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Pain assessment and management in veterinary medicine

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Published in
Frontiers in Veterinary Science

ISSN 1664-8714
ISBN 978-2-8325-5954-3
DOI 10.3389/978-2-8325-5954-3

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Pain assessment and management in veterinary medicine

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Citation

Di Bella, C., Dmitrović, P., Mirra, A., Pennasilico, L., eds. (2025). *Pain assessment and management in veterinary medicine*. Lausanne: Frontiers Media SA.
doi: 10.3389/978-2-8325-5954-3

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frontiers | Frontiers in Veterinary Science

TYPE Editorial
PUBLISHED 17 January 2025
DOI 10.3389/fvets.2024.1549243

OPEN ACCESS

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RECEIVED 20 December 2024
ACCEPTED 30 December 2024
PUBLISHED 17 January 2025

CITATION
Di Bella C, Dmitrović P, Mirra A and Pennasilico L (2025) Editorial: Pain assessment and management in veterinary medicine. *Front. Vet. Sci.* 11:1549243. doi: 10.3389/fvets.2024.1549243

Editorial: Pain assessment and management in veterinary medicine

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KEYWORDS

acute pain, chronic pain, pain diagnosis, pain treatment, veterinary medicine

Editorial on the Research Topic
Pain assessment and management in veterinary medicine

Despite the complexity of identifying and treating painful states in companion animals, several studies have been conducted to explore the topic and provide knowledge to properly manage pain in the veterinary field (1). This Research Topic has collected 10 scientific articles related to the assessment and management of pain in different animal species.

Casas-Alvarado et al. evaluated the nociceptive response to CBD alone and in combination with meloxicam in dogs undergoing ovariohysterectomy, using the technique of infrared pupillometry. In this study, a double result was obtained: (1) the evaluation of nociception by infrared pupillometry could be of valuable aid in dogs affected by acute pain; (2) CBD provides good postoperative analgesia both alone and with the anti-inflammatory, representing a good therapeutic alternative. The pupillometry method for the evaluation of acute pain has also been studied in equine species by Mascaró Triedo et al. in order to evaluate the painful response to nose twitching in horses. In agreement with the previous study, the authors state that the subjects showed pupillary dilation following the manipulation described above. However, one aspect to consider is that the modification of pupil diameter could be influenced by the administration of analgesic drugs such as acepromazine, which promotes pupil dilation, or romifidine, which, instead, inhibits it. To date, there are different pain recognition methods and, among those most described in the literature, the Grimace scales stand out. Chiavaccini et al. published a narrative review that provides an overview of animal pain recognition technologies, starting from the classic Grimace Scales that, although valid, have limitations related to the subjectivity of the assessments and the need for basic operator training, up to the description of automatic pain recognition (APR) based on artificial intelligence. These technologies, thanks to the analysis of facial expressions, body language, vocalizations and physiological parameters, allow to obtain complete data on the patient's pain status and offer a promising progress in veterinary field.

One area of veterinary medicine where pain recognition and management are fundamental is animal experimentation. Ensuring good analgesia in experimental subjects is ethically essential (2). Petrucci et al. evaluated the usefulness of the Nociceptive Withdrawal Reflex (NWR) to assess antinociception following

spinal analgesia (morphine and ropivacaine) in pigs undergoing veno-arterial extracorporeal membrane oxygenation (VA-ECMO). Nociceptive withdrawal reflex thresholds increased significantly following spinal injection, and an effect was seen on average up to 6 h. This study supports the usefulness of the NWR for evaluating antinociception following spinal analgesia in experimental pigs, especially if the evaluation of cardiovascular variables are not reliable, as during VA-ECMO.

Equally complex is the assessment of chronic pain. In dogs, diagnostic capabilities have improved with the introduction of Owner-Reported Outcome Measures. Among these, the Liverpool Osteoarthritis in Dog (LOAD) is widely used. It is a questionnaire to be submitted to the owners of dogs with osteoarthritis (OA). One limitation is that LOAD is distributed in English. The aim of the study by Olcoz et al. was to develop a version of the LOAD in Spanish, equivalent to the English one. This scientific production promotes the use of the LOAD by Spanish-speaking veterinarians, researchers and owners, improving the assessment of chronic OA pain in dogs.

Regarding the treatment of acute and chronic pain, new research has been introduced in both small and large animals with the common goal of improving the quality of life of our pets. Wickstead et al., retrospectively analyzed the incidence of complications related to the application of a wound infusion catheter (WIC) in horses undergoing partial ostectomy of the thoracolumbar vertebral processes, with the aim of documenting whether the presence of the catheter could induce negative events in the post-operative period. The extrapolated results confirm the absence of correlation between the application of the WIC and the development of infections and secondary complications. Based on this, the authors encourage further scientific research on the topic as it could represent a valid tool for the management of pain induced by spinal surgery in equine species. A study on the application of loco-regional analgesia in large animals was also carried out by Interlandi et al., who evaluated the efficacy of the local application of butorphanol alone and in association with lidocaine in calves undergoing umbilical hernia repair. The study demonstrated that both protocols are safe and valid in ensuring good intra- and post-operative analgesia in this species, reducing pain and stress, and increasing animal welfare.

Pain treatments in dogs are largely studied, especially in the field of orthopedic pain. Galosi et al., evaluated the synergistic efficacy of Magnesium Sulfate ($MgSO_4$) and Ketamine in dogs undergoing TPLO. The aim was to evaluate whether $MgSO_4$, acting in potentiating synergism with Ketamine, could provide better perioperative analgesia. The results showed that the ketamine/$MgSO_4$ association resulted in a lower rescue analgesia request and opioid consumption. This protocol could represent a useful analgesic support in dogs affected by orthopedic acute pain.

Instead, regarding the management of chronic pain from orthopedic pathology, such as osteoarthritis, different analgesic protocols have been studied in dogs. A valid example is the study conducted by Enomoto et al., who evaluated the efficacy of the association grapiprant/fish oil/exercise in young dogs affected by OA, for a period of 4 months, demonstrating that the multimodal treatment guaranteed a significant clinical benefit to the treated subjects. Similarly, Kampa et al., compared the efficacy of green-lipped mussel/krill oil, meloxicam, *Biota orientalis* extracts and a placebo (sunflower oil) for 6 weeks in young dogs affected by OA. Results showed a significant clinical benefit in patients that received the anti-inflammatory or the green-lipped mussel/krill oil compared to the other two groups. Data obtained could be significant in defining a suitable multimodal therapy that includes the synergism between multiple molecules.

In conclusion, the results of studies and reviews mentioned include interesting findings on diagnosis and treatment of pain in small and large animals, that contribute to a constant improvement in our clinical and experimental activity.

Author contributions

CD: Writing – original draft. PD: Writing – review & editing. AM: Writing – review & editing. LP: Writing – review & editing.

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

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frontiers | Frontiers in Veterinary Science

TYPE Original Research
PUBLISHED 20 February 2024
DOI 10.3389/fvets.2024.1360926

OPEN ACCESS

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RECEIVED 24 December 2023
ACCEPTED 29 January 2024
PUBLISHED 20 February 2024

CITATION
Olcoz M, Cabezas MÁ and Gómez de Segura IA (2024) Translation and linguistic validation into Spanish of the Owner-Reported Outcome Measure "Liverpool Osteoarthritis in Dogs". *Front. Vet. Sci.* 11:1360926. doi: 10.3389/fvets.2024.1360926

Translation and linguistic validation into Spanish of the Owner-Reported Outcome Measure "Liverpool Osteoarthritis in Dogs"

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Introduction: Assessing chronic pain in dogs has been greatly favoured by the development of Owner-Reported Outcome Measures. Among them, the Liverpool Osteoarthritis in Dogs (LOAD) has been widely used for this purpose. Most of these tools have been written in English and its use by non English natives requires not only translation but also linguistic validation for use by veterinarians and owners. For its use, the LOAD has not undergone translation into Spanish and the objective was to generate a linguistically validated Spanish translation of the LOAD.

Methods: Following the World Health Organisation and the International Society for Pharmacoeconomics and Outcomes Research published guidelines, the original LOAD English version underwent analysis and translation by two native linguists proficient in the target language. Both translations were then reviewed by a third native linguist to identify potential disparities and establish a cohesive translation (reconciliation). Subsequently, an independent linguist, fluent in both English and the target language, conducted the back translation. Finally, the research team compared the original and back translated versions to pinpoint and resolve any significant differences. Following the creation of the translated version, a cognitive debriefing was conducted to assess the questionnaire within the target population.

Results: A total of 89 surveys were distributed to dog owners of varying ages, genders, and socioeconomic backgrounds. Although there were some suggestions and comments, and some adjustments were made, all respondents found the survey to be clear, achieving a linguistic validation of the Spanish LOAD.

KEYWORDS

chronic pain, pain assessment, osteoarthritis, questionnaire, dog, Owner-Reported Outcome Measures

1 Introduction

Osteoarthritis (OA) is the most commonly diagnosed joint disease in veterinary medicine, it is estimated that 20–37% of dogs aged >1 year are affected (1–3). It has a significant negative impact on the well-being and quality of life of patients, characterised by reduced mobility, alterations in activity patterns, changes in behaviour, and considerable healthcare costs (4, 5). Assessing activity and pain in chronic diseases like canine osteoarthritis is challenging because it progresses slowly, and individual effects can be relatively small (6). Additionally, associated clinical signs are more subtle, intermittent, and often have a slow onset, resulting in gradual behaviour changes (7).

For proper osteoarthritis management, it is essential that veterinarians have the appropriate tools to assess and monitor the disease progression in each patient, choose the most appropriate treatments, and study their effectiveness. Various Owner-Reported Outcome Measures (OROMs) have been developed for this purpose, measuring pain and difficulty in performing daily activities (4). These OROMs (also known as Clinical Metrology Instruments) are derived from those used in human medicine, where information is directly collected from the patient (symptoms, health-related quality of life, or functional status) (8). They include sequences of questions or items scored based on the observer's observations or experiences, typically the owner (5).

A relevant OROM among these is the Liverpool Osteoarthritis in Dogs (LOAD) scale. Initially developed to assess dogs with elbow osteoarthritis (6), it has also proven to be useful for assessment of OA in other joints (5, 9–11). LOAD consists of 23 questions, with three related to patient history, seven to lifestyle, and 13 to mobility, evaluating the impact of joint diseases on the patient's daily activity. It provides a "LOAD score" indicating the presence and severity of the patient's joint disease (Mild, 0–10; Moderate, 11–20; Severe, 21–30; Extreme, 31–52).

LOAD was originally written in English but for its global implementation in clinical practise and accessibility to all veterinarians and owners, translation into different languages is mandatory. Linguistic validation is the process by which the cultural adequacy and conceptual equivalence of translated elements are assessed to ensure that the content validity of the original element is not affected by translation (12). This reduces the risk of data invalidity resulting from incorrect translation and ensures that variations in population responses are attributable to genuine differences rather than discrepancies caused by inappropriate data collection methods (13).

LOAD has been translated and psychometrically validated to Portuguese (4) but has not been translated nor validated to Spanish. Therefore, the aim of this study was to develop a conceptually equivalent and culturally relevant version of LOAD for use in Spanish. The main hypothesis was that a valid translation into Spanish (spoken in Spain; hereafter considered as Spanish in the article) of LOAD, culturally and conceptually equivalent to the original English version, could be generated.

2 Materials and methods

Following authorisation from the developer (Dr. John Innes), the translation of the original version of LOAD into Spanish was carried out following the guidelines and recommendations established by the World Health Organisation (WHO), the International Society for Pharmacoeconomics and Outcomes Research (ISPOR) and Beaton et.al (12, 14–16). The selected translators for this task had previously worked on translating another OROM related to osteoarthritis in dogs. Given their experience in the subject matter, it was deemed unnecessary to provide them with detailed information or recommendations on how to carry out the translation.

Initially, two native speakers of the target language (Spanish) independently performed the direct translation (from English to Spanish). One of them was a veterinary professional with technical knowledge and familiarity with LOAD, while the other was an individual with no background in health sciences. Since the LOAD is a questionnaire directed at pet owners without technical knowledge, it was decided to include a non-veterinarian to perform one of the direct translations. Subsequently, a third Spanish linguist and a veterinary professional compared the two direct translations to identify any discrepancies to create a unified direct translation (reconciliation).

In the next step, an independent linguist proficient in native-level English and fluent in the target language drafted the back translation, i.e., translated the unified document in Spanish back to the original language (U.S. English).

Following this, the research team, along with one of the involved linguists, conducted a thorough review to examine and resolve any discrepancies between the direct translation into Spanish, the back translation into English, and the original document. This process also ensured the clarity of wording and translation concepts. If necessary, in case of significant differences or identification of comprehension issues in the target language, a new translation would be undertaken.

Once the translation process was completed, a cognitive analysis was performed to assess the questionnaire in the target population. The questionnaire employed in the study was approved by the institutional ethics committee (Ref: CE_20230511-02_SAL, 11 May 2023). Although the WHO and ISPOR recommend a minimum of five individuals to conduct this cognitive assessment up to 10 individuals per section, a total of 72 surveys were conducted with dog owners of different ages, genders, and socioeconomic characteristics to ensure representation of the target population. They were provided with a questionnaire containing a brief description of the LOAD and the objective of this study. Participants were asked to read the translated version of LOAD, indicate if each question and answer option was clearly understood, and suggest possible alternative responses (phrases or words) that they believed would facilitate comprehension of the question.

Finally, the research team evaluated all the questionnaires to determine if any modifications were necessary and, thus, to ultimately obtain a definitive translation of LOAD in Spanish.

As several modifications were made after the cognitive analysis, it was decided to repeat this analysis with a smaller number of participants ($n = 17$) to check whether the changes made posed any understanding issues.

3 Results

The process of translation and linguistic validation, along with cognitive analysis, allowed the development of a version of LOAD translated into Spanish that is conceptually equivalent to the original English version.

3.1 Translation

The direct or independent translations performed by the two native linguists were very similar, although there were 18 discrepancies between both versions. In the following step, a third native Spanish linguist compared and unified both versions to

create a unified translation, selecting terms from the translation that were deemed more appropriate (considering the original version) or using different terms that were more similar to the original document. For example, in Question 1 of the Lifestyle section, "In the last week, on average, how far has your dog exercised each day?" the response options were written in miles (0–0.06 miles, 0.6–1.2 miles, etc.) in the original version. However, since distance is measured in kilometres in Spain according to the International System of Units, it was decided to convert miles to kilometres (0–1 km, 1–2 km,...), rounding to the nearest figure (0.6 miles = 1 km). Also for this question, the independent translators proposed "*En la última semana, de media, ¿cuánto ejercicio ha hecho su perro cada día*?" but the researchers ultimately decided to translate it as "*En la última semana, de promedio, ¿cuánta distancia ha recorrido su perro cada día*?" as it was considered to make more sense, given that the answers refer to the distance the dog covers during exercise, not the amount of exercise done. Annex I shows all the differences between both independent translations and the unified translation.

In the back-translation or reverse translation step, 57 discrepancies were identified compared to the original version. The research team analysed all discrepancies between the unified translation, the back-translation, and the original version. In case of persisting discrepancies, consultation with the native English-speaking linguist was done. Some changes were considered irrelevant as they were synonyms and could be used interchangeably. For example, the term "*correa*" was back-translated as "lead," while in the original version, it was "leash." Another example is the term "*aceptable*," translated as "acceptable," corresponding to the original term "fair." In other cases, the back-translation did not exactly match the original version, but differences were very subtle and did not alter the statement's meaning. For example, in Question 3 in the Background section, the back-translation was "If you can, make a list of the medications your dog is taking..." while the original document states "If you can, please list any medications that your pet is currently receiving..." In some cases, a decision was made to make a modification based on the back-translation, such as the response option for Question 5 in the Lifestyle section "Over rough ground," which was translated as "*Sobre terreno accidentado*" and back-translated as "Broken ground." It was decided to change it to "*Sobre terreno irregular*" based on this difference between the back-translation and the original version, although finally changed to "*terreno accidentado*" based on the cognitive debriefing (see below). In total, out of 57 differences, only 17 terms or sentences were modified after comparing the back-translation with the original version and observing any significant differences. Annex II shows all the differences between the original version and the back-translation, as well as the modifications made to the final translated version.

3.2 Cognitive debriefing

Once the translation process was completed, a total of 72 dog owners were surveyed to assess the readability and understanding of the LOAD translated into Spanish. Participants were categorised by age, gender, and education level. Of these 72 individuals, 32 were men and 40 were women. The age ranges included were: ≤ 29 years (n = 9), 30–39 years (n = 15), 40–49 years (n = 15), 50–59 years (n = 23), ≥60 years (n = 10). The education level considered included primary school (n = 6), secondary school (n = 10), high school (n = 17), and postgraduate studies (n = 39). Overall, all participants understood without difficulty each element of the LOAD and its response options. Nevertheless, a total of 11 individuals suggested changing some sentences or words to make the reading more straightforward.

Nine participants had difficulty with Question 3: "*¿Cómo hace el ejercicio?*" ("What type of exercise is this?") regarding the response option "*Trabajando*" ("Working"), indicating that they did not understand this concept. The research team decided to add a comment in the final translation: "*Trabajando (perro de trabajo)*" [Working (working dog)] to explain that the physical activity performed was as a working dog. Other alternatives, such as "*perro de asistencia*," "*perro de apoyo*," or "*perro de servicio*" were not considered, as these activities are focused on specific tasks, such as assisting people with disabilities. In addition, three respondents indicated that they did not fully understand this question, not knowing whether it referred to previous Questions 1 ("… how far has your dog exercised each day?") and 2 ("… how many walks has your dog had each day?") or to another specific activity. Another respondent commented that it closely resembled Question 6. "*Durante el ejercicio, ¿cómo lleva a su perro?*" ("At exercise, how is your dog handled?") and did not understand the differences between both questions, suggesting that they should be integrated into one. It was decided to rewrite this question as "*¿Qué tipo de actividad es esta?*" ("What type of activity is this?") because it does refer to the previous questions. Additionally, the term "*ejercicio*" ("exercise") was replaced with "*actividad*" ("activity") since both concepts are synonymous, and in this case, the question is related to the dog's daily activity, not just when exercising.

There was a suggestion to change Question 6 in the Lifestyle section "*Durante el ejercicio, ¿cómo lleva a su perro*?" ("At exercise, how is your dog handled?") to "*Durante el ejercicio, ¿cómo va su perro?*" indicating a grammatical inconsistency in the Spanish translation between the question's subject (the owner) and the response options' subject (the dog). In other words, the question asks how the owner leads their dog (with a leash, without a leash, etc.), but the answers refer to how the dog goes (walks, trots, etc.). The research team confirmed this grammatical discrepancy and decided to modify the question to "*Cuando hace ejercicio, ¿cómo va su perro?*" ("When exercising, how does your dog do?").

One participant indicated that he found the answer to question 5 "*Sobre terreno irregular*" confusing, as the other options (In the forest, in the street...) can also be irregular. The research team decided to re-translate it as "*Sobre terreno accidentado*," to better differentiate the response options and to indicate that this is a rough and complicated terrain.

Two participants proposed that, in Questions 1 "*¿Cómo es la movilidad de su perro en general?*" ("How is your dog's mobility in general?") and 6 "*Cuando hace ejercicio, ¿cómo de activo es su perro?*" ("At exercise, how active is your dog?"), despite correctly understanding the response options, they would change "*Pobre*" and "*Muy pobre*" to "*Mala*" and "*Muy mala*," respectively. Further to this, another participant indicated that did not understand the terms "*Pobre*" and "*Muy pobre*," finding them subjective. The

research team decided to change the terms "*Pobre*" and "*Muy pobre*" to "*Mala*" and "*Muy mala*" both in Question 1 and Question 6, as in Spanish, "*Pobre*" and "*Muy pobre*" are usually used as adjectives to indicate something humble or scarce in economic terms, while "*Mala*" and "*Muy mala*" are adjectives used to indicate a negative value. One of these same participants also suggested that in the response options for Questions 5 "*¿Hasta qué grado su perro muestra rigidez en la extremidad afectada después de estar tumbado?*" ("To what degree does your dog show stiffness in the affected leg after a "lie down"? ") and 12 "*¿Hasta qué grado su perro muestra rigidez en la extremidad afectada después de haber estado tumbado tras el ejercicio?*" ("To what degree does your dog show stiffness in the affected leg after a "lie down" following exercise?"), should be changed from "*Rigidez severa*" ("Severe stiffness") and "*Rigidez extrema*" ("Extreme stiffness") to "*Rigidez grave*" ("Serious stiffness") and "*Rigidez muy grave*" ("Very serious stiffness"), respectively. In this case, in the end it was decided to modify only the option "*Rigidez severa*" to "*Rigidez grave*," since although they are very similar terms and it is not a problem of understanding, grammatically it is more correct to use the adjective "*grave*" in this context than "*severa*" (harsh in treatment or punishment, or rigid in the observance of a rule). Another participant suggested modifying the answer options "*No muy activo*" in question 6 "At exercise, how active is your dog" and "*No muy interesado*" in question 7 "How interested is your dog in exercising" to "*Poco activo*" and "*Poco interesado*," respectively. The researchers accepted this modification because, although there were no problems of understanding, both options are correct and mean the same thing, but in Spanish this grammatical construction ("*Poco interesado*" and "*Poco activo*") is more common than the use of "*No muy activo*" and "*No muy interesado*," which would make them easier to read.

In total, 12 questions and answers were modified after the cognitive analysis. Annex III shows all comments and suggestions from the cognitive analysis, as well as the modifications made.

Once a revised version was produced based on the previous results, a second cognitive analysis including 17 participants was performed. Of these individuals, 10 were women and 7 were men. The age ranges included were: $\leq$ 29 years (n = 2), 30–39 years (n = 5), 40–49 years (n = 3), 50–59 years (n = 4), $\geq$60 years (n = 3). The education level considered included primary school (n = 0), secondary school (n = 1), high school (n = 2), and postgraduate studies (n = 14). In general, all of them understood all the questions and answer options, although there were two participants who suggested some changes in terms of modifying a question to make it more understandable. These changes were discarded as they did not pose a problem of understanding. One of them commented that he could not see any relationship between question 3 of the lifestyle section: "*¿Qué tipo de actividad es esta*?" (What type of exercise is this?) with the answer options: "*Siempre con correa*," "*Casi siempre con correa*," "*Casi siempre sin correa*," "*Siempre sin correa*," and "*Trabajando (perro de trabajo)*" and that she would rephrase the question as "*¿Cómo pasea usted a su perro*? (How you walk your dog?)." This question already raised understanding issues in the first cognitive analysis and had been modified previously. Considering all the comments and despite the fact that the back-translation matched the original version, the research team decided to rewrite the question to "*¿Cómo hace esta actividad*?" Although it differed from the English version (the English translation would be: "How does he/her do this activity?"), this small modification had to be made because if it were translated literally, it would be confusing in Spanish. The Spanish translation of LOAD can be downloaded at https://assets.elanco.com/0cec44ed-3eaa-0009-2029-666567e7e4de/2f12e790-db29-46cc-bed6-4c914f776af9/Spanish_LOAD_24.pdf.

4 Discussion

A translation and linguistic validation of the LOAD into Spanish has been produced. The LOAD is an OROM originally written in English, with only a validated translation into Portuguese (4). The translation has been cognitively tested by owners and the necessary adjustments to the test were made. To implement this tool globally and make it accessible to all veterinarians, translation into different languages, including Spanish, is crucial. This allows its use by a much broader community of dog owners while maintaining the conceptual integrity of the original version.

The observed discrepancies between translations and back-translations mainly focused on choosing a more suitable term that captured the concept or nuance of the original. For example, the term "fair" has various meanings, and within the context of other responses, "*aceptable*" (acceptable) fits better. Another example is the translation of the term "poor"' which lacks the moral connotation of "bad" when translated into Spanish, this moral connotation does not always apply, and the use of "*malo*" better reproduces the original concept, preventing a confusing translation through a mere transliteration. Overall discrepancies were relatively few and easy to review; suggesting the original survey in English was straightforward and easily understandable. More discrepancies were observed in the back translation. However, the English native linguist was not a veterinarian and thus unlikely to use exactly the same technical terms or sentences, although most discrepancies were irrelevant.

A high number of responses were gathered from owners from different gender, ages, and cultural background. Reported doubts or suggestions in the cognitive evaluation were minimal, which was expected as the survey posed short and relatively simple questions, along with straightforward response options. One translation drew attention from several respondents, likely due to being an uncommon term in Spanish. Specifically, the term "working" ("*trabajando*") does not usually apply to dogs engaged in professional activities, and lead to comprehension doubts among respondents in the cognitive evaluation. In this case, and not having identified an easily, unique, understandable equivalent term, it was considered to add a brief explanation ("working dogs" or "*perros de trabajo*"). This simple explanation facilitated understandability from these same respondents afterwards. Perhaps an example of these activities would have been more intuitive, but in this case, a more faithful translation to the original was preferred, avoiding expanding the explanation. Another question that proved to be confusing was number 3 in the lifestyle section, "*¿Cómo hace esta actividad?*" ("What type of exercise is this?"). It had to be translated again several times as several participants did not understand the connexion with other previous questions and did not see any connexion between the question and the answer options.

The research team has opted to rephrase the content from the original version in a manner that ensures it is easily comprehensible to the owners. It was not considered that this would affect the results or significantly modify the questionnaire, since, although the question does not match exactly and is not a literal translation, the meaning and sense of the question is the same as in the English version. It should be noted that the current translation has been performed to meet Spain's cultural requirements. Therefore, minor adjustments may be considered to cope with different Spanish-speaking countries like those in South and Central America, but also in North America. This may include not only the use of country-specific words but also additional adjustments such as the use of miles instead of kilometres.

To achieve a linguistically validated version of the LOAD, recommendations and guidelines published (12, 14–16) were followed. Two native Spanish-speaking linguists were selected for direct translations, one with technical knowledge and familiarity with health sciences, while the other had no training on health sciences. Although guidelines recommend that direct translations should be carried out by healthcare professionals, a second non-technical linguist was included by the research team, considering better reflected the average pet owner in clinical practise. While this decision may have interfered with the translation process, it was considered that the unified or reconciliation translation by veterinarians, has not hindered or prevented a reliable translation of the LOAD into Spanish. In the Portuguese translation of the LOAD (4), a team of veterinarians performed an independent translation without considering a linguist with no medical background. Another limitation of this study is that all participants were from a specific geographic area (Madrid province), and participants from other areas of Spain were not included, potentially introducing sociocultural variables into the results.

Translation guidelines do not specify the characteristics of linguists needed for translations (they should be native speakers of the target language). The linguists in this study did not have specific qualifications or training, which could have influenced the results by overlooking inconsistencies or grammatical errors in the translation. This could have been avoided by including a Spanish or English philologist. On the other hand, the use of a relatively technical yet simple language of OA in dogs suggests that this factor might have less relevance.

5 Conclusion

In conclusion, this study provides a linguistically validated version of the LOAD in Spanish, promoting its use by Spanish-speaking veterinarians and researchers for the assessment and management of chronic pain in dogs. The next step will be to conduct psychometric validation of the Spanish translation of the LOAD to ensure greater reliability and validity.

Data availability statement

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

Ethics statement

The studies involving humans were approved by Research Ethics and Biosafety Committees Universidad Complutense de Madrid (Ref: CE_20230511-02_SAL, 11 May 2023). The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

MO: Writing – original draft, Writing – review & editing. MC: Writing – review & editing, Conceptualisation. IG: Writing – review & editing, Conceptualisation.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This research received external funding from Elanco for supporting open access for this article.

Acknowledgments

Authors would like to thank the University of Liverpool, John Innes, and Elanco for authorising the production of this work, linguists participating in the process of translation and subsequent revisions, and participants who anonymously completed the surveys for the cognitive debriefing.

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

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2024.1360926/full#supplementary-material

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frontiers | Frontiers in Veterinary Science

TYPE Original Research
PUBLISHED 04 July 2024
DOI 10.3389/fvets.2024.1412755

OPEN ACCESS

EDITED BY
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RECEIVED 05 April 2024
ACCEPTED 26 June 2024
PUBLISHED 04 July 2024

CITATION
Mascaró Triedo CE, Karar S, Abunemeh M and Portier K (2024) Effect of nose twitching on the pupillary dilation in awake and anesthetized horses.
Front. Vet. Sci. 11:1412755.
doi: 10.3389/fvets.2024.1412755

Effect of nose twitching on the pupillary dilation in awake and anesthetized horses

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Pupillometry is used in humans to monitor pain, nociception and analgesia. This single-center, non-randomized, non-blinded intervention trial, evaluated the effect of nose twitching on the pupil size in awake, sedated, and anesthetized horses. Pupil height (H) and length (L) were measured before (Be) and after (Af) nose twitching in fourteen non-painful adult awake horses (T0). The percentage of variation (PSV) was calculated (PSVTn=[(TnAf-TnBe)/TnBe]*100). Measurements were repeated (Tn) after acepromazine (0.04mgkg^{-1} IV) (T1), romifidine (0.04mgkg^{-1} IV) (T2), morphine (0.1mgkg^{-1} IV) (T3), after anesthesia induction with diazepam (0.05mgkg^{-1} IV) and ketamine (2.2mgkg^{-1} IV), at the time the horse was placed on the operating table (T4) and when the expiratory fraction of sevoflurane was 2% (T5). HAf vs. HBe, LAf vs. LBe as well as PSVH vs. PSVL at each time were compared with a Mann–Whitney Wilcoxon test. The PSVL and PSVH, as well as HBe and LBe over time were compared with the Skillings-Mack test followed by a Wilcoxon test for paired data to make pairwise comparisons (Tn+1 vs. Tn). In non-sedated horses (T0), the application of the nose twitch induced a significant increase in pupil length (LT0Be: 17.09 [16.05; 19.67] mm versus LT0Af: 19.52 [18.74; 21.40]) mm (p=0.004). Thirty minutes after acepromazine administration (T1), nose twitching induced a significant increase in pupil length (LT1Be: 16.45 [14.80; 18.66] mm versus LT1Af 18.31 [17.20; 20.52] mm) (p=0.016) and height (HT1Be: 8.44 [5.68; 12.04] mm versus HT1Af: 11.09 [7.97; 14.3] mm) (p<0.001). PSVHT1 was significantly greater than PSVLT1 (p=0.025). PSVH was higher at T1 than at T0 (p=0.04). It was also significantly higher at T1 than at T2 (p<0.001). Romifidine induced mydriasis (HT2Be 16.95 [14.73; 18.77] mm versus HT1Be 8.44 [5.68; 12.04] mm) (p<0,001) (LT2Be 19.66 [18.45; 20.41] mm versus LT1Be 16.45 [14.80; 18.66] mm) (p<0.001). The results suggest that nose twitching induced a pupillary dilation in the awake horse. This effect was potentiated after the administration of acepromazine but disappeared after the administration of romifidine.

KEYWORDS

horse, pain assessment, anesthesia, analgesia, pupillometry, pupillary dilation

1 Introduction

Pain can only be expressed by individuals who are alert and able to speak. It is therefore difficult to measure in babies, debilitated and anesthetized individuals. In animals, pain must be assessed by humans, which leads to a bias in interpretation due to the sensitivity of the observer (1).

The subjectivity of pain perception highlights the need for objective tools to assess it, particularly in non-communicative subjects. Pain scales based on behavior, physiological parameters or facial expression have been developed, but remain subject to the subjective interpretation of the observer (1).

Devices have been developed to more objectively measure conscious and unconscious pain (nociception) in humans and animals. They record physiological variables, which are then integrated by algorithms to improve the objectivity of pain measurement (2).

Among these techniques, pupillometry has recently been shown to be an objective and reliable monitor of the level of antinociception in humans, allowing opioid administration to be reduced (2, 3). Pupillometry has also been used to monitor pain in awake patients (4–6).

Pupillometry has many advantages. It is non-invasive, easy to apply, inexpensive to purchase, it does not require advanced analyses, and is reproducible. In addition, data can be obtained in real-time (7).

The principle is based on the fact that painful stimulation reliably causes pupil dilation. This mydriasis occurs simultaneously with changes in the balance of the autonomic nervous system. The regulation of pupillary diameter results from the action of antagonistic muscles (the sphincter muscle and the dilator muscle) present in the iris. The former is modulated by the cholinergic fibers of the parasympathetic system and the latter by the adrenergic fibers of the sympathetic system (8–10).

Furthermore, the amplitude of the pupillary dilatation reflex (PDR) is proportional to the intensity of the nociceptive stimuli. This can be observed in awake and anesthetized patients (3–5). However, some drugs can affect the PDR because they alter the pupil response to a painful stimulus (9, 11). For example, opioids increase parasympathetic activity, which causes contraction of the circular sphincter of the iris and thus induces miosis in humans. In the event of a painful or nociceptive stimulus, they prevent the transmission of the nociceptive signal, thereby inhibiting the PDR (3, 11, 12). Among anesthetic agents, alpha-2-agonists prevent mydriasis in dogs by inhibiting the action of the iris dilator muscle (13, 14). However, alpha-2 agonists may induce mydriasis in several animal species, by activation of alpha-1 receptors in the dilator muscle, although they are not very specific. The sensitivity of the receptors varies according to the species (there are also receptors on the sphincter muscle, in smaller quantities) (11, 15). Systemic ketamine and atropine have a mydriatic effect (16).

To our knowledge, pupil dilation in reaction to a painful/nociceptive stimulus has not yet been studied in animals. In horses, pupil size measurement can be performed under general anesthesia because the eye remains in a central position. Pupillometry could be of particular interest in adapting intraoperative analgesia in this species. Indeed, pain or excessive analgesics administration such as opioids, can have deleterious effects, such as gastro-intestinal ileus (17–19) and can lead to poor recovery, which is the period most at risk during anesthesia in horses (20).

This study aimed to evaluate the effect of the application of a nose twitch (considered as a painful stimulus) to the horse's upper lip, on pupil size in awake and anesthetized horses. We hypothesized that this stimulus induces pupil dilation in awake horses, but that this dilation is affected by some molecules usually included in general anesthesia protocols.

2 Materials and methods

This single-center, non-randomized, unblinded experimental study was carried out at the equine clinic Clinequine (VetAgro Sup's equine university hospital). The study was approved by the Ethical Committee of the National Veterinary School of Lyon (Number 2234, April 12th, 2022). Written consent was obtained from each owner before their horse was included in the study.

2.1 Animals

The target population was horses over 2 years of age, free of acute or chronic pain, admitted to the hospital for elective surgeries under general anesthesia between 1 August 2022 and 30 June 2023. Horses had to be free of ocular disease and not medicated at the time of admission. Horses were required to behave in such a way as to allow the application of the nose twitch to the upper lip without risk to either the horse or the handlers (Figure 1).

The study was carried out in awake horses and at each stage of the general anesthesia protocol. The pupil size was measured with a pupillometer previously modified to fit the horse's eye (AlgiScan®, IDMed, Marseille, France). For each evaluation, two measurements were taken. The first measurement (Be) was taken before the nose twitch was applied. The nose twitch was then applied to the horse's upper lip and the string rotated until it stopped, and a second measurement (Af) was taken immediately. The nose twitch was then removed. These two measurements were compared to assess whether the application of the nose twitch caused a variation in pupil size. The same nose twitch was used in the same way by the same person for all the horses included in the study. Also, for each measurement, a photo of the pupillometer's screen was taken so that a blinded external assistant could check the measurement.

To assess the influence of the anesthetics and analgesics used during the anesthesia protocol on pupil size, the evaluation previously described was repeated after the administration and onset of action of each molecule. Each horse, measured awake, was its control during the study. For each horse, the same eye was measured during the study. To facilitate access to the eye, the left eye was preferentially chosen because the right one was near the wall of the induction box to assist lying down, which made the picture difficult. Horses undergoing surgery requiring left lateral decubitus on the operating table were excluded.

2.2 Procedure

For each horse, six time points (Tn with $n=0$ to 5) were observed, each representing two pupil measurements: before (TnBe) and after (TnAf) the nose twitch was applied (Figure 2).

The values measured for height (H) and length (L), before (Be) and after (Af) the application of the nose twitch at each stage of anesthesia (Tn) were used to calculate a percentage in pupil size variation (PSV) in length (PSVLTn) and in height (PSVHTn). The PSV was calculated, for example for the height H, according to the following formula:

$$PSVHTn = \frac{HTnAf - HTnBe}{HTnBe} \times 100$$

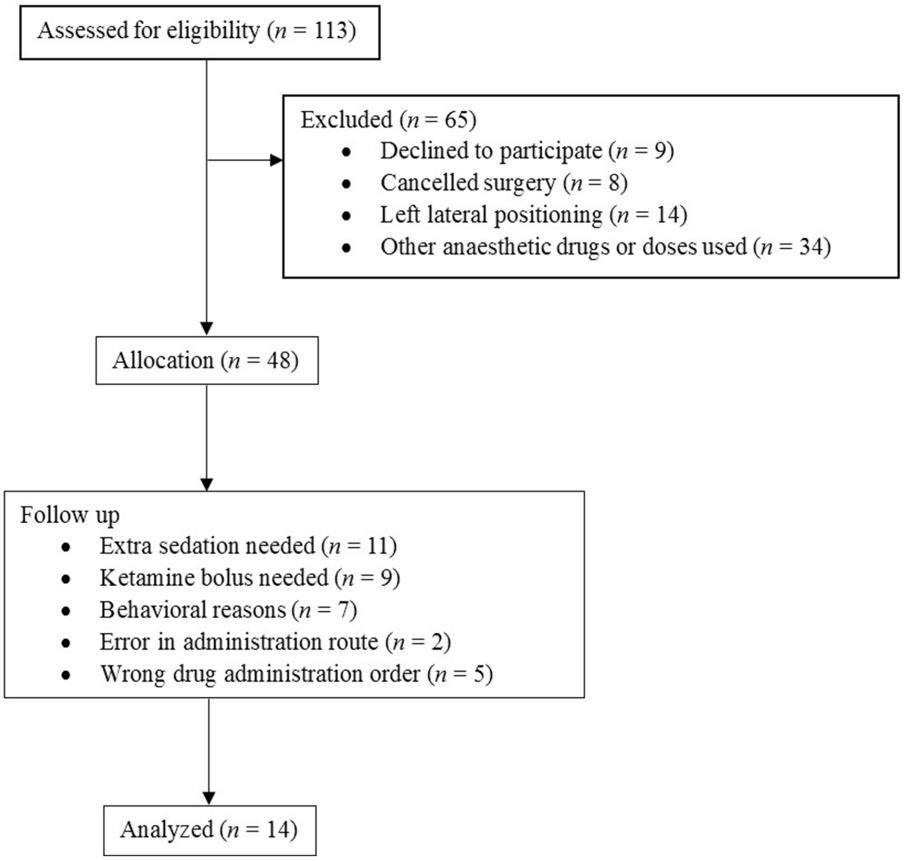

FIGURE 1
Flow diagram. Inclusion and exclusion flow diagram of 113 horses presented into the veterinary hospital for elective surgery.

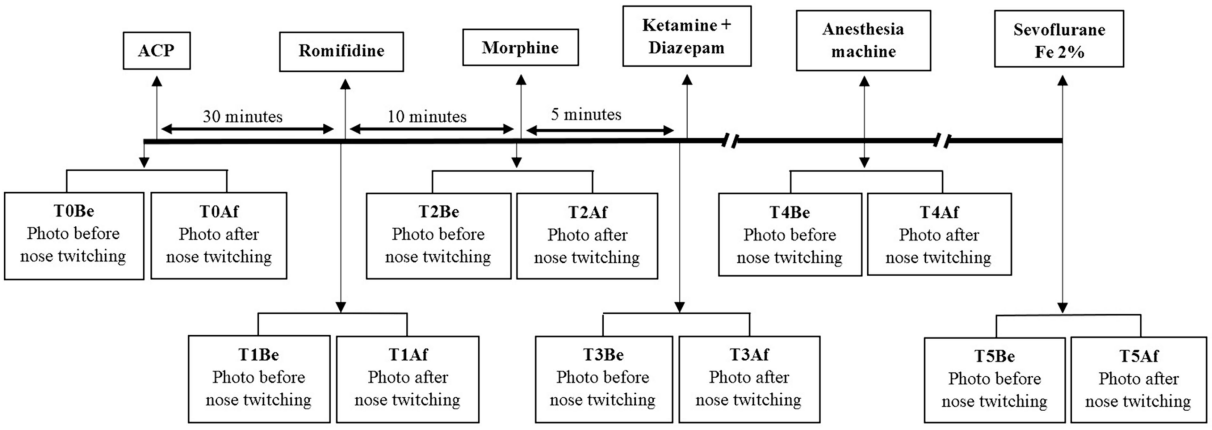

FIGURE 2
Study design. Study timeline of 14 horses included in the study showing the interventions. A first measure (T0) was taken. Premedication with acepromazine (ACP) 40 μg kg^{-1} IV was done and 30 min later, a second measure was taken (T1). Then, romifidine 40 μg kg^{-1} IV was administered and 10 min later, a third measure was taken (T2). After that, morphine 0.1 mg kg^{-1} IV was administered and 5 min later, a fourth measure was taken (T3). Then, the anesthesia induction was done with the administration of ketamine 2.2 mg kg^{-1} IV and diazepam 0.05 mg kg^{-1} IV. Once the horse was connected to the anesthetic machine, a fifth measure was done (T4). After stabilization of a sevoflurane expired fraction of 2%, a last measure was taken (T5).

A 14-gauge catheter (BD Angiocath) was placed in the jugular vein for every horse enrolled in the study. An initial assessment was carried out on the awake horse (T0) in its hospital stall. Each horse was then premedicated with intravenous (IV) acepromazine (0.04 mg kg^{-1}). Thirty minutes later, the horses were taken to the induction stall and the pupillary dilation (PD) evaluation was performed (T1). The horses were then

sedated with intravenous romifidine (0.04 mg kg^{-1}). Ten minutes later, the PD was assessed again (T2). Morphine (0.1 mg kg^{-1} IV) was then slowly administered. The PD was assessed again 5 min after morphine administration (T3). Anesthesia was induced with diazepam (0.05 mg kg^{-1} IV) and ketamine (2.2 mg kg^{-1} IV). Once the horses were lying down, the trachea was intubated and the animals were placed on a padded table and connected to an anesthesia machine in the operating room. As soon as the horse was connected to the anesthesia machine, a new PD assessment was performed (T4).

Mechanical lung ventilation was started immediately (6 breaths per minute, at a tidal volume of 10 mL kg^{-1} and a maximum inspiratory pressure of 30 cmH_2O) and adjusted during anesthesia to maintain a partial pressure of mean expired carbon dioxide (P_ECO_2) between 35 and 45 mmHg. Sevoflurane (in 100% oxygen) was used to maintain anesthesia on a surgical plane. The inspired fraction of sevoflurane (FiSevo) was adjusted during a stabilization period until the expiratory fraction of sevoflurane (FeSevo) reached 2%. At this point, a final PD assessment was performed (T5). As soon as the last PD measurement was made, a continuous constant rate infusion of romifidine (0.04 mg kg^{-1} h^{-1}) was administered concomitantly with the sevoflurane. Doses of ketamine (0.5 mg kg^{-1} IV) were administered if the animal showed spontaneous movement or nystagmus. Horses that required a ketamine bolus during the PD measurements were excluded from the study. Ringer lactate was administered intravenously during anesthesia at a rate of 10 mL kg^{-1} h^{-1}.

Anesthetic monitoring included heart rate (HR) and rhythm, invasive blood pressure (IBP) measured using a catheter placed in the facial artery, respiratory rate (RR), tidal volume (Vt), pulse oximetry, P_ECO_2, FeSevo and FiSevo. Depth of anesthesia was monitored by assessment of clinical signs and absence of spontaneous movement. After the last measurement, dobutamine could be administered intravenously at a rate of 2 to 8 µg kg^{-1} min^{-1} using a syringe pump to maintain mean arterial pressure around 60 mmHg to 70 mmHg. Arterial blood gases were analyzed every hour. At the end of the procedure, the animals were disconnected from the respiratory circuit, transferred to the recovery stall, and placed in lateral decubitus with the lower forelimb pulled forward. Recovery was unassisted. Romifidine (0.02 mg kg^{-1} IV) was administered during recovery if necessary.

2.3 Statistical analysis

Statistical analyses were performed using R software (version 2023.06.1-524). The normality of our data was tested using the distribution of differences for each pair of data (before and after the application of the nose twitch for each time Tn). The data did not follow a normal distribution and were treated by non-parametric tests. Differences were considered statistically significant if $p < 0.05$. The figures are presented in the form of the median and the interquartile range [Q1; Q3].

A power calculation was performed to calculate the minimum sample size required to have an 80% chance of identifying a 25% variation in pupil height and a 15% variation in pupil length. This calculation indicated that 14 horses were required.

For each time Tn, the sizes before and after the application of the nose twitch and the percentages of pupil size variation (PSV) in height and length, were compared using the Mann–Whitney Wilcoxon test for paired data.

To compare the effect of the molecules on the pupil size, we compared PSV in height (PSVHTn) and in length (PSVLTn) for each of the 6 times (T0-5) with the Skillings-Mack test, which is an equivalent of the Friedman test that can be used on a sample with missing or equal data (21). We then used the Mann–Whitney Wilcoxon test for paired data to make pairwise comparisons.

3 Results

The results are presented in Table 1.

Fourteen horses were finally recruited (Figure 1). They were referred to the hospital for various elective surgeries: castration, arthroscopy, electrochemotherapy, bursoscopy, removal of fragments of osteochondritis dissecans, or bone sequestration. The cohort of horses consisted of four mares, three geldings, and seven stallions and had a mean age of 5.6 (±3.7) years.

3.1 Effect of the nose twitch at each measurement time

Before application of the nose twitch and administration of any molecule, the pupil was 17.09 [16.05; 19.67] mm in length (LT0Be) and 11.73 [9.22; 13.91] mm in height (HT0Be).

In non-sedated horses (T0), the application of the nose twitch induced a significant increase in pupil length ($p = 0.004$), while pupil height increased non-significantly ($p = 0.14$). PSVLT0 and PSVHT0 were not significantly different ($p = 0.36$).

TABLE 1 Pupil height (H) and length (L) measured in mm, before (Be) and after (Af) nose twitching.

		T0	T1	T2	T3	T4	T5
H	Be (mm)	11.73 [9.22; 13.91]	8.44 [5.68; 12.04]	16.95 [14.73; 18.77]	17.52 [16.02; 19.98]	16.80 [16.45; 17.80]	16.02 [15.20; 18.63]
	Af (mm)	12.09 [10.47; 14.52]	11.09 [7.97; 14.3]*	17.73 [15.30; 19.13]	17.16 [15.80; 19.59]	17.30 [15.84; 18.16]	17.09 [14.80; 18.63]
	PSV (%)	6.48 [−5.98; 19.02]	38.79 [16.01; 47.09] $^{\$}$	3.24 [0.2; 9.82]$^{\$}$	1.02 [−0.48; 6.68]	−0.47 [−3.75; 5.37]	0.71 [−3.65; 6.16]
L	Be (mm)	17.09 [16.05; 19.67]	16.45 [14.80; 18.66]	19.66 [18.45; 20.41]	19.09 [18.02; 20.99]	17.88 [17.31; 19.45]	17.88 [16.30; 19.27]
	Af (mm)	19.52 [18.74; 21.40]*	18.31 [17.20; 20.52]*	20.31 [19.34; 21.17]	19.88 [19.20; 20.63]	17.80 [17.02; 18.95]	18.16 [16.48; 20.02]
	PSV (%)	12.49 [3.23; 17.67]$^{\#}$	9.63 [4.88; 16.21] $^{\#}$	3.38 [0.72; 7.8]	0.82 [−2.88; 3.62]	−1.19 [−4.46; 2.34]$^{\$}$	2.91 [−6.06; 7.4] $^{\$}$

Percentage in variation in pupil size (PSV) on awake horse (T0), 30 min after acepromazine administration (T1), after romifidine administration (T2), after morphine administration (T3), after ketamine and diazepam administration (T4) and during maintenance of anesthésia with an expired fraction of sevoflurane maintained at 2% (T5). Results are expressed as median and interquartile range [Q1-Q3]. *Significant difference between before and after nose twitching at Tn for H or L. $^{\#}$ Significant difference between PSVL vs. PSVH at Tn. $^{\$}$ Significant difference between PSVTn vs. PSVT0. $^{\$}$ Significant difference between PSVTn vs. PSVTn-1.

Thirty minutes after acepromazine administration (T1), pupil length and height increased significantly after positioning the nose twitch ($p = 0.016$ and $p < 0.001$ respectively). PSVHT1 was significantly higher than in PSVLT1 ($p = 0.025$).

Ten minutes after administration of romifidine (T2), the nose twitch had no significant effect on pupil height and length ($p = 0.13$ and $p = 0.07$ respectively). PSVHT2 was not different from that in PSVLT2 ($p = 0.58$).

Five minutes after morphine administration (T3), pupil length and height did not increase significantly after the nose twitch ($p = 0.13$ and $p = 0.78$ respectively). PSVLT3 and PSVHT3 were not significantly different ($p = 0.19$).

After induction (T4), pupil size did not vary significantly in length ($p = 0.43$) or height ($p = 0.96$) after the nose twitch was fitted. PSVLT4 was not significantly different from PSVHT4 ($p = 0.73$).

When the horses were anesthetized with a FeSevo of 2% (T5), pupil length and height did not vary significantly ($p = 0.59$ and $p = 0.67$ respectively) after the nose twitch was fitted. PSVLT5 and PSVHT5 did not differ ($p = 0.95$).

Two horses were not measured immediately after induction. These horses required more time to settle on the operating table. As a result, the horses had a FeSevo of 2% at the time of measurement. These measurements were considered for time T5. The results described below for time T4 are summarized based on twelve horses instead of the initial fourteen.

3.2 Percentage of pupil size variation (PSV) over time

The PSVL was significantly different overall between the different time periods ($p = 0.005$). However, a two-by-two comparison in chronological order did not reveal any significant difference in pupil length variation between the separate times. The only significant difference concerns PSVL at T4 and T5 compared to PSVL at T0 ($p = 0.001$; $p = 0.03$ respectively).

