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Advancement in equine pain management

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Published in
Frontiers in Pain Research

ISSN 1664-8714
ISBN 978-2-8325-6014-3
DOI 10.3389/978-2-8325-6014-3

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Advancement in equine pain management

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Citation

Chiavaccini, L., Reed, R. A., Spadavecchia, C., eds. (2025). *Advancement in equine pain management*. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-8325-6014-3

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frontiers | Frontiers in Pain Research

TYPE Editorial
PUBLISHED 05 February 2025
DOI 10.3389/fpain.2025.1547764

OPEN ACCESS

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RECEIVED 18 December 2024
ACCEPTED 20 January 2025
PUBLISHED 05 February 2025

CITATION
Chiavaccini L, Reed RA and Spadavecchia C (2025) Editorial: Advancements in equine pain management.
Front. Pain Res. 6:1547764.
doi: 10.3389/fpain.2025.1547764

Editorial: Advancements in equine pain management

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KEYWORDS

equine, pain, drug therapy, quantitative sensory testing (QST), clinical metrology index

Editorial on the Research Topic
Advancement in equine pain management

"Pain is a more terrible lord of mankind than even death itself."—Albert Schweitzer. Pain is a global issue affecting humans and animals, and over the past decade, research into its biology, treatment, and prevention has flourished. However, progress in equine pain research has lagged behind its small animal counterpart. Painful conditions in horses are often overlooked by owners and practitioners, leading to inadequate recognition and management of pain (1–3). Accurate pain assessment is essential for ensuring appropriate analgesia in equine patients. Yet, studies reveal significant gaps in knowledge and action, even among confident horse owners in the UK and US (2). For example, while owners may recognize signs of colic (abdominal pain), their responses to emergencies are often inconsistent. Furthermore, equine pain recognition and treatment remain underexplored in low- and middle-income countries (Laleye et al.), where limited resources, inadequate training, cultural diversity, and language barriers contribute to animals not receiving basic pain treatment. The retrospective study by Laleye et al. demonstrated that delays in recognizing abdominal pain and referring horses for treatment increase mortality and hospital expenses, in Senegal.

Some of this discrepancy in pain recognition stems from the subtle and multifaceted expressions of pain in horses (3). Although several equine pain scales have been developed (4–9), their implementation in practice remains inconsistent. Factors such as lack of exposure, misinterpretation, or overinterpretation of these tools contribute to the gap in pain recognition and treatment (10). For example, Reed et al. showed that the residual effects of general anesthesia may affect the accuracy of facial expression-based pain scoring systems in the hours immediately following anesthetic recovery.

The inherent subjectivity of evaluating pain behavior is a challenge to achieving objective and quantitative pain assessment. A standardized scale for clinical and research applications could address these limitations. In the study of Nowak et al., specific behavioral indicators—such as weight shifting and unstable resting—appeared reliable tools for distinguishing between horses experiencing musculoskeletal pain and those that were pain-free. Severely painful horses displayed reduced feeding and resting behavior while standing, along with increased unstable resting. These findings align with previous research highlighting postural behaviors as dependable pain indicators,

particularly in orthopedic conditions (11, 12). In this issue, Auer et al. refined an equine musculoskeletal pain scale (MPS) by integrating elements of the equine pain face (13, 14), posture, head–neck position, weight-bearing, and weight shifting. This updated MPS provides a comprehensive framework for assessing chronic orthopedic pain in horses and explores incorporating visual information into automated pain recognition systems, offering new avenues for introducing objectivity in pain assessment in veterinary medicine (15).

While subjective pain scales and objective gait analysis systems quantify lameness severity in horses (16, 17), quantitative sensory testing (QST) methods—widely used in human medicine to define pain phenotypes—remain underexplored in equine practice. Mechanisms such as impaired autonomic joint innervation, nociceptive fiber plasticity, and dysfunction of descending pain inhibitory pathways likely contribute to chronic pain persistence. Gisler et al. demonstrated the feasibility and reliability of periarticular pressure pain assessment in healthy horses' distal thoracic limb joints. Their findings showed good repeatability among researchers, suggesting that periarticular pressure mapping could be a valuable complementary diagnostic tool for evaluating and mapping orthopedic pain phenotypes in horses. Furthermore, QST devices hold potential for assessing disruptions in modulatory pathways associated with chronic pain, offering insights into peripheral and central sensitization. For instance, the lip twitch, causing pressure on the upper lip, has been hypothesized to activate opioidergic and non-opioidergic descending modulatory pathways in horses (18). Blum et al. supported this theory by showing that lip-twitch application increased nociceptive withdrawal reflex and thermal pain thresholds in healthy horses. Integrating QST methods and assessments of conditioned pain modulation into equine practice could advance understanding of chronic pain mechanisms, improving diagnostic and therapeutic approaches.

