52.9 ± 18.0 and 44.3 ±

TABLE 1 | Free-gel fraction volume, sperm concentration, viability, and normal morphology of fresh Poitou jackass ejaculates.

Parameters	Jackass 1 (J1)	Jackass 2 (J2)	Range (J1 + J2)		Trimeche et al. (22)	Gupta et al. (18)	Talluri et al. (9)	Kumar (29)	Kumar et al. (19)	Kumar et al. (30)
	Mean ± SD	Mean ± SD	Mean ± SD	95% Confidence interval	Mean ± SD	Mean ± SD[a]	Mean ± SD[a]	Mean ± SD[a]	Mean ± SD	Mean ± SD[a]
Free-gel fraction volume (ml)	52.9 ± 18.0	44.3 ± 14.0	48.6 ± 16.1	40.2–58.0	-	43.7 ± 11.5	64.5 ± 7.1	45.9 ± 23.9	44.4 ± 3.9	47.0 ± 27.2
Sperm concentration (10^6/ml)	552.9 ± 122.8	198.6 ± 55.6	375.7 ± 205.4	268.1–483.3	–	331.56 ± 67.3	262.3 ± 15.9	292.6 ± 22.7	282.1 ± 4.8	274.9 ± 5.6
Viability (%)	77.8 ± 5.5	85.6 ± 5.3	81.7 ± 6.6	78.3–85.1	73.6 ± 7.9	84.3 ± 3.5	85.2 ± 0.8	86.8 ± 9.4	91.8 ± 0.3	87 ± 2.0
Morphology (%)	74.7 ± 6.9	79.9 ± 9.9	77.3 ± 8.7	82.2–93.4	–	–	96.7 ± 0.85	91.9 ± 2.3	91.3 ± 0.26	91 ± 0.6
Number of donkeys	1	1	2		4	5	6	4	4	6
Total number of ejaculates	7	7	14		40	150	60	24	32	48
Donkey age (years)	3	6	3–6		–	–	–	4–6	–	4–7
Donkey weight (kg)	380	450	380–450		–	–	–	–	–	–

[a]*Values of standard error of the mean were transformed to standard deviation.*

14.0 ml of free-gel fraction volume, 552.9 ± 122.8 and 198.6 ± 55.6 × 106/ml of sperm concentration, 77.8 ± 5.5 and 85.6 ± 5.3% of viability, and 74.7 ± 6.9 and 79.9 ± 9.9% of normal morphology, respectively. Concerning the overall means, free-gel fraction volume, viability, and normal morphology showed slight dispersion with values of 48.6 ± 16.1 ml, 81.7 ± 6.6%, and 77.3 ± 8.7%, respectively. In contrast, the overall mean of sperm concentration showed a high dispersion with a value of 375.7 ± 205.4 × 106/ml.

Regarding motility parameters, the mean values for both jackass 1 and 2 were 90.1 ± 6.2 and 85.6 ± 8.6% of total motility and 70.9 ± 8.8 and 56.4 ± 13.9% of progressive motility, respectively, with total and progressive motility overall means of 87.8 ± 7.6 and 63.6 ± 13.5%, respectively. The kinetic evaluation revealed that the sperm of both donkeys exhibited high velocity (VCL: 97.1 ± 10.1, VSL: 62.3 ± 13.1, and VAP 76.6 ± 11.9 μm/s) and linearity (STR: 80.8 ± 5.6, LIN: 64.3 ± 9.8, and WOB: 79.3 ± 7.3%) in their sperm movement.

Biochemical Evaluation

The overall seminal plasma values of pH and osmolarity were 7.42 ± 0.1 and 285.7 ± 6.5 mOsm/kg, respectively. The enzymatic evaluation revealed the presence of active and variable ALP, AST, and GGT activity in the seminal plasma of both Poitou donkeys. Thus, mean values and range for ALP, AST, and GGT were 338.0 ± 81.5 and 206.9 ± 56.5 [231.9 to 362.9], 4.9 ± 0.1 and 3.4 ± 1.2 [3.4 to 4.9], and 301.8 ± 215.5 and 272.0 ± 146.5 [187.1 to 386.7] μkat/L for jackass 1 and 2, respectively (**Table 3**).

Regarding ion concentrations in seminal plasma (**Table 4**), sodium and chloride were the main electrolytes in seminal plasma in both donkeys with overall values of 130.0 ± 21.3 and 128.5 ± 9.3 mmol/L, respectively. Total calcium and inorganic phosphorus overall concentrations were 1.8 ± 0.5 and 1.4 ± 0.6 mmol/L, respectively, with a Ca:Pi ratio of 1.3 ± 0.2. The concentrations of potassium and magnesium for both jackasses were 11.0 ± 1.6 and 2.2 ± 1.1 mmol/L, respectively. Low concentrations of zinc were also observed with a value of 20.9 ± 5.0 μmol/L.

As shown in **Table 5**, the analysis of seminal plasma metabolites revealed that globulins were the main protein of seminal plasma in these jackasses (27.3 ± 6.3 g/L and 28.0 ± 6.5 g/L of total protein). Additionally, low concentrations of cholesterol and glucose in seminal plasma were found in both donkeys, with values of 0.2 ± 0.1 and 0.1 ± 0.1 mmol/L, respectively. The urea concentration in ejaculates of these jackasses was 6.6 ± 0.7 mmol/L.

Post-thawing Sperm Quality and Freezability Analysis

The cooling and freezing rates of the cryopreservation protocol were −0.3° and −5.5°C/min, respectively. Sperm achieved −105.9°C before being immersed in liquid nitrogen (**Figure 1**).

The comparative results are shown in **Figure 2**, in which post-thawed sperm viability, total motility, and progressive motility, as well as all kinetic parameters (except for ALH) were significantly higher when using the Botucrio extender than the HM-0 extender ($p < 0.01$). The only one parameters in which the result using

TABLE 2 | Motility and kinetic parameters of fresh Poitou jackass ejaculates.

Parameters	Jackass 1 (J1)	Jackass 2 (J2)	Range (J1 + J2)		Trimeche et al. (21)	Trimeche et al. (20)	Gupta et al. (18)	Talluri et al. (9)	Kumar (29)	Kumar et al. (19)	Kumar et al. (30)
	Mean ± SD	Mean ± SD	Mean ± SD	95% Confidence interval	Mean ± SD[a]	Mean ± SD[a]	Mean ± SD[a]	Mean ± SD[a]	Mean ± SD[a]	Mean ± SD[a]	Mean ± SD[a]
Total motility (%)	90.1 ± 6.2	85.6 ± 8.6	87.8 ± 7.6	82.2–93.4	71.3 ± 8.4	70.3 ± 7.4	84.9 ± 6.4	–	–	–	84.0 ± 4.3
Progressive motility (%)	70.9 ± 8.8	56.4 ± 13.9	63.6 ± 13.5	56.5–70.7	39.1 ± 5.1	56.2 ± 5.1	75.1 ± 6.9	81.5 ± 0.9	80.1 ± 7.2	88.5 ± 0.4	80 ± 4.4
VCL (μm/s)	103.2 ± 5.4	91.0 ± 10.2	97.1 ± 10.1	89.6–104.6	60.6 ± 7.2	61.3 ± 6.8	–	–	–	–	–
VSL (μm/s)	66.3 ± 12.0	58.3 ± 13.8	62.3 ± 13.1	52.6–72.0	42.0 ± 4.2	45.2 ± 4.52	–	–	–	–	–
VAP (μm/s)	80.2 ± 10.4	72.9 ± 12.9	76.6 ± 11.9	67.8–85.4	38.3 ± 3.1	39.8 ± 3.4	–	–	–	–	–
STR (%)	82.3 ± 5.0	79.3 ± 6.2	80.8 ± 5.6	76.6–85.0	–	–	–	–	–	–	–
LIN (%)	65.0 ± 9.1	63.7 ± 11.1	64.3 ± 9.8	57.1–71.5	74.0 ± 8.7	74.6 ± 6.8	–	–	–	–	–
WOB (%)	78.7 ± 6.9	79.9 ± 8.1	79.3 ± 7.3	73.9–84.7	–	–	–	–	–	–	–
ALH (μm)	3.6 ± 0.4	3.1 ± 0.7	3.3 ± 0.6	2.8–3.8	3.2 ± 0.4	4.1 ± 0.6	–	–	–	–	–
BCF (Hz)	10.1 ± 0.9	8.8 ± 0.7	9.5 ± 1.0	8.8–10.2	13.9 ± 1.9	–	–	–	–	–	–
Number of donkeys	1	1	2		4	4	5	6	4	4	6
Number of ejaculates	7	7	14		40	32	150	60	24	32	48
Donkey age (years)	3	6	3–6		–	3–7	–	–	4–6	-	4–7
Donkey weight (kg)	380	450	380–450		–	300–500	–	–	–	–	–

[a] *Values of standard error of the mean were transformed to standard deviation.*

VCL, curvilinear velocity; VSL, linear velocity; VAP, mean velocity; LIN, linearity coefficient; STR, straightness coefficient; WOB, wobble coefficient; ALH, mean amplitude of lateral head displacement; BCF, frequency of head displacement.