The PSVH was also significantly different overall between the separate times ($p = 0.005$). PSVH was higher at T1 than at T0 ($p = 0.04$). It was also significantly higher at T1 than at T2 ($p < 0.001$).

3.3 Pupil size variation over time

The variation in pupil height and length before the nose twitch application was significantly different overall between the various times ($p < 0.001$; $p < 0.001$ respectively). Romifidine induced mydriasis, shown by a significant increase in height and length (HT2Be versus HT1Be and LT2Be versus LT1Be) ($p < 0.001$; $p < 0.001$ respectively). Height and length remained constant until the end of the protocol: HT3Be versus HT2Be and LT3Be versus LT2Be ($p = 0.31$; $p = 0.05$); HT4Be versus HT3Be and LT4Be versus LT3Be ($p = 0.34$; $p = 0.97$); HT5Be versus HT4Be and LT5Be versus LT4Be ($p = 0.16$; $p = 0.14$).

4 Discussion

This study was carried out on a group of fourteen healthy horses undergoing anesthesia, and showed that twitching the nose produced a pupillary dilation in the awake and the sedated horse with acepromazine. During premedication and general anesthesia induction, this dilation was inhibited by the administration of romifidine and throughout the rest of the anesthesia protocol. In addition, we observed that these molecules had a direct effect on pupil size.

The pupil size values measured in our study are compatible with pupillometric measurements obtained by photography in a study evaluating the mydriatic effect of tropicamide 1% on the horse eye (22).

This study demonstrated that applying the nose twitch resulted in an increase in pupil length in conscious non-sedated horses, triggering a PDR similar to the response observed in the human pupil to pain (11). We indeed assumed that the nose twitch could serve as a painful stimulus. The nose twitch was used to create a reliable, repeatable, and reproducible acute painful/nociceptive stimulus whether the horse was conscious and standing or lying down under general anesthesia. We aimed to standardize the stimulus by having the same person perform the manipulation, using the same nose twitch and the same technique on each horse. However, this pain model cannot be extrapolated to spontaneous pain or chronic pain.

Nevertheless, its mechanism remains controversial and it is not one of the nociceptive stimuli validated for use in horses. The controversy surrounding this method arises due to the description of various mechanisms of action for the nose twitch. Different studies have observed that this method produces pain in the upper lip potentially raising pain tolerance during manipulation of other body parts (23, 24). Conversely, other research has observed an analgesic effect of this method, demonstrating an increase in endogenous β-endorphins, which could enhance pain tolerance (25). It seems difficult to conclude that nose twitch would induce an analgesic effect by the mere argument of a release of β-endorphins. In fact, it has been shown that the release of β-endorphins is a response to a painful or stressful stimulus (26). It has also been hypothesized that the nose twitch could have a deterrent effect, since, when applying this method, if the horse moves, it will experience pain and therefore try to avoid moving (27). Nevertheless, we cannot certify that its effect was effectively and sufficiently painful/nociceptive during our study. A validated scale for an induced pain model, such as the Equine Pain Face, could have been used; this would have allowed for a comparison of the results with pupil dilation.

Our primary expectation was an increase in pupil height rather than length, since radial muscle fibers are more developed on the vertical axis of the horse's iris dilator muscle (28). Despite the trend observed, the small number of horses studied could explain why the height was not significantly different before and after the application of the nose twitch. The stress, which raised the sympathetic tone, may also have led to pupillary dilation before the application of the nose twitch. Indeed, the horse's stress following the operator's first approach could have led to an increase in pupil size before the nose twitch was placed, affecting the first measurement and therefore the amplitude of the dilation induced by the stimulus. Six of them were between two and 3 years old and three of them were admitted for castration. Thus, the approach of the operator and the manipulations carried out at T0 could have been stressful for these young horses with stallion-like behavior. In addition, our measurements showed some variability, which could be explained by individual-dependent stress.

It is interesting to note that the pupil size before the application of the stimulus in the horse tranquillized with acepromazine was smaller

than in the awake horse. This may be the result of acepromazine-induced miosis. Acepromazine is an inhibitor of adrenergic and dopaminergic receptors (29). In dogs, the administration of acepromazine causes miosis (30).

Several mammalian species have adrenergic receptors at the neuromuscular synapses of the iris dilator muscle (28). We can hypothesize here that acepromazine inhibited the adrenergic receptors present in these synapses, leading to the relaxation of the iris dilator muscle. Pupil size was then solely dependent on the iris sphincter muscle, the tonus of which resulted in a decrease in the initial pupil size measured by the pupillometer. Another possible explanation is that under acepromazine-induced tranquillization, the pupil would have retained its normal size on approaching the pupillometer, whereas it would have dilated due to the stress induced by the approach of the machine in the non-sedated horse as mentioned above.

In horses tranquilized with acepromazine, the stimulus led to a more marked increase in pupil height than in pupil length. In addition, the pupillary dilation was greater than in non-sedated horses. Acepromazine therefore seems to potentiate the pupillary dilation induced by the stimulation, perhaps because the pupil size was initially reduced compared to that of an awake horse. The effect of acepromazine is reported to be paroxysmal 15 min after intravenous administration and may last for up to 2 h after injection (31). The thirty-minute gap between the administration of acepromazine and the measurement at T1 means that these measurements can be taken under the action of acepromazine, free from individual variability.

Romifidine appeared to inhibit the pupillary dilation in response to the stimulus. Romifidine is an α2-agonist used in horses for its sedative and analgesic properties (32–34). In humans, α2-agonists such as clonidine or dexmedetomidine reduce pupil size and inhibit the PDR (35, 36). In animals, however, the effects of α2-agonists vary between species. Clonidine causes mydriasis in animals (35). Dexmedetomidine causes miosis in dogs (30) and mydriasis in cats (37). In our study, intravenous injection of romifidine resulted in mydriasis. The mydriasis, already present before applying the nose twitch may have limited the pupillary dilation after its application. Nor can we rule out the possibility that the analgesic effect of romifidine contributed to the reduction in the pupillary dilation.

Five minutes after morphine administration, the painful stimulus did not induce a significant pupillary dilation. Opioids have been shown to reduce PDR in humans in a dose-dependent manner (10, 38). This reduction in pupillary dilation for the same given nociceptive stimulus was achieved within 5 min after administration of an opioid bolus in anaesthetized humans (39). In dogs, morphine induces miosis by exciting the pupil constrictor nuclei (28). On the other hand, in cats, morphine not only has the same effect but also triggers the release of catecholamines, thereby stimulating the sympathetic system and causing mydriasis (28). In horses, the iris sphincter muscle has muscarinic receptors and is blocked by cholinergic antagonists. The iris dilator muscle has adrenergic receptors that could cause contraction of this muscle and pupil dilation. To our knowledge, no study has demonstrated the composition and distribution of these receptors in the horse iris (40, 41). Therefore, it is difficult to conclude on the specific effect of morphine on the horse's pupillary dilation. Nevertheless, we cannot exclude that the effect of romifidine (mydriasis) was still present and may have inhibited the reflex.

After induction of anesthesia, the pupillary dilation was still inhibited. These results are contrary to those observed in humans. One study showed that the PDR persisted following tetanic stimulation in a sample of twenty-four children whose anesthesia had been induced by a bolus of ketamine (42). As for diazepam, its influence on PDR has never been studied to our knowledge. However, it does not appear to influence the control of iris muscles (43). Pupillometry has been used to measure PDR in patients whose anesthesia was maintained with sevoflurane (44) even though pupillary reactivity may be reduced compared with intravenous propofol anesthesia (45). We cannot exclude that the mydriatic effect of romifidine and morphine was still present at these measurement dates.

4.1 This study has several limitations

First, it was chosen to work on horses free of eye conditions, devoid of pain, not receiving drug treatment, and admitted for elective surgery. This point made it possible to exclude altered reactivity of the pupil, any influence of molecules other than those of our anesthetic protocol, the presence of pain before the first measurement at T0, and to have only the application of the nose twitch as only painful stimulation. Our results therefore only apply to this type of patient.

Second, it can take several minutes for the horse's eye to adapt to a new light (46). The measurements were conducted in three different rooms (the hospitalization stall, the induction stall, and the operating room). The measurements were made after an adaptation period; however, the luminosities were different. Even though, ambient light does not affect PDR in humans (8), but the horse's second eye was not hidden during our measurements possibly exhibiting a pupillary light reflex.

Third, the study only involved fourteen horses. The power calculation shows that the findings can be applied to a population of healthy, calm-behaved horses similar to our study group. Nevertheless, the conclusions drawn from our results cannot be generalized to horses in general. The study could hardly be randomized or blinded, nevertheless, photos of the pupillometer screen were taken to validate the measurements with observers unaware of when the photo was taken.

Fourthly, any kind of clinical pain is very different from what can be produced by a nose twitch on the upper lip. Nevertheless, we did not intend to extrapolate experimental pain to clinical pain. We aimed to use a stimulus that was reliable, consistent, and reproducible to create a pain model and to observe the pupil reaction. Initially, we wanted to know whether the pupillometer could be used under the same conditions as in humans, i.e., to check that the level of analgesia is sufficient in response to an acute nociceptive stimulus (i.e., surgery acts under general anesthesia). In other words, to use the pupillometer to measure the quality of per-operative analgesia and not as a measure of pain in general. However, initial trials have shown us that it is difficult to measure per-operative pupillary dilatation in horses. Indeed, even if the eye remains central under anesthesia in this species (unlike that of small animals which tilts) it presents complete mydriasis masking any further pupillary dilatation. We therefore wanted to know whether the horse's eye dilates under the effect of acute pain and, if so, at what point in the anesthesia protocol this dilation was no longer detectable. That's why we have chosen a stimulus that was as reproducible as possible, but also practical to use on an awake non-sedated standing horse, then standing and sedated horse, then

lying down anaesthetized horse. The aim was also to use the same stimulus throughout all stages of anesthesia (premedication, induction, maintenance).

At last, a comparison group with another means of detecting pain and also nociception (since the notion varies throughout the protocol) could have been interesting even essential if the aim was to validate the use of the pupillometer to measure pain. There are not many validated pain scales in equine species. The Equine Pain Face was validated with two experimentally induced pain models (a tourniquet on the antebrachium and topical application of capsaicin). We could indeed have used this scale to compare with the results of pupillometry. However, as the stimulus was not the same, the validity of this grid would have been questionable. Furthermore, every scale is subject to uncertainties (even if validated, they are validated under very precise conditions), and comparing two imperfect systems cannot lead to a conclusion.

5 Conclusion

These elements highlight that pupillary dilation, in response to a stimulation considered painful, exists in horses. Further studies are needed to evaluate the specific effects of α2-agonists and opioids on pupil size and dilation in horses. Furthermore, it would be necessary to be able to overcome the mydriasis observed intraoperatively to use the pupillary dilation to guide anti-nociceptive treatment. This is done using a calibrated nociceptive stimulus (such as tetanic stimulation in humans) and validated in horses. The study of the effects of antinociception guided by pupillometry on the quality of recovery and postoperative complications would then be very interesting.

Data availability statement

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

Ethics statement

The animal studies were approved by Ethical Committee of the National Veterinary School of Lyon (Number 2234, April 12th, 2022). The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

CM: Writing – original draft, Writing – review & editing, Conceptualization, Data curation, Investigation, Methodology. SK: Writing – original draft, Writing – review & editing, Conceptualization, Data curation, Formal analysis, Investigation, Methodology. MA: Writing – review & editing, Conceptualization, Data curation, Investigation, Methodology. KP: Supervision, Writing – original draft, Writing – review & editing, Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Validation.

Funding

The author(s) declare that no financial support was received for the research, authorship, and/or publication of this article.

Acknowledgments

The authors thank IDMed and Frédéric Bernert for loan of equipment used in this study and its adaptation for our patients.

Conflict of interest

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

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

Publisher's note

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frontiers | Frontiers in Veterinary Science

TYPE Original Research
PUBLISHED 04 July 2024
DOI 10.3389/fvets.2024.1380022

OPEN ACCESS

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RECEIVED 31 January 2024
ACCEPTED 17 May 2024
PUBLISHED 04 July 2024

CITATION
Casas-Alvarado A, Martínez-Burnes J, Hernández-Ávalos I, Mora-Medina P, Miranda-Cortés A, Domínguez-Oliva A and Mota-Rojas D (2024) Assessment of the nociceptive response to the use of cannabidiol alone and in combination with meloxicam through infrared pupillometry in female dogs undergoing elective ovariohysterectomy.
Front. Vet. Sci. 11:1380022.
doi: 10.3389/fvets.2024.1380022

Assessment of the nociceptive response to the use of cannabidiol alone and in combination with meloxicam through infrared pupillometry in female dogs undergoing elective ovariohysterectomy

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The negative effects of pain are a constant concern in the surgical management of animals, leading to the search for new drugs or more effective analgesic protocols to control this negative emotion. This study aimed to evaluate the nociceptive response of cannabidiol (CBD) alone and in combination with meloxicam using infrared pupillometry in female dogs undergoing elective ovariohysterectomy (OVH) under isoflurane anesthesia. A total of 60 female dogs of different breeds were included. These dogs were randomly assigned to four study groups according to the treatment: Control Group (G_0: $n = 15$) receiving saline solution; group premedicated with meloxicam at a dose of 0.2 mg Kg^{-1} IV (G_{Melox}: $n = 15$). Postoperatively this drug was used at 0.1 mg Kg^{-1} IV every 24 h; the CBD-treated Group (G_{CBD}: $n = 15$) at a dose of 2 mg Kg^{-1} orally in the preoperative. Postoperatively was administrated every 12 h; and the Group premedicated with the combination of meloxicam and CBD ($G_{Melox/CBD}$: $n = 15$) Meloxicam at a dose of 0.2 mg Kg^{-1} IV preoperatively, and 0.1 mg Kg^{-1} IV during the postoperative. CBD at a dose of 2 mg Kg^{-1} orally in the preoperative, and every 12 h in the postoperative. Treatments were administered for 48 postoperative hours. After OVH, the pupillary neurologic index, pupillary size, minimum diameter (MIN), percentage change, constriction latency (Lat), constriction velocity, and maximum constriction velocity were recorded as pupillometric variables in both eyes during events (E): Baseline (30 min before drug administration), $E_{30\,min}$, E_{1h}, E_{2h}, E_{3h}, E_{4h}, E_{8h}, E_{12h}, E_{24h}, and E_{48h}. The Short-Form of the Glasgow Composite Measure Pain Scale (GCMPS-SF) was used to assess pain during the same events. Overall, it was observed that the pupillometric variables Size, MIN., and Lat. were significantly higher in G_0 compared to the other groups during $E_{30\,min}$, E_{1h}, and E_{2h} ($p = 0.03$), indicating greater pupil dilation in G_0 animals. Additionally, no statistically significant differences were observed

in GCMPS-SF between G_{Melox}, G_{CBD}, and $G_{Melox/CBD}$ during the postoperative period ($p > 0.05$). In contrast, the scores were statistically different compared to G_0 ($p = 0.00001$), where all animals in this group received rescue analgesia at 2 h post-surgery. According to pupillometry and scores on the GCMPS-SF scale, it was observed that monotherapy with cannabidiol provides a similar analgesic effect to meloxicam alone or in combination with cannabidiol to manage acute pain in dogs. Similarly, these findings suggest that infrared pupillometry could be a tool for recognizing acute pain in dogs.

KEYWORDS

pain, pupillometry, dogs, nociception, CBD, meloxicam

1 Introduction

Pain has physiological and emotional/behavioral negative outcomes in animals (1, 2). Therefore, it is a bioethical duty for the veterinarian to acknowledge and alleviate the perception of pain in animals under their care (3–5).

Pain management in companion animals relies on the use of analgesics such as opioids, non-steroidal analgesics (NSAIDs), and local analgesics. These drugs can prevent or decrease pain perception by interrupting some steps in the nociceptive neurobiology (6, 7). Despite the effectiveness of these analgesic drugs in several species, some authors state limitations in their use due to errors in clinical pain recognition, lack of pharmacological knowledge, or the risk of adverse effects (8, 9). For instance, opioids may cause respiratory depression and vasodilation, while NSAIDs may lead to adverse effects such as anorexia, vomiting, diarrhea, and negative consequences on renal and platelet function (10, 11).

An alternative to conventional analgesic drugs to manage pain in companion animals is the use of phytocannabinoid extracts, including cannabidiol (CBD) (12–14). In veterinary medicine, CBD is used as phytocannabinoid extracts (e.g., Sativex and Bedrocan) (15, 16), or synthetic cannabinoids such as CBD or tetrahydrocannabinol (THC). These highly liposoluble molecules interact with cannabinoid (CB) receptors 1 y CB2 (17, 18). Agonisms to CB1 receptors inhibit cAMP synthesis, inducing ion reduction. Consequently, the release of excitatory neurotransmitters (e.g., histamine, serotonin, dopamine, and glutamate) by the Central Nervous System (CSN) is reduced (19). Moreover, agonism of CB2 receptors reduces the inflammatory response induced by pro-inflammatory cytokines (20). It has been proposed that CBD can be used in combination with other drugs such as opioids to potentiate the analgesic effect due to shared mechanisms of action, reducing the dosage and minimizing the side effects of opioids (21–23). For multimodal analgesia, combining NSAIDs and CBD helps to prevent pain perception due to the action of each drug in different steps of the nociceptive pathway. However, there are limited studies evaluating the combination of NSAIDs with CBD during the perioperative period, although some reports indicate the reduction of pain perception in an osteoarthritis model (12, 24, 25).

Pupillometry is considered among the novel technological tools implemented to assess pain in dogs. It is suggested as a technique comparable to traditional methods that reduce evaluator subjectivity by quantitatively measuring pupillary diameter (26). In human medicine, this tool has been shown to objectively recognize pain and assess the efficacy of analgesic protocols to reduce their adverse effects (27). In veterinary medicine, although limited studies have been performed, Mills et al. (28) evaluated pupillometry in 126 healthy dogs to establish the pupillometric reference values for this species, which could help to develop pupillometric indices for pain assessment. Therefore, this study aimed to assess the nociceptive response of CBD alone or in combination with meloxicam through pupillometry in female dogs undergoing elective ovariohysterectomy under isoflurane anesthesia. It was hypothesized that animals receiving CBD alone or in combination with meloxicam would exhibit a lower nociceptive response compared to the use of meloxicam alone.

2 Materials and methods

2.1 Ethical considerations

Before carrying out the study, informed consent was obtained from the animals' owners, authorizing the procedures. All work was performed under Mexico's Official Norm NOM-062-ZOO-1999 guidelines on the technical specifications for animal production, care, and ethical use in applied ethological studies. This project was approved by the Academic Committee of the Ph.D. Program of Biological and Health Sciences (number CBS.066.21). Additionally, this study was conducted following the ARRIVE guidelines and ethical guidelines for the use of animals in experimentation (29, 30). No phase of the study during the surgical procedure or variable collection caused injury, mutilation, or overhandling of the animals.

2.2 Experimental design

Female dogs ($n = 60$) were randomly assigned into four groups according to the treatment: Control group (G_0: $n = 15$) where 1 mL of saline solution was administered IV; Group premedicated with meloxicam (Meloxivet 5 mg/1 mL, Norvet, Mexico) (G_{Melox}: $n = 15$) at a dose of 0.2 mg Kg^{-1} IV, 30 min before surgery. In the postoperative period, meloxicam was administered at 0.1 mg Kg^{-1} every 24 h (31); Group treated with CBD (extract of CBD with 1,000 mg/ 30 mL) (G_{CBD}: $n = 15$) at a dose of 2 mg Kg^{-1} PO every 12 h (12); and Group medicated with the combination of meloxicam (0.2 mg Kg^{-1} IV and 0.1 mg Kg^{-1} every 24 h in the postoperative) and CBD (extract of CBD with 1,000 mg/ 30 mL) (2 mg Kg^{-1} PO every 12 h) ($G_{Melox/CBD}$: $n = 15$).

All treatments were administered 30 min before the start of surgery and in the immediate postoperative period for 48 h.

Pupillometry and the Glasgow Composite Measure Pain Scale (GCMPS-SF) scores were evaluated in the following events: Basal, 1 h before medical instrumentation (E_{Basal}). Postoperative evaluations were performed at 30 min. (E_{30min}), 1 h (E_{1h}), 2 h (E_{2h}), 3 h (E_{3h}), 4 h (E_{4h}), 8 h (E_{8h}), 12 h (E_{12h}), 24 h (E_{24h}), and 48 h (E_{48h}) after surgery.

2.3 Animals

Sixty female dogs of different breeds were included in the present study (21 mixed breed, 9 Chihuahua, 8 Poodle, 7 Pitbull, 5 Schnauzer, 2 Bobtail, 2 Cocker Spaniel, 2 Beagle, 1 Shiba, 1 Golden Retriever, 1 Teckel, and 1 Siberian Husky). Dogs had an average age, body condition score, and body weight of 2 ± 1.5 years, 3/5, and 12.1 ± 2.3 kg, respectively. The sample size was estimated using G*power 3.1.9.7 software (Heinrich-Heine-Universität Düsseldorf, Düsseldorf, Alemania) (32). To determine the sample size for four experimental groups and 10 measurements, an α error of 0.05 was established, with a confidence level of 95%, power (1- α error probability) of 0.95, and a correction among repeated measures of 0.5 (33).

All animals enrolled in the study underwent preanesthetic evaluation through a comprehensive general physical examination and laboratory tests, including complete blood cell count, serum biochemistry, and urinalysis, performed 24 h before surgery. Clinically healthy animals meeting the criteria for an ASA1 anesthetic risk according to the American Society of Anesthesiologists (34) were selected. Patients with ASA2 or higher anesthetic risk were excluded. Brachycephalic breeds, dogs medicated with anticholinergics, and with other conditions causing acute pain, with serious infectious or ocular diseases that could interfere with pupillometric evaluation were also excluded.

2.4 Anesthesia and perioperative management

Elective ovariohysterectomy (OVH) was performed with the previous informed consent of the owner. Dogs had 6-h fasting for food and 4-h fasting for water before the surgical procedure.

Animals were aseptically catheterized in the cephalic vein with a number 20G intravenous catheter. Ringer lactate solution was administered at an infusion rate of 5 mL Kg^{-1} h^{-1} (BeneFusion VP1 Vet, Mindray, Germany) during the surgical procedure (35).

Once catheterized, the animals were premedicated with Dexmedetomidine (Dexdomitor 0.5 mg/ 1 mL, Zoetis, Mexico) at a dose of 1.5 µg Kg^{-1} intravenously (IV). Five minutes after premedication, the dogs presented moderate sedation according to Grint et al. (36)'s sedation score. Anesthetic induction was performed with Propofol (Recofol 1%, Pisa, Mexico) at 2–4 mg Kg^{-1} IV (37). Once an adequate state of unconsciousness was observed (e.g., ventromedial deviation of the eyeball and decreased jaw tone), orotracheal intubation was performed. The orotracheal tube was connected to an anesthetic rebreathing circuit with an oxygen flow of 45 mL Kg^{-1} min^{-1}. Anesthetic maintenance was performed with isoflurane (Sofloran, Pisa, Mexico) vaporized in 100% oxygen, regulating the vaporizer dial initially at 1.8% and modifying the concentration according to the anesthetic depth required to maintain a mean arterial pressure (MAP) between 60 to 90 mmHg, assessed through non-invasive blood pressure. All animals were ventilated with a mechanical ventilator into the anesthesia station (Wato-EX20 vet, Mindray, Germany), using a pressurometric ventilation method controlled at a mean airway pressure (Paw) of 10–15 cmH20 and an I:E ratio of 1:2 during surgery. A respiratory rate of 12 to 20 breaths per minute was established to maintain an EtCO2 of 35–45 mmHg (ePM12VETc/AA, Mindray, Alemania).

The surgical anesthetic depth was assessed through the recognition of clinical signs such as jaw tone relaxation, ventromedial deviation of the eyeball, and the absence of the palpebral reflex. All OVH surgeries were performed by the same surgeon using a midline approach and a triple hemostatic surgical technique. Similarly, all anesthetic procedures were carried out by the same anesthesiologist. The administration of inhalant anesthetics stopped 5 min before surgical wound closure. The end of the surgery was considered after the closure of the surgical incision. Extubating with the reappearance of the cough reflex was performed when patients could successfully sustain spontaneous ventilation and returned the ocular globe to the central position.

2.5 Infrared pupillometry

An automated and portable pupillometer (Neuroptics, NPi 200, United States) was used to measure pupillary size during 60 s in each eye (Figure 1). The following parameters were registered: neurological pupil index (NPi), size, minimum diameter (MIN), percentage of change (% CH), constriction latency (LAT), constriction velocity (CV) and maximum constriction velocity (MCV) (38). Pupillary assessment was performed once in each event. Assessments were performed by a single blinded evaluator.

2.6 Assessment of acute pain

The Glasgow Composite Pain Score – Short Form (GCMPS-SF) was used to assess pain. This scale comprises different behavioral and physiological categories, as well as response to touch, facial expression, vocalization, and mobility. The maximum pain score is 24 points (39). A single and trained evaluator performed all measures. Rescue analgesia with Tramadol (Tramajet 50 mg/ 1 mL; Norvet, Mexico) at 4 mg kg^{-1} IV (40–42) was administered in the postsurgical period when GCMPS-SF score was ≥6 points.

2.7 Statistical analyses

Descriptive statistics were obtained using Graph Pad Prism (ver. 9.5) for all groups (G_0, G_{Melox}, G_{CBD}, $G_{Melox/CBD}$) and all events (E_{Basal}, $E_{30\ min}$, E_{1h}, E_{2h}, E_{3h}, E_{4h}, E_{8h}, E_{12h}, E_{24h}, and E_{48h}). Normality tests were done with the Kolmogorov–Smirnov test for all the variables assessed.

The treatments were considered independent variables, while each of the pupillometric parameters and the post-surgical pain evaluation scores were considered dependent. To evaluate the effects of these variables, a linear mixed model was used.

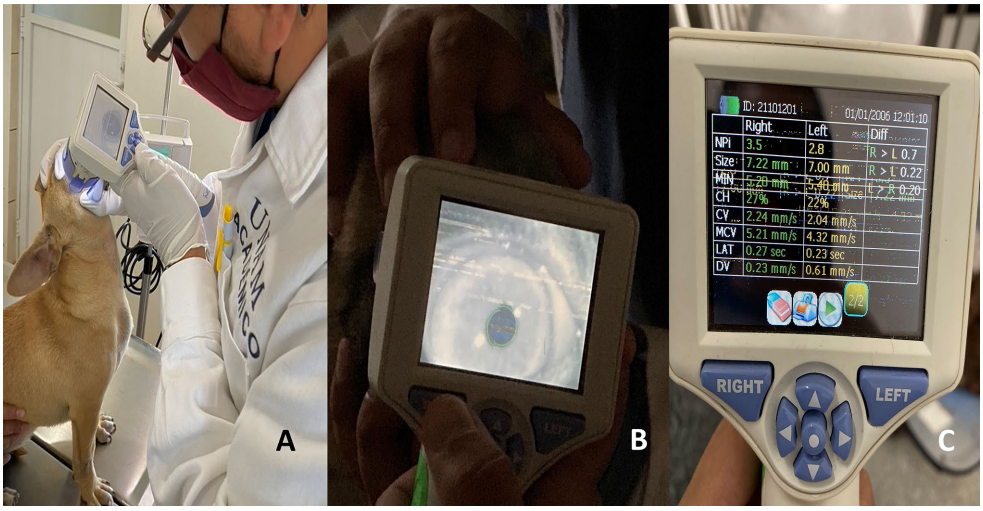

FIGURE 1
Methodology of the infrared pupilometry technique. **(A)** The placement of the pupilometer at a 90° angle in the ocular region is depicted. **(B)** The moment of measuring the pupil diameter using the infrared light camera is shown. From the pupil diameter measurement, 7 different variables are captured, including the neurological pupil index (NPi), size, minimum diameter (MIN), percentage change (% CH), constriction latency (LAT), constriction velocity (CV), and maximum constriction velocity (MCV), as shown in image **C**.

A Tukey *post hoc* test was used to evaluate differences between means. The analysis of sensitivity and specificity was carried out using a receiver operating characteristics (ROC) test using the score obtained in GCMPS-SF as the gold standard. Finally, the linear relationship between study variables was performed using a Pearson correlation test. In all cases, the significance level was set at $p < 0.05$.

3 Results

In the present study, 64 dogs were considered. However, four dogs were excluded: two dogs due to the administration of anticholinergics, one dog due to pyometra, and one dog due to osteoarthritic chronic pain. A total of 60 dogs were included, 21 mixed breeds, 9 Chihuahuas, 8 Poodles, 7 Pitbulls, 5 Schnauzers, 2 Bobtail, 2 Cocker Spaniel, 2 Beagle, 1 Shiba, 1 Golden Retriever, 1 Teckel, and 1 Siberian Husky. In general, the average anesthesia time was 57 ± 8.4 min, surgical time was 24 ± 4.8 min, and extubating time was 13 ± 2.8 min. The main findings of the pupillary assessment show that Size, MIN, and Lat, had significant differences between groups ($p < 0.05$) particularly G_{Melox}, G_{CBD}, and $G_{Melox/CBD}$ with G_0. These differences were observed during the first two postoperative hours. Moreover, all animals in G_0 required rescue analgesia at E_{2h}.

In Table 1, it can be observed that the Size of the right eye (maximum pupil diameter) significantly increased in G_{CBD} during E_{2h} ($p = 0.006$) when comparing basal values in the same group, registering 9.19 ± 0.26 mm. During $E_{2h,}$ the Size of $G_{Melox/CBD}$ was 8.59 ± 0.30 mm, a value that was not statistically significant ($p = 0.47$) in comparison with G_{CBD} (9.19 ± 0.26 mm) and G_{Melox} (9.35 ± 0.20 mm). However, the pupil diameter of G_0 was 9.90 ± 0.07 mm, showing statistically significant differences ($p = 0.003$) with the other experimental groups G_{Melox}, G_{CBD}, $G_{Melox/CBD}$.

In the case of the minimum pupil diameter (MIN) of the right eye, statistically significant differences were reported between study groups during $E_{1h,}$ ($p = 0.01$), $E_{2h,}$ ($p = 0.03$), and $E_{3h,}$ ($p = 0.003$). Animals in G_0 recorded the highest values with 7.27 ± 0.42, 7.33 ± 0.33, and 7.21 ± 0.48 mm at $E_{1h,}$ $E_{2h,}$ and $E_{3h,}$ respectively.

Regarding the latency time of pupillary constriction (Lat), the Lat of G_0 animals increased between 0.08 and 0.18 s compared to the rest of the postsurgical events and the $E_{Basal.}$ from the same experimental group ($p = 0.0001$). Likewise, at E_{30min} and $E_{2h,}$ statistically significant differences between treatments were reported ($p = 0.003$ y $p = 0.02$ respectively). The latency time in G_0 was 0.38 ± 0.03 s during $E_{30min,}$ while at $E_{2h,}$ Lat. was 0.28 ± 0.03. sec. In contrast, values recorded from G_{Melox}, G_{CBD}, $G_{Melox/CBD}$ decreased between 0.16–0.18 and 0.03–0.09 s, respectively, during the evaluation events. Also in Table 1, it can be observed that NPi, CH, CV, and MCV did not have significant differences between treatments and/or events ($p > 0.05$).

Table 2 shows the pupillometric variables of the left eye. Similar to the previously described results, CH, CV, and MCV had no statistical differences between events or between treatments ($p > 0.05$). However, NPi values increased in the left eye (between 0.70–1.00) in all postsurgical events when compared to $E_{Basal.,}$ where a value of 3.60 ± 0.24 ($p = 0.03$) was recorded. For the Size variable in the left eye, the diameter of animals in G_{Melox} at E_{24h} was significantly smaller compared to the rest of the events ($p = 0.006$) and between treatments ($p = 0.002$).

Regarding MIN, dogs in G_0 registered 6.26 ± 0.37 mm during $E_{Basal.}$ This value increased from E_{30min} (7.33 ± 0.40 mm) to 6.92 ± 0.35 mm at E_{48h}, having statistically significant differences between events ($p = 0.005$). Differences between treatments were recorded during E_{30min} ($p = 0.049$) and E_{12h} ($p = 0.049$) where G_{Melox}, G_{CBD}, $G_{Melox/CBD}$ maintained a homogeneous pattern with mean peak constriction values of 5.75 to 6.65 mm. In G_0, the pupil diameter increased from 0.68–1.13 mm during E_{30min} up to 1.93–2.24 mm during $E_{12h.}$

Lat. variable showed values of 0.19 ± 0.00 s in G_0 animals during E_{Basal}; however, this value increased during all postsurgical events, reaching 0.33 ± 0.03 and 0.27 ± 0.02 s at E_{2h} and E_{12h}, respectively.

TABLE 1 Pupillometric values (Mean ± EE) of the right eye pupil in the evaluation events (E) of 60 bitches under elective ovariohysterectomy surgery distributed in 4 study groups: G_0, G_{Melox}, G_{CBD}, $G_{Melox/CBD}$.

Parameters	Treatments	Post-surgical Events										*p* value
		E_{Basal}	$E_{30Min.}$	$E_{1h.}$	$E_{2h.}$	$E_{3h.}$	$E_{4h.}$	$E_{8h.}$	$E_{12h.}$	$E_{24h.}$	$E_{48h.}$	
NPi	G_0 $n = 15$	$4.2^{1,a}$ ± 0.18	$4.3^{1,a}$ ± 0.20	$4.3^{1,a}$ ± 0.20	$4.2^{1,a}$ ± 0.16	$4.3^{1,a}$ ± 0.18	$4.3^{1,a}$ ± 0.17	$4.2^{1,a}$ ± 0.19	$4.6^{1,a}$ ± 0.08	$4.3^{1,a}$ ± 0.16	$4.4^{1,a}$ ± 0.20	$p > 0.05$
	G_{Melox}, $n = 15$	$3.9^{1,a}$ ± 0.25	$4.3^{1,a}$ ± 0.15	$4.2^{1,a}$ ± 0.15	$4.2^{1,a}$ ± 0.14	$4.2^{1,a}$ ± 0.15	$4.2^{1,a}$ ± 0.15	$4.5^{1,a}$ ± 0.14	$4.2^{1,a}$ ± 0.21	$4.2^{1,a}$ ± 0.20	$4.4^{1,a}$ ± 0.14	$p > 0.05$
	G_{CBD} $n = 15$	$4.1^{1,a}$ ± 0.21	$4.3^{1,a}$ ± 0.13	$4.1^{1,a}$ ± 0.14	$4.2^{1,a}$ ± 0.14	$4.5^{1,a}$ ± 0.09	$4.3^{1,a}$ ± 0.14	$4.4^{1,a}$ ± 0.10	$4.4^{1,a}$ ± 0.14	$3.8^{1,a}$ ± 0.13	$4.2^{1,a}$ ± 0.12	$p > 0.05$
	$G_{Melox/CBD}$ $n = 15$	$4.4^{1,a}$ ± 0.11	$4.2^{1,a}$ ± 0.14	$4.3^{1,a}$ ± 0.13	$4.3^{1,a}$ ± 0.11	$4.4^{1,a}$ ± 0.14	$4.5^{1,a}$ ± 0.08	$4.3^{1,a}$ ± 0.12	$4.5^{1,a}$ ± 0.11	$4.0^{1,a}$ ± 0.14	$4.5^{1,a}$ ± 0.08	$p > 0.05$
	P value	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p > 0.05$	
Size (mm.)	G_0 $n = 15$	$9.01^{1,a}$ ± 0.42	$9.53^{1,a}$ ± 0.18	$9.46^{1,a}$ ± 0.16	$9.90^{1,a}$ ± 0.07	$9.73^{1,a}$ ± 0.15	$9.73^{1,a}$ ± 0.15	$9.04^{1,a}$ ± 0.27	$9.88^{1,a}$ ± 0.07	$9.39^{1,a}$ ± 0.16	$9.58^{1,a}$ ± 0.19	$p > 0.05$
	G_{Melox}, $n = 15$	$8.52^{1,a}$ ± 0.42	$9.54^{1,a}$ ± 0.15	$9.52^{1,a}$ ± 0.19	$9.35^{2,a}$ ± 0.20	$9.54^{1,a}$ ± 0.22	$9.47^{1,a}$ ± 0.23	$9.54^{1,a}$ ± 0.15	$9.77^{1,a}$ ± 0.18	$8.94^{1,a}$ ± 0.50	$8.96^{1,a}$ ± 0.29	$p > 0.05$
	G_{CBD} $n = 15$	$8.17^{1,b}$ ± 0.49	$9.02^{1,a}$ ± 0.21	$8.96^{1,a}$ ± 0.24	$9.19^{2,a}$ ± 0.26	$9.06^{1,a}$ ± 0.16	$9.52^{1,a}$ ± 0.20	$9.60^{1,a}$ ± 0.13	$9.58^{1,a}$ ± 0.20	$9.66^{1,a}$ ± 0.21	$9.58^{1,a}$ ± 0.23	**p = 0.006**
	$G_{Melox/CBD}$ $n = 15$	$9.39^{1,a}$ ± 0.37	$8.75^{1,a}$ ± 0.24	$8.73^{1,a}$ ± 0.31	$8.59^{2,a}$ ± 0.30	$9.08^{1,a}$ ± 0.25	$8.93^{1,a}$ ± 0.20	$9.17^{1,a}$ ± 0.23	$9.30^{1,a}$ ± 0.18	$9.44^{1,a}$ ± 0.22	$9.07^{1,a}$ ± 0.36	$p > 0.05$
	P value	$p > 0.05$	$p > 0.05$	$p > 0.05$	**p = 0.003**	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p > 0.05$	
MIN (mm.)	G_0 $n = 15$	$5.89^{1,a}$ ± 0.36	$6.99^{1,a}$ ± 0.38	$7.27^{1,a}$ ± 0.42	$7.33^{1,a}$ ± 0.33	$7.21^{1,a}$ ± 0.48	$6.82^{1,a}$ ± 0.33	$6.08^{1,a}$ ± 0.32	$6.42^{1,a}$ ± 0.39	$6.78^{1,a}$ ± 0.37	$6.95^{1,a}$ ± 0.47	$p > 0.05$
	G_{Melox}, $n = 15$	$6.09^{1,a}$ ± 0.30	$6.43^{1,a}$ ± 0.28	$6.39^{1,a}$ ± 0.24	$6.22^{1,a}$ ± 0.29	$6.38^{1,2,a}$ ± 0.28	$5.98^{1,a}$ ± 0.35	$5.83^{1,a}$ ± 0.33	$5.56^{1,a}$ ± 0.43	$5.43^{1,a}$ ± 0.22	$5.74^{1,a}$ ± 0.23	$p > 0.05$
	G_{CBD} $n = 15$	$5.55^{1,a}$ ± 0.27	$6.03^{1,a}$ ± 0.17	$5.80^{2,a}$ ± 0.20	$5.62^{2,a}$ ± 0.14	$5.82^{1,2,a}$ ± 0.15	$6.04^{1,a}$ ± 0.18	$6.32^{1,a}$ ± 0.33	$6.14^{1,a}$ ± 0.20	$6.29^{1,a}$ ± 0.37	$6.15^{1,a}$ ± 0.27	$p > 0.05$
	$G_{Melox/CBD}$ $n = 15$	$6.00^{1,a}$ ± 0.26	$5.87^{1,a}$ ± 0.28	$5.96^{2,a}$ ± 0.30	$5.98^{3,a}$ ± 0.25	$5.74^{2,a}$ ± 0.31	$5.89^{1,a}$ ± 0.36	$6.55^{1,a}$ ± 0.22	$6.04^{1,a}$ 0.31	$6.47^{1,a}$ ± 0.24	$6.10^{1,a}$ ± 0.25	$p > 0.05$
	P value	$p > 0.05$	$p > 0.05$	**p = 0.01**	**p = 0.03**	**p = 0.003**	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p > 0.05$	
CH (%)	G_0 $n = 15$	$28.40^{1,a}$ ± 2.19	$32.29^{1,a}$ ± 2.70	$36.13^{1,a}$ ± 2.76	$31.50^{1,a}$ ± 2.14	$33.88^{1,a}$ ± 2.08	$33.22^{1,a}$ ± 1.57	$31.20^{1,a}$ ± 2.17	$33.75^{1,a}$ ± 2.44	$33.00^{1,a}$ ± 2.38	$32.25^{1,a}$ ± 3.50	$p > 0.05$
	G_{Melox}, $n = 15$	$28.89^{1,a}$ ± 3.22	$33.36^{1,a}$ ± 2.19	$29.62^{1,a}$ ± 1.73	$29.85^{1,a}$ ± 1.75	$29.09^{1,a}$ ± 1.47	$31.82^{1,a}$ ± 2.12	$31.60^{1,a}$ ± 2.63	$32.27^{1,a}$ ± 2.31	$31.55^{1,a}$ ± 2.96	$35.56^{1,a}$ ± 2.45	$p > 0.05$
	G_{CBD} $n = 15$	$27.82^{1,a}$ ± 1.40	$30.50^{1,a}$ ± 1.75	$29.31^{1,a}$ ± 1.20	$31.54^{1,a}$ ± 1.49	$35.43^{1,a}$ ± 1.65	$33.13^{1,a}$ ± 2.01	$31.79^{1,a}$ ± 1.91	$34.45^{1,a}$ ± 2.11	$27.71^{1,a}$ ± 1.33	$30.43^{1,a}$ ± 1.28	$p > 0.05$
	$G_{Melox/CBD}$ $n = 15$	$28.85^{1,a}$ ± 2.33	$32.17^{1,a}$ ± 1.93	$31.42^{1,a}$ ± 1.23	$33.85^{1,a}$ ± 1.78	$34.00^{1,a}$ ± 2.48	$33.64^{1,a}$ ± 2.85	$30.92^{1,a}$ ± 1.66	$35.09^{1,a}$ ± 2.59	$28.42^{1,a}$ ± 1.14	$33.36^{1,a}$ ± 1.79	$p > 0.05$
	P value	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p > 0.05$	
CV (mm./seg.)	G_0 $n = 15$	$3.59^{1,a}$ ± 0.21	$3.44^{1,a}$ ± 0.32	$3.77^{1,a}$ ± 0.26	$2.91^{1,a}$ ± 0.22	$3.37^{1,a}$ ± 0.18	$3.45^{1,a}$ ± 0.16	$3.34^{1,a}$ ± 0.21	$2.99^{1,a}$ ± 0.29	$3.12^{1,a}$ ± 0.17	$3.06^{1,a}$ ± 0.26	$p > 0.05$
	G_{Melox}, $n = 15$	$3.23^{1,a}$ ± 0.26	$3.43^{1,a}$ ± 0.24	$3.32^{1,a}$ ± 0.20	$3.22^{1,a}$ ± 0.20	$3.08^{1,a}$ ± 0.20	$3.14^{1,a}$ ± 0.20	$3.45^{1,a}$ ± 0.24	$3.45^{1,a}$ ± 0.40	$3.08^{1,a}$ ± 0.25	$3.67^{1,a}$ ± 0.30	$p > 0.05$
	G_{CBD} $n = 15$	$2.56^{1,a}$ ± 0.23	$3.15^{1,a}$ ± 0.24	$3.40^{1,a}$ ± 0.19	$3.35^{1,a}$ ± 0.22	$3.36^{1,a}$ ± 0.20	$3.41^{1,a}$ ± 0.20	$3.37^{1,a}$ ± 0.19	$3.41^{1,a}$ ± 0.23	$2.76^{1,a}$ ± 0.24	$2.88^{1,a}$ ± 0.27	$p > 0.05$
	$G_{Melox/CBD}$ $n = 15$	$3.33^{1,a}$ ± 0.28	$3.19^{1,a}$ ± 0.21	$3.11^{1,a}$ ± 0.28	$3.92^{1,a}$ ± 0.28	$3.82^{1,a}$ ± 0.18	$3.30^{1,a}$ ± 0.28	$3.47^{1,a}$ ± 0.24	$3.37^{1,a}$ ± 0.28	$3.50^{1,a}$ ± 0.24	$3.42^{1,a}$ ± 0.27	$p > 0.05$
	P value	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p > 0.05$	

(Continued)

TABLE 1 (Continued)

Parameters	Treatments	Post-surgical Events										*p* value
		E_{Basal}	$E_{30Min.}$	$E_{1h.}$	$E_{2h.}$	$E_{3h.}$	$E_{4h.}$	$E_{8h.}$	$E_{12h.}$	$E_{24h.}$	$E_{48h.}$	
MCV (mm./seg.)	G_0 $n = 15$	$5.63^{1,a} \pm 0.37$	$5.82^{1,a} \pm 0.39$	$5.96^{1,a} \pm 0.51$	$5.55^{1,a} \pm 0.36$	$5.96^{1,a} \pm 0.32$	$5.42^{1,a} \pm 0.32$	$6.00^{1,a} \pm 0.42$	$5.00^{1,a} \pm 0.51$	$5.27^{1,a} \pm 0.44$	$5.75^{1,a} \pm 0.40$	$p > 0.05$
	G_{Melox}, $n = 15$	$5.35^{1,a} \pm 0.38$	$6.00^{1,a} \pm 0.37$	$6.04^{1,a} \pm 0.23$	$5.83^{1,a} \pm 0.36$	$6.07^{1,a} \pm 0.28$	$6.18^{1,a} \pm 0.34$	$5.80^{1,a} \pm 0.40$	$5.86^{1,a} \pm 0.57$	$5.47^{1,a} \pm 0.56$	$6.16^{1,a} \pm 0.51$	$p > 0.05$
	G_{CBD} $n = 15$	$5.57^{1,a} \pm 0.48$	$5.88^{1,a} \pm 0.42$	$5.92^{1,a} \pm 0.35$	$5.80^{1,a} \pm 0.32$	$6.19^{1,a} \pm 0.33$	$5.43^{1,a} \pm 0.33$	$5.84^{1,a} \pm 0.37$	$5.87^{1,a} \pm 0.39$	$4.88^{1,a} \pm 0.30$	$5.1^{1,a} \pm 0.33$	$p > 0.05$
	$G_{Melox/CBD}$ $n = 15$	$5.47^{1,a} \pm 0.31$	$5.96^{1,a} \pm 0.30$	$5.38^{1,a} \pm 0.25$	$6.25^{1,a} \pm 0.27$	$6.11^{1,a} \pm 0.40$	$5.72^{1,a} \pm 0.39$	$5.64^{1,a} \pm 0.26$	$5.86^{1,a} \pm 0.57$	$5.45^{1,a} \pm 0.26$	$5.90^{1,a} \pm 0.33$	$p > 0.05$
	P value	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p > 0.05$	
Lat (mm./seg.)	G_0 $n = 15$	$0.20^{1,a} \pm 0.01$	$0.38^{1,b} \pm 0.03$	$0.24^{1,b} \pm 0.02$	$0.28^{1,b} \pm 0.03$	$0.24^{1,a} \pm 0.02$	$0.26^{1,a} \pm 0.02$	$0.22^{1,a} \pm 0.02$	$0.26^{1,a} \pm 0.04$	$0.25^{1,a} \pm 0.03$	$0.24^{1,a} \pm 0.02$	**p = 0.0001**
	G_{Melox}, $n = 15$	$0.21^{1,a} \pm 0.01$	$0.22^{2,a} \pm 0.00$	$0.20^{1,a} \pm 0.00$	$0.25^{1,2,a} \pm 0.01$	$0.23^{1,a} \pm 0.00$	$0.20^{1,a} \pm 0.00$	$0.21^{1,a} \pm 0.01$	$0.22^{1,a} \pm 0.01$	$0.22^{1,a} \pm 0.01$	$0.19^{1,a} \pm 0.00$	$p > 0.05$
	G_{CBD} $n = 15$	$0.22^{1,a} \pm 0.01$	$0.22^{2,a} 0.00$	$0.22^{1,a} \pm 0.01$	$0.22^{1,2,a} \pm 0.00$	$0.20^{1,a} \pm 0.01$	$0.23^{1,a} \pm 0.01$	$0.26^{1,a} \pm 0.03$	$0.23^{1,a} \pm 0.01$	$0.24^{1,a} \pm 0.01$	$0.21^{1,a} \pm 0.00$	$p > 0.05$
	$G_{Melox/CBD}$ $n = 15$	$0.22^{1,a} \pm 0.01$	$0.20^{2,a} \pm 0.01$	$0.20^{1,a} \pm 0.00$	$0.19^{2,a} \pm 0.00$	$0.20^{1,a} \pm 0.01$	$0.19^{1,a} \pm 0.01$	$0.21^{1,a} \pm 0.02$	$0.22^{1,a} \pm 0.01$	$0.22^{1,a} \pm 0.01$	$0.20^{1,a} 0.01$	$p > 0.05$
	P value	$p > 0.05$	**p = 0.003**	$p > 0.05$	**p = 0.02**	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p > 0.05$	

[a,b,c]Different literals by row indicate significant differences between events for the same treatment. [1,2,3,4]Different numerals by column indicate significant differences between treatments for the same event. T = treatments (G_0: negative group, G_{Melox}: Meloxicam group, G_{CBD}: Cannabidiol group, $G_{Melox/CBD}$: Mexociam and Cannabidiol group). E: post-surgical events (E_{Basal}: 30 min. Pre-surgery; $E_{30\ min.}$: 30 min post- surgery; $E_{1h.}$: 1 h post-surgery; $E_{2h.}$: 2 h post-surgery; $E_{3h.}$: 3 h post-surgery; $E_{4h.}$: 4 h post-surgery; $E_{8h.}$: 8 h post-surgery; $E_{12h.}$: 12 h post-surgery, $E_{24h.}$: 24 h post-surgery; $E_{48h.}$: 48 h post-surgery). NPi, Neurological pupil index; Size, Maximum pupil size before constriction; MIN, Pupil diameter at peak constriction; CH, Percentage of pupil change. CV, Constriction velocity. MCV, Maximum constriction velocity. Lat., Latency of constriction. Bold values represent statistically significant differences.