Opioids are integral to analgesic protocols due to their high potency and efficacy in treating different pain in human and veterinary medicine. Injectable μ-receptor opioid agonists such as morphine, hydromorphone and methadone are commonly used perioperatively in horses. However, concerns about excitation (19), decreased gastrointestinal motility (20–22), and the conflicting data on the analgesic efficacy of full mu opioids in horses with naturally occurring diseases (23) often deter their use. In this special issue, Paranjape et al. and Reed et al. explored the pharmacokinetics and pharmacodynamics of transdermal buprenorphine and fentanyl in horses. These studies demonstrated good tolerance and prolonged therapeutic plasma concentrations but variable efficacy in increasing thermal or mechanical thresholds. Haralambus et al. investigated the incidence of postoperative colic (PAC), reporting an overall rate of 15.1%. The study also examined opioid use, noting that intraoperative or short-term postoperative administration did not increase PAC rates. However, long-term administration (greater than 24 h) of morphine significantly raised the PAC incidence to 34% ($p = 0.0038$), whereas long-term butorphanol or methadone had no significant effect. These findings underscore the importance of cautious opioid selection in postoperative pain management and use of the lowest effective dose and frequency.

Given the limitations of prolonged opioid treatment, non-pharmacological interventional techniques may offer valuable alternatives for managing chronic pain in horses. In this issue, Amari et al. propose ultrasound-guided radiofrequency ablation of the palmar digital nerve as a potential treatment for horses with chronic distal forelimb lameness. Histopathological findings revealed consistent axonal degeneration, which, in clinical settings, would translate into effective management of chronic pain, as seen in human and veterinary literature.

In conclusion, the Special Issue of "*Advancements in equine pain management*" features 11 articles showcasing the latest progress in understanding and managing equine pain. The collection explores diverse topics, including diagnostic innovations, pharmacological advances, and cutting-edge interventional techniques. While addressing every facet of equine pain in a single issue is impossible, this selection emphasizes novel methodologies and interdisciplinary research to improve equine welfare. Authored by leading experts in the field, these articles provide a comprehensive and accessible overview of equine pain recognition and management, making them invaluable resources for researchers and clinicians. The contributions highlight advancements from foundational science to clinical applications, employing a "bench to bedside" approach that bridges research and practice. We hope this collection will provide an insightful reading and inspire further innovation and collaboration in the ongoing effort to improve the lives of equine patients across the globe.

Author contributions

LC: Conceptualization, Writing – original draft. RR: Conceptualization, Writing – review & editing. CS: Conceptualization, 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.

Generative AI statement

The author(s) declare that no Generative AI was used in the creation of this manuscript.

Publisher's note

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frontiers | Frontiers in Pain Research

TYPE Original Research
PUBLISHED 12 July 2023
DOI 10.3389/fpain.2023.1217034

OPEN ACCESS

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RECEIVED 04 May 2023
ACCEPTED 04 July 2023
PUBLISHED 12 July 2023

CITATION
Reed RA, Krikorian AM, Reynolds RM, Holmes BT, Branning MM, Lemons MB, Barletta M, Quandt JE, Burns CC, Dantino SC and Sakai DM (2023) Post-anesthetic CPS and EQUUS-FAP scores in surgical and non-surgical equine patients: an observational study. Front. Pain Res. 4:1217034. doi: 10.3389/fpain.2023.1217034

Post-anesthetic CPS and EQUUS-FAP scores in surgical and non-surgical equine patients: an observational study