TABLE 3 | pH, osmolarity, and enzyme profile of Poitou jackass seminal plasma.

Parameters	Jackass 1 (J1)	Jackass 2 (J2)	Range (J1 + J2)		Gupta et al. (18)	Kumar (29)	Talluri et al. (9)	Kumar et al. (30)
	Mean ± SD	Mean ± SD	Mean ± SD	95% Confidence interval	Mean ± SD[a]	Mean ± SD[a]	Mean ± SD[a]	Mean ± SD[a]
pH	7.40 ± 0.1	7.45 ± 0.1	7.42 ± 0.1	7.42–7.42	7.31 ± 0.3	7.73 ± 0.2	7.30 ± 0.0	7.45 ± 0.4
Osmolarity (mOsm/kg)	285.7 ± 4.2	285.7 ± 8.7	285.7 ± 6.5	282.0–289.4	–	–	–	–
Enzymes								
ALP (μkat/L)	338.0 ± 81.5	206.9 ± 56.5	297.5 ± 115.8	231.9–362.9	0.9 ± 0.2	-	0.2 ± 0.08	–
AST (μkat/L)	4.9 ± 0.1	3.4 ± 1.2	4.1 ± 1.3	3.4–4.9	–	–	–	–
GGT (μkat/L)	301.8 ± 215.5	272.0 ± 146.5	286.9 ± 176.4	187.1–386.7	–	–	–	–
Number of donkeys	1	1	2		5	4	6	6
Number of ejaculates	6	6	12		150	24	60	48
Donkey age (years)	3	6	3–6		–	4–6	–	4–7
Donkey weight (kg)	380	450	380–450		–	–	–	–

[a]*Values of standard error of the mean were transformed to standard deviation.*
ALP, alkaline phosphatase; AST, aspartate aminotransferase; GGT, gamma-glutamyltransferase.

TABLE 4 | Ion profile of Poitou jackass seminal plasma.

Parameters	Jackass 1 (J1)	Jackass 2 (J2)	Range (J1 + J2)		Gupta et al. (18)	Talluri et al. (9)
	Mean ± SD	Mean ± SD	Mean ± SD	95% Confidence interval	Mean ± SD[a]	Mean ± SD[a]
Total calcium (mmol/L)	2.1 ± 0.6	1.5 ± 0.1	1.8 ± 0.5	1.5–2.1	–	4.5 ± 2.2
Inorganic phosphorus (mmol/L)	1.8 ± 0.6	1.1 ± 0.2	1.4 ± 0.6	1.1–1.7	–	3.8 ± 1.5
Ca:Pi ratio	1.2 ± 0.2	1.4 ± 0.3	1.3 ± 0.2	1.2–1.4	–	–
Magnesium (mmol/L)	2.6 ± 1.5	1.8 ± 0.3	2.2 ± 1.1	1.6–2.8	–	–
Sodium (mmol/L)	116.2 ± 4.3	143.8 ± 22.8	130.0 ± 21.3	118.0–142.0	110.8 ± 5.9	-
Chloride (mmol/L)	121.5 ± 3.0	135.5 ± 8.1	128.5 ± 9.3	123.2–133.8	115.2 ± 5.6	–
Potassium (mmol/L)	12.0 ± 1.5	10.1 ± 1.0	11.0 ± 1.6	18.1–23.7	19.7 ± 0.9	–
Zinc (μmol/L)	23.5±5.8	18.3±2.5	20.9±5.0	18.1–23.7	–	–
Number of donkeys	1	1	2		5	6
Number of ejaculates	6	6	12		150	60
Donkey age (years)	3	6	3–6		–	–
Donkey weight (kg)	380	450	380–450		–	–

[a]*Values of standard error of the mean were transformed to standard deviation.*

TABLE 5 | Metabolite profile of Poitou jackass seminal plasma.

Parameters	Jackass 1 (J1)	Jackass 2 (J2)	Range (J1 + J2)		Gupta et al. (18)	Talluri et al. (9)
	Mean ± SD	Mean ± SD	Mean ± SD	95% Confidence interval	Mean ± SD[a]	Mean ± SD[a]
Cholesterol (mmol/L)	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.1–0.3	16.3 ± 5.6	0.6 ± 0.2
Total protein (g/L)	32.7 ± 4.7	23.3 ± 4.3	28.0 ± 6.5	24.3–31.7	50.4 ± 6.0	40.3 ± 9.0
Globulins (g/L)	32.0 ± 3.9	23.3 ± 4.3	27.3 ± 6.3	23.8–30.8	28.1 ± 4.0	–
Urea (mmol/L)	6.2 ± 0.3	7.0 ± 0.7	6.6 ± 0.7	6.2–7.0	6.2 ± 4.1	–
Glucose (mmol/L)	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.0–0.2	0.5 ± 0.2	1.4 ± 0.6
Number of donkeys	1	1	2		5	6
Number of ejaculates	12	12	12		150	60
Donkey age (years)	3	6	3–6		–	–
Donkey weight (kg)	380	450	380–450		–	–

[a]*Values of standard error of the mean were transformed to standard deviation.*

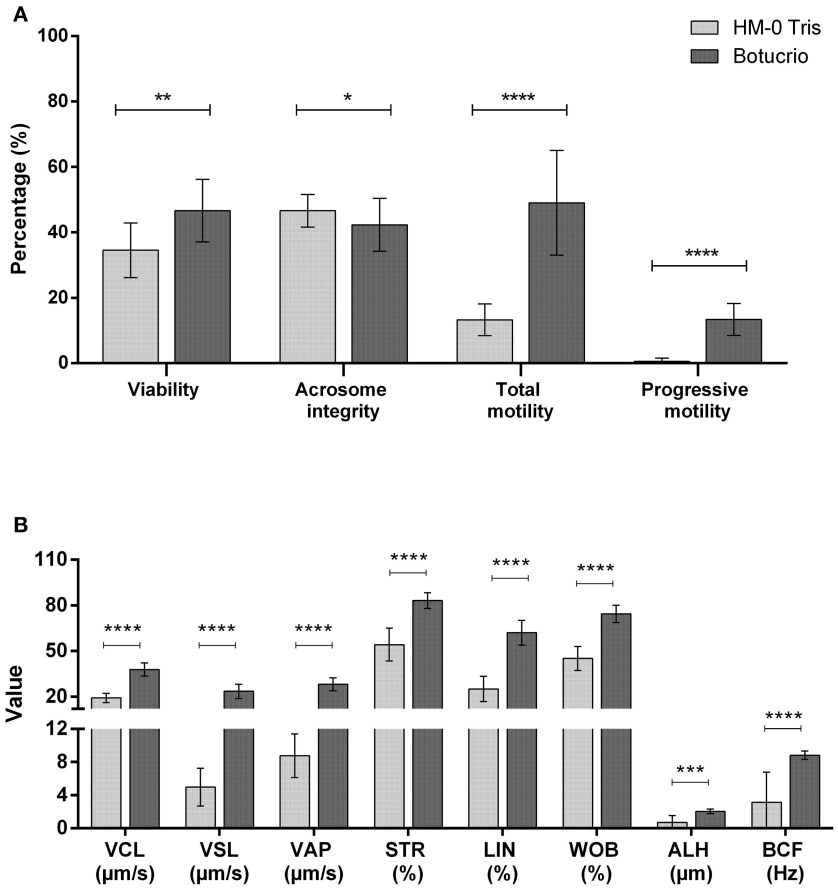


FIGURE 2 | Post-thawed results of Poitou jackass sperm cryopreserved with HM-0 Tris and Botucrio extenders. **(A)** Viability, acrosome integrity, and total and progressive motility. **(B)** Kinetic parameters. VCL, curvilinear velocity; VSL, linear velocity; VAP, mean velocity; LIN, linearity coefficient; STR, straightness coefficient; WOB, wobble coefficient; ALH, mean amplitude of lateral head displacement; BCF, frequency of head displacement. Each bar represents mean ± SD ($n = 14$). Paired *t*-test analysis; significant differences between two extenders (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$) are shown.