Therefore, as observed in the right eye, there was a statistically significant difference between postsurgical events ($p = 0.002$). Moreover, significant differences between treatments at $E_{1h,}$ and E_{2h} were recorded ($p = 0.01$ y $p = 0.003$, respectively), where the Lat. of G_{Melox}, G_{CBD}, $G_{Melox/CBD}$ was lower than G_0 during E_{1h} (average of 0.09 s) and E_{2h} (average of 0.1 s).

GCMPS-SF scores are presented in Table 3. In all groups, scores increased from E_{Basal} to the post-operative period ($p = 0.0001$). However, the highest values were recorded in G_0 during E_{30min}, E_{1h}, E_{2h}, $E_{3h,}$ and E_{4h}, in comparison to the rest of the treatments at the same events ($p = 0.0001$). Furthermore, it was found that the pain scores of G_{Melox}, G_{CBD}, $G_{Melox/CBD}$ did not present differences between groups ($p > 0.05$). Rescue analgesia was administered in one dog included in G_{Melox}, G_{CBD}, $G_{Melox/CBD}$.

Finally, in the ROC analysis, it was determined that the Size variable presented a sensitivity of 77.2% and specificity of 96.9% ($p < 0.0001$), while Lat., had a sensitivity of 94.5% and specificity of 88.1% was obtained ($p < 0.001$). Likewise, MIN had a sensitivity of 98.2% and a specificity of 95.6% was recorded ($p < 0.0001$). No significant correlation between the pupillometric indicators was found (Table 4).

4 Discussion

Among the most significant findings, the pupillometric variables Size, MIN., and Lat. showed higher sensitivity and specificity to identify pain during the postoperative period of dogs undergoing OVH. This suggests that pupillometry is an objective method to recognize acute pain in dogs. The neurophysiological control of the pupil diameter is related to the changes that can be observed in the pupil in animals experiencing pain. Both the sphincter and the dilator muscle control the pupil size. The dilator muscle has sympathetic fibers that increase the pupil diameter or the pupil dilator reflex (27). In humans, pupillometry is currently used to assess pain in pediatrics and traumatology (40–43).

The results indicate that CBD and meloxicam offer equivalent perioperative analgesic quality, without either being superior when these drugs were administered together in the studied animals. Derived from the pupillometric data obtained, it was observed that CBD exhibited similar analgesic activity to meloxicam. This could be explained by the presence of CB1 receptors in neurons of the dorsal horn of the spinal cord (44) and CB2 receptors primarily found in cells of both the immune system and smooth muscle in viscera (45–47). The presence of CB1 and CB2 receptors in the retina, ciliary body, and sympathetic iris fibers has also been suggested (48, 49). Moreover, CBD has a high affinity to CB2 receptors (19).

The analgesic mechanism of action of cannabinoids is mainly by agonism to cannabidiol receptors. The first is the agonism of CB1 receptors, which can induce the activation of Gi/o proteins, inhibiting adenylate cyclase activity and reducing cAMP synthesis. CB1 receptor agonism induces the blockade of voltage-dependent N-type Ca^{2+} channels and an increase in G protein-related K+ channel conductance (19, 50). At the presynaptic level, these actions reduce the release of neurotransmitters such as norepinephrine, histamine, serotonin, dopamine, cholecystokinin, and glutamate in the central nervous system, thereby reducing the perception of nociceptive stimuli (14).

TABLE 2 Pupillometric values (Mean ± EE) of the left eye pupil in the evaluation events (E) of 60 bitches under elective ovariohysterectomy surgery distributed in 4 study groups: G_0, G_{Melox}, G_{CBD}, $G_{Melox/CBD}$.

Parameters	Treatments	Post-surgical events									*P* value
		E_{Basal}	$E_{30Min.}$	$E_{1h.}$	$E_{2h.}$	$E_{3h.}$	$E_{4h.}$	$E_{8h.}$	$E_{12h.}$	$E_{24h.}$	$E_{48h.}$	
NPi	G_0 $n=15$	$3.96^{1,a}$ ± 0.19	$4.04^{1,a}$ ± 0.23	$4.17^{1,a}$ ± 0.31	$3.97^{1,a}$ ± 0.24	$4.35^{1,a}$ ± 0.14	$4.03^{1,a}$ ± 0.16	$4.30^{1,a}$ ± 0.09	$4.24^{1,a}$ ± 0.11	$4.37^{1,a}$ ± 0.11	$4.25^{1,a}$ ± 0.15	$p>0.05$
	G_{Melox}, $n=15$	$3.99^{1,a}$ ± 0.21	$4.32^{1,a}$ ± 0.11	$3.93^{1,a}$ ± 0.24	$4.22^{1,a}$ ± 0.14	$4.31^{1,a}$ ± 0.10	$4.35^{1,a}$ ± 0.10	$4.50^{1,a}$ ± 0.11	$4.25^{1,a}$ ± 0.19	$4.11^{1,a}$ ± 0.13	$3.93^{1,a}$ ± 0.18	$p>0.05$
	G_{CBD} $n=15$	$3.90^{1,a}$ ± 0.19	$3.95^{1,a}$ ± 0.20	$4.43^{1,a}$ ± 0.08	$4.45^{1,a}$ ± 0.09	$4.29^{1,a}$ ± 0.14	$4.47^{1,a}$ ± 0.09	$4.48^{1,a}$ ± 0.14	$4.32^{1,a}$ ± 0.14	$4.14^{1,a}$ ± 0.15	$4.22^{1,a}$ ± 0.14	$p>0.05$
	$G_{Melox/CBD}$ $n=15$	$3.60^{1,a}$ ± 0.24	$4.38^{1,b}$ ± 0.10	$4.30^{1,b}$ ± 0.09	$4.33^{1,b}$ ± 0.10	$4.44^{1,b}$ ± 0.12	$4.60^{1,b}$ ± 0.05	$4.50^{1,b}$ ± 0.08	$4.49^{1,b}$ ± 0.08	$4.50^{1,b}$ ± 0.05	$4.22^{1,b}$ ± 0.13	**p = 0.03**
	P value	$p>0.05$	$p>0.05$	$p>0.05$	$p>0.05$	$p>0.05$	$p>0.05$	$p>0.05$	$p>0.05$	$p>0.05$	$p>0.05$	
Size (mm.)	G_0 $n=15$	$8.90^{1,a}$ ± 0.29	$9.57^{1,a}$ ± 0.21	$9.65^{1,a}$ ± 0.17	$9.79^{1,a}$ ± 0.13	$9.89^{1,a}$ ± 0.05	$9.35^{1,a}$ ± 0.40	$9.66^{1,a}$ ± 0.18	$9.85^{1,a}$ ± 0.08	$9.39^{1,a}$ ± 0.15	$9.53^{1,a}$ ± 0.16	$p>0.05$
	G_{Melox}, $n=15$	$9.26^{1,a}$ ± 0.21	$9.76^{1,a}$ ± 0.09	$9.45^{1,a}$ ± 0.18	$9.44^{1,a}$ ± 0.16	$9.35^{1,a}$ ± 0.25	$8.95^{1,a}$ ± 0.22	$9.29^{1,a}$ ± 0.20	$8.77^{1,a}$ ± 0.31	$7.88^{2,b}$ ± 0.36	$8.82^{1,a}$ ± 0.32	**p = 0.006**
	G_{CBD} $n=15$	$8.77^{1,a}$ ± 0.31	$8.96^{1,a}$ ± 0.24	$9.19^{1,a}$ ± 0.17	$9.42^{1,a}$ ± 0.16	$9.41^{1,a}$ ± 0.18	$9.47^{1,a}$ ± 0.15	$9.52^{1,a}$ ± 0.18	$8.40^{1,a}$ ± 0.29	$9.21^{1,a}$ ± 0.22	$9.62^{1,a}$ ± 0.12	$p>0.05$
	$G_{Melox/CBD}$ $n=15$	$9.07^{1,a}$ ± 0.27	$9.16^{1,a}$ ± 0.17	$9.39^{1,a}$ ± 0.15	$8.80^{1,a}$ ± 0.29	$9.35^{1,a}$ ± 0.24	$9.25^{1,a}$ ± 0.15	$9.25^{1,a}$ ± 0.13	$9.05^{1,a}$ ± 0.22	$9.44^{1,a}$ ± 0.18	$8.58^{1,a}$ ± 0.28	$p>0.05$
	P value	$p>0.05$	$p>0.05$	$p>0.05$	$p>0.05$	$p>0.05$	$p>0.05$	$p>0.05$	$p>0.05$	**p = 0.002**	$p>0.05$	
MIN (mm.)	G_0 $n=15$	$6.26^{1,b}$ ± 0.37	$7.33^{1,a}$ ± 0.40	$6.74^{1,a,b}$ ± 0.64	$6.72^{1,a,b}$ ± 0.51	$6.63^{1,a,b}$ ± 0.45	$6.99^{1,a,b}$ ± 0.36	$6.90^{1,a,b}$ ± 0.49	$7.99^{1,a}$ ± 0.34	$6.70^{1,b}$ ± 0.30	$6.92^{1,a,b}$ ± 0.35	**p = 0.005**
	G_{Melox}, $n=15$	$6.75^{1,a}$ ± 0.30	$6.20^{1,a}$ ± 0.43	$6.12^{1,a}$ ± 0.37	$6.34^{1,a}$ ± 0.35	$5.87^{1,a}$ ± 0.45	$6.10^{1,a}$ ± 0.29	$6.02^{1,a}$ ± 0.34	$6.06^{2,a}$ ± 0.37	$5.10^{1,a}$ ± 0.28	$5.84^{1,a}$ ± 0.35	$p>0.05$
	G_{CBD} $n=15$	$6.30^{1,a}$ ± 0.31	$6.42^{1,a}$ ± 0.29	$6.32^{1,a}$ ± 0.19	$6.39^{1,a}$ ± 0.24	$6.54^{1,a}$ ± 0.28	$6.46^{1,a}$ ± 0.20	$6.33^{1,a}$ ± 0.31	$5.75^{2,a}$ ± 0.32	$6.09^{1,a}$ ± 0.30	$5.84^{1,a}$ ± 0.34	$p>0.05$
	$G_{Melox/CBD}$ $n=15$	$6.55^{1,a}$ ± 0.33	$6.65^{1,a}$ ± 0.22	$6.55^{1,a}$ ± 0.21	$5.75^{1,a}$ ± 0.26	$6.21^{1,a}$ ± 0.30	$6.00^{1,a}$ ± 0.28	$6.09^{1,a}$ ± 0.21	$5.97^{2,a}$ ± 0.26	$6.41^{1,a}$ ± 0.29	$5.79^{1,a}$ ± 0.27	$p>0.05$
	P value	$p>0.05$	**p = 0.049**	$p>0.05$	$p>0.05$	$p>0.05$	$p>0.05$	$p>0.05$	**p = 0.049**	$p>0.05$	$p>0.05$	
CH (%)	G_0 $n=15$	$30.75^{1,a}$ ± 2.88	$30.25^{1,a}$ ± 2.43	$35.71^{1,a}$ ± 2.40	$30.44^{1,a}$ ± 2.42	$31.38^{1,a}$ ± 1.78	$26.44^{1,a}$ ± 1.62	$28.27^{1,a}$ ± 1.65	$28.22^{1,a}$ ± 2.08	$31.44^{1,a}$ ± 1.90	$29.11^{1,a}$ ± 2.74	$p>0.05$
	G_{Melox}, $n=15$	$26.10^{1,a}$ 2.24	$30.86^{1,a}$ ± 1.91	$32.08^{1,a}$ ± 2.97	$30.79^{1,a}$ ± 2.14	$32.07^{1,a}$ ± 2.67	$32.21^{1,a}$ ± 2.00	$32.90^{1,a}$ ± 2.22	$34.18^{1,a}$ ± 2.69	$30.08^{1,a}$ ± 1.69	$30.08^{1,a}$ ± 1.98	$p>0.05$
	G_{CBD} $n=15$	$26.85^{1,a}$ ± 1.20	$30.82^{1,a}$ ± 1.73	$32.29^{1,a}$ ± 1.56	$32.77^{1,a}$ ± 1.18	$33.33^{1,a}$ ± 1.55	$31.93^{1,a}$ ± 1.53	$34.08^{1,a}$ ± 1.80	$33.85^{1,a}$ ± 2.13	$29.20^{1,a}$ ± 1.78	$32.31^{1,a}$ ± 1.46	$p>0.05$
	$G_{Melox/CBD}$ $n=15$	$25.50^{1,a}$ ± 1.29	$30.64^{1,a}$ ± 1.50	$29.67^{1,a}$ ± 1.40	$31.46^{1,a}$ 1.27	$31.92^{1,a}$ ± 2.28	$34.64^{1,a}$ ± 1.67	$32.92^{1,a}$ ± 1.97	$34.00^{1,a}$ ± 1.68	$32.36^{1,a}$ ± 0.88	$31.42^{1,a}$ ± 1.83	$p>0.05$
	P value	$p>0.05$	$p>0.05$	$p>0.05$	$p>0.05$	$p>0.05$	$p>0.05$	$p>0.05$	$p>0.05$	$p>0.05$	$p>0.05$	
CV (mm./seg.)	G_0 $n=15$	$3.82^{1,a}$ ± 0.16	$2.98^{1,a}$ ± 0.29	$2.90^{1,a}$ ± 0.30	$2.65^{1,a}$ ± 0.27	$3.11^{1,a}$ ± 0.25	$3.14^{1,a}$ ± 0.29	$3.16^{1,a}$ ± 0.14	$3.18^{1,a}$ ± 0.24	$3.32^{1,a}$ ± 0.18	$3.44^{1,a}$ ± 0.36	$p>0.05$
	G_{Melox}, $n=15$	$3.16^{1,a}$ ± 0.16	$3.57^{1,a}$ ± 0.21	$3.59^{1,a}$ ± 0.20	$3.14^{1,a}$ ± 0.17	$3.30^{1,a}$ ± 0.21	$3.38^{1,a}$ ± 0.31	$3.27^{1,a}$ ± 0.28	$3.27^{1,a}$ ± 0.22	$3.47^{1,a}$ ± 0.19	$3.28^{1,a}$ ± 0.26	$p>0.05$
	G_{CBD} $n=15$	$2.75^{1,a}$ ± 0.19	$3.35^{1,a}$ ± 0.23	$3.44^{1,a}$ ± 0.20	$3.29^{1,a}$ ± 0.20	$3.27^{1,a}$ ± 0.19	$3.61^{1,a}$ ± 0.19	$3.36^{1,a}$ ± 0.19	$3.73^{1,a}$ ± 0.20	$3.22^{1,a}$ ± 0.18	$3.27^{1,a}$ ± 0.25	$p>0.05$
	$G_{Melox/CBD}$ $n=15$	$3.43^{1,a}$ ± 0.21	$3.17^{1,a}$ ± 0.19	$3.52^{1,a}$ ± 0.21	$3.28^{1,a}$ ± 0.18	$3.50^{1,a}$ ± 0.21	$3.53^{1,a}$ ± 0.21	$3.35^{1,a}$ ± 0.12	$3.47^{1,a}$ ± 0.24	$3.42^{1,a}$ ± 0.24	$3.37^{1,a}$ ± 0.19	$p>0.05$
	P value	$p>0.05$	$p>0.05$	$p>0.05$	$p>0.05$	$p>0.05$	$p>0.05$	$p>0.05$	$p>0.05$	$p>0.05$	$p>0.05$	

(Continued)

TABLE 2 (Continued)

Parameters	Treatments	Post-surgical events										*P* value
		E_{Basal}	$E_{30Min.}$	$E_{1h.}$	$E_{2h.}$	$E_{3h.}$	$E_{4h.}$	$E_{8h.}$	$E_{12h.}$	$E_{24h.}$	$E_{48h.}$	
MCV (mm./seg.)	G_0 $n = 15$	$5.25^{1,a} \pm 0.46$	$4.61^{1,a} \pm 0.54$	$4.62^{1,a} \pm 0.53$	$4.78^{1,a} \pm 0.41$	$4.75^{1,a} \pm 0.44$	$5.26^{1,a} \pm 0.28$	$5.41^{1,a} \pm 0.49$	$5.51^{1,a} \pm 0.56$	$5.31^{1,a} \pm 0.35$	$5.77^{1,a} \pm 0.58$	$p > 0.05$
	G_{Melox}, $n = 15$	$5.16^{1,a} \pm 0.24$	$5.68^{1,a} \pm 0.32$	$5.73^{1,a} \pm 0.28$	$5.84^{1,a} \pm 0.46$	$6.15^{1,a} \pm 0.41$	$6.62^{1,a} \pm 0.42$	$5.93^{1,a} \pm 0.41$	$5.97^{1,a} \pm 0.40$	$5.42^{1,a} \pm 0.37$	$5.46^{1,a} \pm 0.41$	$p > 0.05$
	G_{CBD} $n = 15$	$5.05^{1,a} \pm 0.33$	$5.41^{1,a} \pm 0.32$	$5.89^{1,a} \pm 0.38$	$5.45^{1,a} \pm 0.34$	$5.32^{1,a} \pm 0.41$	$6.29^{1,a} \pm 0.35$	$5.38^{1,a} \pm 0.34$	$5.73^{1,a} \pm 0.41$	$5.10^{1,a} \pm 0.25$	$5.43^{1,a} \pm 0.28$	$p > 0.05$
	$G_{Melox/CBD}$ $n = 15$	$5.22^{1,a} \pm 0.32$	$5.23^{1,a} \pm 0.38$	$6.14^{1,a} \pm 0.33$	$5.43^{1,a} \pm 0.32$	$5.62^{1,a} \pm 0.34$	$5.66^{1,a} \pm 0.25$	$5.69^{1,a} \pm 0.27$	$5.79^{1,a} \pm 0.27$	$5.69^{1,a} \pm 0.37$	$5.90^{1,a} \pm 0.30$	$p > 0.05$
	P value	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p > 0.05$	
Lat. (mm./seg.)	G_0 $n = 15$	$0.19^{1,c} \pm 0.00$	$0.30^{1,a,b} \pm 0.02$	$0.32^{1,a,b,1} \pm 0.03$	$0.33^{1,a,b} \pm 0.03$	$0.27^{1,a,b} \pm 0.03$	$0.26^{1,a,b} \pm 0.01$	$0.27^{1,a,b} \pm 0.01$	$0.27^{1,a,b} \pm 0.02$	$0.22^{1,b} \pm 0.01$	$0.25^{1,a,b} \pm 0.01$	**p = 0.002**
	G_{Melox}, $n = 15$	$0.22^{1,a} \pm 0.01$	$0.25^{1,a} \pm 0.01$	$0.23^{2,a} \pm 0.01$	$0.23^{2,a} \pm 0.01$	$0.23^{1,a} \pm 0.01$	$0.22^{1,a} \pm 0.01$	$0.23^{1,a} \pm 0.02$	$0.22^{1,a} \pm 0.01$	$0.20^{1,a} \pm 0.00$	$0.22^{1,a} \pm 0.01$	$p > 0.05$
	G_{CBD} $n = 15$	$0.23^{1,a} \pm 0.01$	$0.23^{1,a} \pm 0.01$	$0.22^{2,a} \pm 0.00$	$0.26^{1,2,a} \pm 0.01$	$0.22^{1,a} \pm 0.02$	$0.23^{1,a} \pm 0.01$	$0.25^{1,a} \pm 0.01$	$0.21^{1,a} \pm 0.02$	$0.25^{1,a} \pm 0.01$	$0.20^{1,a} \pm 0.00$	$p > 0.05$
	$G_{Melox/CBD}$ $n = 15$	$0.21^{1,a} \pm 0.02$	$0.25^{1,a} \pm 0.02$	$0.23^{2,a} \pm 0.01$	$0.20^{2,a} \pm 0.00$	$0.23^{1,a} \pm 0.01$	$0.23^{1,a} \pm 0.01$	$0.21^{1,a} \pm 0.01$	$0.22^{1,a} \pm 0.01$	$0.23^{1,a} \pm 0.01$	$0.20^{1,a} \pm 0.00$	$p > 0.05$
	P value	$p > 0.05$	$p > 0.05$	**p = 0.01**	**p = 0.003**	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p > 0.05$	

[a,b,c]Different literals by row indicate significant differences between events for the same treatment. [1,2,3,4]Different numerals by column indicate significant differences between treatments for the same event. T = treatments (G_0: negative group, G_{Melox}: Meloxicam group, G_{CBD}: Cannabidiol group, $G_{Melox/CBD}$: Mexociam and Cannabidiol group). E: post-surgical events (E_{Basal}: 30 min. Pre-surgery; $E_{30\,min.}$: 30 min post- surgery; $E_{1h.}$: 1 h post-surgery; $E_{2h.}$: 2 h post-surgery; $E_{3h.}$: 3 h post-surgery; $E_{4h.}$: 4 h post-surgery; $E_{8h.}$: 8 h post-surgery; $E_{12h.}$: 12 h post-surgery, $E_{24h.}$: 24 h post-surgery; $E_{48h.}$: 48 h post-surgery). NPi, Neurological pupil index. Size: Maximum pupil size before constriction. MIN, Pupil diameter at peak constriction; CH, Percentage of pupil change; CV, Constriction velocity; MCV, Maximum constriction velocity; Lat., Latency of constriction. Bold values represent statistically significant differences.

TABLE 3 Pain evaluation scale values (Median ± EE) in the evaluation events (E) of 60 bitches undergoing elective ovariohysterectomy surgeries distributed in 4 study groups: G_0, G_{Melox}, G_{CBD}, $G_{Melox/CBD}$.

Parameter	Treatments	Post-surgical Events										P value
		E_{Basal}	$E_{30Min.}$	$E_{1h.}$	$E_{2h.}$	$E_{3h.}$	$E_{4h.}$	$E_{8h.}$	$E_{12h.}$	$E_{24h.}$	$E_{48h.}$	
GCMPS-SF	G_0 $n = 15$	$0^{1,e} \pm 0$	$8.00^{a,1} \pm 0.83$	$9.00^{a,1} \pm 0.66$	$6.00^{1,a,b} \pm 0.67$	$6.00^{1,a,b,c} \pm 0.60$	$4.00^{1,b,c,d} \pm 0.51$	$3.00^{1,c,d} \pm 0.49$	$3.00^{1,d} \pm 0.36$	$3.00^{1,c,d} \pm 0.38$	$3.00^{1,d} \pm 0.44$	**p < 0.0001**
	G_{Melox}, $n = 15$	$0^{1,b} \pm 0$	$3.00^{2,a} \pm 0.13$	$2.50^{2,a} \pm 0.54$	$2.50^{2,a} \pm 0.34$	$2.50^{2,a} \pm 0.31$	$2.00^{2,a} \pm 0.27$	$1.00^{1,a} \pm 0.25$	$1.00^{1,a} \pm 0.22$	$1.00^{1,a} \pm 0.22$	$1.00^{1,a} \pm 0.19$	**p < 0.0001**
	G_{CBD} $n = 15$	$0^{1,d} \pm 0$	$3.00^{2,a} \pm 0.48$	$3.00^{2,a} \pm 0.68$	$3.00^{2,a,b,c} \pm 0.29$	$3.00^{2,a,b} \pm 0.68$	$3.00^{1,2,a,b,c} \pm 0.43$	$2.00^{1,a,b,c} \pm 0.28$	$3.00^{1,a,b,c} \pm 0.38$	$1.00^{1,b,c} \pm 0.23$	$1.00^{1,c} \pm 0.15$	**p < 0.0001**
	$G_{Melox/CBD}$ $n = 15$	$0^{1,b} \pm 0$	$3.00^{2,a} \pm 0.35$	$3.00^{2,a} \pm 0.53$	$3.00^{2,a} \pm 0.32$	$3.00^{2,a} \pm 0.31$	$1.50^{1,2,a} \pm 0.46$	$1.00^{1,a} \pm 0.27$	$2.00^{1,a} \pm 0.36$	$1.00^{1,a} \pm 0.18$	$1.00^{1,a} \pm 0.27$	**p < 0.0001**
	P value	$p > 0.05$	**p < 0.0001**	**p < 0.0001**	**p < 0.0001**	**p = 0.001**	**p = 0.03**	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p > 0.05$	

[a,b,c]Different literals by row indicate significant differences between events for the same treatment. [1,2,3,4]Different numerals by column indicate significant differences between treatments for the same event. T = treatments (G_0: negative group, G_{Melox}: Meloxicam group, G_{CBD}: Cannabidiol group, $G_{Melox/CBD}$: Mexociam and Cannabidiol group). E: post-surgical events (E_{Basal}: 30 min. Pre-surgery; $E_{30\,min.}$: 30 min post- surgery; $E_{1h.}$: 1 h post-surgery; $E_{2h.}$: 2 h post-surgery; $E_{3h.}$: 3 h post-surgery; $E_{4h.}$: 4 h post-surgery; $E_{8h.}$: 8 h post-surgery; $E_{12h.}$: 12 h post-surgery, $E_{24h.}$: 24 h post-surgery; $E_{48h.}$: 48 h post-surgery). GCMPS, Glasgow Composite Pain Score. Bold values represent statistically significant differences.

Sagar et al. (44) reported that the use of a CB1 receptor agonist decreased Ca^{2+} conductance induced by capsaicin stimulation in dorsal horn neurons of the spinal cord, which could be an explanation for the antinociceptive effect observed in this study.

On the other hand, CB2 agonism could lead to the reduction of an inflammatory response (51) by mediating tumor necrosis factor-alpha (TNF-α) and interleukins from microglia or macrophages (14). Gugliandolo et al. (20) mentioned that the administration of cannabidiol in dogs receiving lipopolysaccharide reduced the presence of interleukin (IL)-10, nuclear factor-kappa B (NF), and the expression of cyclooxygenase 2 (COX-2). Therefore, the reduction in the expression and activity of COX-2 also inhibits the formation of prostaglandins such as prostaglandin E2 (PGE2) and lipoxygenases, subsequently decreasing the expression of proinflammatory metabolites (52). This mechanism of action is also associated with the reduction of proinflammatory cytokine synthesis such as IL-1, IL-8, NFκB, and TNF-α (53, 54). Hence, the evidence suggests that CBD can help to manage or

TABLE 4 Correlation matrix between Glasgow Composite Pain Scale (GCMPS) values of 60 bitches undergoing elective ovariohysterectomy surgeries distributed in 4 study groups: G_0, G_{Melox}, G_{CBD}, $G_{Melox/CBD}$.

Correlation	NPi	Size	MIN	CH	CV	MCV	Lat	GCMPS-SF
Npi	1.00 $p < 0.0001$	0.38 $p = 1.00$	−0.07 $p = 0.08$	0.81 $p = 1.00$	0.00 $p = 0.90$	0.56 $p = 1.00$	−0.11 $p = 0.81$	0.09 $p = 0.03$
Size	0.38 $p = 0.98$	1.00 $p < 0.0001$	0.76 $p = 1.00$	0.10 $p = 1.00$	0.03 $p = 0.39$	0.18 $p = 1.00$	−0.09 $p = 0.03$	0.10 $p = 0.01$
MIN	−0.08 $p = 0.08$	0.76 $p = 1.00$	1.00 $p < 0.0001$	−0.29 $p = 1.00$	0.07 $p = 0.08$	−0,14 $p = 1.00$	−0.07 $p = 0.08$	0.05 $p = 0.24$
CH	0.82 $p = 0.99$	0.10 $p = 0.01$	−0.29 $p = 1.00$	1.00 $p < 0.0001$	0.03 $p = 0.47$	0.50 $p = 1.00$	0.02 $p = 0.59$	0.07 $p = 0.11$
CV	0.01 $p = 0.98$	0.03 $p = 0.39$	0.07 $p = 0.08$	0.03 $p = 1.00$	1.00 $p < 0.0001$	0.03 $p = 1.00$	−0.13 $p = 0.002$	0.03 $p = 0.39$
MCV	0.57 $p = 0.98$	0.18 $p < 0.0001$	−0.14 $p = 0.001$	0.50 $p = 1.00$	0.03 $p = 0.49$	1.00 $p < 0.0001$	−0.06 $p = 0.14$	0.08 $p = 0.05$
Lat	−0.01 $p = 0.98$	−0,09 $p = 0.02$	−0.07 $p = 0.08$	0.02 $p = 1.00$	−0.13 $p = 0.002$	−0.06 $p = 1.00$	1.00 $p < 0.0001$	0.03 $p = 0.42$
GCMPS	0.10 $p = 0.98$	0.10 $p = 0.01$	0.05 $p = 0.24$	0.09 $p = 1.00$	0.03 $p = 0.39$	0.08 $p = 1.00$	0.03 $p = 0.42$	1.00 $p < 0.0001$

GCMPS, Glasgow Composite Pain Score; NPi, Neurological pupil index; Size, Maximum pupil size before constriction; MIN, Pupil diameter at peak constriction; CH, Percentage of pupil change; CV, Constriction velocity; MCV, Maximum constriction velocity; Lat., Latency of constriction.

reduce pain by reducing the inflammatory process, possibly being an additional mechanism of pain control.

The pupillometric data obtained in this study showed the analgesic activity of meloxicam due to the preferential inhibition of COX-2 (55, 56). This isoform of COX is the most active during an inflammatory process and is responsible for the production of prostaglandins (57). The inhibition of COX-2 prevents the increase in phospholipase A2 in dorsal horn neurons of the spinal cord, which can consequently prevent the expression of substance P, serotonin, histamine, PGE2, and proinflammatory cytokines (58–60). Preanesthetic administration of meloxicam can prevent peripheral and central sensitization phenomena during nociceptive events due to its pharmacodynamic properties (61, 62).

During the perception of pain, there is an increase in the activity of the sympathetic nervous system (SNS), so NSAIDs like meloxicam can reduce autonomic activity (63, 64). Hernández-Avalos et al. (65) reported that meloxicam increases parasympathetic tone or PTA index similarly to the use of carprofen and paracetamol by decreasing sympathetic nervous system (SNS) activity. The decrease in SNSi activity due to a predominant parasympathetic tone inhibits the stimulation in the Edinger-Westphal nucleus and, in turn, promotes miosis in the pupil (66), as observed in the present results. This effect explains that G_{Melox} obtained the lowest value in the Size variable compared to the other study groups during E_{24h}. ($p < 0.05$). However, it should be considered that, in dogs, meloxicam's half-life is 24 h, which is why re-administration of meloxicam was necessary at this point to maintain adequate plasma levels and therapeutic effect (67), a situation that could have altered the pupillary response of the study subjects.

CBD, by its agonism to CB1 and CB2 receptors, prevents the transmission of nociceptive stimuli by inhibition of central neurotransmitters. On the other hand, meloxicam modulates PGE2 formation (6). Combining both drugs results in a multimodal analgesia that allows pain control at different points of the nociceptive pathway (55, 68). Thus, this could be the possible explanation for $G_{Melox/CBD}$ having a lower MIN compared to the other groups during E_{3hr} ($p < 0.05$) and would reaffirm the fact that CBD exhibits analgesia similar to meloxicam. Therefore, based on our results, CBD can be used to control acute pain in dogs undergoing abdominal surgery and during the immediate postsurgical period. Similarly, according to the findings regarding infrared pupillometry, it can be suggested that the nociceptive response of dogs undergoing OVH and receiving CBD alone or in combination with meloxicam was similar.

Since pain is a subjective condition, its perception may differ among individuals (69). For this reason, it is suggested to use scales that integrate both behavioral and physiological indicators to recognize pain (70–73). In the present research, pain management during the immediate postoperative period could explain the differences observed in this study during the first hours of post-surgery evaluation, since the use of analgesics at the first signs of pain could help control long-term physiological changes and alter the scale scores (74). The scores obtained show the importance of using analgesics before surgery, which could prevent sensitization phenomena and, thus, pain perception (75).

On the other hand, the presence of a larger Size, MIN, and Lat value in G_0 compared to G_{Melox}, G_{CBD}, and $G_{Melox/CBD}$ suggests that the pupillary response can be used as a method to recognize postoperative pain in dogs. This has been described in dogs, in whom a positive association between pupil diameter and the value obtained in the numerical rating scale was reported, highlighting that its assessment was limited to the presence or absence of the pupillary reflex (76). The possible neurobiological explanation for the increase in pupil diameter is the increase in SNS activity with catecholamine neurosecretion when animals perceive pain (77). Catecholamines have an effect on α1 adrenergic receptors present in the long ciliary fibers of the iris dilator muscle, which activation would lead to pupil dilation (78, 79). This was observed in G_0 animals during E_{2hr}, values that were also associated with increasing scores in the GCMPS-SF. A similar association between pupil diameter and pain scales has been reported in human medicine (43, 80, 81). Therefore, the present findings suggest a possible relationship between pain scales and the pupillary response in animals. Although further research is needed to establish the correlation

between both methods to evaluate pain, the application of pupillometry could help to refine pain assessment in companion animals (4, 8).

Size and MIN represent an increase in the pupil diameter; however, the response to the light stimulus increased both in the left and right eye. This can also be evaluated through Lat, where the highest values were recorded in G_0 in both the left and right eyes, in comparison with G_{Melox}, G_{CBD}, and $G_{Melox/CBD}$. This indicates that the pupil speed is greater when faced with a light stimulus (27). In this sense, Mills et al. (28) suggested that the maximum value of Lat in dogs is 0.30 s, a value that was below the ones reported in the present study, possibly due to nociception. The pupillary response observed in animals during the perception of pain is related to the activation of the Locus Coeruleus, a region that contains pre-motor and excitatory sympathetic neurons that are projected to preganglionic neurons in the Edinger-Westphal nucleus and present in α2 adrenergic receptors. Through sympathoexcitation and parasympathetic inhibition, these fibers cause pupillary dilation, decreasing the response to light (82). Therefore, this could be the first time addressing the influence that these drugs have on the pupil diameter of dogs.

The increase in these values occurred at E_{30min}, E_{1h}, and E_{2h}, when animals in G_0 received rescue analgesia. In this sense, although there could be a residual effect of anesthetics, it is reported that pupil dilation has a positive relation with anesthetic depth (83, 84). This effect could only be observed in G_0 at E_{30min}, in contrast to the G_{Melox}, G_{CBD}, and $G_{Melox/CBD}$ groups, which was not observed at E_{1h}. and E_{2h}. It is necessary to mention that meloxicam has an elimination half-life of 24 h in dogs (67), while CBD has an elimination half-life of 3 to 5 days (14). This coincides with the increase in pain scores assessed with the behavioral-based scale in G_0.

The present findings suggest that pupillometry could be used to recognize pain in dogs subjected to OVH. However, it is necessary to consider that increased values during the immediate postsurgical period where pain control is essential to avoid the physiological consequences of pain might coincide with these critical events (72, 73). This would explain why the pupillometric parameters and pain scores decreased in the subsequent events. Dyson (85) explains that pain control during the first hours after surgery reduces the risk of short- and long-term complications. Therefore, this evidence could lead to corroborating the theory that this tool can be used as an objective and quantitative way of acute pain in animals (86). Additionally, the sensitivity and specificity for MIN and Lat. were greater than 80%, possibly making it a reliable tool for assessing pain in animals. This has been reported in humans, recording a sensitivity of around 100% and a specificity of 77% (80). Regardless of the species, future studies need to consider the clinical application of pupillometry.

Regarding rescue analgesia, it was observed that G_{Melox} and G_{CBD} required more rescue analgesia than $G_{Melox/CBD}$ ($G_{Melox} = 1$, $G_{CBD} = 1$, $G_{Melox/CBD} = 0$). This is due to the effect of multimodal analgesia in which CBD inhibits the nociceptive stimulus while meloxicam negatively alters the nociceptive signal at the peripheral level, preventing pain perception (68, 87). However, when comparing the number of animals that required rescue analgesia in G_{Melox} and G_{CBD}, these were significantly lower than G_0, where all animals received rescue analgesia due to the lack of an analgesic protocol before the surgery. Thus, these observations add to the importance of providing analgesia to dogs before the surgical procedure to avoid pain-related complications during the postoperative period (88). Furthermore, at E_{30min} the increase in pupillometry parameters was related to an increase in the GCMPS score. However, this might be attributed to the residual effect of general anesthetics and sedatives such as α2 agonists (89). Thus, this could be considered a limitation on the use of pupillometry in surgical patients.

One of the main limitations of the present study is that current pupillometry does not consider the anatomical and conformational characteristics of a dog's eyes. For example, the iris pigmentation and morphology might affect the accuracy of pupillometric variables (90). This needs to be established in future research when implementing pupillometry as a complementary tool to assess pain. Another field of research would be implementing pupillometry during other surgical procedures such as trauma surgery where there is a greater risk of pain perception. Other limitation could be the level of fear that awake animals might experience, which needs further study to improve the application of pupillometry in veterinary medicine. Likewise, physiological parameters are not reported during the postoperative period, which can be modified due to the painful experience. This limitation arises from the incorporation of these parameters into another paper derived from the present research. Finally, another important perspective is the correlation with other methods that have been suggested to evaluate pain, such as the physiological parameters, the parasympathetic tone index monitor and infrared thermography (90–98).

5 Conclusion

According to the results obtained through pupillometry and the GCMPS-SF scores, CBD alone or in combination with meloxicam has a similar analgesic effect for the control of acute pain in dogs. The findings of the present study suggest that infrared pupillometry could be implemented as a tool to recognize acute pain in ovariohysterectomized bitches.

Data availability statement

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

Ethics statement

The animal studies were approved by the Ph.D. Program in the Biological and Health Science Academic Committee (number CBS.066.21). The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

AC-A: Writing – review & editing, Writing – original draft, Supervision, Methodology, Formal analysis. JM-B: Writing – review & editing, Writing – original draft, Supervision. IH-Á: Writing – review & editing, Writing – original draft, Supervision, Methodology, Conceptualization. PM-M: Writing – review & editing, Writing – original draft, Supervision, Methodology. AM-C: Writing – review & editing, Writing – original draft, Supervision. AD-O: Writing – review & editing, Writing – original draft, Supervision. DM-R:

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Funding

The author(s) declare that no financial support was received for the research, authorship, and/or publication of this article.

Acknowledgments

This is study is part of the PhD. Thesis by Alejandro Casas-Alvarado, a graduate student in the program of "Doctorado en Ciencias Biológicas y de la Salud" at UAM campus Xochimilco; (CONACYT scholarship no. 927304).

Conflict of interest

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

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2024.1380022/full#supplementary-material

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frontiers | Frontiers in Veterinary Science

TYPE Original Research
PUBLISHED 05 July 2024
DOI 10.3389/fvets.2024.1436308

OPEN ACCESS

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RECEIVED 21 May 2024
ACCEPTED 26 June 2024
PUBLISHED 05 July 2024

CITATION
Wickstead FA, Milner PI and Bardell DA (2024) Use of wound infusion catheters for delivery of local anesthetic following standing partial ostectomy of thoracolumbar vertebral spinous processes in horses is not associated with increased surgical site infections. *Front. Vet. Sci.* 11:1436308. doi: 10.3389/fvets.2024.1436308

Use of wound infusion catheters for delivery of local anesthetic following standing partial ostectomy of thoracolumbar vertebral spinous processes in horses is not associated with increased surgical site infections

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Background: Wound infusion catheters (WICs) have been used in humans and some veterinary species for post-operative local anesthetic administration following a variety of surgical procedures, aiming to reduce post-operative analgesia requirements and improve patient comfort. Benefit in reduction in pain, post-operative analgesia requirements and length of hospital stay are well documented in humans, but use of WICs may not have been widely adopted in veterinary species due to the concern of increased complications, such as dehiscence or surgical site infection (SSI), creating a barrier to their use. This study aimed to evaluate the use of WICs in horses undergoing standing partial ostectomy surgeries, document complications and investigate if the incidence of SSI was equivalent between those horses that did and did not have a WIC.

Methods: Clinical records were searched between January 2010–December 2023 for horses undergoing standing partial ostectomy surgery of thoracolumbar vertebral spinous processes at one institution. Population variables (age, breed, bodyweight), placement of a WIC or not, post-operative complications, analgesia protocols and surgical time were recorded. Horses received up to 0.1 mg kg^{-1} bupivacaine (0.5 mg mL^{-1}) every 6–8 h via the WIC where one was placed. To compare SSI complication incidence between using or not using a WIC, a proportional independent equivalence test was used.

Results: There were 64 horses included in the final analysis with a WIC placed in 29/64 horses (45.3%) and 35/64 (54.7%) having no WIC placed at surgery. Incidence of short-term SSI was 11.4% (no WIC used) and 13.8% (WIC used), respectively. The difference in proportion of SSI between the presence or absence of a WIC was not significant [−0.024 (90% CI −0.181; 0.133); $p = 0.94$].

Conclusion: The incidence of SSIs was equivalent between groups whether a WIC was used or not. WICs should be considered as part of a multi-modal analgesic approach in the post-operative period. Further research into local anesthetic dosing and its impact on rescue analgesia requirements and pain-scores is warranted.

KEYWORDS
wound catheter, local anesthetic, horse, ostectomy, vertebral spinous process

Introduction

Managing post-operative pain in the horse can be a challenge due to the ability to accurately recognize changes in pain status following surgical intervention (1). Often post-operative management involves the use of systemic administration of analgesics which in themselves may result in unwanted side-effects. Additionally, horses affected by conditions resulting in chronic pain have modulated pain pathways, potentially limiting the efficacy of systemically administered drugs (2).

Partial ostectomy is a recognized surgical technique for management of impingement of the vertebral spinous processes ("kissing spines") in the thoracolumbar region. Originally performed under general anesthesia (3, 4) this procedure is now commonly undertaken with regional anesthesia in the standing sedated horse (5, 6). Due to the invasive nature of the procedure and the removal of sections of bone from multiple processes, post-operative complications can include acute post-operative discomfort, swelling and surgical site infection. Providing adequate analgesia in these patients, particularly in the immediate post-operative period is essential but difficult to achieve.

Wound infusion catheters (WICs) have been used routinely for post-operative local anesthetic administration in humans, with significant reductions in opioid requirement noted following surgery (7–9). Use of WICs for provision of post-operative analgesia in dogs and cats following limb amputation, total ear canal ablation and injection site sarcoma resection has been described, but similar benefits are not reported (10–12). The use of WICs has not been widely adopted for post-operative pain management in equine surgery, potentially due to concerns over increased wound complications such as dehiscence, infection and seroma formation. The objective of this study was to evaluate the use of WICs in a cohort of horses undergoing standing partial ostectomy of thoracolumbar vertebral spinous processes, and document post-operative complications compared to horses undergoing the same procedure, but without a WIC being used. Our hypothesis was that there is equivalence in incidence of short-term surgical site infection (SSIs) in horses undergoing partial ostectomy of the vertebral spinous processes whether a WIC was used or not.

Materials and methods

Data collection

Institutional ethical approval (VREC 1423 date: 10.31.23) was obtained for this study. Clinical records for horses admitted to the Leahurst Equine Hospital, University of Liverpool, UK between January 2010 and December 2023 for surgical management of impingement of thoracolumbar spinous processes by partial ostectomy under standing sedation were inspected. Explanatory variables collected included age (years), sex, breed, bodyweight (kg), surgeon, surgical time (minutes), number of spinous processes operated on, post-operative analgesia used, presence of surgical site infection (yes/no), presence of moderate/marked swelling (yes/no), time from surgery to hospital discharge (days). Surgical site infection (SSI) was defined as purulent discharge from the incision, positive bacterial culture from the incision and/or evidence of wound breakdown (or deliberate opening of the surgical incision to allow drainage) (13), prior to hospital discharge. Cases were grouped into whether a WIC was used or not.

Surgical method and wound infusion catheter protocol

All horses were administered acepromazine [0.03 mg kg^{-1} intramuscular (IM), Tranquinervin, Dechra] 45 min prior to surgical preparation. Flunixin meglumine [1.1 mg kg^{-1} intravenous (IV), Finadyne, MSD] and either procaine penicillin (20 mg kg^{-1} IM, Depocillin, MSD), gentamicin sulfate (6.6 mg kg^{-1} IV, Genta-Equine, Dechra), or oxytetracycline (7.5 mg kg^{-1} IV, Engemycin, MSD) were administered 30 min prior to the start of surgery. A cannula was placed in the left jugular vein and horses were administered xylazine (0.5 mg kg^{-1} IV, Virbaxyl, Virbac), romifidine (0.05 mg kg^{-1} IV, Sedivet, Boehringer Ingelheim) or detomidine (0.008 mg kg^{-1} IV, Equimidine, Zoetis) as an intravenous bolus and continued as an intravenous infusion, alongside morphine sulfate (0.2 mg kg^{-1} IV, Morphine sulfate, Martindale Pharma) during the procedure. Mepivicaine hydrochloride [Intra-epicaine (2%), Dechra] was infiltrated around the surgical site (4 mL cm^{-1}; up to 8 mg kg^{-1}) 10 min prior to the start of surgery. Surgery consisted of a midline incision through the skin, subcutis and supraspinous ligament to access the affected spinous processes. Partial ostectomy of relevant processes was performed using a combination of oscillating saw and rongeurs. Closure of the supraspinous ligament, subcuticular and skin layers was then performed. Post-operatively all horses received 4.4 mg kg^{-1} phenylbutazone IV twice daily for 24 h, reducing to 2.2 mg kg^{-1} twice daily PO. Systemic antimicrobials were continued for 3–5 days.

For horses where a WIC was used, a 16 gauge diffusion catheter (Mila, Kentucky, United States) was placed along the length of the resected spinous processes and exited through a separate skin incision approximately 3 cm to the left of the cranial extent of the surgical incision (Figure 1). Catheters had either a 6, 7.5 or 9 inch dispersion length according to length of surgical field (Figure 2) and a 0.2 µm filter (Smiths Medical ASD Inc., Minnesota, United States) was secured to the catheter and a cruciate suture pattern used to anchor the filter in place (Figure 3). Bupivicaine [Marcain (0.5%), AstraZeneca] was administered via the WIC at a volume of 0.3 mL cm^{-1} of surgical wound (equivalent of up to 0.1 mg kg^{-1}), at the end of the procedure and then every 6 h for the first 24 h, followed by every 8 h for the next 24 h, under sterile conditions. The surgical site was covered with a non-absorbent adhesive dressing (Primapore, Smith & Nephew) which was protected with an oversewn stent bandage. Dressings were changed every 12 h and assessed for discharge or dehiscence. The WIC filter was covered with an adhesive dressing (Opsite, Smith & Nephew) and sterile bung. The WIC was

removed after 48 h in all cases. Inclusion of a WIC was non-randomized and based on surgeon preference.

Statistical analysis

Data are presented as mean (95% CI) or median (IQR). Statistical analysis was performed using SPSS statistical software (version 29 for Windows, IBM, Chicago, IL, United States). Distribution of data was assessed using visual inspection of histograms, Q-Q plots and Shapiro–Wilk test for normality and Levene's test for equality of variance. Unpaired student's *t*-test or Mann Whitney *U* test were used for univariable analysis of continuous data. Fisher's exact test was used to compare categorical data between groups. An adjusted *p*-value of 0.05/12 = 0.004 was used after Bonferroni correction. To compare complication incidence of SSI between using/not using a WIC, independent proportional equivalence testing was used (14). Results of the equivalence test are presented as proportional difference with 90% CI based on two one-sided testing with upper and lower limits of equivalence set as +/− 0.20. Post-hoc sample size calculation indicated 28 cases per group were required to achieve a significance of 0.05 and power of 0.8 (15).

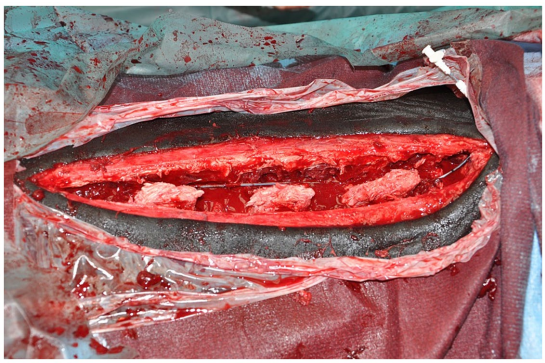

FIGURE 1
Wound infusion catheter *in situ* at end of surgery, prior to closure (cranial is to the right).

Results

Descriptive analysis

There were 64 horses included in the final analysis with a WIC placed in 29/64 horses (45.3%) and 35/64 (54.7%) having no WIC placed at the end of surgery. Mean age of horse included in the study was 9.3 years (95% CI 8.3–10.2) with 38 geldings and 26 mares. Most breeds (34/64) were Thoroughbred or Thoroughbred cross (TB/TBX). There were 11 different surgeons involved with two surgeons performing most surgeries (42 and 32%, respectively). Days to discharge had a median of 5 days (IQR 4–6). The number of vertebral spinous processes operated on were a median of 4 (IQR 3–5), consisting of T13-L3.

There was no significant difference in age, sex, breed, bodyweight, number of spinous processes operated on, surgical time or days to discharge between groups with or without a WIC. There was a significant difference in surgeons using a WIC with one surgeon responsible for using WICs in 24/29 cases ($p < 0.001$). Table 1 summarizes the continuous clinical data for groups with and without a WIC.

Surgical site infection

All horses with WICs tolerated placement and no interference with the catheter post-operatively was reported. SSI was reported in 4/29 (13.8%) horses with a WIC and in 4/35 (11.4%) horses without a WIC. When present, all instances of SSI responded successfully to antimicrobial therapy. The presence, or absence of a WIC did not alter the proportion of horses with SSI [−0.024 (90%CI −0.181; 0.133); $p = 0.94$]. There were no reports of moderate to marked swelling of the surgical site in horses without SSI.