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Background: Equine pain scoring may be affected by the residual effect of anesthetic drugs.
Objectives: To compare pain scores in the hours immediately following anesthetic recovery to baseline pre-anesthetic scores in equine patients undergoing surgical and non-surgical procedures.
Study design: Clinical observational study.
Methods: Fifty adult horses undergoing anesthesia for surgical or non-surgical procedures were enrolled. Horses underwent pain scoring using the Composite Pain Score (CPS) and Equine Utrecht University Scale for Facial Assessment of Pain (EQUUS-FAP) prior to anesthesia (T0) and following anesthetic recovery to standing, every hour for 5 h (T1-T5). Data were analyzed using a generalized linear mixed effects model. A post-hoc Dunnett's test for multiple comparisons was performed for variables where an effect was detected.
Results: Mean (95% confidence interval) CPS scores for T0-T5 were 1.6 (1.2–2.0), 6.8 (6.0–7.6), 5.1 (4.3–5.9), 4.3 (3.4–5.2), 3.7 (2.8–4.6), and 2.8 (2.0–3.6) and EQUUS-FAP scores were 0.6 (0.3–0.9), 3.0 (2.5–3.5), 1.9 (1.6–2.2), 1.1 (0.8–1.4), 0.6 (0.4–0.8), and 0.7 (0.4–1.0), respectively. For the CPS, scores greater than 5, and for the EQUUS-FAP scores greater than 3, are consistent with minor pain. There was no effect of type of procedure (surgical vs non-surgical) on CPS or EQUUS-FAP scores. There was an effect of time with CPS scores significantly greater than baseline at T1-T5 and EQUUS-FAP scores significantly greater than baseline at T1 and T2.
Main limitations: Discomfort caused by hoisting was not quantified and it was difficult to ascertain if this affected the results.
Conclusions: Post-anesthetic pain scores may be influenced by the residual effect of anesthetic agents for as long as 5 h and 2 h for the CPS and EQUUS-FAP, respectively.

KEYWORDS

anesthesia, analgesia, horse, pain score, pain

1. Introduction

Pain scoring is an important component of providing adequate analgesia to equine patients and is one of the clinical recommendations for primary practice made by the British Equine Veterinary Association Analgesia Panel (1). In recent years, several equine pain scoring systems have been investigated and validated for different types of pain. These scoring systems have been reviewed elsewhere (2, 3).

The Equine Composite Pain Score (CPS) and the Equine Utrecht University Scale for Facial Assessment of Pain (EQUUS-FAP) are two systems that have been validated for scoring of orthopedic pain and visceral pain (4–8). However, it is unclear how the residual effects of general anesthesia may affect the accuracy of these pain scoring systems in the hours immediately following anesthetic recovery. Indeed, the effect of general anesthesia on the equine stress response has been well documented (9–11) and it can influence factors assessed in pain scoring systems. The residual effects of general anesthesia on physiologic variables including heart rate, respiratory rate, body temperature, and borborygmi included in the CPS could falsely alter the resulting pain score. Additionally, the effect of anesthesia on patient position, stance, and appearance could falsely increase both CPS and EQUUS-FAP pain scores resulting in unnecessary rescue analgesia and associated systemic adverse effects.

The objective of the study presented here was to determine the effect of general anesthesia on CPS and EQUUS-FAP scores in the hours immediately following recovery in horses undergoing surgical and non-surgical anesthetic episodes. It was hypothesized that pain scores would be significantly higher than baseline immediately following anesthetic recovery regardless of whether the patient underwent a surgical or non-surgical procedure.

2. Materials and methods

2.1. Study design

A prospective observational study of horses presenting to the University of Georgia Veterinary Teaching Hospital for elective anesthesia was performed between May 2022 and August 2022. Ethical approval from the University of Georgia Clinical Research Committee was waived prior to the start of data collection as the study was purely observational and no horse would receive any unique treatment as a result of the study. Inclusion criteria comprised: healthy adult equine patients presenting for elective anesthesia for surgical or non-surgical procedures. Exclusion criteria comprised: behavioral attributes making it unsafe to perform scoring and scheduled time of anesthesia when no investigators would be available for scoring.

Horses were housed in 3.7 × 3.7 m stalls where they acclimated to the hospital for 12–24 h prior to anesthesia. Horses were fed hay and grain thrice daily with the exception of the morning prior to anesthesia when they received a small flake of hay only. Water was made available at all times.