Botucrio extender was significantly lower than with the HM-0 extender was acrosome integrity ($p < 0.05$).

Finally, the freezability analysis (**Figure 3**) has shown that FI of the three parameters considered were significantly higher when the Botucrio extender rather than the HM-0 extender was used (viability: $p < 0.05$; total and progressive motility: $p < 0.0001$).

DISCUSSION

The results of the sperm quality analysis in the present study agree with those of other studies on Poitou jackasses performed in the Northern Hemisphere (9, 18–22, 30). These studies were done under similar conditions of animal number and age (26 animals and 4–7 years of age), but with higher repetitions than in the present study (24–150 ejaculates vs. 14 ejaculates). Despite these similarities, differences were observed in normal morphology and progressive motility values between our study and those mentioned above. The lower normal morphology observed in our study (77.3 vs. >90%) could be due to the criterion used to detect structural abnormalities despite the fact that other authors used the same dye technique (9, 18, 19). The progressive motility reported in our study (63.6%) was higher than that observed by Trimeche et al. (20, 21) (39–56%), but lower than that of the other studies (9, 18, 19, 30), which obtained progressive motility values over 75%. These dissimilarities may be related to differences in the extender composition (21), evaluation objectivity (9, 18), and CASA system configuration (9, 20, 30) used in each study. In addition to these results, this study reported new insights into the kinetic characteristics of Poitou donkey sperm. Thus, the sperm movement of these animals by CASA system showed high velocity and linearity indicators, with values of STR and LIN over 80 and 60%, respectively.

Enzymes, metabolites, and ions of seminal plasma are essential for sperm structure, metabolism, and fertilization. To the best of our knowledge, little information is available about the characterization of seminal parameters of Poitou donkeys. Here, we report a detailed analysis of seminal plasma of the only two pure Poitou sires existing in Chile. This analysis

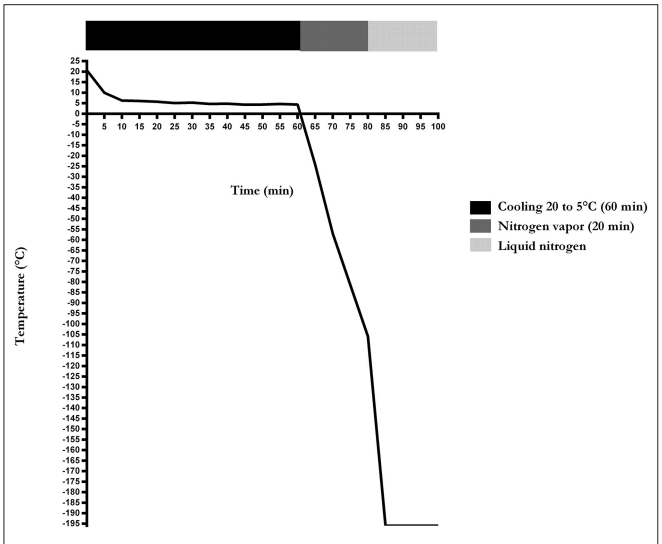


FIGURE 1 | Cooling and freezing rate of sperm cryopreservation of Poitou jackasses ($n = 14$).

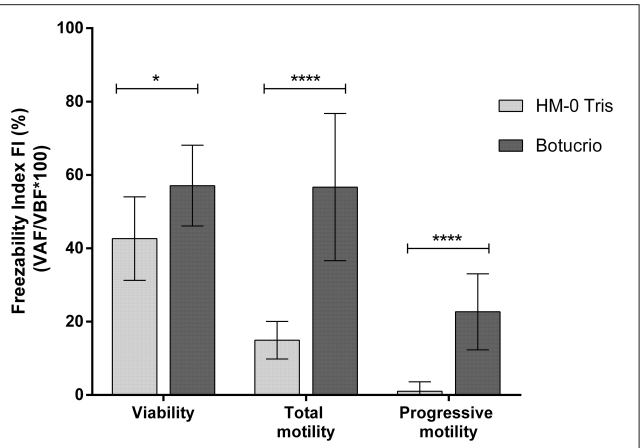


FIGURE 3 | Freezability index (FI) of viability and total motility in Poitou jackass sperm cryopreserved with Botucrio and HM-0 Tris extenders. Each bar represents mean ± SD ($n = 14$). Paired *t*-test analysis; significant differences between two extenders (*$p < 0.05$ and ****$p < 0.0001$) are shown.

included the evaluation of pH, osmolarity, three enzymes, seven ions, and five metabolites of Poitou donkeys aged 3 and 6 years.

The biochemical profile of ions and metabolites in the seminal plasma of the donkeys analyzed here is similar to that reported for this breed in the Northern Hemisphere, where 60–150 ejaculates of 5–6 Poitou jackasses were analyzed (9, 18). However, the results of total protein, total calcium, inorganic phosphorus, and potassium of seminal samples in this study were lower than those reported by these authors. These differences may be related to the geographic location and nutrition of the donkeys among the studies. It is well-known that donkeys have lower maintenance energy requirements than other equids. Donkeys come from semi-arid and mountainous environments with sparse food sources and their digestive tract has adapted to a high-fiber and low-energy diet (31). The cited authors conducted their experiments in India, but they did not specify the exact latitude localization or the nutrition protocol of the studied donkeys. The present study was done at latitude 39°47′32″S, 72°53′34″O in southern Chile, and the donkeys were fed mainly natural pasture and supplemented with alfalfa hay and corn grain twice daily. The soil in southern Chile has a volcanic composition (32), and heavy rainfall during the winter months reduces soil nutrients, such as calcium, phosphorus, and magnesium, which could be implicated in the lower seminal concentrations of these minerals (33, 34). Little investigation has been done to establish the mineral requirements of donkeys (31). Further studies are needed to determine whether the composition of southern Chilean soil affects donkey seminal quality since these animals have been reported to have lower mineral requirements than other equids (35).

Sodium (118.0–142.0 mmol/L) and chloride (123.3–133.8 mmol/L) were the main ions, and globulins (23.8–30.8 g/L) were the main metabolites in the seminal plasma of the donkeys. Also, low concentrations of glucose (0.0–0.2 mmol/L), cholesterol (0.1–0.3 mmol/L), zinc (18.1–23.7 μmol/L), and magnesium (1.6–2.8 mmol/L) were found. Zinc is a micromineral that plays an important role in many protective sperm properties, such as membrane stabilization, and antioxidant and antibacterial functions (36), and a testicular and epididymal origin has been suggested in Equidae (11, 37). Magnesium is a cation related to high seminal plasma quality in boars because it improves viability by reducing sperm membrane damage (38). A prostatic origin of magnesium has also been described in humans (39). Interestingly, to date, no studies have reported zinc and magnesium levels in the seminal plasma of Poitou jackasses. Thus, the present study reports the seminal concentrations of zinc and magnesium in this breed, where the zinc level was similar and the magnesium level was higher than those found in other domestic donkeys by Vyvial et al. (37).

There is little information about the enzymatic activity of seminal plasma in donkeys. The GGT is an important antioxidant in semen that protects sperm against oxidative damage. This enzyme has been correlated with high sperm viability and motility in fresh samples from stallions (11). In bull, seminal GGT has been correlated with high sperm motility, embryo cleavage, and blastocyst rate with post-thawed sperm (40). In humans, low seminal concentrations of seminal GGT have been related to infertility (41). The AST and ALP have been related to membrane sperm integrity in humans (42). In donkeys, AST and ALP are secreted from testis and epididymis, while GGT's origin is not clear because it does not correlate with volume or sperm concentration (37). In stallion, GGT has a testicular and epididymal origin, but since it is species-specific, this information may not apply to jackass (11). The results of our study revealed that the seminal plasma of both donkeys had active and variable ALP, AST, and GGT enzymatic levels, with ALP and GGT showing the highest and most variable activity, in agreement with

observations by Vyvial et al. (37) in seminal plasma of other domestic donkeys. In humans, these enzymes were reported to be significantly higher in seminal plasma than in serum, between 30 and 500 times higher (42), and these molecules might need to be highly concentrated in donkey seminal plasma, like other antioxidant enzymes, in order to protect the sperm (43). We have not found references of seminal AST or GGT concentrations for Poitou donkeys, but these values are apparently normal since AST and GGT levels (AST: 3.4–4.9 μkat/L, GGT: 187.1–386.7 μkat/L) are within the range reported for other domestic donkeys [AST: 0.7–5.1 and GGT: 72.7–1,853 μkat/L; (37)]. The ALP concentration found in this study (231.9–362.9 μkat/L) was significantly higher than that previously reported for Poitou jackasses (9, 18). This difference may be related to the sensitivity of the technique used for ALP detection by the authors because the ALP values obtained in this study were between the normal range reported for domestic donkeys [23.4–542.4 μkat/L; (37)]. ALP values are related to sperm concentration (11, 18) and considered a marker of ejaculation in stallions (17). Therefore, the high activity of ALP observed in both jackasses could be associated with the high sperm concentration, especially that obtained from jackass 1 ($552.9 \pm 122.8 \times 10^6$/ml). Further studies are necessary to determine the importance of these enzymes in the quality of fresh, cooled, and thawed donkey sperm samples and their relation to fertility.