Other post-operative complications

Signs of colic were reported in 2/29 (6.9%) horses with a WIC and in 4/35 (11.4%) horses without (no significant difference between groups, $p = 0.68$). In most cases, signs were described as mild and responded to medical management within 24 h. One horse in the non-WIC group developed persistent and severe colic signs and

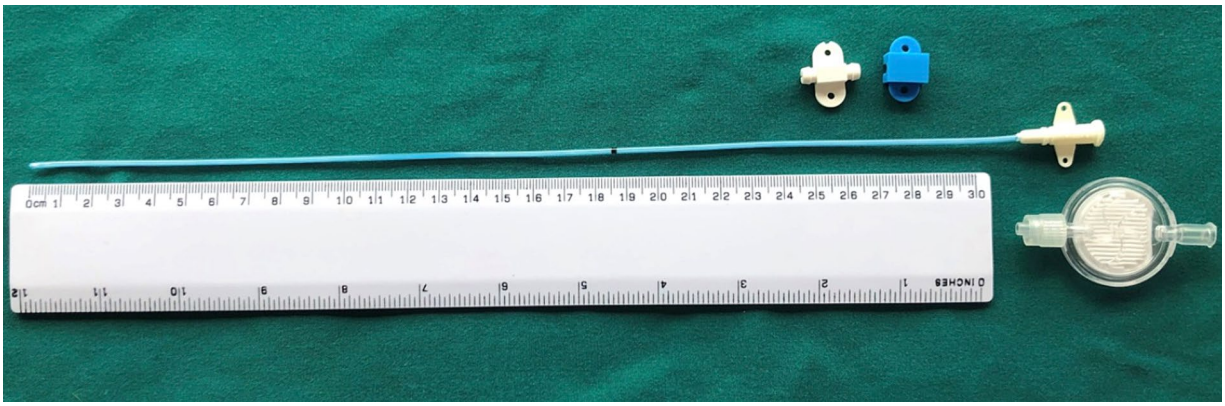

FIGURE 2
Veterinary MILA wound diffusion catheter and filter.

TABLE 1 Univariable analysis of continuous explanatory variables for horses undergoing partial spinous process ostectomy surgery with and without a wound infusion catheter (WIC).

Variable	Non-WIC	WIC	*p*-value
Surgical time (minutes)	111 (96.5–126.2)	107 (94–119.9)	0.66
Days to discharge (days)	5.2 (4.5–6)*	5.7 (4.9–6.4)*	0.24†
Number of SPO	3.5 (3–4)*	4.4 (3.7–5.1)*	0.05†
Bodyweight (kg)	539.0 (514.7–563.3)*	573.4 (541.9–604.8)*	0.07†
Age (years)	9.8 (8.4–11.1)	8.8 (7.4–10.3)	0.33

Independent *t*-tests were used for normally distributed and Mann Whitney *U* tests † for non-normally distributed data. A Bonferroni corrected $p < 0.004$ indicates significance. Data presented as mean (confidence interval 95%) or median (IQR)*. SPO, spinous process ostectomy.

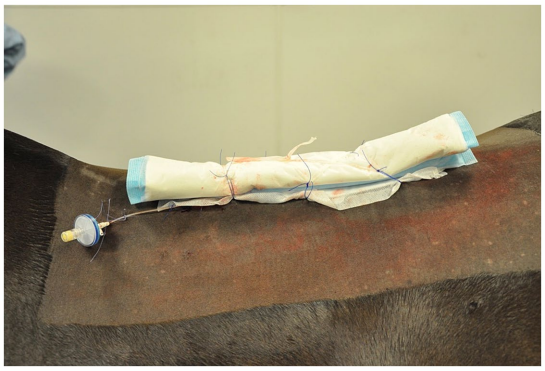

FIGURE 3
Wound infusion catheter and filter in place at end of procedure (cranial is to the left).

underwent exploratory laparotomy 48 h post-surgery, at which time cecal rupture was diagnosed and the horse euthanized. The horse was euthanized, for clinical reasons not related to this study, with IV cinchocaine hydrochloride quinalbarbitone sodium (Somulose, Dechra). All other data from this patient was included for analysis.

Other complications were reported in 2/29 (6.9%) horses with a WIC. One horse developed a head tilt and circling 24 h after WIC removal which resolved spontaneously, and one horse had an elevated heart rate after WIC removal, which responded to phenylbutazone administration and was ascribed to pain. In the non-WIC group, 2/35 (5.7%) horses showed other complications. One horse had an episode of esophageal obstruction which resolved spontaneously, and one horse developed pyrexia for 24 h which resolved without any change in post-operative management.

Discussion

This study found an equivalent incidence of surgical site infections (SSIs) following spinous process ostectomy surgery in horses irrespective of whether a wound infusion catheter (WIC) was placed for administration of bupivacaine for post-operative pain management or not.

Concerns of increased seroma formation, wound drainage, delayed wound healing and increased wound infection rates in relation to the use of WICs have been proposed, but have not been proven in human or veterinary literature (16). Where complications have been reported, they have been described as minor, including seroma formation and patient disconnection from infusion delivery systems, without increased infection rates (10, 12). Abelson et al. (10) reported a 5.5% incidence of SSI with WICs compared to 15% in historic case controls without WICs. No incidents of catheter interference by horses were identified in this study.

Incidence of SSI was 12.5% overall (13.8% in horses with a WIC and 11.4% in those without), in line with previous reports for this surgery (3.5–20%) (4, 15) and similar to studies utilizing WICs in other veterinary species (10, 12, 17). SSIs can lead to wound breakdown, delayed healing, increased patient discomfort, cosmetic and financial implications. All cases in this study received pre-operative antimicrobial treatment which was continued for at least 3 days post-operatively (procaine penicillin, gentamicin sulphate, oxytetracycline). All SSIs resolved with dressing management and antimicrobial therapy based on culture and sensitivity results.

Post-operative colic signs were mild in all cases except one in the non-WIC group, in which cecal rupture occurred resulting in euthanasia of the horse. Colic signs are multi-factorial and include change in housing and management, sedation, pain, stress, and other underlying co-morbidities. Horses that displayed signs of colic had surgery times close to the mean, without excessive use of opioids or alpha-2 agonists. Interestingly, another horse, also in the non-WIC group, developed esophageal obstruction, which resolved spontaneously, making gastrointestinal dysfunction the most frequently reported non-SSI post-operative complication in this study.

Due to the limited duration of action of commonly used local anesthetic agents, repeat administration or continuous infusion is required. Bupivacaine administration in those horses where a WIC was placed, was based on volume per unit length of surgical incision (0.3 mL cm^{-1}, 0.5% bupivacaine) delivered at predefined time points. This was empirically derived and based on surgical closure of dead space requiring minimal volume sufficient to permeate the surgical site and remain below accepted toxic doses, whilst achieving a useful effect. Other authors have reported using lidocaine at 1.2–3 mg kg^{-1} h^{-1} (10, 12) and bupivacaine 1.5 mg kg^{-1} (10, 16) and 0.13–0.21 mg kg^{-1} h^{-1} (17). Effective blockade of the palmar nerves in the equine distal limb has been reported following continuous perineural infusion of 0.5% bupivacaine at 4 mL hr^{-1} (18) and perineural bolus deposition of 3–4 mL of 1.25–2.5 mg mL^{-1} solutions (19). Described in these terms, our dosing regime equates to <0.1 mg kg^{-1} every 6–8 h, so may have been excessively conservative. We utilized commercially available, veterinary specific WICs with variable dispersion lengths (6, 7.5 and 9 inches). Dispersion length was chosen to match incision length as closely as possible, to maximize drug distribution throughout the surgical site, but uniformity of distribution is unknown.

Local anesthetic toxicity can manifest as neurotoxicity, and one horse in the WIC group exhibited neurological signs 24 h after WIC removal. The bupivacaine dosing regime, coupled with the time frame between administration and onset of clinical signs, make it unlikely this was due to systemic absorption of the local anesthetic. The total dose of bupivacaine received was 0.28 mg kg^{-1} over 48 h. Toxic doses

of bupivacaine previously reported vary widely between species and route of administration. In dogs 10 mg kg^{-1} IV resulted in cardiotoxicity (20), whilst in humans cardiovascular collapse requiring resuscitation is reported following 2–3 mg kg^{-1} administered for regional anaesthesia (21). The doses in this study were much lower (up to 0.1 mg kg^{-1}), clinical signs spontaneously resolved rapidly, and the cause remains unknown. Radlinsky et al. (17), using a dosing regime of <0.21 mg kg^{-1} h^{-1} (up to 4.3 mg kg^{-1} day^{-1}) were unable to detect bupivacaine in the plasma of dogs and reported no signs of drug toxicity. Abelson et al. (10) reported one dog with signs of toxicity five hours after starting lidocaine infusion at 1.78 mg kg^{-1} h^{-1}, which resolved after the infusion was discontinued.

Local anesthetics bupivacaine, lidocaine and mepivacaine have been demonstrated to have antibacterial properties. Testing with common equine pathogens inhibited growth up to 93% *in vitro*, with the majority of concentrations achieving bactericidal action, lidocaine being the most potent, followed by bupivacaine, then mepivacaine. Bupivacaine 2.5 mg mL^{-1} (half of the concentration used in this study) inhibited 93% of bacterial isolates (22). Bacterial culture of WIC tips performed by Radlinksy et al. (17) produced positive results in only one dog out of ten. None of the dogs in that report exhibited clinical signs of infection and none required treatment. Perineural catheters placed in the distal limb of horses resulted in swelling which the authors attributed to venous dilation caused by the local anesthetic agent reducing lymphatic drainage and venous return (18, 19). This effect was less apparent with bupivacaine than lidocaine, resolved with discontinuation of infusion and had no further implications (18, 19).

Due to the retrospective nature of this study, we were unable to investigate the impact of WIC use on analgesia requirements, as horses were not routinely pain scored post-operatively and analgesia protocols were largely standardized regardless of WIC use. One horse in the WIC group exhibited an episode of tachycardia which responded to bringing the planned administration of phenylbutazone forward, so was ascribed to a pain response, but this occurred 2 days after WIC removal.

Local anesthetic infiltration of surgical wounds via WICs is reported to improve comfort and reduce opioid requirements in humans undergoing limb surgery, breast surgery, laparoscopy/laparotomy of the abdominopelvic area and cardiothoracic surgery. Greatest benefit is derived in treatment areas of subcutaneous, cutaneous and connective tissue structures, whilst in complex structures such as articulations and areas with multiple innervations, response is less dramatic, but still results in reduced opioid consumption (23–25). In veterinary species, it is unknown if opioid requirements and rescue analgesia differ post-operatively with WIC placement (12, 17). This could be due to several reasons; limited variety of surgical procedures studied; other analgesics used concurrently with the local anesthetic agent, inadequate dosing of local anesthetic, varied anesthetic and analgesic protocols and varied compliance with pain scoring. Additionally, wound inflammation and infection may interfere with local anesthetic efficacy. Some authors have described recording lower pain scores in dogs and horses with WICs, but these differences did not achieve statistical significance and were not sufficient to elicit changes in opioid or rescue analgesia requirements (12, 26).

We were not able to demonstrate an advantage in terms of reduced duration of hospitalization in horses with WICs. The horse with the longest period of hospitalization was in the non-WIC group, but discharge from the hospital was delayed by owner availability, not surgery-related factors. In humans, local anesthetic administration in the post-operative period can reduce length of hospital stay, but this is yet to be demonstrated in veterinary species (27).

This study was subject to limitations common to retrospective analyses, namely similar, but non-standardized sedation and analgesia protocols, inconsistent and largely absent pain scoring, limited and inconsistent clinical notes and lack of long-term follow-up. The use of WICs in horses for post-operative pain management following subtotal spinous process ostectomy surgeries warrants further study. Future work should seek to establish safe and effective local anesthetic administration regimes and determine any potential for reduction in systemic analgesic administration by using rigorous assessment of comfort levels and pain-associated behaviors. If patient comfort is improved by use of WICs and local anesthetic administration, this may lead to quicker hospital discharge, reduced financial costs and improved welfare.

In conclusion, WICs used post-operatively in spinous process ostectomy surgeries did not have a negative impact of incidence of SSI prior to hospital discharge and are easily managed in a hospital setting.

Data availability statement

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

Ethics statement

The animal studies were approved by Veterinary Research Ethics Committee—University of Liverpool. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

FW: Data curation, Investigation, Writing – original draft, Writing – review & editing, Formal analysis. PM: Conceptualization, Supervision, Writing – original draft, Writing – review & editing, Formal analysis. DB: Conceptualization, Supervision, Writing – original draft, Writing – review & editing.

Funding

The author(s) declare that no financial support was received for the research, authorship, and/or publication of this article.

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

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frontiers | Frontiers in Veterinary Science

TYPE Review
PUBLISHED 17 July 2024
DOI 10.3389/fvets.2024.1436795

OPEN ACCESS

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RECEIVED 22 May 2024
ACCEPTED 03 July 2024
PUBLISHED 17 July 2024

CITATION
Chiavaccini L, Gupta A and Chiavaccini G (2024) From facial expressions to algorithms: a narrative review of animal pain recognition technologies. *Front. Vet. Sci.* 11:1436795. doi: 10.3389/fvets.2024.1436795

From facial expressions to algorithms: a narrative review of animal pain recognition technologies

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Facial expressions are essential for communication and emotional expression across species. Despite the improvements brought by tools like the Horse Grimace Scale (HGS) in pain recognition in horses, their reliance on human identification of characteristic traits presents drawbacks such as subjectivity, training requirements, costs, and potential bias. Despite these challenges, the development of facial expression pain scales for animals has been making strides. To address these limitations, Automated Pain Recognition (APR) powered by Artificial Intelligence (AI) offers a promising advancement. Notably, computer vision and machine learning have revolutionized our approach to identifying and addressing pain in non-verbal patients, including animals, with profound implications for both veterinary medicine and animal welfare. By leveraging the capabilities of AI algorithms, we can construct sophisticated models capable of analyzing diverse data inputs, encompassing not only facial expressions but also body language, vocalizations, and physiological signals, to provide precise and objective evaluations of an animal's pain levels. While the advancement of APR holds great promise for improving animal welfare by enabling better pain management, it also brings forth the need to overcome data limitations, ensure ethical practices, and develop robust ground truth measures. This narrative review aimed to provide a comprehensive overview, tracing the journey from the initial application of facial expression recognition for the development of pain scales in animals to the recent application, evolution, and limitations of APR, thereby contributing to understanding this rapidly evolving field.

KEYWORDS

AnimalFACS, computer vision, Convolutional Neural Networks (CNNs), deep learning, facial expressions, machine learning, pain recognition

1 Introduction

In animals and humans, facial expressions play a crucial role as a primary non-verbal method for managing peer interactions and conveying information about emotional states (1). Scientific interest in facial expressions was initiated in the 1860s by Duchenne de Boulogne. However, it is in the last two decades that the utilization of facial expressions for understanding emotional conditions, such as pain, has expanded in both humans and non-human species (2). Notably, it was demonstrated that facial expressions of pain show consistency across ages, genders, cognitive states (e.g., non-communicative patients), and different types of pain and may correlate with self-report of pain in humans (3, 4).

Analyzing facial expressions and body language in animals poses unique challenges absent in human medicine, like data collection, establishing ground truth—that is, determining whether or not the animal is experiencing pain or distress, and navigating the vast array of morphological differences, shapes, and colors present within and across animal species (5, 6). Various scales for interpreting facial expressions in animals have been created in the past decade. The Mouse Grimace Scale (MGS) was the first facial grimace scale for animal pain assessment, developed from studies on emotional contagion in mice, and led to the creation of similar scales for other species, such as the Rat Grimace Scale (RGS) (7, 8). These scales, now developed for 11 species, have been used in various pain models, including surgical procedures and husbandry practices. Despite their usefulness, limitations include the fact that most of these pain scales were developed based on a restricted number of action units (AUs) retrieved from picture-based recognition patterns, as described in more details later.

Computational tools, especially those based on computer vision (CV), provide an attractive alternative. Automated Pain Recognition (APR) is an innovative technology that utilizes image sensors and pain algorithms that employ Artificial Intelligence (AI) techniques to recognize pain in individuals (9, 10). These systems are based on machine learning (ML) techniques to recognize and classify facial expressions associated with pain (11). Machine learning consists of training an algorithm to discern various categories or events (classes). Subsequently, this trained algorithm is utilized to identify categories or events within a new or unknown data set. The application of AI optimized the research on classification algorithms of ML, increasing recognition rates, computing speed and preventing system crashes.

Machine learning and AI can radically change how we recognize and treat pain in non-verbal patients, including animals, with an immense impact on veterinary medicine and animal welfare. By harnessing the power of ML algorithms, we can create sophisticated models that analyze various data inputs, not only facial expressions but also body posture and gesture (12), vocalizations (13), and physiological parameters, to accurately and objectively assess an animal's pain level. This approach will enhance our ability to provide timely and effective pain management, and it will be pivotal in minimizing suffering and improving the overall quality of life for animals under our care.

Therefore, this narrative review aims to focus on the impact of automation in the recognition of animal somatosensory emotions like pain and to provide an update on APR methodologies tested in the veterinary medical field, as well as their differences, advantages, and limitations to date.

2 Facial expression-based (grimace) scales for animal pain assessment

A grimace pain scale assesses animals' pain by evaluating changes in their facial expressions. It is developed through systematic observation and analysis of facial expressions exhibited by animals in response to pain-inducing stimuli. Researchers identify specific facial features associated with pain and create a coding system to quantify these responses objectively. The scale then undergoes validation to establish its reliability and sensitivity.

The MGS was the pioneering facial grimace scale for pain assessment developed for animals, emerging from investigations exploring the possibility of emotional contagion in mice (14). These studies exposed the capacity of mice to discern pain in their counterparts through subtle changes in body language and facial expressions after they were injected intraperitoneally with 0.9% acetic acid (7). Within a short span, the RGS followed suit, its inception marked by experiments conducted on appendicular inflammatory models and a laparotomy model (8, 15). Demonstrating features mirroring those of the MGS—such as orbital tightening, ear changes, and whisker alterations—the RGS exhibited comparable reliability and accuracy. Moreover, it showcased sensitivity to morphine and the ability to quantify pain stemming from inflammatory sources (8). Since their development, rodent grimace scales have been tested in several preclinical pain models, including post-laparotomy (16), post-vasectomy (17), post-thoracotomy (18). Following the initial publications, there has been a swift expansion in both the conversation and application of grimace scales. Grimace scales have been developed for 11 distinct species, including rodents, lagomorph (19), feline (20, 21), equine (22, 23), bovine (24), swine (25, 26), ovine (27, 28), ferrets (29), harbor seals (30), and donkeys (31, 32). As castration is considered one of the most common surgical procedures practiced by veterinarians, it is not surprising that several of these models were based on difference in behavior and posture before and after castration (22, 24, 26, 31, 33–35). However, other husbandry procedures have been used, like tail docking, ear-tagging and microchipping (25, 28, 30). A complete overview of the facial grimace scales developed to date and the painful stimulus used has been reported in Table 1.

These studies collectively share several common limitations. Primarily, a significant inconsistency exists in developing species-specific ethograms associated with pain. An ethogram is a descriptive inventory or catalog of all behaviors or actions exhibited by a particular species or group of animals under specific conditions. But many of these investigations were conducted before establishing a formal codification system for facial expressions in the relevant species, such as the Facial Action Coding System (FACS), which will be elaborated upon in the subsequent paragraph. A wide range of pain models has been employed across these studies, including experimental models (7, 8, 23, 29), clinical or husbandry procedures (24, 26, 31, 34, 35, 40) and observations of spontaneous pain (20, 21, 36–39). Notably, it has been demonstrated that the duration of the noxious stimulus affects the facial expression of pain (14). Langford et al. (7) showed that noxious stimuli lasting between 10 min and 4 h were most likely to elicit a "pain face." Consequently, this would render most transient pain models (30) and chronic pain models (39, 41) inadequate for facial pain detection. Interesting ear notching did not evocate grimace in mice (42) but it did in rabbits (19). Furthermore, potential overlap between pain and other states (sleep, grooming, and illness) has been observed (43, 44). In many cases, animals were assessed both before and after procedures requiring general anesthesia (8, 22, 27, 29, 31). However, studies have shown that the facial expression of pain can remain altered for several hours after inhalant anesthesia in both experimental mice and rats (45, 46) and in horses (47). This effect likely holds for other animal species.

TABLE 1 Overview of facial expression-based pain scales developed to date.

References	Species	Number of animals	Pain stimulus	Inputs	Image evaluation	Validation
Yamada et al. (24)	Bovine	45	Castration	Still images (unspecified number)	Blind observer	No
Orth et al. (31)	Donkey	9	Castration	54 still images	12 observers different experience	No
van Dierendonck et al. (32)		264	Spontaneous pain	Direct observation	Six veterinary graduate students	No
Dalla Costa et al. (22)	Equine	46	Castration	126 still images	Blind experienced observer	No
Gleerup et al. (23)		6	Tourniquet application and capsaicin injection	36 still images	Professional scientific illustrator	No
van Loon and Van Dierendonck (36)		50	Spontaneous colic	Direct observation	Four unblind students	No
VanDierendonck and van Loon (37)		46	Spontaneous colic	Direct observation	Unspecified	No
Dalla Costa et al. (38)		10	Spontaneous laminitis	40 still images and videos	Four blinded observers	No
Holden et al. (20)	Feline	87	Spontaneous pain	16 still images	68 observers with different experience	No
Evangelista et al. (21)		55	Spontaneous pain	110 still images	Four observers different experience	Yes
Reijgwart et al. (29)	Ferret	19	Laparotomy	114 still images	11 observers different experience	No
MacRae et al. (30)	Harbor seal	47	Tagging and microchipping	98 clips	Two observers	No
Langford et al. (7)	Mouse	8–20 per assay	0.9% acetic acid abdominal constriction test and others	64 still images	Seven blinded graduate and undergraduate students	Partially
McLennan et al. (39)	Ovine	73	Spontaneous foot root and mastitis	60 still images	Six blinded observers	Partially
Häger et al. (27)		14	Unilateral osteotomy	66 still images	Six observers various experience	No
Guesgen et al. (28)	(lambs 5–6 weeks old)	7	Tail docking	56 still images	Five observers various experience	No
Keating et al. (19)	Rabbit	8	Ear tattooing	64 still images	10 observers various experience	Partially
Sotocinal et al. (8)	Rat	6–8	Various	104 still images	Five blinded graduate students	No
Di Giminiani et al. (25)	Swine (piglets 3 and 4-day old)	23	Tail docking and castration	94 still pictures (combined)	Unspecified number of experienced observers	No
Viscardi et al. (26)	(piglets 5-day old)	19	Castration	627 still images	One blinded experienced observer	No
Viscardi and Turner (35)	(piglets 5-day old)	60	Castration	511 still images	Four blinded observers	No
Viscardi and Turner (34)	(piglets 5-day old)	120	Castration	1,156 still images	Eight blinded observers	No

Studies have been categorized by species, number of animals used to create the scale, type of pain stimulus, kind of data input, facial information processing, and whether the scale underwent full validation.

The collection of images for facial expression scoring lacked consistency across studies. Despite trained personnel being capable of regularly recording and evaluating animal pain intensity in clinical settings, continuous annotation still needs to be attainable (48). Many studies relied on static images, often arbitrarily extracted from videos of varying durations, or real-time scoring, with manual annotation performed by human researchers. This approach introduced the risk of bias and subjective judgment.

Furthermore, researchers emphasized the necessity of using high-definition video cameras or still cameras to ensure optimal image quality (7, 22, 25, 27, 43) but to avoid the use of bright light or camera flashes (20). The development of the RGS coincided with the introduction of the Rodent Face Finder® free software, designed to streamline the conversion of videos into scorable photographs by capturing frames with optimal optical quality and head positioning (8). Similar approaches have been developed also for horses (49). Typically, images were then pre-processed, with cropping around the head and removal of the background being common practices. However, the impact of background on image interpretation remains untested (43). Subsequently, these still images were presented to blind observers with varying levels of experience to assess inter- and intra-rater variabilities. Notably, observer experience significantly impacted the ability to discern facial features (7, 28, 31, 39). Given animals' inability to verbalize pain and the variability in employed pain models, researchers have typically identified facial changes occurring in more than 25–50% of animals following a painful stimulus as indicative of pain (22, 24, 29). Alternatively, they have relied on the coding of pain AUs recognized by experts in human facial pain expression (7, 23). But it is known that human observers often categorize facial expressions based on emotion, which can influence the process of comparing expressions across different species (50).

Construct validity of the pain scale is typically assessed by comparing the scores of animals experiencing pain vs. those undergoing sham procedures and by reassessing the painful animal before and after treatment. However, in the existing literature these comparisons were often omitted due to ethical concerns with performing invasive veterinary procedures without analgesia (22). Dalla Costa et al. (22) found no differences in the Horse Grimace Scale (HGS) among horses undergoing castration under general anesthesia, regardless of receiving one or two doses of flunixin meglumine. Similarly, there were no differences in the Piglet Grimace Scale (PGS) scores between piglets castrated with and without receiving meloxicam (34) or piglets receiving buprenorphine injections whether undergoing castration or not (35). Even when PGS was refined through 3D landmark geometric morphometrics, neither the PGS nor 3D landmark-based geometric morphometrics were able to identify facial indicators of pain in piglets undergoing castration (51). These findings raise questions about the potential confounding effects of drugs and the reliability of the scale in assessing post-castration pain. While this is not substantiated by the current literature, it is also possible that expressions may not always be an accurate indicator of pain in animals or researchers did not identify the pain ethogram for the species yet.

While animals cannot communicate their pain perception directly, the criterion validity of a pain scale can be assessed by testing it against a gold standard. However, this validation method was rarely conducted in previous studies (21, 27, 39, 41). A pain scale's internal consistency measures its components' coherence. In pain assessment, a scale demonstrates internal consistency if it consistently yields similar scores for the same aspect of pain across its various items or questions. This ensures that all items reliably measure the exact dimension of pain. Internal consistency is typically assessed using statistical methods like Cronbach's α coefficient, with higher values indicating more robust agreement among scale items and more reliable pain measurement. However, internal consistency has been reported only for the Feline Grimace Scale (21). Inter- and intra-rater reliability assess the agreement among different raters (inter-rater reliability) or the same rater over multiple assessments (intra-rater reliability) when using the scale to evaluate pain. Inter-rater reliability ensures consistent results regardless of who administers the scale, ensuring validity and generalizability across different observers. Intra-rater reliability confirms the stability and consistency of the scale's measurements over time, indicating that a rater's assessments are not influenced by variability or bias. The Intraclass Correlation Coefficient (ICC) is widely used to measure reliability, with values <0.50 indicating poor agreement, between 0.50 and 0.75 indicating moderate agreement, between 0.75 and 0.90 indicating good agreement, and above 0.90 indicating excellent agreement (52). Inter-rater ICC values for current facial expression pain scales ranged between 0.57 (26) and 0.92 (27), while intra-rater ICC ranged between 0.64 (24) and 0.90 (21), with considerable variability across facial features. But presenting good rater agreement on a given behavior does not mean that the behavior actually measures a given emotion. Another significant limitation of existing facial pain scales is the need for a cutoff value for treatment determination. van Loon and Van Dierendonck (36) reported that the EQUUS FAP had sensitivity and sensibility for distinguishing colic from no-colic of 87.5 and 88% using a cut-off value of 4 in a scale 0–18, but only of 30 and 64.3% for distinguishing surgical and medical colic with a cut-off at 6. Häger et al. (27) and McLennan and Mahmoud (53) both reported a discrimination accuracy below 70% using two different facial pain scales developed for sheep, denouncing a high number of false positive results and highlighting the need for further refinement and standardization in this area.

3 Facial Action Coding System

The gold standard for objectively assessing changes in facial expressions in human emotion research is the FACS, first published almost half a century ago (54). FACS is a comprehensive, anatomically based system that taxonomizes all visible human facial movements (55, 56). In FACS, the authors assign numbers to refer to the appearance changes associated with 33 facial muscle contractions to each specific facial movement, termed AUs. Each AU is linked to mimetic muscles innervated by the facial nerve and characterized by corresponding changes in facial appearance. Additionally, the system introduced 25 more general head/eye movements termed Action Descriptors (AD), representing broader movements from non-mimetic muscles, which could impact AU identification. Recognizing the interplay between AUs and ADs is emphasized, as their concurrent presence could modify the visual expression of individual movements. The FACS manual offers guidelines for scoring these AUs, supported by a collection of photographs and illustrations for reference. The FACS system revolutionized human research based on facial expression interpretation, finding extensive application in psychology, sociology, and communication. It enabled the objective and systematic recognition of individual facial movements based

on facial anatomy and steered the field away from subjective interpretations of visual displays known for their unreliability. Following the FACS approach, researchers have developed the same system for non-human primates, including orangutans [*Pongo* spp: OrangFACS (55)], chimpanzees chimpanzees [*Pan troglodytes*: ChimpFACS (56)], rhesus macaques [*Macaca mulatta*: MaqFACS (57, 58)], gibbons [Hylobatids, GibbonFACS (59)], marmosets (60), and domesticated mammals such as horses [*Equus caballlus*: EquiFACS (61, 62)], dogs (*Canis familiaris*: DogFACS) (63), and cats (*Felis catus*: CatFACS) (64).

Developing species-specific AnimalFACS involved identifying and documenting every potential facial movement of the species based on observable changes in appearance, consistent with the FACS terminology. Subsequently, the muscular foundation of each movement was confirmed through rigorous anatomical studies (56, 61, 63). This extensive work has interestingly unveiled phylogenetic similarities across species, with those already analyzed for FACS demonstrating a shared muscular foundation of at least 47% of their facial muscles (65). While species may share similar anatomical structures, this correspondence does not invariably translate into analogous facial movements. Specific muscles may be implicated in multiple AUs, while others may exhibit infrequent use, complicating the relationship between anatomy and expression (65). For a more detailed description of all the AUs identified in the different species, the reader is referred to Waller et al. (65). But, while FACS is generally considered reliable for gauging human perception due to the presumed alignment between facial expression production and interpretation, its applicability to non-human animals may be less precise, as third party evaluation is always required. Therefore, it's vital to approach its application cautiously and gather empirical data to ascertain how animals respond to stimuli.

Despite the growing interest in facial expression analysis for evaluating pain and emotion, only a few animal studies applied AnimalFACS. Among small animal species, the FACS system has been scarcely used. In dogs and cats, FACS has been used more commonly for emotion interpretation than specifically for pain determination (66–68). In one study, 932 images from 29 cats undergoing ovariohysterectomy were extracted and manually annotated using 48 landmarks selected according to CatFACS criteria (69). A significant relationship was found between pain-associated Principal Components, which capture facial shape variations, and the UNESP-Botucatu Multidimensional Composite Pain Scale tool (69). However, an intrinsic bias of the study was that the first postoperative assessment, prior to administration of analgesia, was recorded between 30 min and 1 h after general anesthesia, and the role of general anesthesia on facial expression cannot be excluded as it has been previously discussed. A groundbreaking methodology for investigating the facial expressions of ridden horses, known as Facial Expressions of Ridden Horses (FEReq) (70, 71), was developed by integrating species-specific ethograms from previous studies (22, 23) with components of the EquiFACS codification system (61). This ethogram represented a pioneering effort in characterizing changes in facial expressions among ridden horses, demonstrating reasonable consistency across diverse professional backgrounds post-adaptation and training. Although initially limited to analyzing still photographs capturing singular moments, the ethogram was subsequently enhanced with additional markers for assessing general body language and behavior in ridden horses (72). Despite no observed correlation between this improved Ridden Horse Pain Ethogram (RHpE) score and maximum lameness grade before diagnostic anesthesia (Spearman's rho = 0.09, $P = 0.262$) (73), the scale has proven effective in detecting musculoskeletal pain in competitively ridden horses (74, 75). These studies uncovered variations in consistency across horse facial features, particularly noting the eye and muzzle as displaying the least reliability. This stands in contrast to findings by Rashid et al. (62), who repurposed data from Gleerup et al. (23) to employ EquiFACS in describing facial features in pain-related videos. The group suggested that *inner brow raiser* (AU101), *half blink* (AU47), *chin raiser* (AU17), *ear rotator* (EAD104), *eye white increase* (AD1), and *nostril dilator* (AD38) were frequently linked with pain. Moreover, these findings were echoed by a recent study by Ask et al. (76), investigating pain indicators in horses with experimentally induced orthopedic pain. Employing the Composite Orthopedic Pain Scale (77) as the gold standard, the group identified numerous lip and eye-related AUs and ADs as robust predictors of pain. Noteworthy indicators included frequency and duration of *eye closure* (AU143), duration of *blink* (AU145), *upper lid raiser* (AU5), duration of *lower jaw thrust* (AD29), frequency and duration of *lower lip relax* (AD160), frequency of lower *lip depressor* (AU16), frequency of *upper lip raiser* (AU10), frequency and duration of AU17, duration of *lip presser* (AU24), frequency and duration of AD38, and frequency and duration of *lips part* (AU25), among others. Additionally, AU16, AU25, AU47, single ear forward (SEAD101), and EAD104 co-occurred more frequently in horses experiencing orthopedic pain. The study by Rashid et al. (62) also noted an interesting discrepancy in pain detection rates. In still images or video segments lasting 0.04 s, the likelihood of detecting more than three pain AUs was extremely low, contrasting with higher detection rates with a 5 s observation window. This may be explained by the fact that 75% of pain-related AUs in horses lasts between 0.3 and 0.7 s (76). This finding underscores the potential value of using video footage over randomly selected images for pain assessment. However, it's essential to acknowledge limitations in these studies, such as the small number of experimental horses used to build the models and the presumption of pain based solely on evaluations by clinically experienced observers, potentially overlooking influences of stress, tiredness and malaise (44).

One of the limitations of AnimalFACS consist in the limited availability across species and the reliance on manual annotation, necessitating rigorous human training to ensure acceptable inter-rater reliability (78, 79). Debates arose regarding distinctive individual differences, encompassing variations in muscle presence, size, symmetry, disparities in adipose tissue distribution, and even inherent facial asymmetry (65, 80). Notably, present studies using AnimalFACS are limited to quantifying the number of AUs, their combinations, and their temporal duration within a confined observation period (62, 72). However, this approach falls short of capturing the intricate complexity of facial movements. Another fundamental limitation of FACS-based systems is their failure to account for the dynamic shifts in movement or posture that often accompany and enrich facial expressions. So, some

studies have assessed behavioral indicators such as changes in consumption behaviors (time activity budgets for eating, drinking, or sleeping, etc.) (81–83); anticipatory behaviors (84), affiliative behavior (85), agonistic behaviors, and displacement behaviors, amongst others (86).

4 Automated pain recognition

Automated Pain Recognition is a cutting-edge technology aiming to develop objective, standardized, and generalizable instruments for pain assessment in numerous clinical contexts. This innovative approach has the potential to significantly enhance the pain recognition process. Automated Pain Recognition leverages image sensors and pain algorithms, powered by AI techniques, to identify pain in individuals (9, 10). AI, a field encompassing a broad range of symbolic and statistical approaches to learning and reasoning, mimics various aspects of human brain function. Data-driven AI models, such as those used in APR, can overcome the limitations of subjective pain evaluation. Machine learning, CV, fuzzy logic (FL), and natural language processing (NLP) are commonly considered subsets of AI. However, with technological advancements and interdisciplinary research, the boundaries between these subsets often blur. Machine learning, a branch of AI, enables systems to learn and improve their performance through experience without explicit programming. It involves training a computer model on a dataset, allowing it to make predictions or decisions independently. Automated Pain Recognition research has focused on discerning pain and pain intensity within clinical settings (87) and assessing responses to quantitative sensory testing in preclinical research (88, 89). The following paragraphs will briefly outline and summarize the steps involved in APR.

4.1 Data collection

The initial step toward implementing APR involves data collection, a significant challenge in the veterinary field due to the scarcity of available datasets (90). Animals exhibit considerable variability even within the same species, influenced by factors such as breed, age, sex, and neuter status, that may affect the morphometry of the face, especially in adult males (91). These variables can impact the pain-related facial information extracted from images (6, 92, 93). This variability, however, can enhance the learning process of deep learning (DL) models. Exposure to diverse examples and scenarios allows models trained on a broad spectrum of data to generalize well to unseen examples, improving performance in real-world applications. Additionally, variability aids in acquiring robust features applicable across different contexts. With the availability of high-definition cameras and the relatively low demand for image or video quality in CV, recording has become less problematic compared to the past (49). Studies suggest that resolutions of 224 × 224 pixels and frame rates of 25 FPS are sufficient for processing images and videos in modern CV systems (49). Multicamera setups are ideal, especially for coding both sides of the face, as required in laterality studies or to avoid invisibility. Different animal species pose unique challenges. Laboratory animals are usually confined to a limited environment, allowing more control over data acquisition and video recording quality (89, 94, 95). Horses can be manually restrained or confined in a stall (96). Data acquisition for farm animals often occurs in open spaces or farms with uncontrolled light conditions (53, 97).

4.2 Data labeling

The absence of verbal communication in veterinary APR introduces a unique challenge in establishing a ground truth label of pain or emotional state. Unlike human medicine, where self-reporting of pain is feasible, veterinary APR requires third-party assessment of the pain status, preferably utilizing a validated pain scale, but commonly not (Table 2). This has led to the categorization of pain labeling methods in animal APR into behavior-based or stimulus-based annotations (90). The former relies solely on observed behaviors and is typically assessed by human experts (5, 6, 97, 99, 104–106). In contrast, the latter determines the ground truth based on whether the data were recorded during an ongoing stimulus or not (5, 10, 49, 76, 94–96, 99, 100, 107–109). Stimulus-based annotations enable recording the same animal under pain and no pain conditions and offer a potential solution to the challenge of variability in pain perceptions across individuals (110). Therefore, CV and ML methods must acknowledge the inherent bias in their algorithms until a definitive marker for pain is identified.

4.3 Data analysis

Computer vision-based methods operate using data in the form of images or image sequences (videos). This suggests that the system can utilize single frames, aggregate frames (10) or incorporate spatiotemporal representations to account for temporality (94, 98, 105). Utilizing single frames offers greater control and facilitates explainability, although it may result in information loss. Researchers demonstrated that the likelihood of observing more than three pain AUs was negligible in still images extracted from videos of horses undergoing moderate experimental nociceptive stimulus (62). On the other hand, based on Martvel et al. (101), different frame extraction rates may affect the accuracy of the results. Preliminary results in mice (94), horses (98), sheep (105), and cats (101) suggest that extracting spatiotemporal patterns from video data may increase the performance of the model. However, working with videos rather than single-frame input requires substantial computational resources.

The data processing pipeline is developed after the images are collected and the input is either images or videos. The output is typically pain classification, which can be binary pain/no pain or multi-class degree assessment. Often, outputs based on grimace pain scale taxonomy encompass at least three scales [pain not present (0), pain moderately present (1), or pain present (2)] (10, 103). The pipeline can encompass multiple steps and may analyze the entire body or face or focus on specific parts. These two approaches, differing in processing facial information, have been defined as parts-based and holistic methods. For instance, Hummel et al. (99) cropped the equine face based on several

TABLE 2 Overview of datasets featuring facial expressions of pain for automated animal pain assessment to date.

References	Species	Pain stimulus	Data	Input	Part/holistic	Approach	Annotation
Broomé et al. (98)	Equine	Tourniquet application and capsaicin injection	60 video from six healthy horses	Videos	Holistic	Learned	Binary
Broomé et al. (96)		Tourniquet application, capsaicin injection and lipopolysaccharides induced lameness	Dataset from (98) and 90 videos from eight healthy horses	Videos and frames	Holistic	Learned	Binary
Hummel et al. (99)		Induced and unknown	1,854 images of horse heads and 531 images of donkey heads	Frames	Parts-based	Hand-crafted and Learned	HGS and EQUUS-FAP
Lencioni et al. (10)		Castration	3,000 frames from seven healthy horses	Frames	Parts-based	Learned	HGS
Pessanha et al. (5)		Induced or unknown	1,854 images of horse heads	Frames	Parts-based	Hand-crafted and Learned	Adapted from EQUUS-FAP
Feighelstein et al. (100)	Feline	Ovariohysterectomy	464 images from 26 cats	Frames	Holistic	Hand-crafted and Learned	Binary
Feighelstein et al. (6)		Unknown	84 client-owned cats	Frames	Holistic	Hand-crafted and Learned	Binary (based on CMPS-feline)
Martvel et al. (101)		Ovariohysterectomy	54 videos from 27 cats + 72 videos from client-owned cats	Frames	Holistic	Hand-crafted	CMPS-feline
Feighelstein et al. (102)	Lagomorphs	Orthopedic surgery	48 videos from 28 rabbits	Frames	Holistic	Learned	Binary
Tuttle et al. (94)	Murine	Laparotomy	5,771 frames	Frames	Holistic	Learned	Binary
Andresen et al. (95)		Castration	18,273 frames	Frames	Holistic	Learned	Binary
Lu et al. (103)	Ovine	Unknown	480 frames	Frames	Holistic	Hand-crafted	SPFS
Mahmoud et al. (104)		Mastitis and pregnancy toxemia	86 frames	Frames	Parts-based	Hand-crafted	SPFS
Noor et al. (97)		Unknown	2,350 frames	Frames	Holistic	Learned	Binary
Pessanha et al. (105)		Mastitis and pregnancy toxemia	86 frames	Frames	Parts-based	Hand-crafted	SPFS

Studies have been categorized by species, kind of pain stimulus, type of data input, facial information processing, whether the approach was hand-crafted or learned and the kind of pain annotation.

CMPS-feline, Glasgow Feline Composite Measure Pain Scale; EQUUS-FAP, Equine Utrecht University scale for facial pain assessment; HGS, Horse Grimace Scale; SPFS, Sheep Pain Facial Expression Scale.

Regions of Interest (ROIs); the eyes, ears, nostrils, and mouth, respectively, and analyzed them with HOG (Histogram of Oriented Gradients), Local Binary Pattern (LBP), Scale Invariant Feature Transform (SIFT), and DL approach using VGG-16 Convolutional Neural Network (CNN). Similarly, Lencioni et al. (10) employed a parts-based approach in annotating 3,000 images from seven horses of similar breeds and ages undergoing castration. They utilized the HGS (22), where the six parameters were grouped into three different facial parts: ears, eyes, and muzzle. Subsequently, three pain classifier models based on CNN architecture were developed. The outputs of these models were then fused using a fully connected network for an overall pain classification. Recent research employing explainable AI methods to investigate different regions of cat faces suggested that features related to the ears may be the least important (111). In contrast, those associated with the mouth movement were considered the most crucial (6, 49). Similarly, Lu et al. (103) have developed a multilevel pipeline to assess pain in sheep, utilizing the Sheep Facial Expression Pain Scale (39). The authors divided the sheep's face into regions, including eyes, ears, and nose, with further subdivision of the ears into left and right. Symmetric features such as eyes and ears were scored separately and then averaged, while scores for all three facial features (ears, eyes, nose) were averaged again to derive the overall pain score. The task of automatically identifying and localizing specific points or features on an animal's face, such as the eyes, nose, mouth corners, etc., known in CV as

recognition of *key facial points*, poses the initial challenge due to limited datasets in animals (95). Researchers have proposed adapting animal training data to a pre-trained human *key point* detector to address this issue. The approach involved morphing animal faces into human faces and fine-tuning a CNN developed for human *key point* recognition. Surprisingly, this approach has demonstrated promising performance in both equine and ovine faces (112).

4.4 Hand-crafted vs. deep learning

Automated Pain Recognition identifies, understands, and enhances image pain features. Two main approaches have been used for feature extraction.

4.4.1 Hand-crafted features extraction

Before the advent of DL, classical ML relied on hand-crafted features (90). The process involves extracting characteristics from the data using previous knowledge to capture pain-related patterns with facial or bodily landmarks, grimace scale elements, or pose representations. For example, Blumrosen et al. (113) studied four fundamental facial movements to recognize facial actions in macaques: neutral expression, lip smacking, chewing, and random mouth opening. They used unsupervised learning, which does not require manually labeling or annotating the data. In their approach, they utilized eigenfaces to extract features from facial images. Eigenfaces use a mathematical method called Principal Component Analysis (PCA) to capture the statistical patterns present in facial images. Another standard method is the landmark-based (LM-based) approach, which identifies pain-related AUs through manual annotation (7, 10, 94, 103). It provides a mathematical representation of previous findings by human experts concerning certain facial expressions. The system requires preliminary efforts to detect and locate the animal face in an image or video clip and to detect individual AUs. Face detection and alignment are achieved by detecting *key facial points*, which are then transformed into multi-region vectors and fed to a multi-layer perceptron neural network (MLP). For example, Andersen et al. (49) trained individual classifiers to detect 31 AUs, including ADs and ear EADs, in 20,000 EquiFACS-labeled short video clips after cropping the images around a pre-defined ROI to help the classifier focus on the correct anatomical region. But the model did not work for the ear action descriptors. The authors attributed this discrepancy to the many different positions possible for ears, suggesting that ears' position should be examined in spatiotemporal data acquisition (49, 90). Similarly, Feighelstein et al. (100) utilized 48 facial landmarks selected based on the CatFACS and manually annotated for developing their automated model. Landmark-based approaches are by their nature better able to directly measure and thus better account for morphological variability. However, the downside of this route is the resource and effort needed for landmark annotation, given that this requires manual completion (114).

4.4.2 Deep learning approach

Deep Learning approaches are gaining popularity in APR due to their reduced need for annotation and manual feature crafting. Unlike LM-based methods, DL is less sensitive to facial alignment (100), although the accuracy of the models improves with data cleaning (102). Deep learning trains artificial neural networks with many layers to automatically extract hierarchical features from vast datasets like video data. Convolutional Neural Networks (CNNs) are particularly effective for image processing tasks like classification and object recognition, offering superior performance by mapping individual inputs to single outputs. Deep learning relies heavily on large volumes of video data for training (6). Continual advancements in DL methods for APR are expanding the possibilities in the field. CNNs, inspired by the functioning of the retina, consist of various layers, including convolutional layers for feature detection, non-linearity layers to introduce non-linearity, and pooling layers for down-sampling parameters. This architecture culminates in a fully connected layer for final processing, where each node in the output layer connects directly to a node in the previous layer. Among the diverse CNN architectures, the Visual Geometry Group (VGG) 16 architecture, with its 16 convolutional layers, each equipped with 3×3 filters, is particularly notable for its extensive utilization in CV applications. Other advanced neural networks, such as deep residual networks (ResNets), enable the handling of deeper architectures and improved performance (95, 100, 102). These advancements in DL methods have equipped researchers and practitioners with more powerful tools for APR. It is crucial to emphasize the significance of large and diverse datasets in DL methods for APR. While DL methods are often effective, they frequently lack interpretability, which poses a challenge for humans to comprehend their decision-making process.

Building upon the work of Finka et al. (69), Feighelstein et al. (100) explored both LM-based and DL methods in APR for cats, achieving comparable accuracies of around 72%. However, DL approaches faced challenges with highly homogeneous datasets, which affected their performance. The model showed improvement when applied to a more diverse population. A similar limitation was observed by Lencioni et al. (10), who extracted 3,000 frames from seven horses of similar breed and age to classify pain following a painful stimulus and general anesthesia. Using CNN-based individual training models for each facial part, they achieved an accuracy of 90.3% for the ears, 65.5% for the eyes, and 74.5% for the mouth and nostrils, and an overall accuracy of 75.8%. This underscores the need for diverse datasets to enhance the performance of DL methods in APR. When Feighelstein et al. (102) used a DL approach for recognizing pain in 28 rabbits undergoing an orthopedic procedure, the initial "naïve" model trained on all frames achieved an accuracy of over 77%. The performance improved to over 87% when a frame selection method was applied to reduce noise in the dataset (102). Another notable DL model is the deep recurrent video model used by Broomé et al. (96, 98), which utilizes a ConvLSTM layer to analyze spatial and temporal features simultaneously, yielding better results in spatiotemporal representations. Steagall et al. (106) and Martvel et al. (114) introduced a landmark detection CNN-based model to predict

facial landmark positions and pain scores based on the manually annotated FGS.

4.5 Limitations and downfalls in animal APR

Data imbalance is a significant challenge in both classic ML and DL methods. This issue, as highlighted by Broomé et al. (90), occurs when they are fewer instances of one class compared to another, potentially skewing the model accuracy, especially in extreme categories. In the case of animal pain recognition, there are often fewer instances of animals in pain compared to non-painful animals (97, 98). The use of data augmentation techniques, such as synthesizing additional data using 3D models and generative AI (5) has been proposed to address this imbalance. However, the highly individualized nature of pain perception and expression in animals may limit the clinical value of these techniques in animal pain recognition.

Overfitting and underfitting are frequently encountered problems in ML. Overfitting happens when a model excessively learns from the training data, resulting in inadequate performance when applied to new data. On the other hand, underfitting occurs when a model does not perform well even on the training set. Cross-validation techniques mitigate these problems by splitting the data into training, validation, and testing sets. For smaller sample sizes, ensuring that each subject appears in only one part of the data (training, validation, or testing) can be beneficial (6, 102). In DL, it is crucial to reserve a fully held-out test set comprising data from subjects not seen during training to ensure unbiased evaluation. Techniques like leave-one-subject-out cross-validation can help reduce bias by rotating subjects between the training and testing sets (96, 100). Additionally, when training DL models from scratch, the initial setup can be influenced by a random number called a "seed." Different seeds can lead to slightly different results each time the model is trained. To ensure robustness, training and testing are often repeated with different random seeds, and the outcomes are averaged to minimize the impact of random variations. Addressing data imbalance, overfitting, and underfitting is not just a choice but a necessity for improving the accuracy and robustness of ML and DL models in applications such as animal pain recognition. It is a crucial step that cannot be overlooked.

5 Discussion

This narrative review aimed to offer a comprehensive journey through the progression of research on recognizing facial expressions of pain in animals. It began with the rapid advancement of grimace pain scales, moved through the refinement of FACS for various animal species, and culminated in APR. Although APR extends beyond facial cues (98, 101), existing studies' predominant focus has been analyzing pain AUs in datasets crafted through prior facial expression research and annotation.