2.2. Anesthetic events

All horses were anesthetized utilizing a similar anesthetic protocol. Subjects received a pre-anesthetic non-steroidal anti-inflammatory drug (NSAID), either phenylbutazone (2.2 mg/kg; Phenylbutazone; Covetrus, Portland, ME, USA) or flunixin meglumine (1.1 mg/kg; Banamine; Merck Animal Health, Rahway, NJ, USA) IV, based on the clinician's preference, and they were sedated with intravenous (IV) xylazine (1.1 mg/kg; AnaSed; Akorn Animal Health, Gurnee, IL, USA). Immediately prior to induction of anesthesia, horses received hydromorphone (0.04 mg/kg; Akorn Animal Health) IV or butorphanol (0.02 mg/kg; Torbugesic; Zoetis Inc, Parsippany, NJ, USA) IV for surgical and non-surgical procedures, respectively. Anesthesia was induced with ketamine (2.2 mg/kg; VetaKet; Akorn Animal Health) and midazolam (0.05–0.1 mg/kg; Midazolam injection; Hospira Inc., Lake Forest, IL, USA) IV, and maintained with isoflurane (Akorn Animal Health) in 100% oxygen combined with ketamine (1 mg/kg/hr), xylazine (0.5 mg/kg/hr), and lidocaine (2 mg/kg loading dose followed by 3 mg/kg/hr; VetOne, Boise, ID, USA). Following anesthesia, horses were placed in a recovery stall and received xylazine 0.18–0.55 mg/kg IV to delay anesthetic recovery allowing time to expire isoflurane. Thirty-four horses received 0.004–0.02 mg/kg of acepromazine (VetOne, Boise, ID, USA) either prior to induction of anesthesia or prior to recovery. All horses were recovered on a pad or air mattress, with the aid of head and tail ropes.

2.3. Pain scoring

Pain was assessed with both CPS and EQUUS-FAP scoring systems as described elsewhere (4) on the day prior to anesthesia and at 1, 2, 3, 4, and 5 h following anesthetic recovery to standing. All pain scores were performed by 5 veterinary students that received training in regard to the use of both scoring systems. At each timepoint, the assessment was performed independently using both scales by two students simultaneously in a quiet environment. A coin toss was used to randomize the order of the scoring systems at each time point and each horse was scored by the same two students at all time points. Although the students were not masked to the procedure (surgical *vs.* non-surgical), they were unaware of the specific objectives and hypotheses of the study.

The CPS, a simple descriptive scale, requires 5 min to complete and includes physiologic data (physical examination including heart rate, respiratory rate, rectal temperature and digestive sounds), behavioral data (posture, appetite, sweating, kicking at abdomen, pawing at floor, head movements, overall appearance) and response to treatment (interactive behavior, response to palpation). Each variable ranges from 0 to 3 with an overall maximum score of 39. For non-surgical procedures, the scorer

palpated the area of concern that was imaged (e.g., joint associated with lameness). The EQUUS-FAP requires 2 min to complete and includes only quiet observation of the patient. Variables assessed are associated with facial expression and each ranged from 0 to 2 with an overall maximum score of 18.

Additional information recorded included signalment, reason for and duration of anesthesia, duration of recovery, anesthetic agents, and perioperative NSAID. No treatments were administered to any patient by any individual involved in the study. Additional analgesic agents were administered at the discretion of the attending Veterinary Teaching Hospital clinician that was not involved with the study. If additional analgesic agents were administered, the horse was subsequently excluded from data collection.

TABLE 1 Breeds of horses ($n = 50$) included in the study.

Breed	Number enrolled
Warmblood	20
Quarter horse	11
Irish sport horse	4
Thoroughbred	4
Andalusian	2
Trakehner	2
Friesian	1
Welsh cross	1
Pony	1
Cleveland bay	1
Lusitano	1
Morgan	1
Tennessee walker	1

2.4. Data analysis

A priori sample size calculation was performed using G*Power 3.1 (Heinrich-Heine-Universität Dusseldorf, Germany). Pain scoring data from a previous clinical study were utilized for the calculation (12). For the CPS, to detect a difference of 1.7 between baseline pain scores and post anesthetic recovery, with an alpha of 5% and power of 95%, a sample size of 40 horses would be required. Therefore, we aimed to observe 50 horses over the course of the study.

Agreement tables with individual veterinary student observers' scores for CPS and EQUUS-FAP were created to calculate Cohen's Kappa. The agreement was defined as: near perfect if 0.80–1.00, substantial if 0.61–0.80, moderate if 0.41–0.60, fair if 0.21–0.40, and none if 0.00–0.20. The individual scores for each timepoint were averaged to determine the overall score for that time point. A generalized linear mixed model with fixed effects of type of procedure (surgical vs. non-surgical), timepoint, and their interaction was used for the analysis. Horse was included as a random effect. The Dunnett's test for multiple comparisons was used for items where a significant effect was found.