Variations in semen quality between Poitou sires and within the same individual are expected (18). These differences might be influenced by individual variations or the animal age (44, 45). Male donkeys reach puberty between 19 and 20 months of age where sperm in ejaculates can be observed, although at this age they have not completed their full sexual maturation (46). Therefore, both jackasses evaluated in this study were post-pubertal (3 and 6 years old), but there may be differences in sexual maturation between them due to jackass age, as the 3-year-old animal had not reached full sexual maturation in comparison with the older animal. Nipken and Wrobler (47) observed in 5- to 6-year-old donkeys an increase in germ cell number per testis, spermiogenesis, tubular diameter, length and development, and epithelial efficiency relative to 3-year-old donkeys, and proposed that 6 years of age is the plateau of maximal testicular function. Further research in testicular size, testicular blood flow, and serum testosterone concentration is necessary to understand sexual differences among post-pubertal jackasses, as previously done in stallions (48, 49). The variability in sperm quality observed between the jackasses in this study was also noted by Kumar (29) in healthy and fertile donkeys of the same breed and age. This information could explain variations in sperm and seminal results between donkeys and could be considered normal since no differences between ejaculates or donkeys were observed in the post-thawed evaluation (data not shown; $p > 0.05$).

Animal genetic resources need to be preserved as part of sustainable management, especially in endangered breeds. Here, we show that Botucrio extender was superior to HM-0 extender in preserving donkey sperm characteristics based on the three freezability index obtained ($p < 0.05$). HM-0 is a simple Tris-based extender composed by glycerol (3%) and egg yolk (15%) maintained more than 40% of viability after the cryopreservation process. These percentage is 26% lower than the percentage obtained with Botucrio extender, which contains a complex composition of antioxidants and cryoprotectants, in addition to glycerol (1%) and egg yolk (5%) (50). Since the glycerol concentration in both extenders coincided to that reported for donkey semen cryopreservation (22), the sperm membrane protective effect of HM-0 Tris extender, could be influenced by high concentration of lecithin and the low density lipoproteins of the egg yolk (51), which improves the protection in the post-thawed sperm membrane in Poitou jacks (20) and in other donkey breeds (52, 53). As expected, post-thawed motility parameters and the FI of motility were significantly higher when using the Botucrio extender ($p < 0.05$). This extender contains amino acids, carbohydrates, and N-methylformamide, which improve sperm post-thawed motility (20, 22, 50, 54). Further studies are necessary to verify if changes in component concentrations (glycerol and egg yolk) and new additives (amides, carbohydrates and amino acids) on HM-0 formulation improves the freezability index (viability and sperm motility) of donkey sperm using a cryopreservation extender based on Tris-glycerol-egg yolk. Additionally, the freezability analysis revealed that Botucrio extender preserve post-thaw viability ($p < 0.05$) and total and progressive sperm motility ($p < 0.0001$) better than HM-0 Tris extender. This report provides new information to the knowledge of Poitou donkey sperm and cryopreservation in the Southern Hemisphere, which may help donkey breeding and conservation programs to develop strategies to improve the effectiveness of population management for this breed.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was reviewed and approved by Bioethics of Animals for Research (C#151-2014; UACh. Chile) and the Animal Welfare Law-Conicyt (Chile). Written informed consent and protocol of animal manipulation were obtained from Haras Militar Pupunahue (Chilean Army) for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

FE, OU, and AR-R contributed to conception and design of the study. FE, OU, and PS supported the assembly and development of methods and protocols for sampling and analysis of seminal quality parameters. FE organized the database, performed the statistical analysis, and wrote the first draft of the manuscript. AR-R contributed with formal analysis, funding acquisition, supervision, writing, review, and editing of final manuscript. All authors approved the submitted version.

FUNDING

This work was funded by Fondef-Conicyt [Grant D08I1076] and its publication was supported by the Graduate School of the Faculty of Veterinary Sciences and by the VIDCA (Universidad Austral de Chile).

ACKNOWLEDGMENTS

We would like to thank all the staff of Haras Militar Pupunahue (DGFER, Chilean Army), especially the military technicians for their cooperation in this research. We also thank Alejandra Scotti for editing the manuscript.