Pessanha et al. (5) underscored several significant challenges encountered in detecting APR in animals. The first among these challenges is the scarcity of available datasets, a notable contrast to the abundance of databases in the human domain (115). Very few current datasets have been created specifically for CV and APR studies. The majority of researchers have out-sourced their dataset from previous studies (6, 84, 96, 98, 99, 101, 104, 105), with the significant advantage was that most of these dataset were already annotated for pain AUs. However, as highlighted, most of the previously published pain scales based on facial expressions were based on unspecified or artificially created ethograms and they did not undergo complete validation, except for Evangelista et al. (21). Most interestingly, they were developed before or independently from the development of the AnimalFACS dataset for the species. While agreement was often found for many AUs developed before and after AnimalFACS (62, 76), this issue may introduce inherent bias. One solution proposed for overcoming the scarcity of data is data augmentation (5, 100). However, one of the primary ethical concerns is the integrity and representativeness of the augmented data. Augmented data should accurately represent real-world variations, and care should be taken to ensure that the augmented data does not lead to misinterpretations that could result in harm or unnecessary interventions (116). To address the issue of small datasets, open access to datasets and sharing between researchers is crucial. Fairly implementing AI in veterinary care requires integrating inclusivity, openness, and trust principles in biomedical datasets by design. The concept of openly sharing multiple facets of the research process—including data, methods, and results—under terms that allow reuse, redistribution, and reproduction of all findings has given birth to open science, a practice strongly supported by several institutions and funding agencies (49, 117). Secondly, animals may have much more significant facial texture and morphology variation than humans. While initially perceived as a challenge, this may be advantageous when employing a DL approach. Finally and foremost, a significant limitation in animal APR is the need for consistent ground truth. Unlike in humans, where self-reporting of the internal affective state is commonly used, there is no verbal basis for establishing a ground truth label of pain or emotional state in animals. Consequently, animal pain detection heavily relies on third-party (human expert) interpretation, introducing intrinsic bias that cannot be bypassed entirely. One possible strategy for establishing ground truth involves designing or timing the experimental setup to induce pain. However, since pain is a subjective experience, this approach may not eliminate bias. Additionally, the type and duration of pain need to be researched further, as there are postulations about differences in facial expressions of pain between acute nociceptive and chronic pain and on the effects of general anesthesia (14, 47, 95). These hypotheses, for example, could be tested to improve the understanding and detection of pain in animals. Currently, the best way to address this problem is by using fully validated pain scales to discriminate the pain status.

In conclusion, the advancement of animal APR has immense potential for assessing and treating animal pain. However, it requires addressing data scarcity, ensuring the ethical use of augmented data, and developing consistent and validated ground truth assessments. Open science practices and collaboration will be crucial in overcoming these challenges, ultimately improving the welfare of animals in research and clinical settings.

Author contributions

LC: Conceptualization, Data curation, Funding acquisition, Investigation, Resources, Writing – original draft, Writing – review & editing. AG: Investigation, Methodology, Writing – original draft, Writing – review & editing. GC: Investigation, Methodology, Writing – original draft, Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. The project was partially funded by the International Veterinary Academy for Pain Management (PRO00054545).

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

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frontiers | Frontiers in Veterinary Science

TYPE Original Research
PUBLISHED 03 October 2024
DOI 10.3389/fvets.2024.1470957

OPEN ACCESS

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RECEIVED 26 July 2024
ACCEPTED 23 September 2024
PUBLISHED 03 October 2024

CITATION
Interlandi C, Spadola F, Neve VC, Tabbì M, Di Pietro S, Giudice E, Macrì D and Costa GL (2024) Use of butorphanol as a local anaesthetic for pain management in calves undergoing umbilical hernia repair.
Front. Vet. Sci. 11:1470957.
doi: 10.3389/fvets.2024.1470957

Use of butorphanol as a local anaesthetic for pain management in calves undergoing umbilical hernia repair

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The aim of the study was to compare the analgesic efficacy of butorphanol and lidocaine, alone or in combination, in calves undergoing surgical repair of umbilical hernia. The study was conducted in 60 calves of different breeds. Xylazine 0.3mg/kg was administered intramuscularly to all animals in the study. The animals were then divided into three groups (n=20) that received different treatments with lidocaine at 4.5mg/kg and butorphanol at 0.02mg/kg. The L group received lidocaine both by infiltration of the surgical planes and intraperitoneally, the B group received butorphanol both by infiltration of the surgical planes and intraperitoneally, and finally the LB group received lidocaine by infiltration of the surgical planes and butorphanol intraperitoneally. Heart and respiratory rates, haemoglobin oxygen saturation, non-invasive blood pressure and temperature were recorded during surgery. Response to the surgical stimulus was scored on a cumulative numerical scale that included percentage changes in HR, RR and SAP. Postoperative pain was assessed by three independent observers, blinded to treatment, using the UNESP-Botucatu Unidimensional Composite Pain Scale (UNESP-Botucatu UCPS-IV) for the assessment of postoperative pain in cattle. The course of physiological variables was appropriate for patients under anaesthesia. No subject required rescue intraoperative analgesia. In group L, 4 subjects at 40m and 5 subjects at 50m required postoperative rescue analgesia. Both butorphanol alone and the combination of butorphanol and lidocaine showed excellent intraoperative and postoperative scores. Furthermore, this combination did not cause any cardiopulmonary or other adverse effects. Based on the results of this study, both butorphanol alone and the co-administration of butorphanol and lidocaine administered locally proved to be safe and effective in providing adequate and long-lasting analgesia in calves, helping to reduce postoperative discomfort and maintaining adequate animal welfare.

KEYWORDS

calves, umbilical hernia, lidocaine, butorphanol, pain management

Introduction

A hernia is a protrusion of the contents of a body cavity through an accidental or malfunctioning natural opening. Umbilical hernias are the most common congenital defects in calves with an incidence ranging from 1 to 21% (1). As a result, ventral abdominal surgery for umbilical hernia is one of the most requested procedures in calves (2, 3). The combination

of deep sedation and local anaesthesia is the anaesthetic technique of choice for this type of surgery (4). However, in the EU, the list of drugs approved for this purpose in this species is very limited. Although the use of local anaesthetics and opioids administered *in situ* for intraoperative and postoperative pain management is widespread in veterinary medicine, these potent analgesics are still rarely used in cattle.

In damaged peripheral tissues (skin, muscles, joints and viscera), primary afferent neurons (PANs) transduce noxious stimuli into action potentials that are modulated and transmitted to the brain, where they are processed and perceived as "pain" (5). Peripheral opioid receptors (PORs) on PANs represent an important therapeutic target because their inhibition could prevent the transmission of noxious impulses and block the generation of pain in the brain (6, 7).

New multimodal analgesia techniques involve the use of different substances that act synergistically to enhance the effect obtained (8–11).

Butorphanol is an opioid whose mixed agonist–antagonist activity results in analgesia with a lower probability of inducing respiratory depression than pure μ-receptor agonist activity, contributing to a balanced anaesthetic management. Butorphanol has been shown to improve superficial and visceral signs of pain in several species when administered intravenously or epidurally (12–16). However, the analgesic and haemodynamic effects of opioids in the bovine species have not been extensively investigated and documented. The aim of the present study was to compare the analgesic efficacy of butorphanol and lidocaine, administered alone or in combination, by infiltration at the surgical incision site and intraperitoneally during umbilical hernia repair in calves. The hypothesis of the study is that locally administered butorphanol, alone or in combination with lidocaine, may improve surgical pain management in this species.

Materials and methods

Animals and study design

This study was performed in accordance with Legislative Decree no. 26 of 4 March 2014 on Italian animal welfare legislation and was approved by the Institutional Ethics Committee for Animal Welfare of the University of Messina, protocol number 027/2018. Procedures were performed according to national (Italian Law D.M. 116192) and international (EU Directive 2010/63/EU and USA Public Health Service Policy on Humane Care and Use of Laboratory Animals) regulations for the care and use of laboratory animals. Owners were fully informed and gave written consent for their calves to be enrolled.

Sixty ($n = 60$) calves of different breeds (Friesian, Alpine Brown, Modicana, half-breeds) from different local herds undergoing umbilical surgery were included in this study. The selected calves included 28 males and 32 females and the study was conducted during the spring and autumn seasons, with approximately 6 calves enrolled per month. The inclusion criterion was the presence of an umbilical hernia of 8–13 cm in diameter. The exclusion criterion was the presence of an omphalocele or any other pathological condition. Food was withheld for 7 h prior to surgery and access to water was withheld for 3 h prior to surgery. Calves ($n = 60$) were randomly divided into three groups: Lidocaine, L group ($n = 20$); Butorhanol, B group ($n = 20$) and Lidocaine/Butorhanol, LB group ($n = 20$). Animals underwent umbilical hernia repair surgery at their farms.

Treatment administration

A prospective, block-assigned, operator-blinded clinical trial was performed on each calf at the farm of origin. On the day of surgery, the animals were weighed (OCS300, Zoo Piro, Cruto, Calabria, Italy) to determine the appropriate dose of drugs. After a 30-min acclimatisation period, xylazine 0.3 mg/kg (Rompun 2%, Bayer, Leverkusen, Germany) was administered intramuscularly (IM) in the box where the operation would be performed. After 15 min, when sedation and muscle relaxation had been achieved, an intravenous catheter (14G × 5″) was inserted to administer lactated Ringer's solution at a rate of 10 mL/kg/h during surgery. The calves were placed in dorsal recumbency, the umbilical region was aseptically prepared, and a local analgesia protocol was performed. For the local analgesic protocol, the L group received lidocaine 4.5 mg/kg (Lidocaina Cloridrato Esteve 2%, Ecuphar Italia S.r.l., Milan, Lombardy, Italy) both by infiltration of the surgical planes and intraperitoneally, while the B group received butorphanol 0.02 mg/kg (Butorphanol Tartrate, Dolorex 10 mg, Codifa, MSD Animal Health S.r.l., Italy) by infiltration of the surgical planes and intraperitoneally, with the drug dose divided equally between two syringes for both groups. Instead the LB group received lidocaine 4.5 mg/kg only by infiltration of the surgical planes and butorphanol 0.02 mg/kg only intraperitoneally, with each drug administered with its own syringe. To achieve greater diffusion, the volume of each syringe was increased to 40 mL with the addition of saline (0.9% sodium chloride). Infiltration in the umbilical region involved both the skin and muscle planes, while intraperitoneal injection was performed in the hernia sac. Both infiltration of the surgical planes and intraperitoneal administration were performed at different sites.

Umbilical hernia repair

After sedation, the calves were placed dorsally on a padded mattress and the skin over the umbilical region was cleaned, aseptically prepared and infiltrated. The surgeon performing the surgery was the same for all animals. Open herniorrhaphy (2, 3) was performed through an elliptical skin incision and the adhesions of the parietal peritoneum to the skin were released using both blunt and sharp dissection. After repositioning of the abdominal organs, the hernia ring was refreshed and the horizontal interrupted mattress suture with 2–0 chromic catgut was placed on the peritoneum and supported with an autologous flap from the hernia sac. A single interrupted suture was placed on the subcutis (2–0 chromic catgut) and the skin was conformed to the surgical wound and sutured (2–0 nylon).

Measurement of physiological parameters

Heart rate (HR), haemoglobin oxygen saturation (SpO_2, %) and systolic, diastolic and mean arterial pressure (SAP, DAP, MAP) were

measured using a multiparameter monitor (EDAN Instruments Italy, Napoli, Campania, Italy). Heart rate and SpO_2 were measured with a pulse oximeter, while blood pressure was measured with a special cuff placed at the base of the tail, approximately 30–40% of the tail circumference (oscillometric method). Respiratory rate (RR) was determined by counting thoracic excursions per minute. Body temperature was measured using a digital thermometer inserted into the rectum for a few seconds (Digital Veterinary Thermometer, GIMA). Parameters were recorded at T_0 (basal values) before xylazine administration. Local anaesthesia was then administered at T_1 (15 min after xylazine administration). Finally, surgery was started at T_2 (10 min after local anaesthesia administration). From T_2, parameters were recorded at 5-min intervals: T_3 (5 min after the start of surgery), T_4 (10 min), T_5 (15 min), T_6 (20 min), T_7 (25 min), T_8 (30 min), and T_9 (35 min) (intraoperative time). Measurements were also taken at T10 upon awakening, when the calf stood. To assess the response to intraoperative noxious stimulation, we used a cumulative numerical scale that considered percentage changes in HR, RR and SAP compared to T_1 (15 min after xylazine administration) according to the following procedure: (time point value—T_1 value)/T_1 value × 100 = % change. Scores were scored as follows: Score 0 = no change; 1 = increase ≤10%; 2 = increase >10% but ≤20%; 3 = increase >20% but ≤30%; 4 = increase >30%. Scores were assigned by assessors blinded to treatment. A final score ranging from a minimum of 0 to a maximum of 12 was obtained by summing the scores of the selected variables. If HR, RR and SAP increased by more than 20%, to a score of 6 or higher, rescue analgesia was administered (11, 17–19), the surgical area was infiltrated and sprayed intraperitoneally with 2 mg/kg lidocaine 2%.

Post-operative pain assessment

Postoperative pain was assessed by three independent observers, blinded to treatment, using the UNESP-Botucatu Unidimensional Composite Pain Scale (UNESP-Botucatu UCPS-IV) for the assessment of postoperative pain in cattle. Pain scores range from no pain (score 0) to severe pain (score 10). The assessment was made at 10 (T_{10}), 20 (T_{20}), 30 (T_{30}), 40 (T_{40}), and 50 (T_{50}) minutes after the calves were returned to a standing position. The questionnaire consists of 5 behavioural categories (items) that assess locomotion, spontaneous behaviour, activity, appetite and various behaviours that the animal may exhibit. The items are numbered in ascending order of pain intensity (20–23). The cut-off point for the post-operative pain score was ≥4; if the calf exceeded this value, it received 3.3 mg/kg flunixin meglumine IV (Finadyne, Schering-Plough Animal Health, Oss, The Netherlands) as rescue analgesia.

Statistical analysis

A sample size calculation was performed to determine the number of cattle required for this study. Sample size was calculated using G*Power 3.1 software (Hein-rich-Heine-Universitat Düsseldorf, Düsseldorf, Germany). An effect size (f) of 0.45, a significance level (α) of 0.05, and a power (1-β) of 0.85 were assumed using the Anova Fixed Effects, omnibus, one-way test. Statistical analyses were performed using commercially available software (GraphPad Prism version 8.2.1; GraphPad Software Inc., La Jolla, CA, USA, and SPSS version 15.0; SPSS Inc., Chicago, IL, USA). Data were analysed for normality using the Shapiro–Wilk test and reported as mean ± SD or median (range), as appropriate. One-way ANOVA followed by Friedman's test was used to assess differences between groups for demographic data. Clinical parameters (HR, SpO_2, SAP, MAP, DAP, RR, T) were analysed by two-way repeated measures ANOVA.

Bonferroni post-hoc pairwise comparison test between least squares means was used when statistical differences were present. Scores were reported as mean +/− standard deviation (SD). To confirm content and construct validity, pain scores related to responses to noxious intraoperative stimulation and the UNESP-Botucatu Unidimensional Composite Pain Scale were summarised as median, minimum and maximum values. Scores were compared within and between groups using Friedman's test. Kendall's coefficient of concordance W was calculated to measure the degree of agreement between observers. At each time point measured, the scores of the three observers were averaged. Values of $p \leq 0.05$ were considered significant for all analyses.

Results

Sixty-four ($n = 64$) calves with umbilical hernia were studied over a period of 12 months. Of these, four ($n = 4$) were excluded from the study due to co-morbidities. No significant differences in age, weight, body condition score and operative time were found between the calves enrolled (Table 1).

In each group, 20 calves were required to detect a statistically significant a difference, actual power was 0.86. All enrolled animals completed the study, and all recoveries were uneventful. No surgical complications were reported. The interobserver agreement was high ($W = 1$).

Within groups, HR values were statistically lower in the B and LB groups compared to T_0 at several time points ($p < 0.001$), whereas no significant difference was found in the L group. A statistically significant difference was found between groups for HR values. B group showed a significant decrease compared to L group ($p < 0.001$)

TABLE 1 Demographic data from all groups.

Variable	Group B	Group L	Group LB	*p* value
Weight (kg)	86 (65/99)	88 (75/94)	85 (65/95)	0.55
Age (months)	3 (1/4)	3 (1/4)	3 (1/4)	0.16
Body condition score	4 (3/5)	4 (3/5)	4 (3/5)	0.81
Surgery time (minutes)	46 (40/56)	49 (40/57)	47 (40/56)	0.14

Differences in weight, age, body condition score, and operative time in calves treated topically with butorphanol (B), lidocaine (L) alone, or the butorphanol/lidocaine combination (LB). Results are expressed as median (interquartile range, IQR).

and LB group ($p < 0.001$) from T_1 to T_{10}. The LB group had intermediate values between the B and L groups.

Within groups, RR values were significantly higher in the B and LB groups compared to T_0 at certain time points (T_1, T_2, T_9; $p < 0.001$), whereas a significant decrease was observed in the L group from T_3 to T_9 ($p < 0.001$). Among the groups, the B and LB groups showed significantly higher RR values than the L group at certain time points (at T7 with the B group and from T6 to T10 with the LB group; $p < 0.001$). Blood pressure values (SAP, DAP, MAP) during surgery showed a significant reduction compared to baseline (T_0) at almost all time points in each group ($p < 0.001$). Comparison between groups showed that the B and LB groups had higher blood pressure values than the L group at certain time points ($p < 0.001$). SpO_2 did not vary significantly between groups at any time point, with optimal values always maintained around 95%. Body temperature did not vary between groups (Table 2).

The intraoperative noxious stimulation response scale showed a significant reduction from T_3 to T_{10} compared to baseline in the B and L groups ($p < 0.001$). The LB group showed a significant reduction from T_3 to T_7 ($p < 0.001$). Between groups, the B group showed significant differences from the L group at many time points (T_1, T_2, T_4, T_5, T_6, T_9, $p < 0.001$), while the LB group only showed a difference at T_9 ($p < 0.01$). Comparison between the L and LB groups showed significant differences at T_1 and T_6 ($p < 0.001$). In the intraoperative, rescue analgesia was not required in any case (Table 3).

The time from start of surgery to recovery of the animals to standing was significantly different between B, LB and L groups ($p < 0.000$) and was 180 min (160/210; 185 ± 15.5) B group, 128 min (95/180; 131 ± 25.6) L group and 192 min (160/240; 196 ± 23.1) LB group (Figure 1).

The assessment of the postoperative pain score using the UNESP-Botucatu Unidimensional Composite Pain Scale showed a significant variation in the B and LB groups at 40 m and 50 m ($p < 0.001$), remaining ≤4 throughout the observation period. In the L group, UNESP-Botucatu showed changes along the time span from 20 to 50 m ($p < 0.001$), with 4 subjects at 40 m and 5 subjects at 50 m

TABLE 2 Effect of xylazine (0.3 mg/kg IM) followed by: butorphanol (0.02 mg/kg) or lidocaine (4.5 mg/kg) or lidocaine/butorphanol combination administered locally on heart rate (HR), respiratory rate (RR), systolic, diastolic, mean arterial pressure (SAP, DAP, MAP) and body temperature in calves undergoing umbilical hernia repair.

		T_0	T_1	T_2	T_3	T_4	T_5	T_6	T_7	T_8	T_9	T_{10}
HR (beats/min)	B	64 ± 6	52 ± 8*	50 ± 6*	48 ± 5*	49 ± 5*	50 ± 4*	51 ± 3*	53 ± 4*	50 ± 4*	49 ± 4*	52 ± 3*
	L	62 ± 6	67 ± 5♣	64 ± 6♣	67 ± 4♣	71 ± 4♣	74 ± 2♣	75 ± 3♣	75 ± 2♣	69 ± 6♣	69 ± 6♣	74 ± 3♣
	LB	70 ± 3	61 ± 4*♣◊	64 ± 6*♣	62 ± 6*♣	65 ± 5♣	69 ± 4♣	70 ± 4♣	63 ± 4*♣	64 ± 4*♣	63 ± 5*♣	67 ± 3♣
RR (breaths/min)	B	40 ± 5	44 ± 6*	48 ± 0.8*	41 ± 6	40 ± 7	40 ± 6	39 ± 5	39 ± 4	40 ± 6	37 ± 3	36 ± 4
	L	44 ± 5	48 ± 5	48 ± 7	40 ± 6*	41 ± 6*	37 ± 4*	37 ± 3*	33 ± 3*♣	36 ± 3*	36 ± 4*	33 ± 3*
	LB	44 ± 5	48 ± 7*	50 ± 4*	45 ± 5	44 ± 5	41 ± 4	45 ± 4◊	45 ± 7◊	44 ± 6◊	48 ± 6*♣◊	45 ± 5♣◊
SAP (mmHg)	B	136 ± 9	127 ± 6*	112 ± 8*	98 ± 7*	106 ± 9*	103 ± 8*	107 ± 10*	106 ± 9*	110 ± 8*	136 ± 9	144 ± 11*
	L	136 ± 11	129 ± 11	112 ± 9*	102 ± 6*	106 ± 7*	103 ± 8*	102 ± 8*	103 ± 9*	105 ± 0*	110 ± 8*♣	104 ± 7*♣
	LB	144 ± 10♣◊	136 ± 10*	114 ± 7*	108 ± 5*♣	100 ± 5*	101 ± 7*	99 ± 8*	103 ± 8*	104 ± 9*	112 ± 8*♣	115 ± 7*♣◊
MAP (mmHg)	B	120 ± 10	98 ± 4*	82 ± 7*	79 ± 5*	81 ± 6*	82 ± 6*	83 ± 8*	88 ± 6*	87 ± 5*	105 ± 7*	124 ± 4
	L	123 ± 4♣	117 ± 5*♣	83 ± 7*	89 ± 3*♣	91 ± 3*♣	68 ± 9*♣	72 ± 8*♣	69 ± 8*♣	78 ± 6*♣	93 ± 3*♣	86 ± 4*♣
	LB	120 ± 8	106 ± 5*♣	91 ± 6*♣◊	87 ± 5*♣	78 ± 3*◊	81 ± 3*◊	76 ± 3*♣◊	83 ± 3*♣◊	82 ± 3*♣◊	93 ± 5*♣	90 ± 3*♣◊
DAP (mmHg)	B	71 ± 3	62 ± 3*	59 ± 4*	49 ± 4*	55 ± 3*	50 ± 5*	52 ± 3*	57 ± 3*	58 ± 3*	70 ± 4	107 ± 7*
	L	76 ± 3	73 ± 3♣	55 ± 4*	59 ± 4*♣	63 ± 3*♣	41 ± 3*♣	41 ± 2*♣	38 ± 1*♣	48 ± 3*♣	65 ± 5*♣	55 ± 4*♣
	LB	79 ± 4	71 ± 4*♣◊	65 ± 5*♣◊	55 ± 9*♣	46 ± 5*◊	51 ± 3*◊	49 ± 2*♣	51 ± 3*◊	52 ± 3*	65 ± 3*♣	63 ± 4*♣
T° (°C)	B	39.6 ± 1									38.5 ± 5	
	L	39.4 ± 2									38.4 ± 6	
	LB	39.2 ± 1									38.3 ± 4	

*Significantly different from baseline within treatment. ♣Significantly different from corresponding time point between group B and L or LB groups. ◊Significant difference between the L group and the LB group. Two-way ANOVA for repeated measures, followed by Bonferroni test was used to evaluate the changes along the timeline and differences among groups. GraphPad Prism automatically corrects nonparametric data by transforming them into their base 10 logarithms. $p < 0.05$ was considered significant.

TABLE 3 Cumulative intraoperative score for responses to noxious stimulation.

	T_1	T_2	T_3	T_4	T_5	T_6	T_7	T_8	T_9	T_{10}
B	1 (0/3)	2 (0/4)	0 (0/0)*	0 (0/0)*	0 (0/1)*	0 (0/1)*	0 (0/1)*	0 (0/1)*	0 (0/1)*	0 (0/1)*
L	2.5 (0/4)♣	1 (0/3)*♣	1 (0/2)*	1 (0/2)*♣	1 (0/2)*♣	1 (0/3)*♣	1 (0/3)*	1 (0/3)*	1 (0/3)*	1 (0/3)*♣
LB	1 (0/3)◊	1 (0/4)	0 (0/2)*	0 (0/2)*	0 (0/1)*	1 (0/2)*◊	0 (0/2)*	0 (0/2)	1 (0/3)♣	0 (0/2)

Values are expressed as median and interquartile ranges (IQR). Percentage change, at various times, in HR, RR and SAP compared to T_1 (15 min after xylazine administration). *Statistical differences of HR, RR and SAP compared to T_1 (15 min after xylazine administration) in each group ($p < 0.001$). ♣Statistical differences between group B vs. groups L and LB ($p < 0.001$); ◊Statistical difference between group L vs. group LB ($p < 0.001$).

requiring rescue analgesia by administration of 3.3 mg/kg intravenous flunixin meglumine (Finadyne, Schering-Plough Animal Health, Oss, The Netherlands). Comparison of the groups with respect to UNESP scores showed a significant difference between B and L ($p < 0.001$) and between LB and L ($p < 0.001$), as the scores of B and LB were lower than those of L throughout the postoperative period (Table 4).

Discussion

Attitudes towards pain management in small animals are evolving and there is ample evidence that pain management is helpful in improving postoperative recovery (11). In contrast, pain management in large animals, particularly cattle, still appears to be suboptimal (24, 25). This is due to several factors, including a lack of knowledge about pain recognition, the belief that cattle have a higher pain threshold than other species, and economic considerations that limit the use of certain drugs (17, 24, 26). In addition, few analgesics are approved for use in food-producing animals (Commission Regulation (EU) no. 37/2010). For these reasons, intraoperative and postoperative pain management in these species is particularly challenging (9). To meet this challenge, extensive research is needed to investigate new practical and cost-effective strategies for pain relief in cattle using analgesic molecules currently available in veterinary medicine (27).

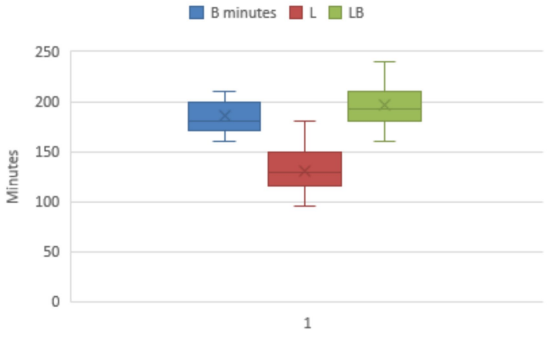

FIGURE 1
Time from the start of the surgery to the animals' recovery of the standing position. There was a significant difference between group B and group LB in relation to group L ($p < 0.000$).

Despite some known adverse effects on the central nervous system (CNS), including sedation, euphoria, dysphoria and arousal, and disadvantages related to the cost and regulation of their possession, opioids are the most effective analgesics available for pain management in veterinary medicine (28). New insights in recent years into the peripheral endogenous opioid system (PEOS) offer the possibility of developing new therapeutic strategies to exploit the analgesic effect of opioids, while minimising adverse systemic effects. The PEOS consists of peripheral opioid receptors (PORs) and peripheral leukocyte-derived opioids (PLDO). Tissue lesions and associated inflammation, such as during surgical tissue dissection, increase the concentration of PLDO-secreting leukocytes, but also the number and efficacy of PORs expressed on primary afferent neurons (PANs) (28). This upregulation of PORs is accompanied by sprouting of new peripheral sensory nerve terminals, alteration of the perineural barrier and reduction of pH. Taken together, these mechanisms enhance the interaction between opioid receptors and G-proteins, thereby increasing the antinociceptive efficacy of opioids in peripheral tissues (29–31). Several experimental and clinical studies have demonstrated the peripheral efficacy of opioids. For example, preservative-free morphine can be administered to canine and equine joints after arthroscopy or arthrotomy to provide analgesia via PORs (28). Other studies have shown that local application of the opioid receptor agonists μ (MOR), δ (DOR) and κ (KOR) produces significantly greater analgesia in injured tissue than in healthy tissue, both in animal models and in humans (32–34). Furthermore, while endogenous analgesia is mediated by both central and peripheral opioid receptors in the early hours, it is predominantly mediated by PORs in the later phases (35). Thus, the analgesic efficacy of peripheral opioids increases significantly with the duration of tissue injury, as observed in animal models of neuropathic, visceral, thermal, bone and oncological pain (6).

Although not a traditional local anaesthetic, in this study we wanted to investigate the potential local analgesic efficacy of butorphanol. This is the first study to investigate the use of locally butorphanol alone or in combination with lidocaine in calves sedated with xylazine for umbilical hernia surgery. The results of the present study suggest that both butorphanol and the butorphanol-lidocaine combination may provide satisfactory intraoperative and postoperative pain management and may therefore be a reasonable alternative to lidocaine alone for maintaining analgesia in calves undergoing routine surgery such as umbilical hernia repair.

TABLE 4 Results off UNESP-Botucatu Unidimensional Composite Pain Scale for comparision of postoperative pain, performed at 10 (T_{10}), 20 (T_{20}), 30 (T_{30}), 40 (T_{40}) and 50 (T_{50}) minutes after the calves readopted a standing position.

	Group B	Group L	Group LB		
Minutes	Median (IQR)	Median (IQR)	Median (IQR)	Score min/max	*p*-value
T_{10}	0 (0/1)	0 (0/0)	0 (0/0)	(0–10)	n.s.
T_{20}	0 (0/1)	1 (0/2)*♣	0 (0/0)◊	(0–10)	$p < 0.001$
T_{30}	0 (0/1)	1 (0/3)*♣	0 (0/1)◊	(0–10)	$p < 0.001$
T_{40}	1 (0/2)*	3 (2/4)*♣	0 (0/1)*♣◊	(0–10)	$p < 0.001$
T_{50}	1 (0/2)*	3 (3/4)*♣	1 (0/2)*◊	(0–10)	$p < 0.001$

Scores are reported as median (interquartile range, IQR), minimum and maximum values are reported. *Statistical differences of scores compared to T10 in each group ($p < 0.001$). ♣Statistical differences between group B vs. groups L and LB ($p < 0.001$); ◊Statistical difference between group L vs. group LB ($p < 0.001$).

Opioids are commonly used in multimodal analgesic regimens in veterinary medicine to improve pain relief, and combination with an alpha-2 agonist enhances the effect (36). Butorphanol is an opioid that produces analgesia through its κ-receptor partial agonist and μ-receptor antagonist actions, which are particularly important for pain management in calves (27). The pharmacokinetic and pharmacodynamic, cardiovascular effects and analgesic activity of butorphanol in calves are poorly reported since most studies have evaluated its adjuvant effects in combination with sedatives (alpha-2 agonists such as xylazine and detomidine), analgesic-dissociative drugs (ketamine) and inhalational anaesthetics (sevoflurane and isoflurane) (8–10, 27, 37).

The pharmacological properties and analgesic efficacy of butorphanol when administered alone have only recently been studied (9). Quantitative evaluation of antinociceptive activity in healthy calves confirmed a statistically significant antinociceptive effect of butorphanol, associated with marked arousal. Co-administration of detomidine abolished the excitatory effect and induced significant sedation, enhancing the antinociceptive effect of butorphanol and the resulting analgesia. However, the authors hypothesised that the mild antinociceptive effect of butorphanol alone, when administered systemically, would not be sufficient during surgical procedures performed routinely in cattle (9).

For this reason, although it remains unclear whether butorphanol alone can affect heart rate, its combination with sedatives is necessary to achieve adequate levels of analgesia and requires constant and careful monitoring of cardiorespiratory parameters. The combination of butorphanol with xylazine reduced the doses required for effective analgesia and increased the overall sedative effect (9, 10). In our study we did not observe any excitatory behaviour in calves treated with topical butorphanol. The time from the start of surgery to recovery of the upright position was different in the three groups, with groups B and LB recovering the upright position in a longer time than group L, which recovered the upright position in a shorter time, which could be related to a potentiating effect of butorphanol with the alpha2-agonist (10, 38).

When xylazine was co-administered with lidocaine in a distal paravertebral block, a significantly longer duration of anaesthesia was observed compared with lidocaine alone. Our results are consistent with previous studies showing that the addition of an alpha2-adrenoceptor agonist also prolongs the duration of local anaesthesia after epidural administration in various species (39, 40). It is likely that the lower scores and longer recovery time of the quadrupeds in groups B and BL were due to the systemic absorption of butorphanol after local injection. Previously, some authors reported that the use of butorphanol (0.1 mg/kg) in combination with IM xylazine (0.2 mg/kg) provided good pain control in calves between 4 and 6 weeks of age (41). In contrast, other authors reported that calves sedated with IM xylazine (0.7 mg/kg) and blocked with procaine showed clear signs of pain (42).

Intravenous administration of butorphanol at 0.2 mg/kg to calves anaesthetised with 3.7% sevoflurane was associated with a decrease in heart rate (HR) and blood pressure (SAP, MAP and DAP) (36), whereas administration of CRI (constant rate infusion) at 20 µg/kg/min to calves anaesthetised with 1.4% isoflurane did not produce clinically relevant changes in haemodynamic values (8). Several authors have reported a statistically significant decrease in heart rate after intravenous administration of various alpha-2 agonists and opioids (9, 10, 22); this effect was also observed in our study with the use of xylazine. The mean HRs for treatment B were significantly lower during the observation period than for the other treatments (groups L and LB). The observed change was relatively small, and bradycardia was not observed in any subject in group B. The normal range of bovine heart rate is reported to be between 38 and 96 bpm, and although the subjects monitored were calves, they were within these parameters (39). Therefore, this result may be insignificant from a physiological and clinical point of view. A significant increase in RR has been described when butorphanol is injected into the subarachnoid space (43) and our results also showed an increase in RR at certain time points in the B and LB groups.

One study evaluated the efficacy of combining morphine with lidocaine and ketamine in calves undergoing routine umbilical herniorrhaphy, with good results in patient management and adequate postoperative analgesia, but the cost and technical support to monitor and maintain CRI (constant rate infusion) makes this protocol infeasible in the field (44). Adverse behavioural effects of butorphanol have been observed in horses, including ataxia and stimulation of locomotor activity. The effects are transient and dose-dependent and are mainly observed after intravenous bolus injections of high doses (0.1 to 0.5 mg/kg); in fact, the same effects were minimised during continuous infusion compared with a single butorphanol injection (16). Other studies have observed that intravenous butorphanol (0.1 mg/kg) has analgesic potential in neonatal and older foals, with no apparent adverse behavioural effects, for the management of painful somatic conditions (45). In our study, we did not observe any side effects in calves, which may be related to the mode of administration of the drug and its wide tissue distribution, typical of opiates, which mainly determines its effect at the site of administration. Determining the cut-off point for rescue analgesia is an additional requirement to assist the veterinarian in making appropriate clinical decisions regarding analgesic therapy in the postoperative period (22). Recognition and measurement of postoperative pain are therefore critical in determining the need for and effectiveness of postoperative analgesia and rescue analgesia. Several scales for the assessment of pain in farm animals such as cattle, sheep and pigs have been reported in the literature. Among the different scales, the UCAPS (UNESP-Botucatu Unidimensional Composite Pain Scale for assessing postoperative pain in cattle), the USAPS (UNESP-Botucatu Sheep Acute Composite Pain Scale) and the UPAPS (UNESP-Botucatu Pig Composite Acute Pain Scale) showed the highest overall strength of evidence for construct validity, criterion validity and reliability (46). The UNESP-Botucatu unidimensional scale for the assessment of postoperative pain is a valid, reliable and repeatable instrument that has been used in both cattle (20, 23) and other species such as pig (47), horse (48) and cat (49). In this study, a cut-off score of $\geq$4 on the Botucatu Unidimensional Composite Pain Scale was chosen *a priori* to

resort to post-operative rescue analgesia. This score was established considering the clinical assessment, even if the score was below the established cut-off point (20, 23). Only in a few subjects treated with lidocaine (group L) did we have to resort to rescue anaesthesia at T_{40} and T_{50}; this may be related to the reduction/disappearance of the effect of the local anaesthetic.

It has been reported in the literature that lower ambient temperatures lead to a greater decrease in body temperature in subjects after sedation (50). Subjects in all groups experienced a decrease in temperature, but the parameters remained within optimal ranges, probably because our study was conducted in mild environmental conditions (average daily ambient temperature of around 18°C) (51, 52). Thus, in our case, sedation with xylazine seems to be appropriate for calves to avoid the detrimental effects of cold stress and could help prevent calves from contracting diseases such as respiratory infections or diarrhoea shortly after surgery (38, 53). Cagnardi et al. compared the sedative effects and pharmacokinetics of intravenous dexmedetomidine with those of xylazine. The results obtained were comparable to those observed with xylazine. We can therefore hypothesise that the use of other alpha-2 agonists may also be associated with the local administration of butorphanol (54).

Despite the encouraging results, this preliminary study has some limitations. The variable amount of hernial adhesions observed among the animals and the resulting variability in the surgical manipulations required may have influenced the amount of noxious stimuli the animals were exposed to. The lack of a control group with intramuscular butorphanol prevents direct comparison with local administration in terms of analgesic efficacy and adverse effects. The lack of monitoring of plasma levels of butorphanol after local administration prevents verification of possible systemic absorption and assessment of the elimination period. Regarding the management costs of the protocol presented in this study, the authors do not believe that the use of butorphanol alone or in combination with lidocaine will increase therapeutic costs, given the savings in analgesic or anti-inflammatory drugs in the postoperative period and the low doses used, which have been shown to be effective in pain management.

Conclusion

The results of this study suggest that local administration of both butorphanol alone and the butorphanol-lidocaine combination may be a viable alternative for intraoperative and postoperative pain management, and thus maintaining an adequate level of comfort, in calves undergoing surgery. Both butorphanol alone and the butorphanol-lidocaine combination at the doses used in this study produced effective analgesia in terms of intensity and duration, as evidenced by optimal intraoperative and postoperative scores. In addition, both treatments were safe, with no cardiopulmonary, excitatory or other adverse effects. Further research is needed to fully understand the pharmacodynamics and pharmacokinetics of butorphanol when administered locally, to establish a dosage range, and to determine potential applications in other types of surgery or other production categories in this species.

Data availability statement

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

Ethics statement

The animal studies were approved by the Institutional Ethics Committee for Animal Welfare of the University of Messina, protocol number 027/2018. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

CI: Conceptualization, Investigation, Methodology, Writing – original draft, Writing – review & editing. FS: Investigation, Writing – review & editing. VN: Investigation, Writing – review & editing. MT: Data curation, Writing – original draft, Writing – review & editing. SP: Data curation, Writing – original draft, Writing – review & editing. EG: Data curation, Writing – review & editing. DM: Methodology, Writing – review & editing. GC: Conceptualization, Investigation, Writing – review & editing.

Funding

The author(s) declare that financial support was received for the research, authorship, and/or publication of this article.

Acknowledgments

The authors acknowledge support from the University of Messina through the APC initiative.

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

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frontiers | Frontiers in Veterinary Science

TYPE Original Research
PUBLISHED 10 October 2024
DOI 10.3389/fvets.2024.1464549

OPEN ACCESS

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RECEIVED 14 July 2024
ACCEPTED 17 September 2024
PUBLISHED 10 October 2024

CITATION
Kampa N, Kaenkangploo D, Jitpean S, Srithunyarat T, Seesupa S, Hoisang S, Yongvanit K, Kamlangchai P, Tuchpramuk P and Lascelles BDX (2024) Evaluation of the comparative efficacy of green lipped mussel plus krill oil extracts (EAB-277), *Biota orientalis* extracts or NSAIDs for the treatment of dogs with osteoarthritis associated pain: a blinded, placebo-controlled study. *Front. Vet. Sci.* 11:1464549. doi: 10.3389/fvets.2024.1464549

Evaluation of the comparative efficacy of green lipped mussel plus krill oil extracts (EAB-277), *Biota orientalis* extracts or NSAIDs for the treatment of dogs with osteoarthritis associated pain: a blinded, placebo-controlled study

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Introduction: With little to no regulation of the supplement markets and a paucity of quality information regarding clinical utility of individual marketed supplements, it is difficult for veterinarians to provide any evidence-based recommendations to owners. The current study aimed to provide clinically useful comparative efficacy data on certain marketed supplements.

Methods: Using a prospective, block-randomized, double-blinded, placebo-controlled design, one hundred and one pet dogs with clinical hip OA-associated pain with one side worse than the other (index limb) were randomly assigned to one of four treatment groups: Green lipped Mussel plus Krill oil extracts (Antinol® Rapid, EAB-277); *Biota orientalis* extracts (4CYTE™ Epiitalis® Forte); an NSAID (meloxicam); or placebo (sunflower oil). Peak vertical force (PVF, expressed as a percentage of bodyweight) of the index limb, orthopedic assessment score (OAS) and hematology and blood chemistry values were evaluated before treatment (week 0), at 2, 4 and 6 weeks during treatment.

Results: At 6 weeks, the changes from baseline in PVF of the index limb in the EAB-277 and meloxicam groups were significantly greater than the change in the placebo and 4CYTE™ groups, and the placebo and 4CYTE groups were not different from each other. At 6 weeks, there were significant differences between the groups for overall OAS scores with the lowest scores (least impairment) in the EAB-277 and meloxicam groups, followed by the 4CYTE group and then the placebo group.

Discussion: Results of this study indicate that meloxicam and EAB-277 have significant objectively measured benefits in managing OA-related pain in dogs compared to placebo, but 4CYTE does not differ from placebo.

KEYWORDS

OA, marine based fatty acid, omega 3, NSAID, gait analysis, PVF

Introduction

Osteoarthritis (OA) is characterized by the progressive deterioration of one or more of the component tissues of the joint. This deterioration can be associated with pain and this pain has widespread, cumulative negative effects on multiple domains including mobility, the ability to perform the activities of daily living, musculoskeletal health and sensory processing which together negatively impact a dog's quality of life (1, 2). Recent data suggest that approximately 37% of dogs in the population may exhibit OA and related clinical signs due to pain (3). Additionally, new information has demonstrated that radiographically visible OA is very common in young dogs (8 months-4 years) with approximately 40% being affected radiographically and ~ 16% having associated pain of a moderate level or greater (4). Clearly, OA and associated pain is a common condition of dogs.

Managing OA pain in dogs typically involves a multimodal approach, including pain management, weight management, physical therapy, nutritional support and potentially surgical intervention in severe cases (5, 6). Non-steroidal anti-inflammatory drugs (NSAIDs) or anti-nerve growth factor monoclonal antibodies are recommend as the first line pharmacological therapy for dogs with chronic pain (7, 8). Omega-3 fatty acids are also recommended as a first line non-pharmacological option, with non-omega-3 based supplements related to 'tier 3' (7, 8). Despite this recommendation, with so many supplements available, little to no regulation of the supplement markets and a paucity of quality information regarding clinical utility of individual marketed supplements, it is difficult for veterinarians to provide any evidence-based recommendations. The current study aimed to provide clinically useful comparative efficacy data on certain marketed supplements.

EAB-277 (Antinol Rapid®) is the proprietary combination of phospholipids extracted from krill oil together with lipid fractions from the Green Lipped Mussel (PCSO-524™). Several studies have shown an apparent benefit of PCSO-524 for canine OA pain (9–13). Krill oil, extracted from krill, a small red-colored crustacean inhabiting the Antarctic, has been suggested to have advantages over fish oil due to its higher phospholipid-bound EPA and DHA content. A study in humans revealed that krill oil alleviated pain symptoms in adults with mild knee discomfort (14). Additionally, a recent blinded, placebo-controlled study using objective outcome measures concluded EAB-277 showed effectiveness for OA pain in dogs (13).

Extracts from the plant *Biota orientalis* are commonly used in Chinese herbal medicine (15). In traditional medicine, it has been used in the treatment of liver diseases, bullous bronchitis, psoriasis, enuresis, amenorrhea, cystitis, uterine carcinomas, diarrhea, and rheumatism (16). Preclinical studies conducted *in vitro* and unspecified *in vivo* studies have revealed the antioxidant (17) and anti-inflammatory (18, 19). In a pilot work, the effectiveness of hydrolyzed oil extract from *Biota orientalis* seeds (hBO/Epiitalis®, Interpath Pty Ltd) was investigated in humans with knee pain due to OA and results suggested efficacy (20). Epiitalis® is a proprietary oil extract from the plant *Biota orientalis*. A pilot study of 4CYTE™ Epiitalis® Forte reported significant improvements in both objective measures of limb use and subjective quality of life questionnaire scores in a population of dogs with pre-existing lameness due to joint OA (21), however no control group was included in this open label study. In a subsequent study, 4CYTE Canine (containing Epiitalis® plus three marine-derived ingredients) was reported to be non-inferior to carprofen over a 28-day study in dogs with OA pain (22).

We hypothesized that the commercially available supplement preparations Antinol® Rapid and 4CYTE™ Epiitalis® Forte would have beneficial effects in treating OA pain in dogs as compared to placebo and benchmarked against a positive control, the NSAID meloxicam.

Materials and methods

Study design

This study was a prospective, block-randomized, double-blinded, placebo-controlled clinical trial in client-owned (pet) dogs. Approval for the study protocol was obtained from the Institutional Animal Care and Use Committee of Khon Kaen University (IACUC-KKU-53/64). Throughout the study duration, the dogs remained in the care of their owners. Prior to commencement, each owner received a detailed explanation of the study, and consent was obtained through signed consent forms. The study took place at the Veterinary Teaching Hospital (VTH), Faculty of Veterinary Medicine, Khon Kaen University (KKU), Thailand, spanning from 2021 to 2023.

Sample size estimation

The sample size was estimated based on the change in peak vertical force (PVF) observed in a prior study evaluating the efficacy of EAB-277 in dogs with hip osteoarthritis (13). Changes in PVF after 4 weeks of treatment were + 3.90, +4.17, +3.08, and + 0.08 for the PCSO-524, EAB-277, Carprofen, and placebo groups, respectively. A type I error probability was set at 0.05 and power at 0.80 (1 minus the probability of type II error) were specified. Utilizing G*Power software (version 3.1.9.3, Heinrich Heine University Düsseldorf, Germany) for repeated measurement trials, it was determined that a sample size of 25 dogs per group was necessary.

Animals

Pet dogs, regardless of breed or sex, were eligible for participation in the study if they met the following criteria: at least 18 months old, weighing at least 15 kg, having a body condition score ranging from 3 to 9 (on a 9-point scale system), and exhibiting hematology and blood chemistry values within normal ranges. Additionally, the medical history had to include disability as reported by their owners and considered by the veterinarian as being due to OA pain; dogs were required to have clinical signs of hindlimb lameness due to hip OA pain; hip joint pain during examination by a study veterinarian; and radiographic evidence of OA in one or both hip joints that were found to be painful upon examination. Furthermore, dogs were required to be capable of trotting across a force plate for gait analysis. The hindlimb with the lowest value of peak vertical force (PVF) was denoted as the index limb at the initial evaluation (see section on gait analysis).

Dogs were not eligible if: they could not trot across the force platform; were lame or impaired due to an orthopedic condition that was not OA, had undergone any joint surgery within the preceding 6 months, displayed clinically detectable neurological deficits or systemic diseases, or if they were pregnant or lactating bitches.

Study protocol

The study protocol was written prior to the start of the study and agreed upon by all investigators. It was not publicly registered. Dogs were recruited to the VTH by outreach to local practitioners. Each dog underwent a full physical, orthopedic and neurological examination (conducted by SH), and the orthopedic assessment scores (OAS) were documented (Table 1). Radiographs of the hips were obtained and interpreted by a single radiologist (NK). Radiographic severity was evaluated based on established criteria outlined in previous publications (23) (Table 2).

Prior to starting the study, dogs were required to undergo a 2-week washout period for NSAIDs and joint supplements, and a 4-week washout period for corticosteroids. Throughout the study, no additional analgesic therapies were allowed. The diet type and quantity, as well as the daily activities of the study dogs, were kept consistent throughout the study period.

Each dog and its owner made four visits to the hospital: one for screening and enrollment prior to treatment, and then at 2, 4 and 6 weeks post-treatment. During each visit, ground reaction force measurements of the hindlimbs were recorded, and orthopedic evaluations were performed. Samples for complete blood count and serum chemistry, as well as urine for urinalysis, were obtained at each time point. Dogs were permitted to withdraw from the study for any reason, at any time, at the discretion of the researchers, the attending veterinarian, or the owners. If dogs withdrew from the study, they received treatment as determined appropriate by the referring veterinarian.

Treatment groups, allocation and blinding methods

Enrolled dogs were categorized into two groups (mild and moderate severity groupings) based on the severity of signs associated with OA pain using the overall orthopedic assessment score (see Table 1). Within each severity classification, dogs were randomly assigned to treatment groups. The allocation of treatments was carried out by the trial coordinator, who was not involved in assessing the dogs. Both the investigators collecting data and the dog owners were kept unaware of the treatment assignments. The trial coordinator provided guidance to the owners on the administration of treatments, including instructions on how and when to administer them. The treatments were provided in their original manufactured capsule or tablet form, distributed in unlabeled containers. The placebo consisted of capsules containing sunflower oil, prepared to match the appearance of EAB-277.

Using computer generated random numbers, dogs were randomly assigned to one of the four groups:

1 Antinol® Rapid (EAB-277) (Pharmalink International Co. Ltd.), administered orally at a dosage of 1 capsule per 10 kg body weight twice daily for a duration of 6 weeks.
2 4CYTE™ Epiitalis® Forte gel (Interpath Co. Ltd.) administered once daily at the dose recommended by the manufacturer (1.0 mL for 10–20 kg, 1.5 mL for 20–30 kg, 2.0 mL for 30–40 kg and 2.5 mL for 40–50 kg) for a period of 6 weeks.

TABLE 1 Assessment system used in the orthopedic evaluation (Orthopedic Assessment Scores, OAS) (24).

Criterion	Clinical evaluation
Lameness	1. Walks normally
	2. Slightly lame when walking
	3. Moderately lame when walking
	4. Severely lame when walking
	5. Reluctant to rise and will not walk more than five paces
Joint mobility	1. Full range of motion
	2. Mild limitation (10–20%) in range of motion; no crepitus
	3. Mild limitation (10–20%) in range of motion; with crepitus
	4. Moderate limitation (20–50%) in range of motion; with crepitus
	5. Severe limitation (>50%) in range of motion; with crepitus
Pain on palpation	1. None
	2. Mild signs; dog turns head in recognition
	3. Moderate signs; dog pulls limb away
	4. Severe signs; dog vocalizes or becomes aggressive
	5. Dog will not allow palpation
Weight-bearing	1. Equal on all limbs standing and walking
	2. Normal standing; favors affected limb when walking
	3. Partial weight-bearing standing and walking
	4. Partial weight-bearing standing; non-weight-bearing walking
	5. Non-weight-bearing standing and walking
Overall score of clinical condition	1. Not affected
	2. Mildly affected
	3. Moderately affected
	4. Severely affected
	5. Very severely affected

Each part of the OAS was scored and analyzed separately.