3. Results

3.1. Summary of subjects and procedures

A total of 70 horses met the inclusion criteria with 20 horses excluded due to lack of availability of investigators for post anesthetic assessment, resulting in a total sample size of 50 horses. The study population included: 33 geldings, 14 mares, and 3 stallions of various breeds (Table 1), with a mean ± SD age of 11 ± 6 years and weighing 546 ± 67 kg. Twenty-six horses underwent surgical procedures and 24 non-surgical procedures. Of the former, 21 were orthopedic surgeries (arthroscopy, tenoscopy, neurectomy, dorsal spinous process excision, tooth extraction) and 5 were soft tissue surgeries (wound explore/debride, castration, removal of scirrhous cord, tumor ablation, enucleation). The non-surgical procedures included 22 for magnetic resonance imaging (MRI) and 2 for computed tomography (CT) scans. No complications were noted for any of the anesthetic events and all horses recovered successfully to standing.

3.2. Pain scores

For the CPS and the EQUUS-FAP, Cohen's Kappa (95% confidence interval) was 0.95 (0.94–0.97) and 0.78 (0.71–0.84), respectively.

CPS scores are presented in Figure 1. There was no effect of type of procedure (surgical vs. non-surgical) ($p = 0.6841$); however, there was an effect of time ($p < 0.001$), with hours 1–5 being significantly greater than baseline ($p < 0.001$ for all). In regard to individual categories of the scoring system, there was no effect of type of procedure, but there was a significant effect of time ($p < 0.006$ for all). Physiologic scores were significantly greater than baseline at hours 1–5 ($p < 0.02$ for all), behavioral scores were significantly greater than baseline at hours 1–4

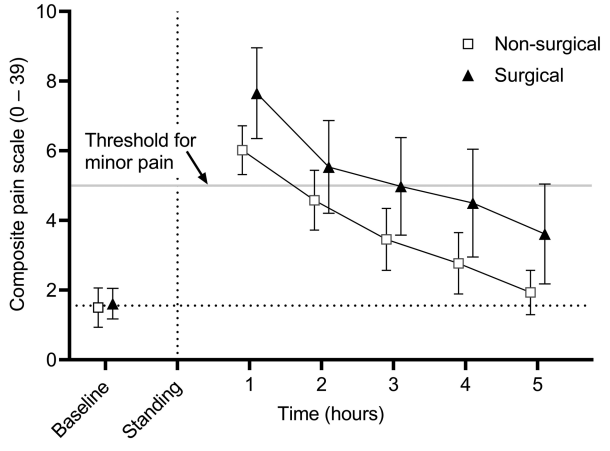

FIGURE 1
Mean (± 95% confidence interval) composite pain scores (CPS) in 50 adult horses undergoing surgical or non-surgical procedures prior to anesthesia (baseline) and in the hours immediately following recovery to standing. Overall scores are significantly greater than baseline at hours 1-5 following anesthetic recovery ($p < 0.001$). There was no significant effect of reason for anesthesia.

($p < 0.011$ for all), and response to treatment was significantly greater than baseline at hour 1 ($p = 0.024$). There was no interaction between reason for anesthesia and time for any of the analyses.

EQUUS-FAP scores are presented in Figure 2. There was no effect of type of procedure (surgical vs. non-surgical) ($p = 0.7942$); however, there was a significant effect of time ($p < 0.001$) with hours 1 and 2 being significantly greater than baseline ($p < 0.001$ for both). There was no interaction between reason for anesthesia and time.

There was no effect of anesthetic time or recovery time on CPS ($p = 0.619$ and $p = 0.2411$, respectively) and on EQUUS-FAP ($p = 0.220$, $p = 0.1821$, respectively). No horse received additional analgesic agents within the period of data collection.

4. Discussion

In the study presented here, all horses, regardless of reason for anesthesia, were assigned higher CPS and EQUUS-FAP pain scores in the hours immediately following anesthetic recovery to standing in comparison to baseline. In previous studies evaluating the efficacy of these scoring systems in horses undergoing general anesthesia, the first assessment occurred no earlier than 4 h following anesthetic recovery (4, 5, 13). Indeed, van Loon et al. evaluated the role of general anesthesia on CPS after surgical and non-surgical procedures and found no effect. However, in that study, the first pain score was recorded at 4 h post-anesthetic recovery and therefore it is possible that any effect prior to that time point had been missed. Additionally, only 6 horses were included in the non-surgical group and the authors did not report the results of a power analysis, citing small sample size as a limitation to the study.