REFERENCES

1. Kugler W. *Added Value of Donkey Breeds in Europe. Project Report January 2013*. Saint Gallen, Germany (2013). Available online at: http://www.agrobiodiversity.net/topic_network/donkey/Donkey/Donkey-ReportEN.pdf
2. Camillo F, Rota A, Biagini L, Tesi M, Fanelli D, Panzani D. The current situation and trend of donkey industry in Europe. *J Equine Vet Sci.* (2018) 65:44–9. doi: 10.1016/j.jevs.2017.11.008
3. Kugler W, Grunenfelder HP, Broxham E. *Donkey Breeds in Europe*. St. Gallen: Monitoring Institute for Rare Breeds and Seeds in Europe (2008).
4. Food and Agriculture Organization of the United Nations. *Domestic Animal Diversity-Information System (FAO DAD-IS). Baudet du Poitou/France (Ass)*. Rome, Italy (2020). Available at: http://www.fao.org/dad-is/browse-by-country-and-species/es/
5. L'âne MC. *Toujours utilisé en maraîchage en France?* Bordeaux: Mémoire de fin d'études. diplôme d'Ingénieur diplômé de l'Institut upérieur d'Agriculture-Yncréa Hauts-de-France (2020).
6. Gicquel E, Boettcher P, Besbes B, Furre S, Fernández J, Danchin-Burge C, et al. Impact of conservation measures on demography and genetic variability of livestock breeds*. *Animal.* (2020) 14:670–80. doi: 10.1017/S1751731119002672
7. Food and Agriculture Organization Corporate Statistical Database (FAOSTAT). *Live Animals: Asses in World*. Rome, Italy (2021). Available online at: http://www.fao.org/faostat/en/#data/TA/visualize
8. Lagos J, Tadich TA. Hematological and biochemical reference intervals for mules in Chile. *Front Vet Sci.* (2019) 6:400. doi: 10.3389/fvets.2019.00400
9. Talluri TR, Mal G, Ravi SK. Biochemical components of seminal plasma and their correlation to the fresh seminal characteristics in Marwari stallions and Poitou jacks. *Vet World.* (2017) 10:214–20. doi: 10.14202/vetworld.2017.214-220
10. de Morais RN, Mucciolo RG, Viana WG. Biologia reprodutiva de jumentos. III. pH, osmolalidade e níveis de eletrólitos no sêmen. *Brazilian J Vet Res Anim Sci.* (1994) 31:145–51. doi: 10.11606/issn.1678-4456.bjvras.1994.52060
11. Pesch S, Bergmann M, Bostedt H. Determination of some enzymes and macro- and microelements in stallion seminal plasma and their correlations to semen quality. *Theriogenology.* (2006) 66:307–13. doi: 10.1016/j.theriogenology.2005.11.015
12. Pal Y, Legha RA, Tandon SN. Comparative assessment of seminal characteristics of horse and donkey stallions. *Indian J Anim Sci.* (2009) 79:1028–9.
13. Holt WV, Fazeli A. Sperm selection in the female mammalian reproductive tract. Focus on the oviduct: hypotheses, mechanisms, and new opportunities. *Theriogenology.* (2016) 85:105–12. doi: 10.1016/j.theriogenology.2015.07.019
14. Juyena NS, Stelletta C. Seminal plasma: an essential attribute to spermatozoa. *J Androl.* (2012) 33:536–51. doi: 10.2164/jandrol.110.012583
15. Fagundes B, Van Tilburg MF, Souza GV, Caiado J, Barreto MA, Silva JF. Effect of addition of concentrated proteins and seminal plasma low molecular weight proteins in freezing and thawing of equine semen. *Acta Biomédica Bras.* (2011) 2:7. doi: 10.18571/acbm.002
16. Al-Essawe EM, Wallgren M, Wulf M, Aurich C, Macías-García B, Sjunnesson Y, et al. Seminal plasma influences the fertilizing potential of cryopreserved stallion sperm. *Theriogenology.* (2018) 115:99–107. doi: 10.1016/j.theriogenology.2018.04.021
17. Turner RM, McDonnell SM. Alkaline phosphatase in stallion semen: characterization and clinical applications. *Theriogenology.* (2003) 60:1–10. doi: 10.1016/S0093-691X(02)00956-1
18. Gupta A, Singh R, Mamtal, Singhl M, Pall Y. Physical and biochemical studies in Jack's semen. *Ann Arid Zone.* (2008) 47:79–87.
19. Kumar R, Ravi S, Dholpuria S, Purohit G, Pushp MK, Solanki S, et al. Study on fresh semen characteristics in Poitou donkeys. *J Entomol Zool Stud.* (2018) 6:227–9. doi: 10.22271/j.ento.6.1.77.1
20. Trimeche A, Anton M, Renard P, Gandemer G, Tainturier D. Quail egg yolk: a novel cryoprotectant for the freeze preservation of Poitou Jackass sperm. *Cryobiology.* (1997) 34:385–93. doi: 10.1006/cryo.1997.2009
21. Trimeche A, Renard P, Le Lannou D, Barrière P, Tainturier D. Improvement of motility of post-thaw Poitou Jackass sperm using glutamine. *Theriogenology.* (1996) 45:1015–27. doi: 10.1016/0093-691X(96)00029-5
22. Trimeche A, Renard P, Tainturier D. A procedure for Poitou Jackass sperm cryopreservation. *Theriogenology.* (1998) 50:793–806. doi: 10.1016/S0093-691X(98)00184-8
23. Kumar R, Dholpuria S, Purohit GN, Ravi SK, Solanki S, Talluri TR. Additives effect of alpha-tocopherol, pentoxifylline and tau-rine on post thaw semen in Poitou donkeys. *Vet Pract.* (2018) 19:241–4.
24. Legha RA, Talluri TR, Pal Y. Effect of thawing temperature and duration on post- thaw seminal characteristics in exotic donkeys Effect of thawing temperature and duration on post-thaw seminal characteristics in exotic donkeys. *J Entomol Zool Stud.* (2020) 8:1124–6.
25. Walbornn SR, Love CC, Blanchard TL, Brinsko SP, Varner DD. The effect of dual-hemisphere breeding on stallion fertility. *Theriogenology.* (2017) 94:8–14. doi: 10.1016/j.theriogenology.2017.02.003
26. Murcia-Robayo RY, Jouanisson E, Beauchamp G, Diaw M. Effects of staining method and clinician experience on the evaluation of stallion sperm morphology. *Anim Reprod Sci.* (2018) 188:165–9. doi: 10.1016/j.anireprosci.2017.11.021
27. Córdova A, Strobel P, Vallejo A, Valenzuela P, Ulloa O, Burgos RA, et al. Use of hypometabolic TRIS extenders and high cooling rate refrigeration for cryopreservation of stallion sperm: presence and sensitivity of 5′ AMP-activated protein kinase (AMPK). *Cryobiology.* (2014) 69:473–81. doi: 10.1016/j.cryobiol.2014.10.008
28. Ramírez AR, Castro MA, Angulo C, Ramió L, Rivera MM, Torres M, et al. The presence and function of dopamine type 2 receptors in boar sperm: a possible role for dopamine in viability, capacitation, and modulation of sperm motility 1. *Biol Reprod.* (2009) 80:753–61. doi: 10.1095/biolreprod.108.070961
29. Kumar P, Kumar R, Mehta JS, Chaudhary AK, Ravi SK, Mehta SC, et al. Ameliorative Effect of Ascorbic Acid and Glutathione in Combating the Cryoinjuries During Cryopreservation of Exotic Jack Semen. *J Equine Vet Sci.* (2019) 81:102796. doi: 10.1016/j.jevs.2019.102796
30. Kumar P, Mehta JS, Ravi SK, Dedar RK, Purohit GN, Legha RA, et al. Cholesterol loaded cyclodextrin supplementation enhances the cholesterol-to-phospholipid ratio and diminishes oxidative stress in jack spermatozoa during cryopreservation. *J Equine Vet Sci.* (2020) 94:103237. doi: 10.1016/j.jevs.2020.103237
31. Burden FA, Bell N. Donkey nutrition and malnutrition. *Vet Clin North Am Equine Pract.* (2019) 35:469–79. doi: 10.1016/j.cveq.2019.08.004
32. Dec D, Dörner J, Balocchi O. Temporal and spatial variability of structure dependent properties of a volcanic ash soil under pasture in southern Chile. *Chil J Agric Res.* (2011) 71:293–303. doi: 10.4067/S0718-58392011000200015
33. Mora ML, Baeza G, Pizarro C, Demanet R. Effect of calcitic and dolomitic lime on physicochemical properties of a Chilean Andisol. *Commun Soil Sci Plant Anal.* (1999) 30:427–39. doi: 10.1080/00103629909370214
34. Mora ML, Alfaro MA, Jarvis SC, Demanet R, Cartes P. Soil aluminium availability in Andisols of southern Chile and its effect on forage

production and animal metabolism. *Soil Use Manag.* (2006) 22:95–101. doi: 10.1111/j.1475-2743.2006.00011.x
35. Bazzano M, McLean A, Tesei B, Gallina E, Laus F. Selenium and vitamin E concentrations in a healthy donkey population in Central Italy. *J Equine Vet Sci.* (2019) 78:112–6. doi: 10.1016/j.jevs.2019.04.003
36. Edström AML, Malm J, Frohm B, Martellini JA, Giwercman A, Mörgelin M, et al. The major bactericidal activity of human seminal plasma is zinc-dependent and derived from fragmentation of the semenogelins. *J Immunol.* (2008) 181:3413–21. doi: 10.4049/jimmunol.181.5.3413
37. Vyvial M, Horáčková E, Sedlinská M, Jánová E, Krisová Š, Mráčková M. Determination of selected components in seminal plasma of donkey stallions and their correlation to semen quality parameters. *Acta Vet Brno.* (2019) 88:377–84. doi: 10.2754/avb201988040377
38. López Rodríguez A, Rijsselaere T, Beek J, Vyt P, Van Soom A, Maes D. Boar seminal plasma components and their relation with semen quality. *Syst Biol Reprod Med.* (2013) 59:5–12. doi: 10.3109/19396368.2012.725120
39. Valsa J, Skandhan KP, Khan PS, Sumangala B, Gondalia M. BASIC SCIENCE split ejaculation study: semen parameters and calcium and magnesium in seminal plasma. *Cent Eur J Urol.* (2012) 65:216–8. doi: 10.5173/ceju.2012.04.art7
40. Pero ME, Lombardi P, Longobardi V, Boccia L, Vassalotti G, Zicarelli L, et al. Influence of γ-glutamyltransferase and alkaline phosphatase activity on *in vitro* fertilisation of bovine frozen/thawed semen. *Ital J Anim Sci.* (2017) 16:390–2. doi: 10.1080/1828051X.2017.1290509
41. Ai-LNaqeeb AA, Fakhrildin MBMR. Determination of serum and seminal Gamma Glutamyl transferase (GGT) on sperm parameter in infertile patients. *Indian J Public Heal Res Dev.* (2019) 10:1405. doi: 10.5958/0976-5506.2019.02644.5
42. Feng R-X, Lu J-C, Zhang H-Y, Lü N-Q. A pilot comparative study of 26 biochemical markers in seminal plasma and serum in infertile men. *Biomed Res Int.* (2015) 2015:1–7. doi: 10.1155/2015/805328
43. Papas M, Catalan J, Barranco I, Arroyo L, Bassols A, Yeste M, et al. Total and specific activities of superoxide dismutase (SOD) in seminal plasma are related with the cryotolerance of jackass spermatozoa. *Cryobiology.* (2020) 92:109–16. doi: 10.1016/j.cryobiol.2019.11.043
44. Dorado J, Acha D, Ortiz I, Gálvez MJ, Carrasco JJ, Díaz B, et al. Relationship between conventional semen characteristics, sperm motility patterns and fertility of Andalusian donkeys (*Equus asinus*). *Anim Reprod Sci.* (2013) 143:64–71. doi: 10.1016/j.anireprosci.2013.10.003
45. Atroshchenko MM, Kudlaeva AM, Fomina MA, Kalashnikov VV, Zaitcev AM, Denisova OV, et al. Analysis of seminal plasma biochemical parameters and sperm cryostability in different age groups of stallions. *IOP Conf Ser Earth Environ Sci.* (2019) 341:012162. doi: 10.1088/1755-1315/341/1/012162
46. Rota A, Puddu B, Sabatini C, Panzani D, Lainé A-L, Camillo F. Reproductive parameters of donkey jacks undergoing puberty. *Anim Reprod Sci.* (2018) 192:119–25. doi: 10.1016/j.anireprosci.2018.02.021
47. Nipken C, Wrobel KH. A quantitative morphological study of age-related changes in the donkey testis in the period between puberty and senium. *Andrologia.* (2009) 29:149–61. doi: 10.1111/j.1439-0272.1997.tb00310.x
48. Johnson L, Neaves WB. Age-related changes in the leydig cell population, seminiferous tubules, and sperm production in stallions 1. *Biol Reprod.* (1981) 24:703–12. doi: 10.1095/biolreprod24.3.703
49. Johnson L, Thompson DL. Age-related and seasonal variation in the sertoli cell population, daily sperm production and serum concentrations of follicle-stimulating hormone, luteinizing hormone and testosterone in stallions 1. *Biol Reprod.* (1983) 29:777–89. doi: 10.1095/biolreprod29.3.777
50. Samper JC, Garcia A. Post-thaw characteristics and fertility of stallion semen frozen in extenders with different cryoprotectants. *Anim Reprod Sci.* (2008) 107:348–9. doi: 10.1016/j.anireprosci.2008.05.125
51. Barbas JP, Mascarenhas RD. Cryopreservation of domestic animal sperm cells. *Cell Tissue Bank.* (2009) 10:49–62. doi: 10.1007/s10561-008-9081-4
52. 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
53. Zhang H, Ye H, Shao Y, Wu S, Yu J, Ji C, et al. The effects of egg yolk concentration and particle size on donkey semen preservation. *J Equine Vet Sci.* (2018) 65:19–24. doi: 10.1016/j.jevs.2018.03.002
54. Dorado J, Hidalgo M, Acha D, Ortiz I, Bottrel M, Azcona F, et al. Cryopreservation of Andalusian donkey (*Equus asinus*) spermatozoa: use of alternative energy sources in the freezing extender affects post-thaw sperm motility patterns but not DNA stability. *Anim Reprod Sci.* (2019) 208:106126. doi: 10.1016/j.anireprosci.2019.106126