TABLE 2 Scoring system for the radiographic evidence of osteoarthritis (2)

Articulation	Radiographic sign	Score
Hip	Osteophytes and sclerosis absent	0 (none)
	Acetabular remodeling, Morgan line, slight neck remodeling and slight femoral head sclerosis	1 (mild)
	Acetabular remodeling and osteophytosis, neck remodeling, enthesiophytosis, and femoral head sclerosis	2 (moderate)
	Advanced acetabular and neck remodeling, severe osteophytosis and advanced femoral head sclerosis	3 (severe)

3 Meloxicam (Metacam®, Boeringher Ingelheim Co. Ltd.) administered orally at a dose of 0.2 mg/kg on the first day, followed by 0.1 mg/kg orally, every 24 h, for 6 weeks
4 Placebo capsules containing sunflower oil were administered at the same dosage as prescribed in group 1.

Outcome measures

Ground reaction force measurement: peak vertical force

Gait analysis was performed using dual in series biomechanical strain gage force plates (Advanced Mechanical Technology®, AMTI Model OR6-6, Watertown, MA, United States); 40 × 60 cm size each embedded in the middle of a 8-m-long walkway. Dogs were trotted across the force plates by trained handlers. The signals from the force plates were acquired and processed through dedicated gait analysis software (ToMoCoFPm, Toso System Inc.®, Saitama, Japan) and peak vertical force (PVF) values extracted. Velocity was measured by four laser sensors mounted 50 cm apart, spanning a distance on either side of the force plates. Velocity was limited to a range of 1.7–2.2 m/s and acceleration range within 0.5 m/s^2 throughout the study. A video camera (Panasonic HC-V180, Panasonic, Japan) recorded each pass to confirm appropriate foot strikes of each limb. The valid trial was defined as the forelimb followed by the ipsilateral hindlimb striking the center of the force plate. The initial PVF value was reported in Newton meter (Nm), then was normalized to body weight, and expressed as a percentage of total body weight for each limb. The mean value of PVF at each evaluation time point was derived from the average of the first five valid trials collected. The hindlimb with the lowest value of PVF was denoted as the index limb at the initial evaluation (before treatment) and the index limb was followed for improvement of limb function during the study period.

Orthopedic assessment scores

Following gait analysis at each time point, an orthopedic evaluation was conducted, and Orthopedic Assessment Scores (OAS) were documented. The OAS system, initially proposed by Moreau et al. (2) and later modified by McCarthy et al. (24), includes assessments of lameness, joint mobility, pain upon palpation, weight-bearing, and overall impact, with scoring criteria detailed in Table 1. Although it has not been formally defined or tested, a category change of '1' is considered clinically relevant.

Hematology and blood chemistry evaluations

A blood sample was collected from each dog before treatment and during every visit. Complete blood count (CBC) and serum biochemistry profiles were assessed. The serum biochemistry analysis consisted of blood urea nitrogen (BUN), creatinine, alanine aminotransferase (ALT), alkaline phosphatase (ALK), total protein, albumin, globulin, and the albumin:globulin ratio.

Statistical analysis

Prior to treatment (week 0), the homogeneity of variables among groups was assessed. Categorical data such as sex, body condition score, affected limb side, affected joint, radiographic score, and OAS were analyzed using the Chi-square test. Continuous and ordinal data including age, body weight, lameness score, pain score, joint mobility score, weight-bearing score, overall score, and PVF index limb were evaluated using one-way analysis of variance (ANOVA) for normally distributed data and the Kruskal-Wallis test for non-normally distributed data or ordinal data. The experimental unit was each individual dog.

The primary outcome, PVF of the index limb (PVF_{index}) expressed as a percentage of total bodyweight, was utilized to calculate changes in PVF_{index} at each time point relative to baseline (week 0) ($deltaPVF_{index}$). The effect of treatment on PVF_{index}, $deltaPVF_{index}$, and OAS (including lameness score, pain score, joint mobility score, weight-bearing score, and overall score) was explored using linear mixed models with repeated measurements. Treatment group, visit time, and their interaction were considered as fixed factors, while the subject's response measured at multiple time points was treated as a random factor with unstructured variance components. Simple effects between treatment groups at different time points and contrasts between visits within each group were examined using the CONTRAST options with Bonferroni adjustment. The minimal detectable change at the 95% confidence interval (MDC95), previously proposed by Moreau with a cutoff value of an increase in PVF >2.0% body weight was used to distinguish responders (25). All statistical analyses were performed using the STATA software (STATA v18, University licensed, StataCorp LLC, Texas, United States), and statistical significance was determined at a p-value of less than 0.05.

Results

Following screening, a total of 101 dogs were included in the study, distributed across treatment groups as follows: 26 in the EAB-277 group, 25 in the 4CYTE™ group, 24 in the meloxicam group, and 26 in the placebo group. All enrolled dogs were included in all analyses. Of these, 66 were male and 35 were female, with average (mean ± SD) age, body weight, and body condition score (BCS) (median, range) of

5.23 ± 2.63 years, 32.83 ± 9.55 kg, and 4 (6), respectively. Ten breeds of dogs participated, including Alaskan Malamute, American Bully, Beagle, German Shepherd, Golden Retriever, Labrador Retriever, Samoyed, Siberian Husky, Thai Native, and mixed breeds. Golden and Labrador Retrievers were the predominant breeds, accounting for 41 and 21% of the total, respectively.

On clinical examination, 42 dogs predominantly exhibited lameness in the right hindlimb, while 59 dogs exhibited lameness in the left hindlimb. Of these, 54 dogs were classified as having mild OA, while 47 were classified as having moderate OA. Radiographic assessment revealed bilateral hip osteoarthritis (OA) lesions (radiographic score of hip ≥1) in 71 dogs and unilateral lesions in 30 dogs. Characteristics of the dogs, including sex, body condition score, affected limb side, unilateral or bilateral affection, radiographic severity score, OA classification, Orthopedic Assessment Scores (OAS), and PVF_{index} at baseline (week 0), are presented in Table 3. There were no significant differences between the four treatment groups ($p > 0.05$) for any variable. The hematology and blood chemistry values of all dogs were within normal limits during the study period of 6 weeks. There were no unexpected adverse events.

Force plate gait analysis: peak vertical force

Velocity at each time point showed no differences either between or within groups (Supplementary Table S1).

There was a notable and significant overall effect of treatment ($p < 0.001$) and time ($p = 0.016$) on the change in the primary outcome measure PVF_{index}. Specifically, the dogs in the EAB-277 and meloxicam groups showed increases in the mean $deltaPVF_{index}$ from week 0 over time, whereas the 4CYTE™ group demonstrated minimal change and the placebo group exhibited no change throughout the study period. By week 2 post-treatment, dogs in the meloxicam group showed a significant increase in PVF_{index} compared to pre-treatment levels (Supplementary Table S2), with a mean $deltaPVF_{index}$ (3.15 ± 3.87) that was significantly higher than in the placebo group (−1.29 ± 3.00) (Table 4; Figure 1). Following 4 weeks of treatment, both the EAB-277 and meloxicam groups showed a significant increase in PVF_{index} compared to baseline (Supplementary Table S2). The mean $deltaPVF_{index}$ was 2.13 ± 4.28 in the EAB-277 group, 1.23 ± 4.52 in the 4CYTE™ group, and 3.36 ± 3.67 in the meloxicam group, with the latter significantly higher than the placebo group (−0.18 ± 3.10) (Table 4; Figure 1). At the final observation point (week 6), both the EAB-277 and meloxicam groups had significantly greater changes in PVF_{index} compared to baseline, similar to the results at week 4 (Supplementary Table S2). The mean $deltaPVF_{index}$ for the EAB-277 (3.83 ± 3.08) and meloxicam (4.87 ± 3.07) groups was significantly higher than that of the 4CYTE™ group (0.43 ± 3.67) and the placebo group (−0.77 ± 3.14) (Table 4; Figure 1). Using the MDC95 as a cut-off value ±2.0% PVF of body weight, the percentage of responders in each treatment group (EAB-277, 4CYTETM, Meloxicam and placebo) was 69.23, 40.00, 79.19, and 7.69%, respectively (Table 5).

Orthopedic assessment scores

The lameness scores in the EAB-277 and Meloxicam groups exhibited significant decreases compared to pre-treatment levels, while scores in the 4CYTE™and placebo groups remained unchanged throughout the study period (Supplementary Table S3). Additionally, the pain scores in the Meloxicam group were consistently lower at all visits compared to pre-treatment, with the lowest scores observed during weeks 4 and 6 following treatment (Supplementary Table S4). Joint mobility scores significantly decreased in the EAB-277, 4CYTE™, and Meloxicam groups at weeks 2, 4, and 6 post-treatment (Supplementary Table S5). Similarly, bearing scores significantly decreased at weeks 2, 4, and 6 post-treatment compared to pre-treatment levels in the EAB-277 and Meloxicam groups (Supplementary Table S6).

Finally, the overall scores of the EAB-277 and Meloxicam groups showed significant decreases compared to pre-treatment levels, with both groups achieving their lowest scores at 6 weeks post-treatment (Table 6). At 6 weeks, there were significant differences between the groups, with scores being lowest (decreased clinical signs) in the EAB-277, 4CYTE and meloxicam groups compared to the placebo group. However, the degree of change in overall scores was quite small, likely reflecting the subjective nature of the assessments and the very coarse scale.

Discussion

This study selected dogs with hip joint osteoarthritis, with one leg more affected than the other, and used objective gait analysis – measurement of the ground reaction force peak vertical force – to assess the efficacy of two supplements compared to the NSAID meloxicam and to placebo, over a 6-week period. Overall, we found PVF increased over time (limb use improved) in both the EAB-277 and Meloxicam groups, whereas there was minimal improvement in the 4CYTE™ group and no change in the placebo group throughout the study. Positive effects were seen earliest in the meloxicam group (by week 2) and then in the EAB-277 group (by week 4). Conversely, 4CYTE™ and placebo did not exhibit positive treatment effects based on PVF measurements. Interestingly, at both week 4 and 6 post-treatment, the change in PVF for EAB-277 was similar to that of the Meloxicam group. Overall, the results show a clear benefit of EAB-277 and meloxicam in improving limb use in dogs over a 6-week period. In this study, both a positive control (the NSAID, meloxicam) and a negative control (placebo) were included to contextualize PVF changes in the other groups.

The results from gait analysis were supported by the subjective assessments across lameness, pain, joint mobility and weight-bearing scores. As well as the improvements in these parameters seen in the EAB-277 and meloxicam groups over time, joint mobility and weight bearing were assessed as being significantly improved compared to baseline in the 4CYTE™ at 6 weeks. However, across these parameters, only the pain score and the overall assessment scores showed significant group effects, favoring EAB-277 and meloxicam at week 6 for pain, and favoring all three treatment groups versus placebo for the overall score. Overall, the findings suggest potential benefits of EAB-277, 4CYTE™, and meloxicam in managing OA-related pain in dogs, as evaluated by the OAS.

In this study, we found no improvement of the objective assessment of GRFs with 4CYTE™. One previous study demonstrated significant improvements in both objective measures of limb use (TPI% [total pressure index]) using a pressure sensitive mat

10.3389/fvets.2024.1464549

TABLE 3 Subject characteristics and data at prior treatment (week 0) of treatment groups and comparison.

Variable	EAB-277	4CYTE™	Meloxicam	Placebo	*p*- value
	n = 26	*n* = 25	*n* = 24	*n* = 26	
Categorical variables*					
Sex					
Male	17	17	17	15	0.784
Female	9	8	7	11	
BCS					
3	1	1	1	0	0.955
4	16	16	16	16	
5	6	5	5	5	
7	1	2	2	4	
9	2	1	0	1	
Side of affected limb					
Right	14	7	9	12	0.273
Left	12	18	15	14	
Affected joint					
Unilateral	8	7	7	8	0.996
Bilateral	18	18	17	18	
Radiographic score (index limb)					
1	10	8	6	8	0.954
2	9	9	8	9	
3	7	8	10	9	
Radiographic score (contralateral limb)					
0	8	7	7	8	0.976
1	7	5	4	4	
2	7	8	6	7	
3	4	5	7	7	
OA classification					
Mild OA	16	12	12	14	0.777
Moderate OA	10	13	12	12	
Continuous variables (mean±SD)**					
Age (years)	4.94 ± 2.52	5.24 ± 2.68	5.58 ± 2.81	5.19 ± 2.64	0.843
Body weight (kg)	32.06 ± 8.96	33.22 ± 8.55	33.13 ± 8.70	34.21 ± 9.27	0.804
PVF index limb	58.44 ± 7.13	61.34 ± 7.99	59.22 ± 7.45	60.46 ± 8.82	0.568
Non-parametric variable* (median, range)**					
Lameness score	3.0 (2.0)	2.0 (3.0)	3.0 (3.0)	2.0 (2.0)	0.297
Pain score	2.0 (3.0)	2.0 (2.0)	2.0 (2.0)	2.0 (3.0)	0.379
Joint mobility score	2.0 (3.0)	2.0 (2.0)	2.0 (2.0)	2.0 (2.0)	0.878
Bearing score	2.0 (2.0)	2.0 (2.0)	2.0 (2.0)	2.0 (1.0)	0.306
Overall score	2.0 (2.0)	2.0 (1.0)	2.5 (2.0)	2.0 (1.0)	0.886

BCS, body condition score; OAS, Orthopedic Assessment Scores. *Chi-square tests. **One-way ANOVA. ***Kruskal-Wallis tests.

(GAITRite® Portable Walkway System) and subjective quality of life questionnaire scores (HCPI) in dogs with pre-existing lameness due to joint OA (21). However, this open-label study did not include a control group which makes it impossible to assess whether the changes seen were truly due to treatment, or the natural variation in impact of pain over time. In contrast, our study was a randomized, placebo-controlled trial that included both positive and negative control groups. Although the different gait analysis system was used for the objective assessment, both placebo and 4CYTE™ group's PVF showed no significant change after the study was completed (6 weeks).

TABLE 4 The mean deltaPVF$_{index}$ ± Standard Deviation [95% confident interval] for dogs in each group at 2, 4 and 6 weeks following initiation of treatment.

Visit time	EAB-277	4CYTE™	Meloxicam	Placebo
	n = 26	*n* = 25	*n* = 24	*n* = 26
Week 0 (PVF)	58.44 ± 7.13	61.34 ± 7.99	59.22 ± 7.45	60.46 ± 8.82
Week 2 mean change	1.40 ± 4.27 [a,b]	0.79 ± 4.51 [a,b]	3.15 ± 3.87* [a]	−1.29 ± 3.00 [b]
	[−0.04, 2.84]	[−0.68, 2.25]	[1.65, 4.64]	[−2.73, 0.14]
Week 4 mean change	2.13 ± 4.28* [a,b]	1.23 ± 4.52 [a,b]	3.36 ± 3.67* [a]	−0.18 ± 3.10 [b]
	[0.57, 3.45]	[−0.24, 2.69]	[1.86, 4.86]	[−1.61, 1.26]
Week 6 mean change	3.83 ± 3.08* [a]	0.43 ± 3.67 [b]	4.87 ± 3.07* [a]	−0.77 ± 3.1 [b]
	[2.28, 5.17]	[−1.03, 1.91]	[3.36, 6.37]	[−2.22, 0.67]

Week 0 (prior treatment) absolute PVF values are also shown. *The 95% confident interval of estimated mean difference (deltaPVF$_{index}$) not covered the zero value indicates that the value of the mean change in PVF of the index limb was significantly different ($p < 0.05$) from the week 0 within that treatment group. Between group comparisons are shown in Figure 1. Differences in lower letter superscript (a, b) indicate significant differences between groups for mean deltaPVF$_{index}$.

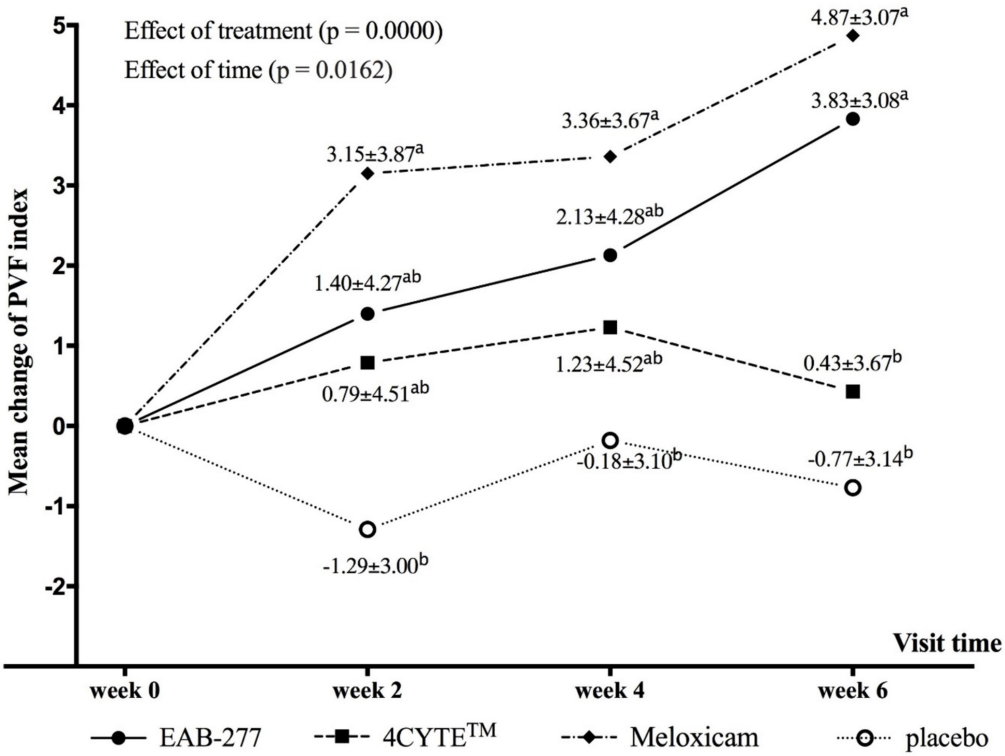

FIGURE 1
Graphical representation of the mean (±standard deviation) change from baseline for PVF (deltaPVF$_{index}$) in each group during the study period. Different in lower letter superscript (a, b) indicate significant differences between groups for mean deltaPVF$_{index}$.

Our results regarding the efficacy of meloxicam, an NSAID, align with previous studies (1, 26–28). Meloxicam exhibited a rapid response in terms of increasing limb use (as measured by ground reaction forces), with significant improvement observed within 2 weeks of treatment evidenced by a PVF change of 3.15 ± 3.87. Our currently reported results for meloxicam are also similar to a study in dogs treated with carprofen for 2 weeks where a change in PVF (%BW) of 3.2 ± 0.8 (significant improvement) was seen (29), and similar to those from another OA study (9) involving Firocoxib, where the PVF change in the index limb was 3.03 ± 4.67 and 3.25 ± 4.13 after 2 and 4 weeks of treatment, respectively (9). The results of EAB-277 in this study were similar to those of the previous studies (13); the PVF change after 6 weeks of treatment was 3.83 ± 3.08, slightly lower than the 4.45 ± 4.23 observed in the previous study.

No work has been done to define the minimal clinically important difference (MCID) with respect to ground reaction forces. We are working on defining the MCID for change in PVF (in separate work) in dogs with multi-joint OA pain. In this study, responder analysis was evaluated using a previously determined cut-off value of ±2.0% PVF change (25, 30). Meloxicam had the highest percentage of response

TABLE 5 Percentage of responders (PVF change >2%) and non-responders (PVF change <2%) in each treatment group (EAB-277, 4CYTETM, Meloxicam and placebo) at week 6 after treatment, with a comparison between groups.

Group	Responders	Nonresponders	*p*- value*
EAB-277	69.23 (18/26) [48.21–85.67]	30.77 (8/26) [14.32–51.78]	<0.001
4CYTE™	40.00 (10/25) [21.12–61.33]	60.00 (15/25) [38.66–78.87]	
Meloxicam	79.17 (19/24) [57.84–92.86]	20.83 (5/24) [7.13–42.15]	
placebo	7.69 (2/26) [0.94–25.13]	92.31 (24/26) [74.86–99.05]	

Data are presented in % (number of dogs responding/total number of dogs) (95% confidence intervals). *Analysis by Chi square test.

TABLE 6 The 'overall scores' of the orthopedic assessment score (median, range) for the treatment groups prior to treatment (week 0), week 2, 4 and 6 after treatment.

Visit time	EAB-277	4CYTE™	Meloxicam	placebo	*p*- value for between group comparisons
	n = 26	*n* = 25	*n* = 24	*n* = 26	
week 0	3.0 (2.0)	2.0 (3.0)	3.0 (3.0)	2.0 (2.0)	0.797
week 2	2.0 (3.0)	2.0 (1.0)	2.0 (1.0)*	2.0 (1.0)	0.997
week 4	2.0 (2.0)*	2.0 (1.0)	2.0 (2.0)*	2.0 (1.0)	0.453
week 6	2.0 (2.0)[a]*	2.0 (2.0)[a]	2.0 (2.0)[a]*	2.0 (2.0)[b]	0.027

[a,b]Different in lower letter superscript indicates significant differences between groups at time point. *Indicates the score in point time (week) significantly different ($p < 0.05$) from the value of week 0 in each treatment group.

rate at 79.19%, followed by EAB-277 at 69.23%, 4CYTE™ at 40.00%, and placebo at 7.69%.

Overall, our results clearly indicated little to no positive effects associated with placebo. Further, the results from the positive and negative control groups give us confidence in interpreting the effects of administration of each of the supplements we evaluated, EAB-277 and 4CYTE™: the changes observed with meloxicam and EAB-277 were significantly different from the placebo group, strongly suggesting a clinically significant improvement. Given our inclusion criteria and the results in our positive and negative control groups, we believe our results are generalizable to the broader population of dogs with OA pain.

Our study had several limitations. Although clearly recommended in current pain management guidelines (7, 31), our study did not employ clinical metrology instruments (CMIs), or client reported outcome measures (CROMs) for assessing OA pain. There are several CMIs that have been developed, validated, and reported for measuring the severity of OA in dogs such as the Liverpool Osteoarthritis in Dogs (LOAD) instrument (32), the Canine Brief Pain Inventory (CBPI) (33), the Helsinki Chronic Pain Index (HCPI) (34). Owners must complete questionnaires, necessitating their understanding of the questions, which should also align with the local culture and context. A recent study in Thailand (9) that employed the CBPI suggested that the translated version might not have been fully comprehended. Our pilot experience with the LOAD indicated that, even after translation, the questions might not have been suitable for the Thai culture. Ideally, each CMI should be validated +/− adapted for each new language and culture. Therefore, CMIs were not used in this study as none have been validated in the Thai language and culture. Unlike CMIs, ground reaction forces (GRFs) measured using a force plate provide an objective assessment and have been utilized as a proxy measure of joint pain in dogs with appendicular joint OA (29, 35–39). Additionally, the duration of the study was only 6 weeks and it is possible that over longer durations of administration of supplements, greater effects may be seen. Extending the study duration may provide more comprehensive information about supplements' effects on OA pain, however our results clearly indicate positive effects for EAB-277, but not 4CYTE, over a 6 week period. Finally, many times supplements are used together with NSAIDs, but we did not have a group evaluating combined treatment. Future research should evaluate the combination of EAB-277 with an NSAID to test for potential synergistic effects in multimodal therapy management.

Conclusion

In dogs with painful OA, we found that PVF increased over time (indicating improved limb use) in both the EAB-277 and meloxicam groups, while there was minimal improvement in either the placebo or 4CYTE™ group. At 6 weeks there were significant differences between the groups in improvement in limb use, with improvement in the meloxicam and EAB-277 groups being significantly greater than in the placebo and 4CYTE groups. These results, combined with the subjective orthopedic assessments of lameness, pain, joint mobility, and weight-bearing scores, suggest that meloxicam and EAB-277 have clear benefits in managing OA-related pain in dogs, with equivocal evidence for a beneficial effect of 4CYTE™.

Data availability statement

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

Ethics statement

The animal studies were approved by Institutional Animal Care and Use Committee of Khon Kaen University (IACUCKKU-53/64).

The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

NK: Conceptualization, Methodology, Project administration, Supervision, Writing – original draft, Writing – review & editing, Formal analysis, Data curation, Funding acquisition, Investigation, Resources. DK: Data curation, Writing – original draft, Writing – review & editing. SJ: Data curation, Writing – review & editing, Writing – original draft. TS: Data curation, Writing – review & editing, Writing – original draft. SS: Data curation, Writing – original draft, Writing – review & editing, Formal analysis. SH: Data curation, Writing – original draft, Writing – review & editing. KY: Data curation, Writing – original draft, Writing – review & editing. PK: Data curation, Writing – original draft, Writing – review & editing. PT: Data curation, Writing – original draft, Writing – review & editing. BL: Writing – original draft, Writing – review & editing, Conceptualization, Formal analysis, Methodology, Project administration, Supervision.

Funding

The author(s) declare that financial support was received for the research, authorship, and/or publication of this article. This work was funded by Pharmalink International Ltd., Central, Hong Kong and supported by the Faculty of Veterinary Medicine, Khon Kaen University. The authors declare that 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.

Acknowledgments

The authors would like to thank the owners of the studied dogs. Clinicians and veterinary assistants in the Veterinary Teaching Hospital, Faculty of Veterinary Medicine, Khon Kaen University for their technical assistance.

Conflict of interest

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

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2024.1464549/full#supplementary-material

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frontiers | Frontiers in Veterinary Science

TYPE Original Research
PUBLISHED 24 October 2024
DOI 10.3389/fvets.2024.1461628

OPEN ACCESS

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RECEIVED 08 July 2024
ACCEPTED 27 September 2024
PUBLISHED 24 October 2024

CITATION
Enomoto M, Hash J, Cole T, Porcel Sanchez MD, Thomson A, Perry E, Aker S, Nakanishi-Hester A, Haupt E, Opperman L, Roe S, Thompson NA, Innes JF and Lascelles BDX (2024) Response to treatment with grapiprant as part of a standard multimodal regimen in young dogs with appendicular joint osteoarthritis associated pain.
Front. Vet. Sci. 11:1461628.
doi: 10.3389/fvets.2024.1461628

Response to treatment with grapiprant as part of a standard multimodal regimen in young dogs with appendicular joint osteoarthritis associated pain

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Introduction: The response to medical management of young dogs with osteoarthritis (OA) associated pain has not been evaluated. Using an open-label design, the effectiveness, over a 4-month period, of standardized management (grapiprant/fish oil/exercise) for treating OA pain in young dogs was evaluated.

Methods: Included dogs were 9 months-4 years of age; ≥3.6 kg body weight; had ≥1 appendicular joint with radiographic OA and obvious joint pain; had a Liverpool Osteoarthritis in Dogs (LOAD) score of ≥5. The non-steroidal anti-inflammatory piprant (grapiprant) was given at the recommended dose daily, omega-3 fatty acid supplementation was initiated at 100 mg/kg and then increased to 200 mg/kg daily, and leash exercise was gradually increased to a target of 60 min daily. Client-reported outcome measures (CROMs) and force plate gait analysis were collected at baseline and monthly for 4 months. The index limb was defined as the most severely affected limb at baseline.

Results: Forty-eight dogs were enrolled (mean ± SD age of 30.7 ± 10.7 months). Hips, elbows, and stifles were commonly affected. Medication and supplement compliance was excellent (≥95% of target administered), and treatments were well-tolerated. CROMs showed significant improvement over time and at each time point. Overall, peak vertical force (PVF) increased significantly (<0.001), and vertical impulse increased numerically. Increase in PVF from baseline was significant at all time points except 4-months.

Discussion: This study demonstrates a clinically meaningful benefit of a multimodal treatment regimen over a 4-month period for young dogs (<4 years old) with OA-pain. Future work should determine if early, effective treatment is of long-term benefit.

KEYWORDS

osteoarthritis, pain, dog, grapiprant, force plate, fish oil, exercise

Introduction

Osteoarthritis (OA) is a degenerative joint disease, often resulting in chronic pain (1). OA associated pain adversely affects multiple dimensions such as gait, function, and sleep (2), and its management can be challenging due to complex relationships between peripheral disease, nervous system input and changes over time, and comorbidities (3, 4). However, management is likely to be less complex earlier in the course of the disease. Therefore, earlier, effective treatment of OA-pain may better control joint pain and the longer-term negative impacts of joint pain on multiple dimensions, although this concept has not been tested. In the treatment of canine OA pain, a multimodal approach has been recommended including pharmacological agents, dietary modulation, and exercise and rehabilitation therapy (5).

In dogs, OA is thought to be initiated primarily by developmental joint disease (1). Recently, our group found that 40% of young dogs between 8 months and 4 years old had radiographic OA (rOA) in one or more appendicular joints and 40–60% of those dogs had joint pain (≥ moderate or mild pain, respectively) in one or more of the rOA joints (6).

Despite the high prevalence of OA-pain in young dogs and potential benefits of early treatment, no studies have evaluated the response to multimodal OA treatment in young dogs with OA-pain. The multimodal OA-pain treatment regimen employed in this study consisted of a nonsteroidal anti-inflammatory drug (NSAID), a nutritional supplement (omega-3 fatty acid supplement), and modification of exercise. Grapiprant is registered for the treatment of OA pain and is a non-COX-inhibiting, piprant class NSAID with a good safety profile (7). Omega-3 supplements are considered to be associated with efficacy in canine OA pain (5, 8, 9). Regular, low-impact controlled exercise is recommended to support movement, muscle strength and to help control body weight (10). The aim of this study was to assess the effectiveness of this standardized management plan for treating the clinical signs of OA in young dogs using objective and subjective outcome measures.

Materials and methods

Study design

This study was an open label evaluation of the response to multimodal treatment over a 4-month period in young dogs (9 months to 4 years old) with clinical signs associated with OA. The *in vivo* portion of the study was performed between June 2020 and November 2022. NC State University Institutional Animal Care and Use Committee (IACUC) approved this study and all procedures (IACUC#19-604-O), and the study was approved by the Hospital Clinical Studies Review Board. All dog owners signed a written consent form following a detailed verbal explanation of the study protocol.

Sample size calculation

In this study, the primary outcome measure was the Liverpool OsteoArthritis in Dogs (LOAD) owner assessment. Based on pilot data from clinical management of dogs with OA pain, we expected a decrease of 4.5 points in the LOAD scores and pre- and post-treatment standard deviations of 6.77 and 7.60 respectively, and these data indicated that 80% power would be achieved with a sample size of 40 dogs. These data were from dogs ~8 years old.

Recruitment

The study aimed to recruit 50 young dogs with clinical signs associated with OA. Osteoarthritic young dogs with clinical signs associated with OA that were identified in the previous prevalence study (6) were invited to participate in the current study. Additionally, young dogs with lameness due to OA pain were recruited to the study by advertisements via NCSU websites, e-mails to local practices and NC State employees, local radio advertisements and via CVM social media (Twitter and Facebook). Recruitment proved difficult (likely mainly due to the changes induced by COVID), and so Visionaire[1] was employed and recruitment successfully completed via a targeted Facebook campaign.

Case selection

To be eligible for the study, dogs were required to be between 9 months and 4 years of age at the time of recruitment, and ≥ 3.6 kg body weight. Dogs were required to have clinical signs of OA-associated joint pain confirmed by gait evaluation, veterinary assessment, and radiographic evidence, be in general good health or have stable chronic conditions and able to complete the study in the opinion of the veterinarian. Health status was assessed by physical examination, medical history, and clinical pathology evaluations (complete blood count, serum biochemistry profile, and urinalysis including sediment examination). The recruited dogs were also required to have a LOAD score of ≥5 and at least one joint with radiographic evidence of OA and a pain score of ≥2 out of 4 (moderate pain).

Dogs that had clinically relevant abnormal clinical pathology findings, spinal orthopedic abnormalities, or neurologic abnormalities that affected gait were excluded. Other exclusion criteria were concomitant disorders that may have affected evaluations for the study, and other joint diseases (such as immune-mediated joint disease). Dogs that had had major surgery within 1 month, or cruciate ligament surgery within 3 months were excluded, as were dogs that had surgeries that could confound the evaluation of OA pain (acute inflammatory pain due to surgery). A required wash-out period was at least 3 weeks for NSAIDs or short-acting steroids, and 4 weeks for long-acting steroids.

Brief description of the study timeline

Owners signed an owner consent form before any study activities and then dogs were screened to see if they met the inclusion or exclusion criteria. A brief description of study outline is shown in

1 https://vrande.com

Supplementary file 1. Outcome measures were performed, and blood and urine were collected for clinical pathology evaluations. Following veterinary assessments (physical, orthopedic, neurological), dogs were sedated for radiographs of all joints. Veterinary assessments and outcome measures (owner questionnaires, gait analysis) were also performed every month for 4 months. Blood work and urinalysis were repeated at the end of the study visit.

Orthopedic examination

Physical, orthopedic, and neurologic examinations were performed, and data were captured. During the orthopedic examination, every joint of each limb was examined by a veterinarian experienced in evaluating pain associated with OA in dogs (ME), and joints were graded for pain, crepitus, effusion, and thickening. The manus and pes were considered as one joint region for evaluation purposes. Other appendicular joints evaluated were carpus, elbow, shoulder, tarsus, stifle, and hip. Spinal column segments were examined and graded for pain. The axial skeleton was evaluated by dividing the spine into cervical, thoracic (T1-9), thoraco-lumbar (T10-L6), and lumbosacral regions. Scores for pain ranged from 0 to 4. Assessments for crepitus, effusion, thickening, and range of motion were recorded, but not used in analysis. Scores were recorded on the Joint Evaluation Scoring SystEm canine (11). At screening, the Canine OsteoArthritis Staging Tool (COAST) was used for staging the impact of OA on patients (12). Based on the published papers, the items considered as the risk factors for OA in this study were orthopedic disease without radiographic evidence of OA (e.g. hip subluxation), traumatic joint injury/surgery, certain breed, overweight (BCS≥7) (13, 14).

Radiography

Radiographs were taken under sedation with a mu-opiate combined with alpha-2 adrenergic agonist, for example, hydromorphone 0.05–0.1 mg/kg/IV and dexmedetomidine 0.003–0.005 mg/kg/IV. However, the choice of drug and dose was adapted according to the dog's health condition. Orthogonal views of all appendicular joints and lateral views of the spine were taken. To minimize ionizing radiation exposure, where appropriate, radiographs were centered on the midpoint of the limb or spinal segment to reduce the number of individual exposures used. A subjective overall severity score was assigned to each joint based on a numerical rating scale where 0 = no radiographic abnormalities identified and 10 = most severe radiographic OA, as described previously (15, 16). Radiographs were assessed using a DICOM viewer (Horos ver. 3.3.6) by a veterinarian experienced in evaluating canine OA (ME).

Treatment

Grapiprant was administered orally every 24 h according to the approved dosing chart to achieve a target dose of 2 mg/kg (7). Owners were instructed to give the dose 1 h before a meal and at approximately the same time each day. Compliance with dosing was evaluated by reconciling the returned pills with what was dispensed. 100 mg/kg of omega-3 fatty acid (fish oil; Nature Made, Viva naturals, Nutrigold) was added to the diet for the first week and 200 mg/kg of omega-3 fatty acid from the second week to the end of the study. The exercise protocol suggested varied based on the starting point of leash exercise for each case. Owners were advised to gradually increase leash exercise to 30 min twice daily or the equivalent thereof (adding 5–10 min of exercise every week). If a dog received leash-walking exercise for 60 min daily at the screening visit, no change was applied.

Outcome measures

Client reported outcome measures (CROMs)

CROMs were used as previously described. The Liverpool OsteoArthritis in Dogs (LOAD) and Canine Brief Pain Inventory (CBPI) have been shown to be valid measures of the impact of OA-pain in dogs (17–21). Sleep and Nighttime Restlessness Evaluation Score Questionnaire version 2.0 (SNoRE) was used to collect data regarding sleep quality (22). The CROMs were completed by the dog owner. For the LOAD, the sum of each item score was calculated. For CBPI [pain severity scores (PSS) and pain interference scores (PIS)] and the SNoRE, the average of each item was calculated. A reduction of ≥4 in LOAD scores was defined as "minimal clinically-important differences (MCIDs)" as suggested by previous studies (23, 24).

Ground reaction forces (GRFs) measurement using a force plate (FP)

Inclusion criteria were not optimized for collection of data using a force plate (FP); therefore, FP data were collected only if the dogs 'fit' the FP system (i.e., were of a size such that GRF data would be collected using the FPs). GRFs were collected using dual in series FPs (AMTI, Watertown, MA, USA) and custom software (Sharon software, Dewitt, MI, USA). Velocity and acceleration were measured by means of five photoelectric cells placed 0.5 m apart and coupled with a triggered timer system (25). The dogs were trotted across the FPs at a velocity of 1.7–2.1 m/s and acceleration of each dog was restricted to mean acceleration at baseline ±0.5 m/s. A trial from which data was retained for analysis consisted of a full forefoot strike on each FP without another foot being on the plate at the same time, followed by an ipsilateral hindfoot strike in the same fashion on each FP. Thus, data from all four limbs were obtained in a single pass. A single trained observer evaluated each foot strike and subsequent force profile and determined whether or not the trial should be retained. A single handler gaited all the dogs for each trial and timepoint. Five valid trials were collected for each dog at each timepoint. Peak vertical force (PVF) and vertical impulse (VI) were the GRFs extracted, and the means of the five trials at each visit were used for analysis. All forces were normalized to body weight and expressed as a percentage of bodyweight. In all dogs from which GRF data were collected, an index limb (the most severely affected) was identified based on clinical signs of joint pain, muscle atrophy and limb use (regardless of if multiple limbs were affected). The change from baseline in PVF and VI of the index limb was calculated for analysis.

Adverse events (AEs)

The owners were asked to report any unusual events during the study period. An AE was defined as any observation, undesirable

experience, or reaction in animals that was unfavorable and unintended and occurred after the initiation of treatment, whether or not considered to be related to any treatment. Blood work and urinalysis were repeated at the end of the study visit.

The list of hematology parameters evaluated were: white blood cells, red blood cells, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, red blood cell distribution width, reticulocytes count and its percentage, mean platelet volume, plateletcrit, platelet count, segmented neutrophils, lymphocytes, atypical lymphocytes, monocytes, eosinophils, neutrophils, basophils, packed cell volume, and plasma protein. Biochemistry parameters evaluated were: glucose, blood urea nitrogen, creatinine, phosphorus, calcium, magnesium, total protein, albumin, globulin, albumin/globulin ratio, cholesterol, total bilirubin, alkaline phosphatase, alanine transaminase, aspartate aminotransferase, gamma-glutamyl transferase, creatine kinase, sodium, potassium, chloride, bicarbonate, anion gap, sodium/potassium ratio, osmolality, amylase, lipase, hemolysis, and lipemia. Urinalysis parameters evaluated were: dipstick (Ph, protein, glucose, ketone, bilirubin, blood), color, urine specific gravity, white blood cell, fat, and sediment.

Statistical analysis

All statistical analyses were performed using R version 4.2.2 or JMP software (JMP pro 16; SAS), $\alpha = 0.05$ as our cutoff for statistical significance. For the CROM and GRF data, linear mixed models were fit with scores/variables as responses and time as a covariate with a random intercept for each patient. Each score/variable was also compared to baseline at each timepoint via Wilcoxon signed rank test with a Bonferroni correction then applied within each timepoint. For clinical pathology evaluations, the fit model function was used to compare the results collected before and after the study. An adjustment was made for multiple comparisons for clinical pathology evaluations.

Results

Fifty-six dogs were assessed for eligibility for the study (Figure 1). Forty-eight dogs of 15 different breeds were enrolled into the study and 39 dogs completed the study. Nine dogs dropped out of the study prior to completion but efficacy data were included up to the time of dropout. One dog dropped out due to multiple GI issues, but the remainder were unrelated to treatment (elective surgery, progressive ligament disease, aggressive behavior, hit by car, myoglobinuria).

Across all 48 dogs, mean (± SD) age, body weight, and body condition score were 30.7 ± 10.7 months, 30.5 ± 11.5 kg, and 5.4 ± 0.9, respectively. Six dogs were intact male, and 20 dogs were neutered male; 3 dogs were intact female, and 19 dogs were spayed female (Table 1). The most common breeds were mixed ($n = 23$), German Shepherd ($n = 5$), and Labrador Retriever ($n = 5$) (for full list of breeds see Supplementary file 2). Radiographically, the most commonly affected joints in order were hip, elbow, stifle and tarsus (Figure 2). Radiographic OA was present in one joint in 3 dogs, two joints in 15 dogs, 3–4 joints in 19 dogs, and ≥ 5 joints in 10 dogs (whole-body radiographs were not performed for one dog due to a heart problem). Mild or greater pain was detected in 71.5% of joints with rOA; pain was detected in one joint in 8 dogs, two joints in 22 dogs, 3–4 joints in 17 dogs. Nine dogs were recruited from the prevalence study (6) and 39 dogs were enrolled via study advertisement. Patient characteristics for these two groups are detailed in Table 2; LOAD scores, CBPI scores, and COAST stage were significantly lower in the dogs identified during the prevalence study than those identified via study advertisement.

Both overall and monthly compliance for medication/supplement were excellent. Overall, 97% of grapiprant/fish oil prescribed was utilized, and monthly compliance averaged ≥95%. The mean ± SD dose of fish oil was 91.7 ± 9.9 mg/kg for the first week and 196.0 ± 16.3 mg/kg from the second week. The average duration of exercise before treatment was approximately 40 min. Two dogs were reported to be unable to follow the exercise recommendation due to brachycephalic breed or progressive cruciate ligament rupture. The other owners reported they adhered to the exercise regimen, however, actual exercise undertaken was not recorded.

Outcome measures

LOAD and SNoRE scores significantly ($p < 0.001$) improved over time. CBPI scores improved over time but did not fit the statistical model due to clustering around zero (Table 3). Scores each month were significantly improved compared to baseline for all the CROMs (Figures 3A–D). Using MCID values for LOAD, MCIDs were achieved in 42.2, 53.7, 53.7, and 43.6% of the patients at 1 month, 2 months, 3 months, and 4 months after the treatment, respectively.

The veterinarian-assessed joint pain score of the index limb significantly decreased over time overall ($p = 0.046$) but the change from baseline did not reach significance at any time point after correcting for multiple comparisons (Supplementary file 3). In twenty-five dogs GRF data could be collected. There was a significant increase in the peak vertical force (PVF, <0.001), and a numerical increase (not significant) in vertical impulse (VI, $p = 0.209$) over time (Table 4). The PVF increased an average of 1.02 percentage points per time point. The increase in PVF was significant at all time points compared to baseline except at 4-months (Figure 4; Supplementary file 4).

Adverse events

Although not all incidences were considered related to the treatment given, gastrointestinal AEs were in line with expectations for NSAIDs and fish oil use (vomiting, $n = 7$; diarrhea, $n = 3$; hyporexia, $n = 2$). The reported vomiting was, for the most part, a single occurrence, and classified as mild. In one dog, vomiting was classified as moderate for multiple episodes of vomiting a day for several days. This event was likely to be associated with the treatments given and the dog was withdrawn from the study. Another dog vomited a red color liquid several times just before the 2 months recheck, however, the dog was reported to have been chewing on a red color ink pen the day before the visit. Due to this reason, the association with the treatment was concluded as unlikely. However, this dog was withdrawn from the study. In one dog vomiting was treated by the regular veterinarian with maropitant (3 doses) and metronidazole (10 doses). All reported diarrhea instances were single

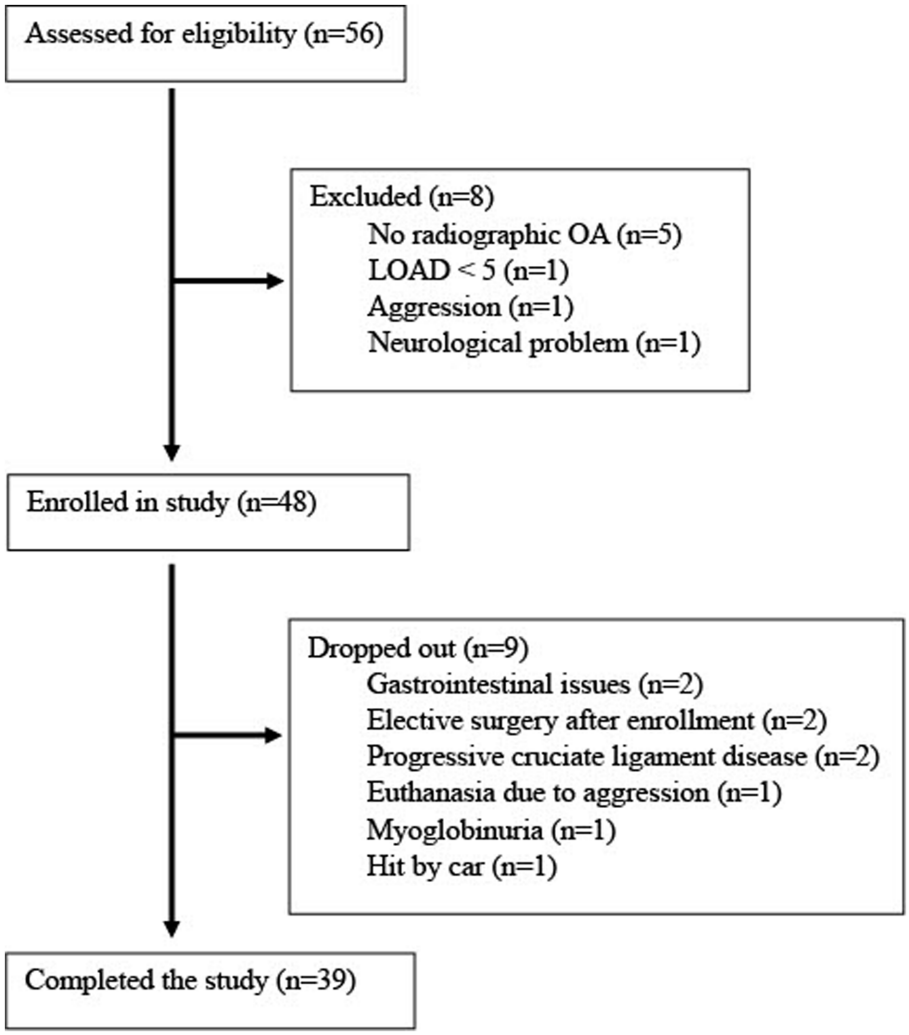

FIGURE 1
Flowchart of the clinical trial selection process showing the number of study patients at each selection process. OA, osteoarthritis; LOAD, Liverpool OsteoArthritis in Dogs.

TABLE 1 Mean ± SD (range) values of all the dogs enrolled in the study ($n = 48$).

	Mean ± SD (range)
Age (months)	30.7 ± 10.7 (11.0–51.0)
Sex	M: 6, F: 3, MC: 20, FS: 19
Body weight (kg)	30.5 ± 11.5 (9.0–68.9)
BCS (1–9)	5.4 ± 0.9 (4–8)
CBPI PSS	2.4 ± 1.9 (0–6.0)
CBPI PIS	2.6 ± 2.1 (0–7.6)
LOAD	16.1 ± 7.4 (5–33)
SNoRE	3.9 ± 1.3 (1.8–7.4)
COAST	3.3 ± 0.6 (2–4)

M, Male; F, Female; MC, Male castrated; FS, Female spayed; BCS, Body condition score; CBPI, Canine Brief Pain Index; PSS, Pain Severity Score; PIS, Pain Interference Score; LOAD, Liverpool OsteoArthritis in Dogs; SNoRE, Sleep and Nighttime Restlessness Evaluation Score; COAST, Canine OsteoArthritis Staging Tool.

occurrences and classified as mild. One dog was treated with probiotics and fiber supplements (5 days), but other cases resolved without treatment.

Other AEs are listed in Figure 1 and were considered unlikely to be related to the treatment given. The mean and median clinical pathology evaluations for screening visit and the end of study visit were within reference ranges. A statistically significant difference was identified between those visits for glucose (decreased), blood urea nitrogen (increased), creatinine (increased), and phosphate (decreased) when adjusting for multiple comparisons. However, only 1 dog was outside the normal range for each of these parameters (see Supplementary file 5), and no dog had values outside of the reference range in ≥ two of the tests above. No significant differences in any of the other parameters evaluated. Clinically meaningful changes were not seen in any dogs except for the dog who had myoglobinuria at its 1 month recheck. However, myoglobinuria was thought to be associated with a prolonged bout of unusually vigorous play with other dogs and it was reported that a similar episode had occurred prior to this study.

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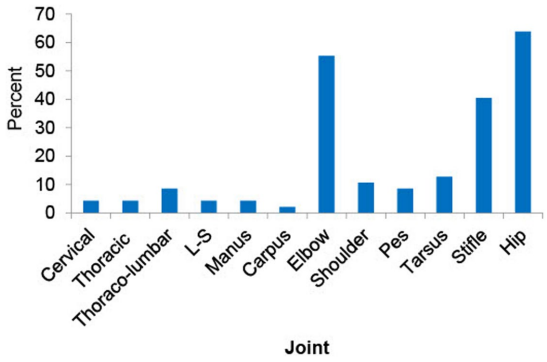

FIGURE 2
This figure shows the prevalence of radiographic osteoarthritis (rOA) across joints. The most commonly affected joints in order were hip, elbow, stifle and tarsus. L-S: lumbo-sacral joint.

TABLE 2 Mean ± SD (range) values of signalment and client-reported outcome measures in dogs transferred from the prevalence study and recruited specifically for this study.