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

REVIEW
published: 01 December 2021
doi: 10.3389/fvets.2021.669423

Pharmacologically Induced Ex Copula Ejaculation in Horses and Donkeys

Afroza Khanam[1], *Ayman A. Swelum*[2,3] *and Firdous A. Khan*[1]*

[1] Department of Large Animal Medicine and Surgery, School of Veterinary Medicine, St. George's University, True Blue, Grenada, [2] Department of Theriogenology, Faculty of Veterinary Medicine, Zagazig University, Zagazig, Egypt, [3] Department of Animal Production, College of Food and Agriculture Sciences, King Saud University, Riyadh, Saudi Arabia

Pharmacologically induced ex copula ejaculation is a method used for collection of semen when the traditional methods of semen collection are not feasible. Common indications for this method include health issues that either preclude the physical act of mating or result in impaired erection and ejaculation. The method also offers an alternative when there is a lack of equipment and facilities required for semen collection using the conventional artificial vagina method. A variety of pharmacological protocols for ex copula ejaculation in stallions have been evaluated in both research and clinical settings with no serious side-effects reported. In general, these protocols included tricyclic antidepressants, alpha-2 adrenergic receptor agonists, and smooth muscle stimulators, either individually or in combination. Although there is a lot of variation in the ejaculatory rates among studies, a combination of imipramine and xylazine appears to be an effective option for inducing ejaculation in stallions. In cases where this protocol is not successful, collections should be reattempted using a combination of imipramine, detomidine, and oxytocin. Regardless of the protocol used, a quiet environment with minimal disturbance is associated with a better outcome. In contrast to the body of literature available on pharmacologically induced ex copula ejaculation in horses, only a few studies have been published so far on this topic in donkeys. Further studies are warranted to investigate whether pharmacologically induced ex copula ejaculation is an effective method of semen collection in jacks and to compare different pharmacological protocols for inducing ejaculation in jacks.

Keywords: equine, imipramine, xylazine, detomidine, semen

OPEN ACCESS

Edited by:
Amy Katherine McLean, University of California, Davis, United States

Reviewed by:
Nei Moreira, Federal University of Paraná, Brazil
Jordi Miro, Universitat Autònoma de Barcelona, Spain
Sue M. McDonnell, University of Pennsylvania, United States

***Correspondence:**
Firdous A. Khan
fkhan8@sgu.edu

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

Received: *18 February 2021*
Accepted: *10 November 2021*
Published: *01 December 2021*

Citation:
Khanam A, Swelum AA and Khan FA (2021) Pharmacologically Induced Ex Copula Ejaculation in Horses and Donkeys. Front. Vet. Sci. 8:669423. doi: 10.3389/fvets.2021.669423

INTRODUCTION

Semen collection in horses is commonly performed using an artificial vagina with the stallion mounting either a jump mare or a phantom/dummy. Pharmacologically induced ex copula ejaculation is an alternative method of semen collection used in stallions with musculoskeletal, neurological, or behavioral abnormalities that preclude the physical act of mounting and copulation or interfere with erection and ejaculation. Documented clinical examples include stallions with lameness (1), disability due to multiple causes including chronic obstructive pulmonary disease, cardiac disease, and chronic musculoskeletal disease of the forelimbs (2), paraphimosis and erectile dysfunction (3), idiopathic ejaculatory dysfunction (4) and ejaculatory failure associated with

aortic-iliac thrombosis (5). Several research studies have been conducted in the past to evaluate different pharmacological protocols for inducing ejaculation in stallions and this topic continues to be an area of active research. In contrast to horses, pharmacologically induced ex copula ejaculation has not been investigated extensively in donkeys, and results from the few studies reported so far are inconsistent. The present article aims to review the published literature on pharmacologically induced ex copula ejaculation in horses and donkeys.

PHARMACOLOGICALLY INDUCED EX COPULA EJACULATION IN HORSES

A variety of drugs and their combinations have been used to induce ex copula ejaculation in horses. Broadly speaking, these drugs include tricyclic antidepressants, alpha-2 adrenergic receptor agonists, and smooth muscle stimulators. As discussed in the subsections below, several protocols have been evaluated with respect to their efficacy in inducing ejaculation in stallions.

Imipramine Alone Protocol

The initial attempts at ex copula ejaculation in stallions were based on the previously documented effects of antidepressants such as imipramine and clomipramine on sexual excitement and ejaculation in men (6, 7). These effects are attributed to imipramine- or clomipramine-mediated inhibition of norepinephrine reuptake, resulting in promotion of alpha-adrenergic activity (8). In a study involving five male horses (one inexperienced young stallion, two mature normal breeding stallions, one 5-year-old stallion with erection and ejaculatory dysfunction, and one long-term gelding), imipramine hydrochloride administered orally at a dose range of 100–600 mg twice a day resulted in repeated erection and masturbation (9). In the same study, the authors reported a similar response when imipramine was administered intravenously over a dose range of 50–1,000 mg. Moreover, the intravenous treatment resulted in ejaculation in 3 out of 28 treatment attempts (11% ejaculation rate) in the four intact stallions. The ejaculations were observed in one of the two mature stallions and the stallion with erection and ejaculatory dysfunction. No serious side effects were reported in either oral or intravenous imipramine treatments. However, higher doses were associated with mild ataxia and drowsiness. Apart from the study by McDonnell et al. (9), the use of imipramine alone for inducing ejaculation in stallions has not received any attention, presumably owing to the low ejaculation rate observed with this protocol.

Xylazine Alone Protocol

Xylazine is an α2-adrenergic agonist that mediates its effects mainly through stimulation of central α2-adrenergic receptors. This stimulation decreases the neurotransmission of norepinephrine and dopamine (10). The administration of xylazine alone for ex copula ejaculation in horses has been reported in both research (11, 12) and clinical settings (5). In a study involving 28 stallions (12 horses and 16 ponies), xylazine was administered intravenously at a dose rate of 0.7 mg/kg and the stallions were left undisturbed for 30 min (11). Each stallion underwent a trial with sexual prestimulation and a trial without sexual prestimulation. Ejaculation was reported in 15 (27%) of the 56 trials. Eleven of the ejaculations were reported with sexual prestimulation and four without sexual prestimulation, suggesting a beneficial effect of sexual prestimulation in this protocol. A much higher ejaculation rate [10 out of 11 attempts (91%)] after xylazine administration was reported in a case report involving two stallions with ejaculatory failure associated with aortic-iliac thrombosis (5). The authors attributed the higher ejaculation rate to relatively longer periods of prestimulation used in the clinical cases and to a speculated hypersensitivity to alpha adrenergic stimulation in stallions with ejaculatory dysfunction. In a more recent study involving 12 mature stallions (12), xylazine was administered intravenously at a dose rate of 0.7 mg/kg and each stallion underwent two trials. Ejaculation was reported in 4 of 24 trials (17%). Absence of sexual prestimulation might have contributed to the lower ejaculation rate in this study. Semen quality of the ejaculates induced by xylazine was reported to be mostly similar to the ejaculates collected from the stallions using artificial vagina (11, 12). Apart from mild to heavy standing sedation (11, 12), no other side effects were reported in any of the studies with this protocol.

Imipramine and Xylazine Combination Protocol

As discussed above, both imipramine and xylazine promote alpha-adrenergic activity. Several studies have investigated the effectiveness of combined treatment with imipramine and xylazine in causing ex copula ejaculation in stallions. In a study involving 8 sexually experienced pony stallions (13), imipramine hydrochloride was administered intravenously at a dose rate of 2 mg/kg followed 10 min later by intravenous injection of 0.3 mg/kg xylazine hydrochloride. In a total of 48 trials, each stallion underwent six treatment trials conducted at 4-day intervals and three of the six trials involved sexual prestimulation using an ovariectomized pony mare. The combined treatment resulted in erection, masturbation, and ejaculation in 34 (71%), 25 (52%), and 16 (33%) of the 48 trials. Out of the 16 ejaculations, six occurred within the 10 min after imipramine administration or before xylazine administration. Sexual prestimulation did not appear to have a beneficial effect. On the contrary, there was a tendency for a reduced likelihood of ejaculation in the trials that were preceded by sexual stimulation (25% with prestimulation vs. 42% without prestimulation).

In another study (14) using a combination of imipramine and xylazine in five sexually experienced pony stallions, ejaculation was observed in 9 (53%) out of 17 trials. A difference between this study and the previous one (13) was that xylazine was administered only if ejaculation did not occur within 60 min of imipramine administration. Seven ejaculations occurred within that timeframe and two ejaculations occurred after the administration of xylazine. Another difference was that the interval between trials in this study was shorter (2–3 days) than the interval (4 days) in the previous study. The authors suggested that the higher ejaculation rate may be attributed in part to lesser disturbance after treatment in the latter study.

Although imipramine is the most frequently used antidepressant for ex copula ejaculation in horses, clomipramine administered intravenously at a dose rate of 2.2 mg/kg followed 55 min later by xylazine (0.5 mg/kg intravenously) has also been reported to induce ejaculation in a stallion with fractured radius (1).

Oral administration of imipramine combined with intravenous administration of xylazine has also been tested in multiple studies. Johnston and DeLuca (15) in a study involving six stallions administered imipramine at a dose rate of 0.8–2.5 mg/kg followed 1–3 h later by intravenous injection of xylazine (0.3 mg/kg intravenously). Ejaculation was reported in six out of 14 trials (57%). In more recent studies, imipramine hydrochloride was administered orally at a dose rate of 3 mg/kg followed 2 h later by intravenous administration of xylazine hydrochloride at a dose rate of 0.7 mg/kg (12, 16). The former study (16) involved eight sexually mature stallions (six ponies and two horses). Each stallion underwent eight trials at intervals of 2–3 days. Ejaculation was reported in 44 of the 64 trials (68%). The latter study (12) involved 12 mature stallions and an additional half dose of xylazine was administered if ejaculation did not occur within 10 min of the first xylazine injection. Ejaculation was reported in seven of 24 trials (29%). An ejaculation rate of 33% (five out of 15 attempts) was reported after oral administration of imipramine hydrochloride (3 mg/kg) followed 2 h later by intravenous injection of xylazine (0.25 mg/kg) in a 20-year-old Quarter Horse with paraphimosis secondary to priapism (3).

In general, ejaculates collected using the imipramine and xylazine combination had higher sperm concentration, higher total sperm number, lower total volume and pH, and similar total and progressive motility when compared with the baseline in copula ejaculates from the stallions (13, 14, 16). Side effects reported with the protocol included sialorrhea (12), mild hemolysis (13), and mild to heavy standing sedation (12, 13).

Detomidine-Based Protocols

Similar to xylazine, detomidine is an alpha-adrenergic agonist that has been tested with respect to its effectiveness in inducing ejaculation in horses. In a study involving 12 mature stallions (12), detomidine administered alone (0.02 mg/kg intravenously) or 2 h after administration of imipramine (3 mg/kg orally) did not result in any ejaculations from 24 trails of each treatment (0%). However, when administered in combination with oxytocin (20 IU intravenously) or imipramine (3 mg/kg orally) and oxytocin (20 IU intravenously), ejaculation was reported in two out of 24 attempts (8.3%) and seven out of 24 trials (29%), respectively. The combination of imipramine, detomidine, and oxytocin was also reported to be effective in a 7-year-old Quarter Horse stallion with ejaculatory dysfunction (4). Detomidine alone was reported to be effective in causing ex copula ejaculations in a 4-year-old pony stallion (17). After sexual prestimulation, detomidine hydrochloride was administered (0.02 mg/kg intravenously) followed 5 min later by a second injection (0.01 mg/kg). The authors reported seven ejaculations in the stallion using this approach. This contrasts with the lack of ejaculation with detomidine alone reported by Cavalero et al. (12). The difference may be attributed to the sexual prestimulation used before detomidine administration by Rowley et al. (17).

Ejaculates collected using a combination of detomidine and oxytocin or a combination of imipramine, detomidine and oxytocin had lower volume and higher sperm concentration than in copula ejaculates. Apart from a mild standing sedation, no other side effects were reported during or after detomidine-induced ex copula ejaculation (12).

Prostaglandin F2alpha-Based Protocol

Prostaglandin F2alpha (PGF2α), a smooth muscle stimulator, has been tested in a study that involved eight pony stallions (18). Based on individual stallion titration trials, a dose range of 0.01–0.15 mg/kg intramuscularly resulted in ejaculation in 75% of the collection attempts. However, this protocol is not used commonly due to the side effects associated with PGF2α treatment (abdominal cramping, sweating, and urine dripping), resulting in discomfort to the stallion and increased chances of urine contamination of the semen (16).

PHARMACOLOGICALLY INDUCED EX COPULA EJACULATION IN DONKEYS

Semen collection in jacks (male donkeys) is performed mainly for artificial insemination of jennies or mares either immediately after the collection or after cooling or cryopreservation of the collected semen. Apart from being an important part of the breeding soundness evaluation, semen collection aids in diagnosis and prevention of venereal diseases (19). Jacks can be collected using an artificial vagina, similar to stallions (20). However, compared to stallions, jacks are more difficult to train to mount a phantom because of a high latency to erection and mounting (21). Therefore, pharmacologically induced ex copula ejaculation could be a useful alternative to traditional methods of semen collection in jacks. To date, only four studies have investigated ex copula ejaculation in jacks (19, 22–24). The pharmacological regimens used in these studies to induce ejaculation included imipramine, butorphanol, xylazine, and detomidine.

Using oral administration of imipramine hydrochloride (3 mg/kg) followed 2 h later by intravenous injection of xylazine hydrochloride (1.1 mg/kg) in five jacks, Naoman and Ali (23) reported 29 ejaculates from 30 trials (96.6%). The collected ejaculates were of good quality and the interval from xylazine injection to ejaculation ranged from 5 to 10 min to 1 h, presumably due to biological variation and different arousal levels of the animals. In contrast to the high success rate reported by Naoman and Ali (23), extremely poor responses were reported in three other studies (19, 22, 24). Sghiri et al. (22) reported ejaculation in only one out of 55 jacks (1.8%) despite using combinations of different imipramine (2 or 3 mg/kg) and xylazine (0.44 or 0.66 or 0.70 mg/kg) dosages, and different time intervals (1 or 2 h) between imipramine and xylazine administration. Mráčková et al. (19) reported ejaculation in zero out of 10 jacks (0%) using xylazine hydrochloride (0.66 mg/kg intravenously), and in two out of 10 jacks (20%) using detomidine

hydrochloride (0.02 mg/kg intramuscularly) followed 15 min later by its half dose (0.01 mg/kg intramuscularly) in cases where the first dose did not result in ejaculation. In another study by the same group (24), a combination of imipramine (3 mg/kg orally) and xylazine (0.66 mg/kg intravenously), and a combination of butorphanol (0.02 mg/kg intravenously) and xylazine (0.33 mg/kg intravenously) were tested. However, ejaculation was reported in zero out of nine jacks (0%) with each of the two combinations. The differences in ejaculation rate between the studies might be attributed to a relatively higher dose of xylazine and younger age of the jacks (2–4 years) in the study by Naoman and Ali (23). Since the jacks in that study were housed under more controlled conditions and used in pilot trials before the actual study, it can also be presumed that the donkeys were more acclimatized and less stressed, which could have contributed to the higher ejaculation rate. Although PGF2α has not been tested for ex copula ejaculation in jacks, it has been shown to hasten the process of in copula semen collection by reducing the intervals to erection and ejaculation (25).