	Prevalence study ($n = 9$)	Recruited for this study ($n = 39$)	p-value
Age (months)	32.4 ± 13.8 (14.0–45.0)	30.7 ± 10.0 (11.0–51.0)	0.66
Sex	M: 1, F: 0, MC: 6, FS: 2	M: 5, F: 3, MC: 14, FS: 17	0.30
Body weight (kg)	29.3 ± 7.5 (19.3–41.8)	30.8 ± 12.2 (9.0–68.9)	0.74
BCS (1–9)	5.4 ± 1.2 (4–7)	5.4 ± 0.8 (4–8)	0.86
CBPI PSS	1.1 ± 1.6 (0–4.8)	2.7 ± 1.8 (0–6.0)	**0.0145**
CBPI PIS	0.87 ± 1.4 (0–4.2)	3.0 ± 2.0 (0–7.6)	**0.0048**
LOAD	9.3 ± 4.8 (5–19)	17.6 ± 6.9 (5–33)	**0.0015**
SNoRE	3.9 ± 1.0 (2.6–5.4)	4.0 ± 1.4 (1.8–7.4)	0.93
COAST	2.9 ± 0.6 (2–4)	3.4 ± 0.6 (2–4)	**0.0172**
Radiographic OA score*	10.3 ± 7.1 (2.0–24.0)	10.7 ± 7.7 (1.0–29.0)	0.90
Number of joints affected	3.6 ± 2.0 (1.0–7.0)	3.3 ± 1.6 (1.0–8.0)	0.74

M, Male; F, Female; MC, Male castrated; FS, Female spayed; BCS, Body condition score; CBPI, Canine Brief Pain Index; PSS, Pain Severity Score; PIS, Pain Interference Score; LOAD, Liverpool OsteoArthritis in Dogs; SNoRE, Sleep and Nighttime Restlessness Evaluation Score; COAST, Canine OsteoArthritis Staging Tool.
$p < 0.05$ considered as significant (bolded).
*Whole body x-rays were not taken on 1 dog due to potential heart problem (this dog was removed from analysis), and the right hip joint was not scored due to femoral head and neck osteotomy/total hip replacement on 3 dogs (all recruited specifically for Part II: these dogs are still included in analysis).

Discussion

In this study, we evaluated the effectiveness of grapiprant as part of a standardized management plan for OA pain in young dogs using subjective owner assessments and objective gait analysis. The results showed that owner-assessed OA-associated clinical signs and objectively measured limb-use were significantly improved over a 4-month period in young dogs with OA-pain undergoing a standardized treatment regimen. This study showed that in an 'open label' context (similar to the situation in clinical practice) the combined treatment regimen appears to be effective and well-tolerated as a standardized multimodal management plan for treatment of OA-associated clinical signs and disability in young dogs.

The joint pain score of the index joint significantly decreased over 4 months of the study period following the treatment. Our assumption is that this was due to treatment. It must be remembered however that none of the joint pain scoring systems, including ours, have been sufficiently validated (5) and this was an open-label study. Ideally, a validated assessment tool should be used to conclude a treatment effect, and a placebo comparator group should be included. In interpreting our results, we are making the assumption that the joint pain score would have stayed the same had treatment not been instituted. In young dogs, the authors' clinical experience suggests that a period of improvement in clinical signs and a reduction in assessed joint pain, can be associated with joint disease progression from acute to chronic; hip dysplasia is a prime example of this. However, this clinical experience has not been carefully documented. In this study, the dogs were of various ages and different clinical histories and it is unlikely that they were all enrolled at the precise time of acute to chronic transition.

CBPI, LOAD, and SNoRE have been validated as subjective measures to assess pain and/or clinical signs associated with OA in dogs (17–21). To evaluate the efficacy of new analgesics, two analytical methods have been commonly used; reduction of scores (pain; disability) from baseline and binary outcomes (success failure) (26, 27). In this study, based on CROM data, overall, pain and associated clinical signs were significantly improved with treatment. In published placebo-controlled studies investigating the efficacy of NSAIDs in dogs, a 20–40% reduction in LOAD or CBPI scores has been documented with treatment (19, 27). Although this study was an open-label study and many dogs enrolled in this study were only mildly affected, a similar degree of improvement was observed.

Binary outcome (e.g., success/failure designation) thresholds have been suggested for CBPI and LOAD changes over time (23, 24, 26). For CBPI, treatment success and failure have been defined, with success defined as a reduction of ≥1 in PSS and ≥2 in PIS from baseline (26). However, these criteria were made based on older dogs who were more impaired, so it is unknown how relevant these cut-offs for success/failure are in this young dog population. Furthermore, the starting point of CBPI was quite low in our study, and thus this approach was not applied to our data. However, as shown above, the percent change from baseline in CBPI PSS and PIS were statistically significant (Figures 3A–D). More recently, a reduction of ≥4 in LOAD scores from baseline was suggested as the MCID (23, 24). When this approach was applied to our data, approximately half of the dogs reached MCIDs at each time point following our standardized management plan.

One of the limitations of this study is that the CROMs used (e.g., LOAD, CBPI) were developed using older dog populations to quantify the severity and impact of chronic pain in dogs with OA, but were not designed to detect subtle and early signs of dog mobility issues; the LOAD was developed using dogs of mean ages 7.9 years and the CBPI was developed using dogs >5 years of age. Recently, the GenPup-M, a novel CROM, was published and it was suggested that it may be able

TABLE 3 Mean ± SD (range) values of client-reported outcome measures at each time point.

	Screening ($n = 48$)	Post-1M ($n = 44$)	Post-2M ($n = 40$)	Post-3M ($n = 40$)	Post-4M ($n = 39$)	p-value
LOAD (0–53)	16.1 ± 7.3 (5–33)	13.3 ± 7.3 (3–32)	12.2 ± 6.4 (2–24)	11.6 ± 7.3 (1–26)	11.9 ± 8.0 (2–31)	**< 0.001**
CBPI PSS (0–10)	2.4 ± 1.9 (0–6.0)	1.9 ± 1.8 (0–6.0)	1.6 ± 1.6 (0–6.5)	1.4 ± 1.6 (0–5.3)	1.4 ± 1.8 (0–6.5)	Not fit
CBPI PIS (0–10)	2.6 ± 2.1 (0–7.6)	1.8 ± 2.0 (0–7.0)	1.5 ± 1.5 (0–5.0)	1.3 ± 1.7 (0–6.7)	1.3 ± 1.8 (0–7.1)	Not fit
SNoRE (0–10)	3.9 ± 1.3 (1.8–7.4)	3.5 ± 1.5 (1.2–9.0)	3.1 ± 1.3 (1.4–6.2)	3.3 ± 1.6 (1.2–7.8)	3.0 ± 1.5 (1.2–6.6)	**< 0.001**

CBPI, Canine Brief Pain Index; PSS, Pain Severity Score; PIS, Pain Interference Score; LOAD, Liverpool OsteoArthritis in Dogs; SNoRE, Sleep and Nighttime Restlessness Evaluation Score; M, month. $p < 0.05$ considered as significant (bolded).

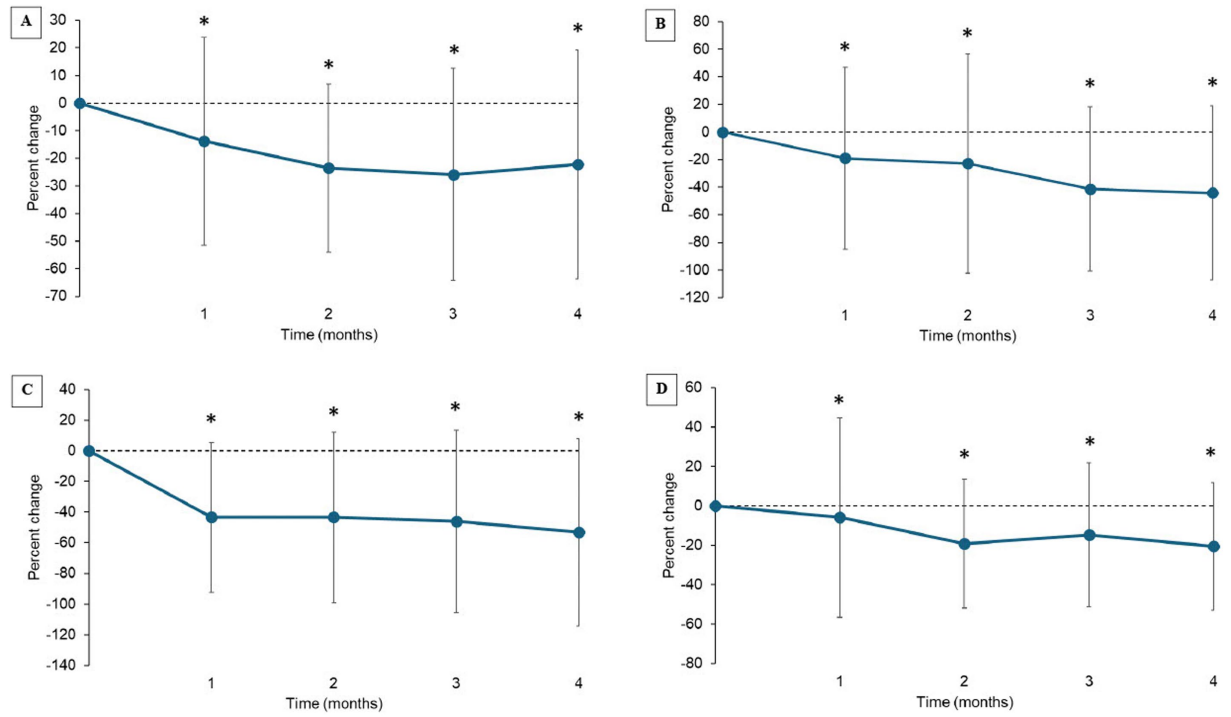

FIGURE 3
(A–D) Percent change from baseline in client-reported outcomes measures (Mean ± SD). **(A)** Liverpool OsteoArthritis in Dogs (LOAD); **(B)** Canine Brief Pain Inventory (CBPI) Pain Severity Score (PSS), **(C)** CBPI Pain Interference Score (PIS); **(D)** Sleep and Nighttime and Restlessness Evaluation Score (SNORE). *indicates significant difference from baseline ($p < 0.05$).

to identify early mobility changes in dogs (28). However, this instrument was not available when this study was performed, and it has not been tested in a young dog population.

Gait analysis has been validated as an objective measure of changes in limb use as it relates to joint pain in dogs (27, 29, 30). In particular, peak vertical force (PVF) and vertical impulse (VI) have been used to determine efficacy of therapeutics in OA studies. A recent review paper suggested that a change from baseline in both PVF and VI over a short time period (less than 6 months) of 3.5% of baseline values (not %body weight (BW) change) is the minimum value that should be considered clinically important (29). In this current study, using force plate data, the change from baseline in PVF was an increase of 4.1–9.4% of the baseline values (Figure 4). In one study, change from baseline in PVF and VI was reported after 2 weeks of carprofen in dogs that appeared to be demographically similar to this current young dog cohort except for age. Although the data collection time point was different, the results were similar to the current study; the mean change in PVF was 3.2%BW and VI was 0.32%BW in that study and in the current study, the mean change in PVF and VI varied over the 4 months between 2.8 and 5.8%BW and 0.09 and 0.54%BW, respectively. The increase in PVF from baseline was not significant at the 4-month time point. There are several potential reasons for this; firstly, this may be due to natural fluctuation of this limb use measure. Secondly, and importantly, in this study, dogs were not enrolled based on an obvious single limb lameness (the majority of dogs had two or more joints affected) nor enrolled based on the ability to collect GRF data on our equipment. Gait analysis is an ideal outcome measure if a dog has lameness in a single limb. If multiple limbs are affected, dogs usually have complex gait abnormalities, and it is more challenging to interpret gait data especially when a systemic intervention is used because the

TABLE 4 Mean ± SD (range) values of gait variables at each time point.

	Time point				
	Screening (n = 25)	Post-1M (n = 24)	Post-2M (n = 24)	Post-3M (n = 25)	Post-4M (n = 23)
PVF (%BW)	77.3 ± 20.2 (45.9–121.8)	80.3 ± 19.8 (38.1–126.2)	81.8 ± 18.3 (54.1–122.2)	83.1 ± 19.5 (52.4–125.1)	79.6 ± 19.1 (47.8–5.3)
VI (%BW)	11.4 ± 3.9 (4.9–18.1)	11.5 ± 3.8 (4.6–18.4)	11.7 ± 3.6 (6.5–17.9)	12.0 ± 4.0 (6.1–19.0)	11.2 ± 3.7 (5.4–18.3)

PVF, Peak vertical force; VI, Vertical impulse; M, month.

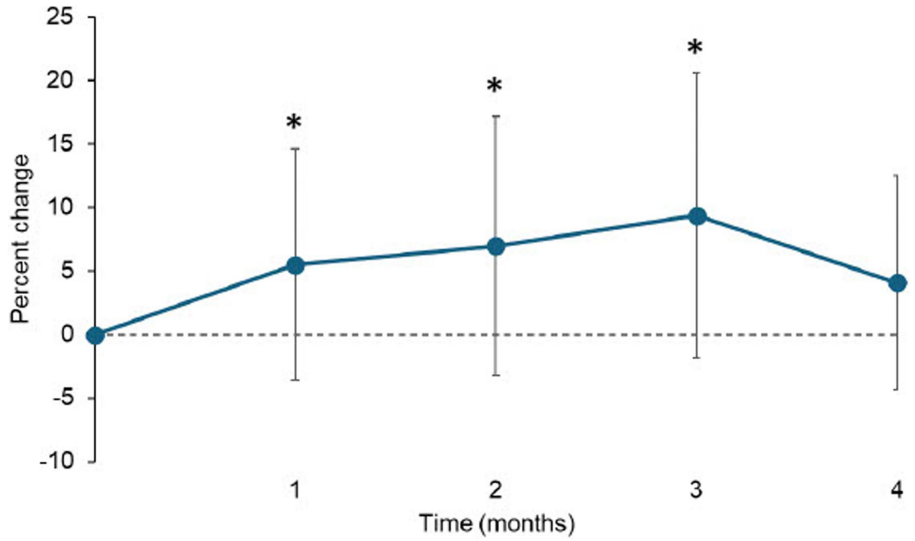

FIGURE 4
Percent change from baseline in the peak vertical force of the most affected limb (Mean ± SD). *indicates significant difference from baseline ($p < 0.05$).

intervention should affect all painful sites. Therefore, limb use changes are likely to occur across all limbs, and it may have been that the 'index' limb benefited most initially, but then other areas benefited, reflected in an apparent decrease in improvement in the index limb. Overall, an important fact to remember is that the percentage change from baseline in PVF of the index limb was above suggested meaningful change throughout the study period. Overall, the GRF data from the current study supports the conclusion that the standardized OA management plan improved limb use in young dogs. It is also possible that following an improvement in dogs' mobility and ability to perform activities, changes in management of the dogs, including other types of exercise that were instigated, may have played a role.

There was a significant difference in LOAD, CBPI, and COAST between the dogs invited from the prevalence study and dogs recruited specifically for this study. Further analysis of COAST data revealed that the difference in COAST scores between these two cohorts of dogs was driven by the owners' assessment. The dogs in the prevalence study were randomly selected from a database and their owners asked to visit the hospital for their dogs to receive a "health screen" (did not know their dogs' joint health) while the owners of the dogs recruited for this study knew their dogs' joint health (confirmed/suspected) and their dogs had a mobility issue. This may highlight that awareness of joint health status affects CROMs scores significantly, which was recently reported in cats (31).

From the perspective of being able to prove efficacy of the treatment regimen tested, the major limitation of this study is the lack of a placebo-group. Generally, a study needs to have a matched placebo treated group to be able to make strong conclusions about the efficacy of a treatment. This study was designed to look at the adherence to, and acceptance of, a standardized multimodal management plan for treatment of OA pain in young dogs and generate initial data on whether young dogs with OA-pain appear to respond to the treatment.

This open-label, pilot study demonstrates that young dogs (≤4 years old) derive a clinically meaningful benefit from a standardized multimodal management of OA-pain over a 4-month period. Future work should replicate these findings and confirm efficacy over placebo, and determine if such early, effective treatment is of long-term benefit.

Data availability statement

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

Ethics statement

The animal studies were approved by NC State University Institutional Animal Care and Use Committee. The studies were

conducted in accordance with the local legislation and institutional requirements. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

ME: Writing – original draft, Writing – review & editing. JH: Writing – review & editing. TC: Writing – review & editing. MP: Writing – review & editing. AT: Writing – review & editing. EP: Writing – review & editing. SA: Writing – review & editing. AN-H: Writing – review & editing. EH: Formal analysis, Validation, Writing – review & editing. LO: Formal analysis, Writing – review & editing. SR: Writing – review & editing. NT: Writing – review & editing. JI: Writing – review & editing. BL: Writing – review & editing, Writing – original draft.

Funding

The author(s) declare that financial support was received for the research, authorship, and/or publication of this article.

Acknowledgments

We acknowledge Drs. Abi Taylor, Amy Snyder, Mariea Ross-Estrada, Kelli Ferris, Diane Deresienski, and Erin Frey for helping with the recruitment. We thank James Robertson for assisting with the statistical analysis.

Conflict of interest

The authors declare that this study received funding from Elanco Animal Health (NC State Grant # 2020-0044).

The funder was not involved in the study design, collection, analysis, interpretation of data, or the decision to submit it for publication. However, the manuscript went through an internal review and approval process at Elanco Animal Health before submission to check for potential disclosure of confidential information.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2024.1461628/full#supplementary-material

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frontiers | Frontiers in Veterinary Science

TYPE Original Research
PUBLISHED 30 October 2024
DOI 10.3389/fvets.2024.1453673

OPEN ACCESS

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RECEIVED 23 June 2024
ACCEPTED 07 October 2024
PUBLISHED 30 October 2024

CITATION
Galosi M, Pennasilico L, Piccionello AP, Serino F, Tosi F, Sassaroli S, Riccio V, Angorini A, Salvaggio A and Di Bella C (2024) Effects of a synergic interaction between magnesium sulphate and ketamine on the perioperative nociception in dogs undergoing tibial plateau leveling osteotomy: a pilot study.
Front. Vet. Sci. 11:1453673.
doi: 10.3389/fvets.2024.1453673

Effects of a synergic interaction between magnesium sulphate and ketamine on the perioperative nociception in dogs undergoing tibial plateau leveling osteotomy: a pilot study

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Introduction: Magnesium Sulphate ($MgSO_4$) is commonly used in human medicine for the management of perioperative pain in different types of procedures. However, in veterinary medicine, the use of $MgSO_4$ has not been evaluated for its analgesic efficacy in dogs, which has generated conflicts of opinion in this area of veterinary anesthesiology. The aim of this study was to evaluate the perioperative analgesic efficacy of $MgSO_4$ in combination with Ketamine in dogs undergoing Tibial Plateau Leveling Osteotomy (TPLO). Our hypothesis is that $MgSO_4$ plus ketamine have a synergistic action in the management of intra-and postoperative pain.

Methods: Twenty adult mixed breed dogs with average age 5.9 ± 2.6 years and weight 27.8 ± 9.2 kg were included in this prospective, clinical, randomized study. Dogs were randomly assigned to two groups. The MK group received ketamine (0.5 mg/kg as starting bolus followed by continuous infusion rate at 1 mg/kg/h). At the end of the ketamine bolus, $MgSO_4$ (50 mg/kg over 15 min) was administered by the same route, followed by a constant rate infusion (CRI) at 15 mg/kg/h, IV. K group received a bolus of ketamine followed by a CRI at the same dosage described in MK group. Main cardiorespiratory parameters were recorded 10 min before the start of surgery (BASE), after the ketamine bolus (T1) and the $MgSO_4$ bolus (T2), during the skin incision (SKIN), the osteotomy (OSTEOTOMY) and skin suturing (SUTURE). In the postoperative period, the short form of Glasgow Composite Pain scale (SF-CMPS) was used to assess pain at 30, 60, 120, and 180 min after extubation (Post30, Post60, Post120, and Post180, respectively). The main blood electrolytes (Mg^{2+}, Ca^{2+}, Na^{+}, K^{+}) were analyzed at BASE, T2, OSTEOTOMY, SUTURE and T3 (one hour after stopping $MgSO_4$ infusion). Number of rescue analgesia and administration times were recorded both in the intra-and postoperative period.

Results: In K group 7 out of 10 dogs required intraoperatory rescue analgesia compared to MK group (3/10). Furthermore, mean arterial pressure (MAP) and heart rate (HR) were significantly higher at OSTEOTOMY compared to BASE time in both groups. In the postoperative period, at T120, ICMPS-SF score was higher in K group than MK group.

10.3389/fvets.2024.1453673

Conclusion: The administration of $MgSO_4$ could guarantee better analgesia in the perioperative period in dogs undergoing TPLO, performing a synergistic action with ketamine.

KEYWORDS

magnesium sulphate, ketamine, analgesia, orthopedic surgery, dogs

1 Introduction

The magnesium is an important cation that involved in several biological process such as gating of calcium channels, hormone receptor binding, transmembrane ion flux, but also muscle contraction, neuronal activity, control of vasomotor tone, cardiac excitability and neurotransmitter release (1). In all these functions, magnesium acts like a physiologic calcium antagonist (2). In recent years, it has been hypothesized that magnesium may have an analgesic action (3). However, it does not have a direct antinociceptive action, but it inhibits the entry of Ca^{2+} ions into cells, blocking NMDA (N-Methyl-D-Aspartate) receptors, with a consequent indirect analgesic effect (4). Specifically, NMDA receptors are ion channels expressed by the nervous system (NS), whose activation is directly involved in the induction of central sensitization and potentiation of short-and long-lasting pain (5, 6). NMDA receptors are opened by pro-algogenic neuropeptides such as glutamate and substance P, which induce membrane depolarization. In contrast, NMDA antagonists, like magnesium and ketamine, act non-competitively block the ion channel, preventing its opening (7, 8).

Despite these assumptions, over the last 25 years, the use of perioperative constant rate infusion (CRI) of magnesium sulphate ($MgSO_4$) in human medicine has led to conflicting results. Several studies state that the administration of $MgSO_4$ infusion guarantees better management of acute pain and reduces the dose of opioids required in the intra-and postoperative period both in soft tissue surgeries, such as laparoscopic cholecystectomies, gynecological procedures and in orthopedics (e.g., lower limb orthopedic surgeries) (9–11). On the other hand, Seong-Hoon et al., state that the use of $MgSO_4$ has no analgesic action in patients undergoing hysterectomy (12). Similarly, Durmus et al., demonstrated that patients, undergoing elective surgery who received infused magnesium, required elevated sevoflurane minimum alveolar concentrations (MAC) (13).

In veterinary medicine, references about the use of $MgSO_4$ are very few and incomplete (14). Many studies describe its application in association with local anesthetics, in order to obtain longer duration of regional analgesia (15–18). As regards its endovenous use, the studies performed are not encouraging. Roja et al., demonstrated that the administration of magnesium in dogs undergoing ovariohysterectomy does not reduce the MAC of isoflurane and does not improve postoperative pain management (19). In agreement with them, Johnson et al., studied the effectiveness of magnesium infusion in association with propofol, not highlighting any advantage in its use (20). Differently, Anagnostu et al., evaluated the thiopental dose-sparing effects of magnesium sulphate in dogs, demonstrating that, administered at a dosage of 12 mg/kg/h, it is effective to reduces the volume of thiopental used, but inducing secondary side effects (nausea and vomit) (21).

Recently, other authors hypothesized that, since ketamine and magnesium act on different sites of the NMDA receptor, the combination of both drugs could lead to a synergic effect, obtaining excellent results (reduced amount of morphine administered, better quality of awakening and high satisfaction score during the first post-operative night) (22, 23). However, in the veterinary field, there are no scientific studies to prove it.

Based on the evidence described in human and veterinary medicine, the authors' opinion is thata more suitable application of magnesium sulphate could be in association with another NMDA antagonist, such as ketamine, to enhance its efficacy. For this purpose the objective of this study was to evaluate the trans-surgical and post-surgical analgesic efficacy of $MgSO_4$ in combination with ketamine in dogs under TPLO. Main physiological parameters were monitored during the intraoperative period, and, at the end of the procedure, the short form of Glasgow Composite Pain scale was used to evaluate postoperative pain (24). Furthermore, to identify any electrolyte imbalances, the blood level of Mg^{2+} and main electrolytes were monitored by blood gas analysis at different times of the study. Our hypothesis is that the use of $MgSO_4$ may play a synergistic role with ketamine in enhancing intra-and postoperative analgesia in dogs affected by acute somatic pain.

2 Materials and methods

This randomized, prospective, blinded clinical study was approved by the Ethics Committee for Clinical Studies on Animal Patients of the University of Camerino, Italy (Prot. 06/2022). Furthermore, all owners were informed about the study and signed an appropriate consent form.

2.1 Animals

This study involved twenty adult mixed breed dogs (Table 1), conducted at the University Veterinary Teaching Hospital of Camerino between September 2022 and June 2023, to undergo TPLO (Tibial

TABLE 1 Distribution of dog breeds in the two study groups undergoing tibial plateau leveling osteotomy.

Breed	K group	MK group
Mixed breed	1	3
American bull	1	1
Rottweiler	0	1
Golden retriever	2	2
Labrador retriever	1	1
Siberian husky	2	0
Gordon setter	0	1
Staffordshire bull terrier	0	1
Boxer	1	0
Shiba Inu	1	0
English pointer	1	0

K, group that received ketamine; MK, group that received ketamine plus $MgSO_4$.

Plateau Leveling Osteotomy) surgery for ligament rupture cranial cruciate. All animals underwent physical examination and blood tests, including complete blood count, biochemistry, and coagulation profile. Exclusion criteria were: aggressive subjects, presence of pain not related to the above-mentioned orthopedic disease, dogs with coagulopathies and/or cardiovascular, hepatic, and renal diseases, pregnant females and obese and/or cachectic dogs (BCS < 3/5 or BCS > 3.5/5). Subjects with cranial cruciate ligament rupture requiring surgical resolution, free of other pathologies, assessed as ASA (American Society of Anesthesiologists) classification class II (25).

2.2 Anesthetic protocol

All animals were fasted for 12 h before surgery, while free access to water was maintained.

All dogs were premedicated with methadone (0.3 mg/kg; Semfortan®, Dechra Italia; 10 mg/mL) administered intramuscularly (IM). Then, the cephalic vein was cannulated for intravenous (IV) administration of medications and fluids (Ringer Lactate solution, 5 mL/kg/h; B Braun, Italy). Thirty minutes before the start of surgery and every 90 min until the end of surgery, cefazolin sodium (20 mg/kg, Cefazolin, Zoetis S.r.l.) was injected intravenously (IV).

During the induction of general anesthesia, patients were preoxygenated with pure oxygen by face mask, then, IV propofol (3–5 mg/kg; Proposure®, Boehringer Ingelheim Animal Healt Italia S.p.A; 10 mg/mL) was administered until adequate muscle relaxation was achieved (muscle relaxation of the limbs, relaxation of the jaws, and loss of the pedal reflex). All dogs were intubated, connected to a circular breathing system and maintained under general anesthesia with isoflurane (IsoFlo, Zoetis S.r.l, Milan, Italy) in a mixture of oxygen and air, maintaining an inspired fraction of O_2 between 65 and 70% (FiO_2 65–70%). These were also mechanically ventilated in volume-controlled mode (Datex-Ohmeda S/5 Avance, Madison, Wisconsin, USA). The settings were 12 mL/kg tidal volume (VT), inspired to exhaled ratio (I:E ratio) 1:2, respiratory rate (RR) variable on the basis of end-tidal carbon dioxide ($EtCO_2$ = 35–45 mmHg) and Positive End Expiratory Pressure (PEEP) = 0 cmH_2O.

When an adequate anesthetic depth level was achieved, a 22-gauge cannula was inserted into the dorsal pedal artery for measurement of systolic, mean, and diastolic blood pressure (SAP, MAP, and DAP, respectively; mmHg) and collection of arterial blood samples for the evaluation of blood electrolytes. The VetStat Electrolyte Blood Gas Analyzer was used for the analysis of Na^+, K^+, Ca^{2+} and Cl^- (Idexx VetStat 8 Plus Cassettes, Idexx Laboratories Italia Srl, Italy; Idexx VetStat Ionized Calcium Cassettes, Idexx Laboratories Italia Srl, Italy) and the Catalyst One Analyzer (single Mg slice, Idexx Laboratories Italia Srl, Italy). The pressure transducer was positioned at the level of the right atrium and zeroed to atmospheric pressure. A multiparametric monitor (BeneView T8, Mindray Medical S.r.l) was used to assess the main cardiovascular and respiratory parameters. Specifically, the following data were manually collected every five minutes during the entire procedure: heart rate (HR; beats/min); invasive SAP, MAP and DAP, peripheral capillary oxygen hemoglobin saturation (SpO_2; %); RR (breath/min), peak and plateau inspiratory pressure (Ppeak and Pplat, cmH_2O), $EtCO_2$, end-tidal concentration of isoflurane (EtIso; %); inspired fraction of isoflurane (FiIso; %); minimum alveolar concentration of isoflurane (MAC; %); and temperature (T, °C). A possible lightening of the anesthetic plan was clinically evaluated (presence of the eyelid reflex and position of the eyeball) and was managed by administering 1 mg/kg of propofol IV or increasing the FiIso. In case of hypotensive events continuing for 1 min (MAP <60 mmHg), a bolus of crystalloids was first administered (5 mL/kg in 5 min) (26). If subjects were not responsive to fluids, a CRI of norepinephrine was started (0.1–0.2 µg/kg/min) (Norepinephrine Tartrate 2 mg/mL, S.A.L.F., Bergamo, Italy). Moreover, if HR was <50 beats/min for more than one minute, atropine sulphate (0.01 mg/kg; Atropine Sulfate 1 mg/mL, Fatro Spa, Italy) was administered IV.

At the end of the procedure, dogs were awakened and monitored in their cage and the affected limb was bandaged with a modified Robert-Jones bandage. After extubation, hypothermia (T < 37°C) and severe/moderate hypoxemia (SpO_2 < 95%) were managed with suitable thermal support and supplementary oxygen (flow by, face mask or CPAP helmet), respectively. In addition, one hour after the extubation, all dogs received a subcutaneous administration of non-steroidal anti-inflammatory (carprofen 4 mg/kg; Rimadyl 50 mg/mL, Zoetis S.r.l) The TPLO performed in this study were performed by the same expert surgeon.

2.3 Study protocol

After the pre-anesthesiologic examination, all dogs were sorted into two groups using a random number generator (Microsoft® Excel®; Microsoft 365 MSO 2021, Italy):

- K group (10 dogs) received a bolus of ketamine (0.5 mg/kg), administered IV over 2 min, followed by a CRI of the same drug (1 mg/kg/h).
- MK group (10 dogs) received a bolus of ketamine administered at the same dosage described in the previous group. In addition, at the end of the bolus and at the same time as starting the ketamine infusion, $MgSO_4$ (50 mg/kg) was administered IV over 15 min. Subsequently, a CRI of $MgSO_4$ was also applied (15 mg/kg/h). Both infusions were stopped at the end of surgery (19).

2.3.1 Intraoperative assessment

The main hemodynamic and respiratory parameters (HR, RR, SAP, MAP, DAP, SpO_2, MAC, T°, $EtCO_2$) were recorded in both groups at BASELINE (10 min before the ketamine bolus), T1 (end of ketamine bolus), T2 (end of the $MgSO_4$ bolus in the MK group and 15 min after the ketamine bolus in the K group), SKIN (skin incision), OSTEOTOMY (the corrective osteotomy), and SUTURE (suture of the skin plane). For both groups the end of the infusions coincided with the SUTURE time.

Furthermore, in the intraoperative period, arterial blood samples were obtained from the dorsal pedal artery for the evaluation of the main electrolytes (Mg^{2+}, Ca^{2+}, Na^+, K^+) by blood gas analysis. The samples were collected at BASELINE, T2 and SUTURE times. In MK group, a further sampling was performed at the OSTEOTOMY time, in order to monitor the blood Mg^{2+} concentration. During the surgery, an increase in HR or MAP greater than 20% above T2 for more than one minute was considered a nociceptive autonomic response to surgical stimulation; in this case, the CRI of ketamine was increased to 2 mg/kg/h. However, if the above-mentioned parameters did not fall within the expected

ranges in the following 10 min or a second nociceptive peak was recorded, a bolus of fentanyl (1 μg/kg IV, Fentadon, Dechra Italia) was administered and the data was noted (25). If the fentanyl bolus was not sufficient, a CRI of this was initiated, and the patient was excluded from the study. The duration of surgery (from the first incision to the end of the skin suture) and anesthesia (duration of isoflurane administration) were recorded. Additionally, any anesthetic or surgical complications during the procedure and extubation time (from the end of anesthesia to removal of the endotracheal tube) were noted.

2.3.2 Postoperative assessment

Physiological parameters (HR, RR, MAP and T°) were recorded 30, 60, 120 and 180 min after extubation (POST30, POST60, POST120 and POST180, respectively). Specifically, as regards MAP, it was monitored non-invasively (SunTech Vet 25 Blood Pressure Monitor, Ancyon Italia Srl, Italy) using specific cuffs based on the diameter of the dog's limb according to the indications of the manufacturer (Suntech Bayonet Blood Pressure Cuffs, Alcyon Italia Srl, Italy) positioned in all subjects at the level of the radial artery (left forelimb). The measurements were taken keeping the limb raised, at the level of the cardiac area. Before manipulating dogs, the short form of the Glasgow Composite Measure Pain Scale (SF-CMPS) was used to assess the presence of pain in this study phase. As indicated by the pain scale itself, it was excluded the question B relating to walking ability, since all subjects had orthopedic pathology. The score attributable to pain was therefore not 6/24 but, 5/20. If dogs reached this score, a bolus of methadone (0.3 mg/kg) would be administered (IM) and the monitoring would be stopped. The time of administration of the rescue analgesia was also recorded. In addition, a final blood gas analysis was performed one hour after the end of the $MgSO_4$ infusion (T3) in order to evaluate the blood concentration of Mg + and the adequacy of electrolyte balance.

3 Statistical analysis

MedCalc 9.0 software (MedCalc version 9.2.10) was used to perform the statistical analysis. All data resulted normally distributed based on the Kolmogorov–Smirnov test and they were compared between groups and at different study times. Cardinal data were analyzed with an independent t-test to compare between groups. ANOVA for repeated measures was used to compare the study times within group. Ordinal variables were analyzed with Kruskal-Wallis test to obtain a comparison between the two groups and Friedman test was used to perform a comparison between the study times within each group. Results for cardinal variables are presented as mean ± standard deviation and ordinal variables as median (minimum – maximum). Instead, for the yes/no variables (intra-and postoperative rescue analgesia), Fisher's exact test was used. A p value <0.05 were considered statistically significant.

4 Results

Twenty-one dogs were considered for this study. During the pre-anesthesiologic evaluation, one subject was excluded for not meeting the inclusion criteria (cardiopathy not previously investigated), as reported according to the CONSORT Statement 2010 for randomized clinical trials (27) (Figure 1).

There were no significant differences in the age (MK = 6.6 ± 2.67 years; K = 5.3 ± 2.66 years) and weight (MK = 28.1 ± 9.03 kg; K = 27.67 ± 9.34 kg) of dogs included in the study. Likewise, no differences were found in the duration of surgery (MK = 94.1 ± 8.6 min; K = 88 ± 10.6 min) and anesthesia (MK = 156.2 ± 36.3 min; K = 140.9 ± 36.8 min).

4.1 Intraoperative assessment

There were no significant differences in RR, $EtCO_2$, MAC and T between the two groups at all times of the study. The HR, in K group, was significantly lower at BASE ($p = 0.043$), T1 ($p = 0.042$), T2 ($p = 0.048$) and SKIN ($p = 0.036$) compared to OSTEOTOMY time. In the same way, in both groups, the MAP was statistically higher at OSTEOTOMY compared to BASE, T1, T2 and SKIN times ($p < 0.01$). Moreover, at SUTURE, it was higher than BASE [K ($p = 0.034$); MK ($p = 0.048$)] and T1 [K ($p = 0.044$); MK ($p = 0.035$)] times. In both groups, DAP was higher at OSTEOTOMY and SUTURE compared BASE, T1, T2 and SKIN times ($p < 0.01$) (Table 2).

Regarding the rescue analgesia administered, in both groups, it was necessary to increase the CRI of ketamine (7 out of 10 dogs). However, in K group, 7 out of 10 patients also required a bolus of fentanyl while, in the MK group, only 3 out of 10 (Table 3; Figure 2).

4.2 Postoperative assessment

The mean ± SD of physiologic parameters are reported in Table 4. HR, MAP and RR did not show significant differences between groups at any study times. At T180 time, the number of patients who required rescue analgesia was significantly greater in the K group (8/10) than MK group (4/10) (Table 4). In agreement with this result, 120 min after extubation, the SF-CMPS score was significantly higher in the K group compared to MK (Figure 3).

4.3 Blood electrolyte assessment

The analysis of blood electrolytes showed no statistically significant differences in the K group at all study times. In contrast, in the MK group, Mg^{2+} was significantly higher at T1, T2, OSTEOTOMY, SUTURE, and T3 times compared to BASE (Figure 4).

5 Discussion

This study aimed to evaluate the synergistic efficacy of magnesium sulphate, in combination with ketamine, in dogs undergoing orthopedic surgery. Our results showed that there were fewer analgesic rescues in MK compared to the K group. Furthermore, in the postoperative period, patients who received $MgSO_4$ plus ketamine obtained a lower value on the SF-CMPS. To the authors' knowledge, this is the first study to confirm the potentiating action of magnesium on the antinociceptive efficacy of ketamine in dogs.

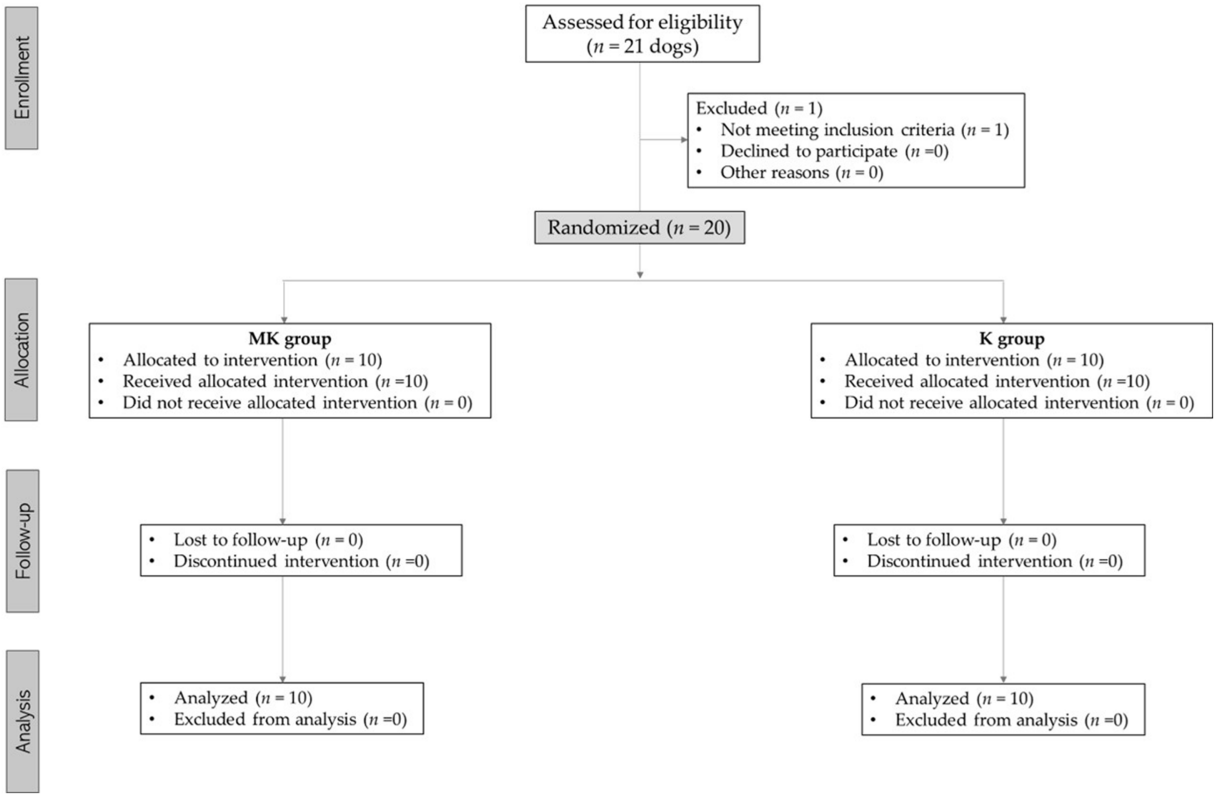

FIGURE 1
Consolidated standards of reporting trials (CONSORT) flow diagram for subjects included in this study.

TABLE 2 Mean ± SD of cardiovascular and respiratory parameters at different time points during the study in dogs undergoing tibial plateau leveling osteotomy (TPLO) with magnesium sulphate and ketamine.

Parameter	Group	Base	T1	T2	Skin	Osteotomy	Suture
HR (beats/min)	K	68.9 ± 16.6°	68.2 ± 10.9°	69.7 ± 9.8°	67.4 ± 9.3°	92 ± 25.6	78.4 ± 19.7
	MK	78.3 ± 23.3	73.2 ± 23	66.6 ± 21.4	66 ± 17.2	83.7 ± 24.8	72.3 ± 27.9
RR (breaths/min)	K	10.1 ± 3.5	9.6 ± 4.5	10.4 ± 4.7	10.4 ± 4.7	12.7 ± 5.6	13.5 ± 5.5
	MK	13.1 ± 4.1	11.4 ± 4.08	11.7 ± 4.4	11.7 ± 4.4	12.8 ± 3.6	13 ± 3.7
SAP (mmHg)	K	114.2 ± 28.5	117.7 ± 30.6	114.2 ± 31.5	122.3 ± 34	135.8 ± 26.4	126 ± 26.7
	MK	107 ± 19.07	101.9 ± 16.7	117.8 ± 11.6	109.4 ± 21.5	135.4 ± 27.8	125.8 ± 12.4
(mmHg)	K	73.8 ± 19.3°*	74.6 ± 24.1°*	78.1 ± 25.2°	78.3 ± 22.2°	100.3 ± 21.2	90 ± 23.1
	MK	76.7 ± 17.9°*	72.8 ± 14.2°*	84.6 ± 15.08°	83.3 ± 18.5°	115.6 ± 26.5	96.5 ± 11.8
DAP (mmHg)	K	57.5 ± 19.4°*	53.7 ± 18.1°*	57.6 ± 19.4°*	60.2 ± 18.1°*	78.7 ± 17.3	72.7 ± 22.7
	MK	54 ± 7.7°*	50 ± 10°*	61.4 ± 7.7°*	57.2 ± 13.4°*	85.2 ± 28.9	79.2 ± 11.9
$ETCO_2$ (mmHg)	K	36.4 ± 7.1	34.8 ± 3.08	35.9 ± 3.1	37.6 ± 2.01	40.7 ± 3.09	40.1 ± 5.7
	MK	40.5 ± 7.1	38.9 ± 5.7	38.2 ± 4.8	38.7 ± 4.5	37.3 ± 5.9	40.3 ± 4.5
MAC (%)	K	0.97 ± 0.2	0.95 ± 0.1	0.97 ± 0.1	0.94 ± 0.2	0.98 ± 0.3	0.90 ± 0.2
	MK	0.75 ± 0.1	0.78 ± 0.1	0.85 ± 0.2	0.86 ± 0.2	0.92 ± 0.2	0.89 ± 0.2
T (°C)	K	37.08 ± 0.6	36.8 ± 0.8	36.7 ± 0.7	36.5 ± 0.9	36.3 ± 1.1	36.2 ± 1.06
	MK	37.1 ± 0.6	37.05 ± 0.6	36.5 ± 0.7	36.4 ± 0.7	36.04 ± 0.8	35.4 ± 0.8

Significant differences are indicated by p° compared to OSTEOTOMY and p* compared to SUTURE times. K, group that received ketamine; MK, group that received ketamine plus $MgSO_4$; BASELINE, 10 min before the ketamine bolus; T1, end of ketamine bolus; T2, end of the $MgSO_4$ bolus in the MK group and 15 min after the ketamine bolus in the K group; SKIN, skin incision; OSTEOTOMY, the corrective osteotomy; SUTURE, suture of the skin plane.

A previous study performed in dogs undergoing ovariohysterectomy demonstrated that $MgSO_4$ has no benefit in intraoperative pain management (19). Our results are partially in agreement with Rioja et al. (19). In fact, we highlighted that the requirement for rescue analgesia was lower in the group that received both $MgSO_4$ and ketamine, however, we still recorded a

TABLE 3 Number of dogs requiring ketamine and fentanyl rescue analgesia during the intraoperative period.

Intraoperative rescue	Ketamine	Fentanyl
K (n°)	7/10	7/10
MK (n°)	5/10	3/10*

$p^* < 0.01$ differences between groups. K, group that received ketamine; MK, group that received ketamine plus $MgSO_4$.

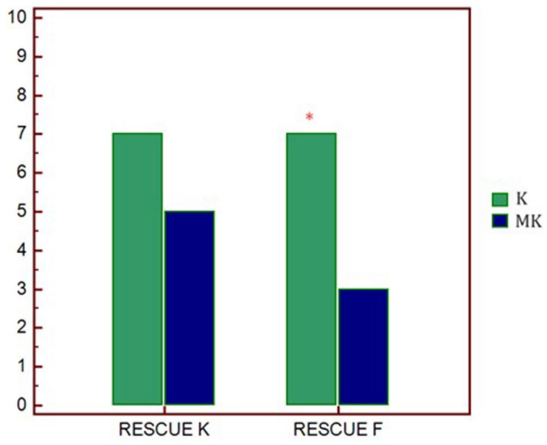

FIGURE 2
Graphical representation of the request for rescue analgesia in the two study groups. $p^* < 0.01$ differences between groups. K, group that received ketamine; MK, group that received ketamine plus $MgSO_4$; RESCUE K, number of ketamine rescues administered; RESCUE F, number of fentanyl rescues administered.

significant increase in MAP, DAP and HR during the osteotomy. Our hypothesis is that, in our study, unlike Rioja et al., we did not use $MgSO_4$ alone, but in association with another NMDA antagonist. The enhanced synergism between the two provided better analgesic coverage and, therefore, a reduced need for rescue interventions. This agrees with Queiroz-Castro et al. who demonstrated that the magnesium-ketamine association provides a better sparing effect on isoflurane compared to magnesium alone in goats subjected to experimental nociceptive stimulation (28). The actual mechanisms of interaction between the two drugs are not fully understood, however, experimental model studies have shown that the synergy between $MgSO_4$ and ketamine is mainly due to the different mechanisms of action of the two molecules (29, 30). In fact, magnesium blocks the flow of calcium and antagonizes the NMDA receptor channels, while ketamine binds to the phencyclidine binding site of NMDA receptors and modifies them through allosteric mechanisms. Furthermore, it is known that ketamine interacts with calcium and sodium channels, dopamine receptors, cholinergic transmission, noradrenergic and serotonergic reuptake, carrying out opioid-related and anti-inflammatory effects (31). On the other hand, magnesium has been shown to reduce the activity of other presynaptic and postsynaptic calcium channels, modulate the release of some neurotransmitters and influence sodium and potassium currents, interfering in the physiological action of membrane potentials (1, 32). These data support our hypothesis, however, probably, during severe pain stimulation (OSTEOTOMY), this association is not sufficient to provide a suitable antinociceptive action (19, 28).

Therefore, the results obtained show that magnesium, although carrying out a synergistic strengthening action with ketamine, is not sufficient to counteract acute nociceptive phenomena during orthopedic surgery. In our study we chose an increase in the CRI of ketamine to 2 mg/kg/h as the first rescue analgesia, however, it would be interesting to evaluate whether, by using higher doses of this drug from the beginning of the procedure, we could modulate and improve the action of magnesium. Higher concentrations of ketamine could have bound to the phencycline binding site more effectively, thus allowing an increase in Mg^{2+} concentration to improve the quality of receptor blockade. This would also support the hypothesis of Vujovic et al., who stated that magnesium has greater analgesic efficacy if ketamine is administered before it. On the other hand, it is weaker when magnesium is administered first. The explanation for this concept could be that magnesium is able to temporarily block NMDA channels, preventing the binding of ketamine and, therefore, its antinociceptive action (29, 30).

NMDA receptors play a key role in the modulation of pain and different inflammatory responses, preventing central sensitization caused by peripheral nociceptive stimulation (33). Having recognized the different mechanism of action of ketamine and magnesium at the level of NMDA receptors, their synergistic action still needs to be further investigated to better understand the mechanisms. From the results obtained in the intraoperative period we can therefore affirm that the magnesium/ketamine association provided good analgesia during the surgical procedure, such as to reduce the request for rescue analgesia, however, it seems not to be able to fully regulate the hemodynamic response to acute nociceptive stimuli and the consequent sympathetic activation.

Regarding the results obtained in the post-operative period, the subjects who had received an infusion of $MgSO_4$ required rescue analgesia later than the K group, furthermore, they showed reduced SF-GMPSG scores at all times of the study and significantly lower at 120 min after extubation. In addition, at 180 min, 4/10 patients in the MK group received rescue analgesia, compared to the K group, in which the majority (8/10) required it. Our results agree with previous studies in which the authors demonstrated the effectiveness of NMDA antagonists on the management of postoperative pain and on the prevention of the development of pathological alterations of the nociceptive pathways, with consequent manifestation of hyperalgesia and allodynia. Kanta et al., in an experimental study on a mouse model, showed for the first time how the administration of magnesium inhibits the expression of the glutamate ionotropic receptor NMDA type subunit 1 (Grin1), thus reducing the development of hyperalgesia and chronic postoperative pain (34). Similarly, other authors, described the effectiveness of NMDA antagonists in the management of chronic pain, favoring the significant reduction in the use of opioids in the postoperative period (35–37).

.As regards the choice of the dose of magnesium sulphate administered, there are no guidelines in veterinary medicine. For this reason, we chose the dosages previously used by Rioja et al. in their study (19). In addition, blood Mg^{2+} concentrations were monitored to understand its trend at different study times. The main risk that can be incurred by administering high concentrations of $MgSO_4$ in bolus is secondary hypermagnesemia. This may cause nausea, vomiting, hypocalcaemia and cardiac arrhythmias. In our study, no side effects

TABLE 4 Main postoperative parameters monitored during tibial plateau leveling osteotomy (TPLO) in the Ketamine and $MgSO_4$ plus Ketamine groups.