CONCLUSIONS

Although pharmacologically induced ex copula ejaculation is a practical method for semen collection in stallions, a considerable variation in the ejaculatory rate has been reported between different protocols. There is also a significant individual variation between stallions. Some stallions respond to protocols involving xylazine while others respond to protocols involving detomidine. Therefore, multiple trials using different protocols should be performed in cases of failure to obtain semen in clinical situations. While the effects of prestimulation on ejaculation rate are inconsistent between protocols, a quiet environment with minimal disturbance during the pharmacological induction generally has a positive effect on the ejaculation rate. The comparable semen quality to in-copula ejaculations and the absence of serious side effects makes the pharmacologically induced approach a good alternative for stallions from which semen collection is not possible using the traditional approach. In contrast to the body of literature available on pharmacologically induced ex copula ejaculation in horses, there is very limited information on this topic in donkeys. Due to the limited information and inconsistent results observed in the few studies that have been conducted so far, it would be too early to determine if pharmacologically induced ex copula ejaculation is an effective method of semen collection in jacks. Further studies are warranted to evaluate and compare different pharmacological protocols for inducing ejaculation in jacks. Lastly, it might be worth exploring whether ex copula ejaculation can be used as an alternative method for semen collection in wild equids and other wild animal species.

AUTHOR CONTRIBUTIONS

AK and FK conceptualized the article. AK searched literature and wrote the first draft of the section on horses. AS searched literature and wrote the first draft of the section on donkeys. FK edited the drafts and prepared the final version of the manuscript. All authors contributed to the article and approved the submitted version.

ACKNOWLEDGMENTS

The authors are thankful to their universities for the time and resources used in preparation of the manuscript.

REFERENCES

1. Turner RM, McDonnell SM, Hawkins JF. Use of pharmacologically induced ejaculation to obtain semen from a stallion with a fractured radius. *J Am Vet Med Assoc.* (1995) 206:1906–8.
2. Card CE, Manning ST, Bowman P, Leibel T. Pregnancies from imipramine and xylazine-induced ex copula ejaculation in a disabled stallion. *Can Vet J.* (1997) 38:171–4.
3. Feary DJ, Moffett PD, Bruemmer JE, Southwood L, McCue P, Niswender KD, et al. Chemical ejaculation and cryopreservation of semen from a breeding stallion with paraphimosis secondary to priapism and hemorrhagic colitis. *Equine Vet Educ.* (2010) 17:299–304. doi: 10.1111/j.2042-3292.2005.tb00396.x
4. Cavalero TMS, Segabinazzi LGTM, Scheeren VFC, Canuto LEF, Gobato MLM, Papa FF. Alternative protocol using imipramine, detomidine, and oxytocin for semen collection in stallion with ejaculatory dysfunction. *J Equine Vet Sci.* (2020) 93:103205. doi: 10.1016/j.jevs.2020.103205
5. McDonnell SM, Love CC, Martin BB, Reef VB, Kenney RM. Ejaculatory failure associated with aortic-iliac thrombosis in two stallions. *J Am Vet Med Assoc.* (1992) 200:954–7.
6. Beaumont G. Sexual side-effects of clomipramine (Anafranil). *J Int Med Res.* (1977) 5:37–44.
7. Petrie WM. Sexual effects of antidepressents and psychomotor stimulant drugs. *Mod Probl Pharmacopsychiatry.* (1980) 15:77–90. doi: 10.1159/000402337
8. McDonnell SM. Ejaculation. Physiology and dysfunction. *Vet Clin North Am Equine Pract.* (1992) 8:57–70. doi: 10.1016/S0749-0739(17)30466-2
9. McDonnell SM, Garcia MC, Kenney RM, Van Arsdalen KN. Imipramine-induced erection, masturbation, and ejaculation in male horses. *Pharmacol Biochem Behav.* (1987) 27:187–91. doi: 10.1016/0091-3057(87)90495-3
10. Shi XX, Yin BS, Yang P, Chen H, Li X, Su LX, et al. Xylazine activates adenosine monophosphate-activated protein kinase pathway in the central nervous system of rats. *PLoS ONE.* (2016) 11:e0153169. doi: 10.1371/journal.pone.0153169
11. McDonnell SM, Love CC. Xylazine-induced ex copula ejaculation in stallions. *Theriogenology.* (1991) 36:73–6. doi: 10.1016/0093-691X(91)90435-G
12. Cavalero TMS, Papa FO, Schmith RA, Scheeren VFC, Canuto LEF, Gobato MLM, et al. Protocols using detomidine and oxytocin induce ex copula ejaculation in stallions. *Theriogenology.* (2019) 140:93–8. doi: 10.1016/j.theriogenology.2019.08.024
13. McDonnell SM, Odian MJ. Imipramine and xylazine-induced ex copula ejaculation in stallions. *Theriogenology.* (1994) 41:1005–10. doi: 10.1016/S0093-691X(05)80023-8
14. McDonnell SM, Oristaglio Turner RM. Post-thaw motility and longevity of motility of imipramine-induced ejaculates of pony stallions. *Theriogenology.* (1994) 42:475–81. doi: 10.1016/0093-691X(94)90685-C
15. Johnston PF, DeLuca JL. Chemical ejaculation of stallions after administration of oral imipramine followed by intravenous xylazine. *Proc Am Assoc Equine Pract.* (1998) 43:59–62.
16. McDonnell SM. Oral imipramine and intravenous xylazine for pharmacologically-induced ex copula ejaculation in stallions. *Anim Reprod Sci.* (2001) 68:153–9. doi: 10.1016/S0378-4320(01)00152-X

17. Rowley DD, Lock TF, Shipley CF. Fertility of detomidine HCl-induced ex copula-ejaculated stallion semen. *Proc Am Assoc Equine Pract.* (1999) 45:221–3.
18. McDonell SM. Pharmacologically induced ex copula ejaculation in stallions. *Proc 12th Int Cong Anim Reprod.* (1991) 1894–6.
19. Mráckova M, Markéta Sedlinská, Zdenka Blahová. The reliability of two different protocols for pharmacologically induced ejaculation in donkeys (*Equus asinus*). *J Equine Vet Sci.* (2013) 33:1121–3. doi: 10.1016/j.jevs.2013.04.014
20. Canisso IF, Panzani D, Miró J, Ellerbrock RE. Key aspects of donkey and mule reproduction. *Vet Clin North Am Equine Pract.* (2019) 35:607–42. doi: 10.1016/j.cveq.2019.08.014
21. Canisso IF, Davies Morel MCG, McDonnell S. Strategies for the management of donkey jacks in intensive breeding systems. *Equine Vet Educ.* (2009) 21:652–9. doi: 10.2746/095777309X479058
22. Sghiri A, Tibary A, El Idrissi R. Behavioral response to imipramine/xylazine treatment in jackass. In: Bakkoury M, Dakkak A, editors. *Proceedings of the 9th International Congress of World Equine Veterinary Association.* Marrakech (2006). p. 348–50.
23. Naoman UT, Ali AJ. Oral imipramine and intravenous xylazine for pharmacologically-induced ejaculation in donkeys. *Iraqi J Vet Sci.* (2012) 26:81–3. doi: 10.33899/ijvs.2012.67461
24. Mráčková M, Hodinová K, Vyvial M, Horáčková E, Sedlinská, M. Failure of pharmacologically - induced ejaculation in donkeys (Equus Asinus) under field conditions: a test of two different treatment protocols. *Israel J Vet Med.* (2017) 72:35–8.
25. Panzani D, Quaresma M, Fanelli D, Camillo F, Moroni R, Rota A, et al. Hastening time to ejaculation in donkey jacks treated with the PGF2α analog, cloprostenol sodium. *Animals.* (2020) 10:2231. doi: 10.3390/ani10122231

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.

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