Parameters	Groups	T30	T60	T120	T180
HR (beats/min)	K	89.3 ± 16.1	95.8 ± 14.4	94.8 ± 17.6	96.5 ± 12.02
	MK	96.5 ± 14.9	101.6 ± 17.4	94.5 ± 16.2	104.8 ± 16.03
(mmHg)	K	106.5 ± 20.1	110.4 ± 17.1	109.5 ± 26	84 ± 8.4
	MK	107.1 ± 11.5	111.6 ± 14.1	119.5 ± 26.03	103.4 ± 20
RR (breaths/min)	K	26.4 ± 11	31.5 ± 5.9	33.6 ± 8.2	30 ± 8.4
	MK	28.5 ± 13.9	32.5 ± 13.9	38.6 ± 13.5	40 ± 14.1
RESCUE (n°)	K	0/10	3/10	4/10	8/10
	MK	0/10	2/10	3/10	4/10*

Significant differences (p* 0.047) between groups. K, group that received ketamine; MK = group that received ketamine plus $MgSO_4$.

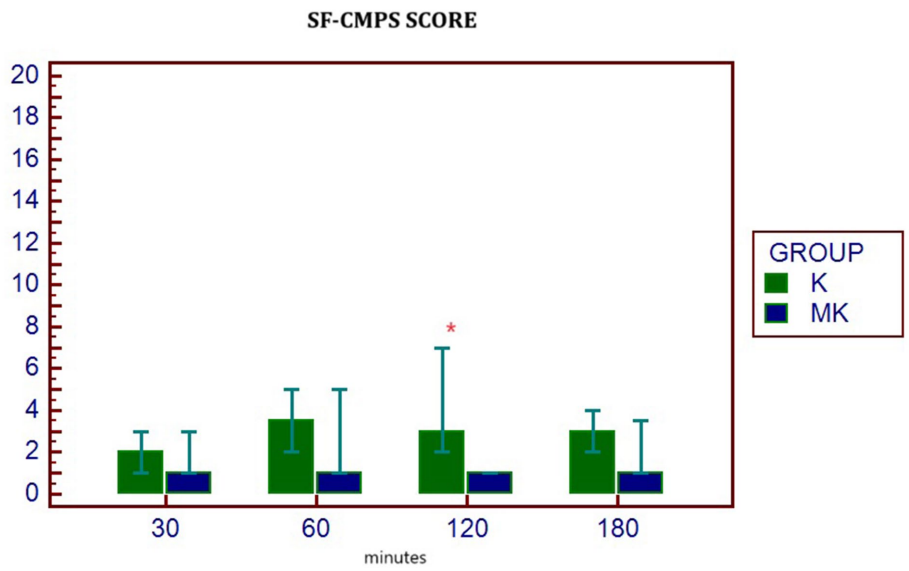

FIGURE 3
Median (min – max) of SF-GMPS values in the two groups at different postoperative study times. p* 0.047 differences between groups. K, group that received ketamine; MK, group that received ketamine plus $MgSO_4$; POST30, POST60, POST120, and POST180 = 30, 60, 120, and 180 min after extubation, respectively.

were highlighted at the chosen doses, however, blood concentrations significantly increased at T2 time, and then gradually decreased until T3 (although remaining significantly higher than the BASE time). Hypermagnesemia is easily managed by patients with normal renal function thanks to the rapid renal excretion of magnesium, however, it is important to know the hematobiochemical condition before choosing to administer this drug (21).

The authors considered some limitations in this study. The first is the absence of a group treated only with $MgSO_4$. This would allow us to better understand the synergistic action carried out with ketamine rather than identifying the analgesic action of $MgSO_4$ alone. Furthermore, it would have been interesting to monitor blood magnesium concentrations until BASE concentrations returned, however, it was not possible to perform further blood sampling due to the clinical nature of the study. Another limitation to consider is that the quality of the dogs' awakening was not assessed. Considering the side effects that ketamine can induce (dysphoria, spasms, delirium) in the awakening phase, this component would be important to define, especially if we choose to use moderately high continuous infusion dosages (e.g., 2 mg/kg/h). Finally, we consider the absence of SAP and DAP evaluation in the postoperative period as a limitation of the study, since, for completeness, it would have been more appropriate to record all data relating to blood pressure.

In conclusion, the preliminary data presented in this study demonstrates for the first time the existence of a synergism between $MgSO_4$ and ketamine in the management of pain in dogs undergoing TPLO surgery. In fact, the intraoperative co-administration of ketamine and $MgSO_4$ seems to be more effective in reducing pain and opioid consumption than an analgesic protocol with ketamine alone. However, it is the authors' opinion that, as already demonstrated, locoregional anesthesia represents the gold standard for pain management during this type of surgical procedure (38, 39). Furthermore, although a synergism between $MgSO_4$ and ketamine was detected, this does not seem to be sufficient to completely manage severe intraoperative nociceptive stimuli, therefore, we believe that analgesic integration with $MgSO_4$ infusion could represent a useful analgesic support mainly in the postoperative period and in the prevention of central sensitization following nociceptive stimulation.

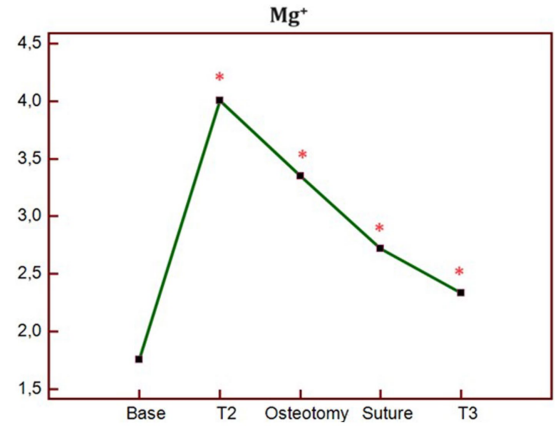

FIGURE 4
Trend of blood concentration of Mg + at different times of study in MK group. $p^* < 0.05$ differences compared to BASE. BASELINE, 10 min before the ketamine bolus; T1, end of ketamine bolus; T2, end of the $MgSO_4$ bolus in the MK group and 15 min after the ketamine bolus in the K group; SKIN, skin incision; OSTEOTOMY, the corrective osteotomy; SUTURE, suture of the skin plane.

Increasing the number of cases and including a third group that will only be administered $MgSO_4$ will help us get more data and better understand this topic.

Data availability statement

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

Ethics statement

The animal studies were approved by Ethics Committee for Clinical Studies on Animal Patients of the University of Camerino, Italy. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

MG: Data curation, Methodology, Validation, Writing – original draft. LP: Formal analysis, Methodology, Writing – review & editing. AP: Investigation, Writing – review & editing. FS: Data curation, Methodology, Writing – review & editing. FT: Data curation, Validation, Writing – original draft. SS: Investigation, Writing – review & editing. VR: Investigation, Writing – review & editing. AA: Writing – original draft. AS: Investigation, Writing – review & editing. CB: Conceptualization, Formal analysis, Supervision, Writing – review & editing.

Funding

The author(s) declare that no financial support was received for the research, authorship, and/or publication of this article.

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frontiers | Frontiers in Veterinary Science

TYPE Original Research
PUBLISHED 11 December 2024
DOI 10.3389/fvets.2024.1449297

OPEN ACCESS

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RECEIVED 14 June 2024
ACCEPTED 19 November 2024
PUBLISHED 11 December 2024

CITATION
Petrucci M, Spadavecchia C, Bachmann KF, Berger D, Mirra A and Casoni D (2024) The nociceptive withdrawal reflex during spinal analgesia in pigs undergoing veno-arterial extracorporeal membrane oxygenation: a prospective observational study.
Front. Vet. Sci. 11:1449297.
doi: 10.3389/fvets.2024.1449297

The nociceptive withdrawal reflex during spinal analgesia in pigs undergoing veno-arterial extracorporeal membrane oxygenation: a prospective observational study

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Introduction: Use of veno-arterial extracorporeal membrane oxygenation (VA-ECMO) is still in the focus of research, in which pigs are commonly involved. During VA-ECMO, cardiovascular parameters are artificially manipulated and therefore not reliable indicators of nociception. Nociceptive withdrawal reflex (NWR) thresholds can be a suitable alternative in such a context. This study aimed at recording and comparing NWR thresholds before and after administering spinal analgesia in healthy pigs undergoing VA-ECMO.

Methods: Sixteen pigs were sedated with a mixture of ketamine, midazolam, and methadone; general anesthesia was induced with propofol and maintained with propofol and fentanyl in continuous rate infusion. Before surgery, ropivacaine 0.75% and morphine (RM) were injected via a spinal catheter (T13-L1). Nociceptive withdrawal reflex thresholds were recorded before RM (baseline) and at 40 min, end of surgery, 240, 300, 360, 420 and 480 min afterward. If after spinal analgesia NWR thresholds increased ≥20% from their baseline values, the increase was deemed clinically relevant. If NWR thresholds decreased at least 20% from their baseline values, ropivacaine alone was injected (rescue analgesia). Thresholds were compared with baseline using ANOVA on Ranks followed by Dunn's method. At each time point, the number of pigs showing a clinically relevant increase in thresholds, thresholds higher than the maximum stimulation intensity and the need of rescue analgesia, was assessed. Nine animals were included in the final data analysis.

Results: A clinically relevant increase of the thresholds was achieved in all the pigs at 240 min after the injection of RM. A statistically significant increase in NWR thresholds was found at 300 and 360 min (p = 0.009 and 0.048, respectively) compared to baseline. Rescue analgesia was required at 300 (one pig) and 420 (two pigs) and 480 (one pig) minutes.

Discussion and conclusion: Nociceptive withdrawal reflex thresholds increased significantly, both clinically and statistically following spinal injection. Their increase suggests that the combination of spinal morphine and ropivacaine can

last on average up to 6 h. Particularly in those scenarios where cardiovascular variables are unreliable, NWR thresholds could be useful for evaluating antinociception following spinal analgesia in pigs.

KEYWORDS

NWR, neuraxial analgesia, nociception, swine, ropivacaine, morphine, VA-ECMOe

1 Introduction

Extracorporeal membrane oxygenation (ECMO) is a rescue therapy for patients who experience severe pulmonary and cardiac dysfunction (1). It supports the function of the heart and the lungs and provides adequate blood flow to the organs (2). It may be used during high-risk cardiac, thoracic or trauma surgeries (3) and during cardio-pulmonary resuscitation (4).

As clinical application of ECMO and weaning strategies (i.e., discontinuation of ECMO support) are still under investigation, pigs are commonly used as animal model due to their anatomical and physiological similarities to humans (5–7). If central ECMO is investigated, the heart must be accessed to establish cannulation of the ascending aorta and the right atrium. Therefore, sternotomy is required, which is recognized to be particularly painful, both intra and post-operatively (8). A correct assessment and treatment of nociception in animal experimentation must be guaranteed for both ethical and legal reasons. Therefore, the adoption of a solid and efficacious antinociceptive and analgesic strategy is required (8, 9). While the use of spinal analgesia is highly debated in cardiac surgery due to inherent risks associated with anticoagulation, it may be the preferred choice in experimental settings with terminal anesthesia, as risks related to the post-operative period (e.g., epidural hematoma) have not to be accounted for (10). In these cases, the ability of neuraxial anesthesia to attenuate the stress response to surgery (11) may overcome the potential side effects and provide hemodynamic stability. This is pivotal importance to avoid uncontrolled cardiovascular modifications associated with nociceptive stimuli, which would act as confounders.

Ropivacaine, an amido-amide local anesthetic, has been shown to provide adequate antinociception, cardiovascular stability and to reduce opioid consumption in major surgeries when administered epidurally (12). Moreover, when combined with μ opioids for both spinal and epidural administration, its antinociceptive activity is enhanced (12–15). While the analgesia duration of ropivacaine 0.75% alone can be up to 6 h when administered spinally in humans (16), to the authors' knowledge, no information is available in pigs.

Nociception under general anesthesia is commonly assessed through the evaluation of cardio-respiratory parameters in both humans and veterinary species (17, 18). In pigs undergoing isoflurane anesthesia, mean arterial pressure (MAP) variation has been shown to be superior to other variables as indicator of nociception (19). However, as the extracorporeal flow is a major determinant of MAP during veno-arterial extracorporeal membrane oxygenation (VA-ECMO), potential modifications in the sympathetic system are not mirrored, and MAP is not helpful in evaluating the adequacy of the antinociception.

The nociceptive withdrawal reflex (NWR) is a polysynaptic spinal reflex, which results in the withdrawal of an area of the body in response to a potential tissue damage, with the aim to prevent it (20). The afferent section of the reflex arc is formed by Aδ and C fibers, which, when stimulated by a nociceptive stimulus, transmit it to the spinal cord. The efferent branch of the reflex is formed by motor neurons, which, once activated, generate a contraction of corresponding muscles. The NWR can be experimentally elicited by transcutaneous electrical stimulation of a sensory peripheral nerve and the electromyographic response from the involved muscles can be recorded and assessed (21). This technique has already been investigated in several animal species (22–24), including pigs (25), to test analgesic and anesthetic drugs' ability to modify nociceptive thresholds (26, 27). No reports concerning NWR thresholds in pigs receiving spinal analgesia under general anesthesia have been published so far.

The aim of this study was to define and compare the NWR thresholds before and after the administration of spinal analgesia in pigs undergoing VA-ECMO. We hypothesized that the NWR thresholds would increase following spinal analgesia. Additionally, we hypothesized that tracking thresholds over time could provide insights into the duration of spinal analgesia.

2 Materials and methods

2.1 Animals

Sixteen pigs (*Suis scrofa domesticus*, Schweizer Edelschwein), six males and ten females, aged 14.5 ± 1.5 weeks, weighted 44.6 ± 3.2 kg were enrolled. These animals were purchased for an experimental study aiming to estimate pulmonary blood flow and right ventricular function in VA-ECMO using a modified Fick principle of thermodilution technique (5, 28). Animals could be included in the study if they showed a normal growth curve and normal appetite, and if they had no history of coughing, fever, diarrhea and antibiotic therapy in the previous 15 days. Pigs were transported 72 h prior to anesthesia from the farm of origin to the Vetsuisse Faculty, University of Bern, where they were housed in single boxes (1.45 m^2) enriched with straw bedding and ropes. A light/dark cycle of 12 h was set, and maintenance of visual, olfactory, and auditory contact with co-mates was always guaranteed. Pigs were fed twice per day with *ad libitum* access to water. Food but not water was withdrawn 12 h before general anesthesia. The study was reviewed and approved by the Committee for Animal Experiments of the Canton of Bern, Switzerland (permission number: BE 111/18). For reporting all the performed procedures, the ARRIVE guidelines (Animals in Research: Reporting of *In Vivo* Experiments) were followed.

2.2 Anesthetic protocol

After clinical examination and American Society of Anesthesiology (ASA) physical status classification (16 pigs ASA 1),

15 mg/kg ketamine (Narketan, Vetoquinol AG, Bern, Switzerland), 0.5 mg/kg midazolam (Dormicum; Roche CH, Switzerland) and 0.2 mg/kg methadone (Methadon Streuli; Streuli Pharma AG, Switzerland) were mixed in the same syringe and injected intramuscularly at the level of the cleido-occipital muscle. The pigs were left undisturbed for 15 min and then lifted on a table for preparation and disinfection once sedation was deemed adequate. Oxygen was supplemented through a non-tight face mask (4–6 liters/min) until tracheal intubation was achieved. Pulse rate (PR) and oxygen saturation (SpO_2) were monitored continuously through a pulse-oximeter probe placed on the lips. A venous catheter (20 Gauge, BD Ventflon Pro Safety, Switzerland) was placed in the auricular vein, fluid therapy (Ringer lactate's solution) was started at 5 mL/kg/h and induction of general anesthesia was achieved with intravenous (IV) administration of propofol (Propofol 10 mg/mL, Fresenius Kabi, Switzerland) 1–4 mg/kg to effect. After endotracheal intubation, anesthesia was maintained with continuous rate infusion of propofol started at 150–200 µg/kg/min and fentanyl (5–10 µg/kg/h), and the endotracheal tube was connected to a circle breathing system. Pigs were mechanically ventilated in volume-controlled mode setting tidal volume (TV) at 10 mL/kg, positive end expiratory pressure (PEEP) at 5 cmH_2O and fraction of inspired oxygen (FiO_2) at 60% (C6 Ventilator, Hamilton Medical, Bonaduz, Switzerland). An arterial catheter (20 Gauge) was then placed in the coccygeal artery to monitor invasive blood pressure. During the entire duration of anesthesia, SpO_2, invasive blood pressure, central venous pressure, electrocardiogram, esophageal temperature, inhaled and exhaled carbon dioxide (CO_2, mmHg) and oxygen (O_2) inspired fraction (%) level, palpebral reflex, eye position, jaw tone, electroencephalogram and bispectral index were continuously monitored (GE Healthcare Carescape B850, Anandic Medical System, Switzerland) and recorded every 5 min on an anesthetic sheet. Before sternotomy, amiodarone (Cordarone 150 mg/3 mL, Sanofi Aventis, Switzerland) 150 mg was administered IV over 30 min to prevent occurrence of lethal arrythmias. Noradrenaline (Noradrenaline 1 mg/mL, Sintetica AG, Switzerland) 0.05–0.1 µg/kg/min and adrenaline (Adrenalin 1 mg/mL, Sintetica AG, Switzerland) 0.1–0.2 µg/kg/min continuous rate infusion were administered to effect to maintain a mean arterial pressure (MAP) above 60 mmHg, when necessary. The infusion rate of propofol was titrated to a minimum of 100 µg/kg/h over time when burst suppressions were repeatedly noticed.

2.3 Nociceptive withdrawal reflex (NWR)

Nociceptive withdrawal reflex thresholds were obtained through the Pain Tracker device (Dolosys GmbH, Germany).

Once in the operating theater, skin preparation of the right hindlimb was performed to place the self-adhesive surface electrodes. Hair was clipped and shaved, and the skin was treated with abrasive tape, designed to remove the non-conductive skin layer, to achieve a better trace quality. The skin was then cleansed with betadine and alcohol, and carefully dried. Thereafter, stimulating (self-adhesive surface electrodes) and recording electrodes (subdermal needle electrodes) were positioned over the nerve digitalis plantaris and over the muscle tibialis cranialis, respectively. If the machine reported a good impedance value, the electrodes were secured in place.

Nociceptive withdrawal reflex was assessed through an established validated threshold tracking algorithm using transcutaneous electrical stimulation. The time window considered for analysis of the electromyographic response (EMG) (nociceptive reflex) was set between 80 and 240 milliseconds. Stimuli were delivered with 5 constant current square-wave pulses, with a duration of 1 millisecond and a frequency of 200 Hertz. The pain tracker monitor showed the real time NWR thresholds and their trend on the screen and stored the dataset on an external storage device. Moreover, thresholds were recorded on an on-purpose made experimental sheet.

2.4 Surgical procedure

The surgical procedure has been previously described and published (5). Briefly, lidocaine 1% (2 mg/kg) was injected before incision of the ventral cervical region; central venous catheters were then placed in both external jugular veins, and an arterial line was placed in the carotid artery. Afterwards, pigs underwent thoracotomy for setting up central VA-ECMO. Medial sternotomy was performed, and pericardium was opened. Thoracic vessels were then prepared, and, after administration of 80 IE/kg sodium heparin, the right atrium and ascending aorta were cannulated and connected to an ECMO circuit (Stockert SCPC Centrifugal Pump, Germany & Capiox FX15 Oxygenator, Terumo, USA). At the end of the procedure, thoracic cavity and pericardium were closed. During the whole duration of the surgery, activated clotting time was maintained between 180 and 220 s (3 times baseline) with a titrated infusion of heparin.

2.5 Spinal analgesia

To place a spinal catheter, the pig was positioned in sternal recumbency with the hindlimbs hyper flexed. An 18 Gauge (G) Tuohy needle was inserted on the median line, at the level of the intervertebral space between the last lumbar and the first sacral vertebra. Correct positioning of the needle was assessed by the presence of cerebrospinal fluid in the hub of the needle. Thereafter, the spinal catheter was introduced through the Tuohy needle and advanced to the thoraco-lumbar junction (T13-L1). The position of the tip of the catheter was verified by fluoroscopy.

Spinal analgesia was provided before starting the surgery of the cervical region, with 0.75 mg/kg ropivacaine 0.75% (Ropivacaine Fresenius 7.5 mg/mL, Fresenius Kabi AG, Switzerland) and 0.1 mg/kg morphine (Morphine HCl 2 mg/mL, Sintetica AG, Switzerland) up to a total volume of 0.1 mL/kg. The drugs were administered through the spinal catheter (Espocan, B Braun Medical AG, Switzerland) and the catheter flushed with 2 mL NaCl 0.9%.

2.6 Experimental protocol

Nociceptive withdrawal reflex thresholds were measured at different time points (TP) before and after spinal administration of ropivacaine and morphine (RM), as depicted in Figure 1.

If NWR thresholds showed an increase ≥20% from the baseline values, the increase was deemed clinically relevant. Conversely, if thresholds showed a decrease ≥20% from their baseline, rescue

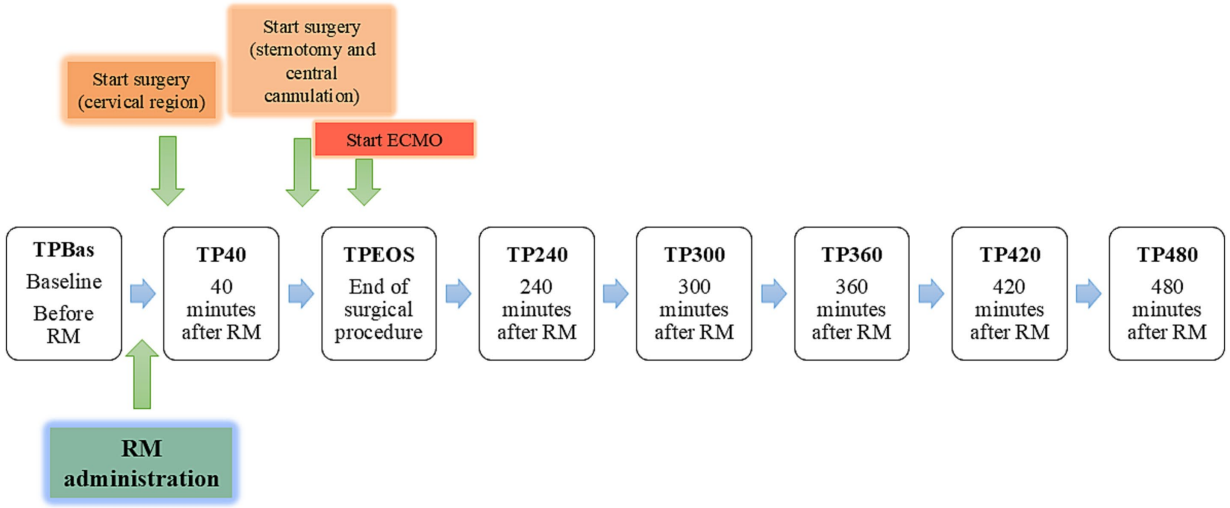

FIGURE 1
Graphic representation of the different time points (TPs) related to the administration of ropivacaine and morphine (RM). ECMO: extracorporeal membrane oxygenation.

analgesia was provided with administration of 0.75 mg/kg ropivacaine 0.75% in the spinal catheter.

Heart rate (HR) and mean arterial pressure (MAP) were not influenced by VA-ECMO until TP40. Surgical procedures (placement of catheters in the cervical region and thoracotomy) started immediately after RM and lasted until TPEOS. Following TPEOS, pigs were maintained on VA-ECMO until the end of the trial.

During the whole study, at each TP and after determination of the NWR threshold, nociceptive flexor response to claw pinching and palpebral reflex were evaluated. Claw pinching was performed with a surgical clamp on the second or third hind toe (alternated) of the left hindlimb, connected to a spring balance, pulled until 60 Newton was reached and hold for 15 s. Pigs' response to claw pinching was recorded as positive in case of withdrawal of the limb. Palpebral reflex was recorded as positive (if present) or negative (if absent).

2.7 Data analysis

Statistical analysis was performed with SigmaStat 4.0 (Point Richmond; CA, USA) and R Studio Statistical Software (version 4.3.2; R Foundation for Statistical Computing, Austria).

For each TP, NWR thresholds recorded by the Pain Tracker device over 1 min were averaged and used for statistical analysis. Data recorded and stored by the device were the first source of data retrieving, whereas, if the electronic storage failed, thresholds were retrieved from the experimental sheet. When recorded threshold were higher than the maximum stimulation intensity delivered by the device (150 mA), the value of 153 mA (the next step in the algorithm of measures) was considered for analysis. The thresholds recorded after rescue analgesia administration were not included in the statistical analysis.

Normality of the data was assessed with the Shapiro–Wilk test. Data are presented as mean ± standard deviation (SD) if normally distributed, and as median and interquartile range (IQR) [25th; 75th] if not normally distributed.

Thresholds were compared with baseline (TPBas) using ANOVA on Ranks test followed by Dunn's method (non-parametric test). Moreover, at each TP, the number of pigs showing (a) a clinically relevant increase in thresholds, (b) thresholds higher than the maximum stimulation intensity and (c) the need of rescue analgesia was calculated.

The time between sedation and RM, and between induction of general anesthesia and RM was calculated.

Values of HR and MAP at TP1 and TP2 were compared using the Paired T-Test (parametric test) and Wilcoxon Signed Rank test (non-parametric test), respectively.

A two-tailed p-value ≤ 0.05 was considered statistically significant.

3 Results

Nine pigs were included in the final data analysis. The first three pigs were used as pilot animals for feasibility assessment and method refinement, while four pigs were excluded due to absence of baseline measurement ($n = 3$) and missed fluoroscopic control of the spinal catheter ($n = 1$). Median and IQR [25th; 75th] of NWR thresholds recorded at different TPs following RM are reported in Table 1 and Figure 2. Due to reason related to the main experiment some measurements were not performed and details are reported in Figure 3. Due to device error, measurements from TP300 on are not reported in pig 3. Thresholds were retrieved by the experimental sheet six times (in three pigs at TP2, one at TP240, one at TP300, and one at TP420). The final number of animals in which NWR thresholds were recorded at each TP is reported in Table 1.

A statistically significant increase in NWR thresholds was found at TP300 and TP360 ($p = 0.009$ and 0.048, respectively) compared to TPBas (Figure 2).

Number of animals showing: (1) a clinically relevant increase in NWR thresholds across TPs, (2) NWR thresholds above 150 mA, (3)

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TABLE 1 Median and interquartile range [25th; 75th] of nociceptive withdrawal reflex thresholds recorded at the different time points (TP) after spinal analgesia administration.

Time point	Number of observations	Median (mA)	Interquartile range [25th; 75th] (mA)
TPBas	9	78.3	[48.6; 109,4]
TP40	7	153	[95; 153]
TPEOS	9	153	[123.1; 153]
TP240	9	153	[103.7; 153]
TP300*	7	153	[153; 153]
TP360*	7	153	[113.4; 153]
TP420	5	153	[91.7; 153]
TP480	5	140.5	[70.9; 153]

mA, milliampere. *Statistically significant difference compared to TPBas (baseline).

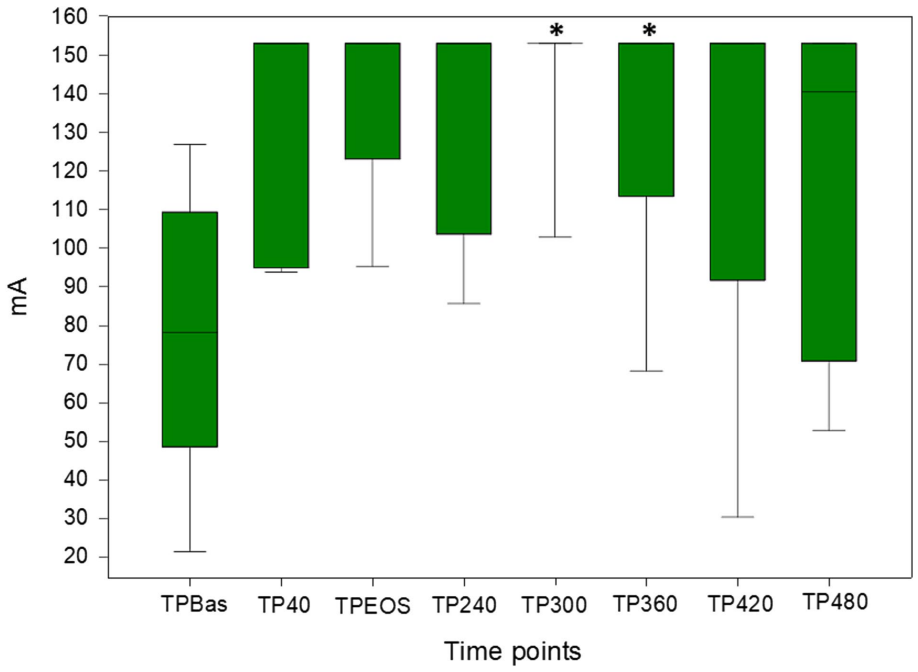

FIGURE 2
Box plots of nociceptive withdrawal reflex thresholds recorded at the different time points following spinal analgesia administration (ropivacaine and morphine: RM). Values reported as median and interquartile range [25th; 75th]. *statistically significant difference. mA: milliampere.

need of rescue analgesia, is reported in Table 2. Furthermore, trends of NWR thresholds modification across TPs per single animal is represented in Figure 3.

Time between sedation and RM, and induction of general anesthesia and RM was 98 ± 6 and 64 ± 7 min, respectively.

No statistically significant differences were found for both HR ($p = 0.713$) and MAP ($p = 0.57$) between TPBas and TP40 (Figures 4, 5).

Noradrenaline was needed in five animals during thoracic surgery, in one animal shortly before sternotomy, and in three after connection to the VA-ECMO. Adrenaline was needed in one animal during thoracic surgery, in three animals after connection to the VA-ECMO and in five animals after multiple weaning from the VA-ECMO.

Positive nociceptive flexor responses (claw pinching) were found only in one animal at TP300 (NWR threshold >150 mA). No presence of palpebral reflex was recorded at any TP.

4 Discussion

In the present study, we observed that NWR thresholds increased following administration of spinal analgesia in pigs undergoing general anesthesia for VA-ECMO. Nociceptive withdrawal reflex thresholds increased in a clinically meaningful way already at 40 min after spinal injection in all but one animal, reached their maximal values at 300 min, and remained clinically

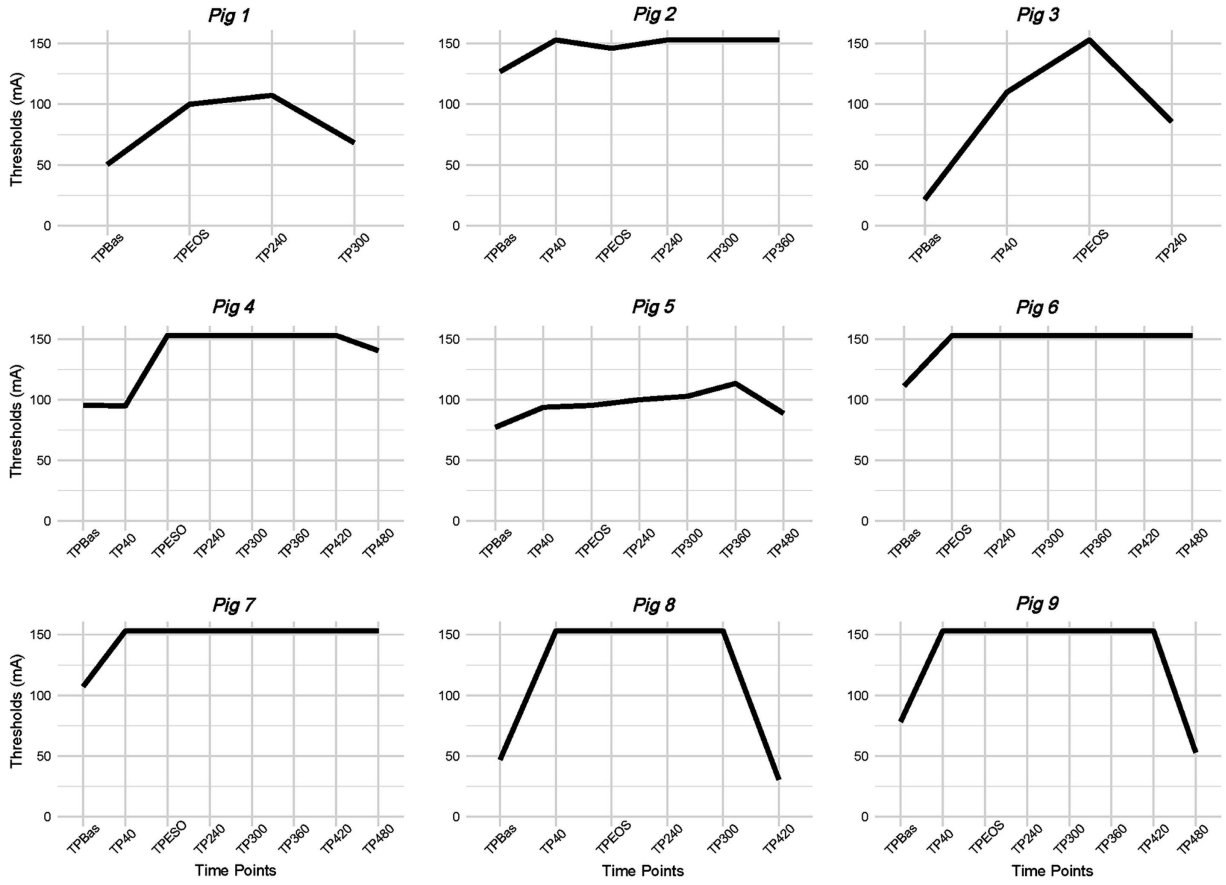

FIGURE 3
Nociceptive withdrawal reflex (NWR) thresholds modification across time points (TP) for each pig. mA: milliampere.

TABLE 2 Number of animals for each time point (TP) which showed a clinically relevant nociceptive withdrawal reflex (NWR) threshold increase [≥ 20% baseline (TPBas)], reached NWR thresholds above 150 mA, and needed rescue analgesia.

	TP40	TPEOS	TP240	TP300	TP360	TP420	TP480
NWR thresholds increase ≥ 20% baseline (TPBas)	*n* = 6/7	*n* = 8/9	*n* = 9/9	*n* = 7/7	*n* = 7/7	*n* = 4/5	*n* = 3/5
NWR thresholds > 150 mA	*n* = 4/7	*n* = 6/9	*n* = 6/9	*n* = 6/7	*n* = 5/7	*n* = 4/5	*n* = 2/5
Rescue analgesia	*n* = 0	*n* = 0	*n* = 0	*n* = 1	*n* = 0	*n* = 2	*n* = 1

and statistically significantly higher up to 360 min. After this time point, NWR thresholds showed a decrease, the extent and the time of which were scattered. Rescue analgesia was needed in four animals. The evolution of recorded thresholds suggests that the combination of spinal morphine and ropivacaine can last on average up to 6 h. Overall, the findings of this study suggest that determination of the NWR thresholds can be of support in the evaluation of intra-operative nociception in pigs following spinal analgesia. To our knowledge, this is the first report investigating NWR threshold following spinal analgesia in this species. Previous studies in veterinary medicine have reported the use of the NWR to investigate the analgesic effect of different drugs administered systemically in both awake (22, 24, 26, 27, 29, 30) and anesthetized animals (31, 32). Furthermore, its usefulness in predicting movements in responses to nociceptive stimuli has been evaluated and demonstrated in anesthetized pigs (33) and humans (34–36).

Duration of the analgesic effect of sole ropivacaine was found to be 176.0 ± 109.0 min in a previous study in anesthetized dogs (37) and 358 [IQR 238; 538] minutes in humans (16) following spinal administration of ropivacaine 1% (dosage: 1 mg/kg) and 0.75% (dosage: 22.5 mg total), respectively. No data have been published about the analgesic duration of ropivacaine combined with morphine injected spinally in veterinary species. However, based on previous studies where this combination was used epidurally in dogs, an extension of the analgesic effect can be expected (38, 39). In the present study, following spinal analgesia, an NWR thresholds increase was found to be present until 360 min following injection in all but one animal, while in four animals lasted up to the last recording performed (480 min after spinal injection). The suggested duration should be confirmed by a higher number of observations and supported by pharmacodynamic and pharmacokinetic (PD/PK) information. Furthermore, the effects of the VA-ECMO on the PD/PK

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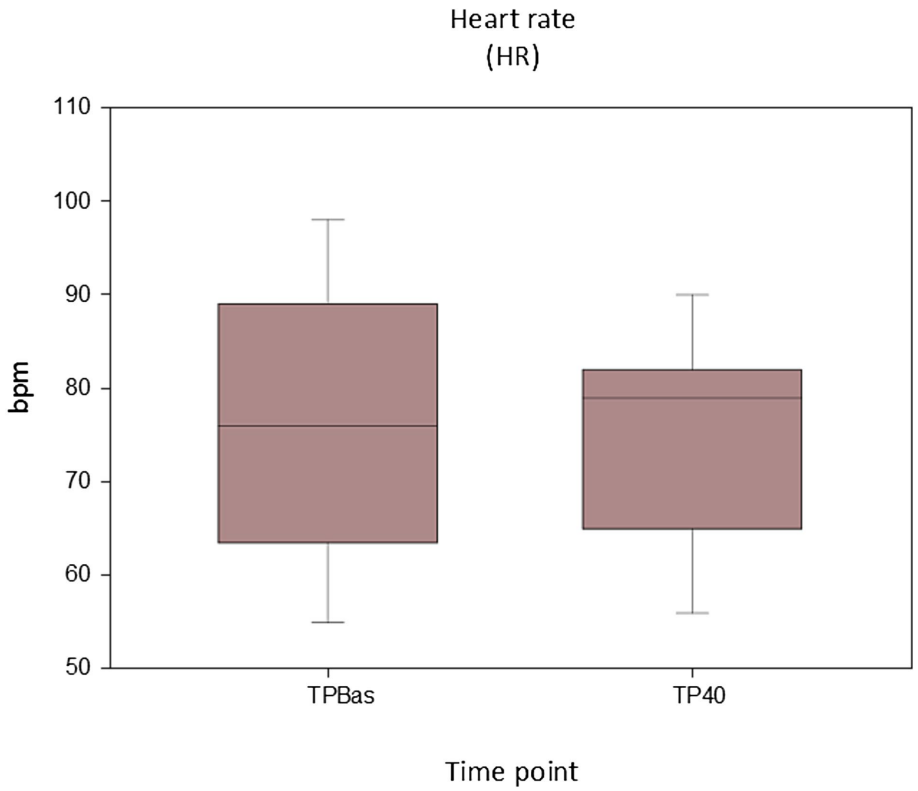

FIGURE 4
Values of heart rate recorded at baseline (TPBas) and 40 min after spinal injection (TP40). Unit: beats per minute (bpm).

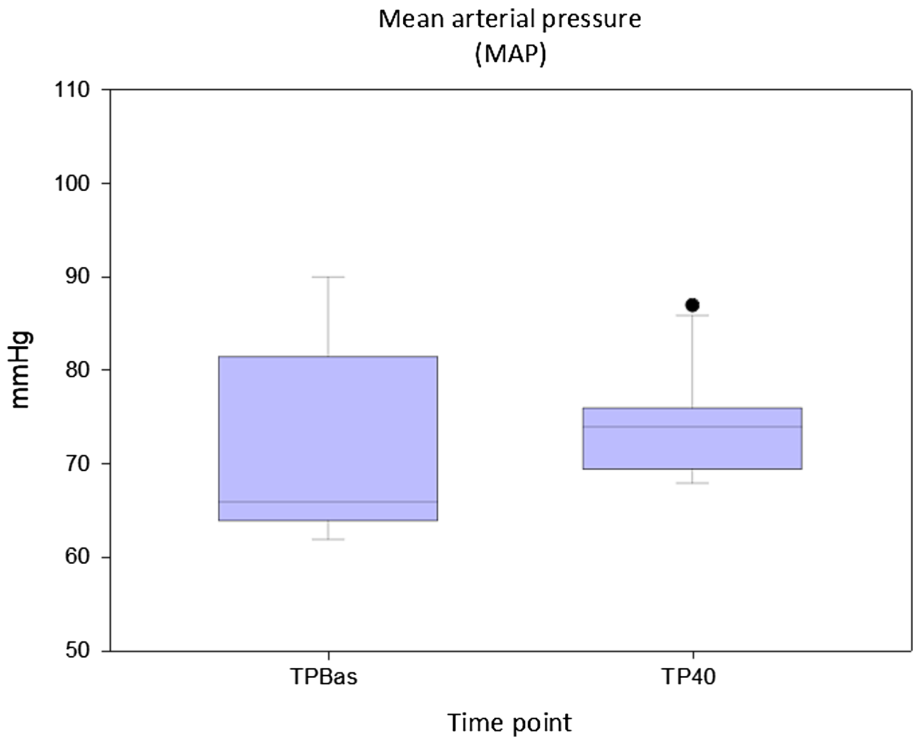

FIGURE 5
Values of mean arterial pressure at recorded at baseline (TPBas) and 40 min after spinal injection (TP40). Unit: millimeters of mercury (mmHg).

of intrathecal injected drugs is supposably negligible, but no studies have been conducted so far.

Recently, NWR thresholds in awake pigs have been reported: 7.2 [IQR 4.9; 10.5] mA (40). Similar thresholds have also been described in other species, such as horses (5.7 [IQR 5; 8.5] mA) (26) and humans (5.14 [IQR 3.03; 8.83] mA) (41). In our trial, NWR thresholds in awake animals were not assessed, and we considered as baseline the thresholds recorded in the context of a balanced anesthesia protocol. This was done to verify the hypothesis that the spinal injection of ropivacaine and morphine would increase them when general anesthesia was already at a stable depth. Our baseline values of 78.3 [IQR 48.6; 109.4] mA are not surprising in pigs receiving a protocol of balanced anesthesia. It is indeed known that the different drugs used for achieving sedation and inducing or maintaining anesthesia have an influence on the NWR (25, 32, 42, 43). Ketamine, injected in our trial in sedation, is known to be able to increase stimulation intensity required to evoke NWR in ponies (44) and a marked antinociceptive effect on high intensity nociceptive electrical stimuli has also been recognized in humans (45). In our study, when baseline thresholds were recorded, 98 ± 6 min after IM injection of ketamine, methadone, and midazolam, the plasmatic concentration of ketamine was not known. In pigs the mean half-life of 15 mg/kg ketamine injected IM was 140 min, although the duration of the anesthetic time was limited to 9–48 min in young animals, and 78–88 min in adult sows (46). Unfortunately, information regarding the onset and duration and analgesia effect is unreported (46). In humans, the association with diazepam brought about an increase of ketamine plasma concentration and a decrease of its clearance (47). We can only speculate a similar effect in pigs and assume that the baseline threshold might have been influenced by its injection. The hypnotic effect of propofol, which in our case was administered throughout the entire anesthesia, has been showed to increase NWR thresholds in humans undergoing computer-controlled propofol IV infusion at increasing dosages (median value recorded after loss of consciousness: 23.75 mA) (36). Similar trends have also been found in pigs by Mirra et al. (40), with thresholds never exceeding values of 29.4 [IQR 21.8; 35.3] mA when sole propofol was administered even though a deep anesthetic level was reached. The effect of fentanyl on the NWR was investigated in pigs undergoing isoflurane anesthesia: when used as sole analgesic, both an increase (5 μg/kg/h) and a decrease (40, 80 and 160 μg/kg/h) of the NWR area under the curve was observed (38). However, different time windows (20–100 milliseconds) to assess the reflex were selected, and recording was performed in the front limb (32).

Spinal administration of ropivacaine and morphine might lead to both sensor and motor fibers blockade, therefore elevation of the NWR thresholds can be due to both decreased peripheral sensitivity as well as motor block. The influence of the two components cannot be distinguished via the methodology used in the present study to track the thresholds. The NWR recording electrodes were positioned only at the level of the hindlimb. One could argue that high NWR thresholds could have reflected a caudal spread of the ropivacaine, accompanied by the blockade of the sensory and motor fibers innervating the hindlimb, rather than an adequate antinociception during thoracic surgery. However, in support of a cephalad spread of the spinal analgesia and therefore adequate antinociception during thoracic surgery, there are two findings. First, the high NWR thresholds recorded during and after surgery are supportive of absence of nociception during surgery. The doses of fentanyl infused during thoracic surgery are not considered antinociceptive in pigs, in which doses of 35 μg/kg/h (48) or 50 μg/kg/h (49) were needed to provide analgesia during surgical manipulations in combination to similar doses of propofol. Second, MAP and HR, before being artificially modulated, were overall stable during surgical manipulation, supporting the absence of nociceptive sympathetic stimulation as well. Further confirmation of the latter is the need of noradrenaline during surgery in six animals.

In this study, response to claw pinching was assessed regularly as a part of clinical evaluation of depth of anesthesia and recorded at each time point following determination of NWR thresholds. Motor response to claw pinching is a technique that has been frequently used to evaluate both analgesia and depth of anesthesia in pigs (19, 50, 51), however, its usefulness has been recently questioned (52). In our study, claw pinching could never be elicited but in one animal at one single time point. In this pig at 300 min after ropivacaine and morphine injection, the NWR was elicited at 150 mA, suggesting motor block and sensory block not matching in this specific circumstance. Being an isolated case, more extensive comments are not appropriate.

Cardiovascular variables, in particular MAP, has been shown to be the most reliable indicators of nociception in pigs under general anesthesia (19). In our animals, evaluation of cardiovascular parameters was reliable until TP40 following RM; 10–30 min after this time point, the VA-ECMO system was started. Stable HR and MAP were found between TPBas and TP40, reflecting adequate analgesia, and revealing no cardiovascular negative effects linked to the spinal injection. No studies investigating cardiovascular modifications due to spinal ropivacaine and morphine administration in pigs have been found, but a trial investigating the cardiovascular impact of lidocaine 2% administered spinally in experimental pigs found that its cardiovascular impact in normovolemic animals was minimal (53). Our results are in line with these findings.

This study has some limitations that needs to be acknowledged. First, no *a priori* sample size calculation was performed, and all the subjects included in the trial to evaluate VA-ECMO were recruited accordingly. Second, NWR thresholds were recorded only in the hindlimb while no records in the front limb were performed. Therefore, the effect of spinal analgesia on the sensory and motor fibers proximal to the thoracic area could not be assessed. Third, the time windows on the EMG for recognizing an NWR was set between 80 and 240 ms based on a previous investigation in pigs (33) but no clear guidelines have been established yet. Lastly, as the maximal stimulation intensity (150 mA) of the device was unmodifiable, we needed to attribute an arbitrary NWR thresholds value (153 mA), when the upper stimulation limit was overcome.

5 Conclusion

Nociceptive withdrawal reflex thresholds increased after spinal administration of ropivacaine and morphine. This finding supports their usefulness for evaluating antinociception following spinal analgesia in pigs, particularly in those cases in which cardiovascular variables cannot be evaluated, as during VA-ECMO. Inclusion of this monitoring technique might improve our ability to evaluate antinociception during general anesthesia following spinal analgesia.

Future studies are warranted to assess the NWR thresholds' performance in pigs receiving different systemic anesthetic/analgesic protocols.

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 at: https://doi.org/10.5281/zenodo.11394587.

Ethics statement

The animal study was approved by the Committee for Animal Experiments of the Canton of Bern, Switzerland (permission number: BE 111/18). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

MP: Data curation, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. CS: Methodology, Writing – original draft, Writing – review & editing. KB: Funding acquisition, Investigation, Resources, Writing – original draft, Writing – review & editing. DB: Funding acquisition, Investigation, Writing – original draft, Writing – review & editing. AM: Writing – original draft, Writing – review & editing, Methodology. DC: Conceptualization, Data curation, Investigation, Methodology, Supervision, Writing – original draft, Writing – review & editing.

Funding

The author(s) declare that financial support was received for the research, authorship, and/or publication of this article. The main study in which pigs were enrolled (VA-ECMO) was funded by the "Stiftung für Forschung in Anästhesiologie und Intensivmedizin" (Bern, Switzerland; Nr 26/2018) awarded to KB and DB, and by the "Fondation Johanna Dürmüller- Bol" (Muri bei Bern, Switzerland; Nr 481) awarded to KB.

Acknowledgments

The authors would like to thank Kay Nettelbeck for his fundamental technical support.

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

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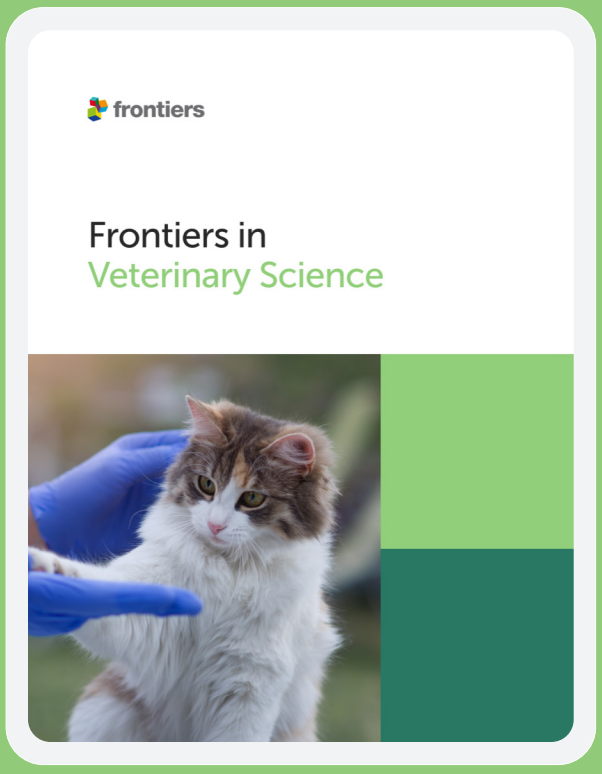

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