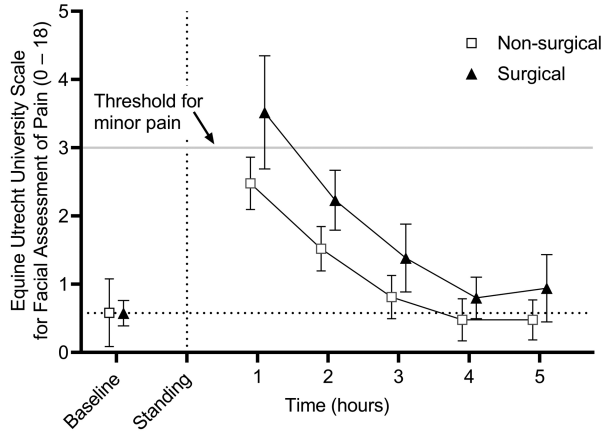

FIGURE 2
Mean (± 95% confidence interval) equine Utrecht university scale for facial assessment of pain (EQUUS-FAP) scores in 50 adult horses undergoing surgical or non-surgical procedures prior to anesthesia (baseline) and in the hours immediately following recovery to standing. Overall scores are significantly greater than baseline at hours 1 and 2 post anesthesia recovery ($p < 0.001$ for both). There was no significant effect of reason for anesthesia.

In a study comparing hydromorphone and butorphanol for analgesia in horses undergoing elective arthroscopy, it was found that horses receiving hydromorphone had CPS scores significantly higher than baseline at 2 h post-anesthetic recovery but returned to baseline at 4 h. Meanwhile, horses receiving butorphanol scored significantly higher than baseline at both 2 and 4 h post anesthetic recovery (12). However, this increase was not observed with the EQUUS-FAP system and the authors speculated that the residual effects of general anesthesia affected the CPS scores at the 2 h timepoint. A similar observation was made in a study comparing buprenorphine and butorphanol for pain management in equine patients undergoing elective surgery. Horses receiving buprenorphine had lower pain scores than butorphanol when using a simple descriptive scale, but this effect did not occur until 3 h following recovery (14). Opioids are known to cause temporary central nervous system excitation immediately following administration in unsedated, non-painful horses (15–17). It is possible that these initial elevated scores were affected by the opioids. However, in the present study the first post-anesthetic pain score was at least 2 h after opioid administration for all horses in the study, and a residual effect of these drugs after this time was unlikely.

The stress response to general anesthesia in horses has been well described with evidence of a substantial adrenocortical response with various anesthetic protocols and patient scenarios (9–11). This stress response may have contributed to the effects on the physiologic measures of the CPS observed here. Partial intravenous anesthesia with ketamine and morphine infusions has been shown to cause increased sympathetic response in comparison to dexmedetomidine infusion with morphine or remifentanil (9). Post-anesthetic cardiopulmonary variables were not monitored in that study, so it is unknown if the enhanced sympathetic tone resulted in altered physiologic response following anesthetic recovery. Therefore, it is possible that augmented sympathetic tone from persisting effects of ketamine in the present study could have contributed to the physiologic and behavioral CPS scores. In cats anesthetized with either alfaxalone or ketamine, post-anesthetic composite pain scale scores were higher in the ketamine group compared to the alfaxalone group (18). In the present study, horses received ketamine for induction of anesthesia and as part of a partial intravenous anesthesia protocol and it is possible that the effect observed may be at least partially attributed to ketamine. However, the horses received several anesthetic agents and it is impossible to determine the role of ketamine in the elevated pain scores observed and further studies are needed to establish this effect.

Difficulty in assessing immediate post-anesthetic pain has also been noted in humans. Ledowski et al. studied the use of a heart rate variability based analgesia nociception index (ANI) as a non-verbal method for post-anesthetic pain assessment in people undergoing non-emergency surgery (19). ANI was compared to the standard self-assessment numeric rating scale (NRS). It was found that ANI was unable to establish different states of acute postoperative pain and the correlation with NRS was deemed weak.

One of the components of pain score validation is construct validity, which is the ability of the test to measure the concept it

was designed to evaluate. A component of construct validity is discriminant validity, showing the ability of the scale to measure only the construct it is designed to evaluate and no other constructs that may be existing simultaneously (20). In our study, the residual effects of anesthesia appear to be captured by both the CPS and EQUUS-FAP scales, resulting in elevated pain scores and revealing weakness in the discriminant validity of the scoring systems in the hours immediately following anesthetic recovery.

Equine CPS scores of 5–8 are considered to be consistent with mild pain, 8–10 moderate pain, and >10 severe pain (4). On average, horses included in this study received scores consistent with mild pain at 1 and 2 h before falling below a score of 5. EQUUS-FAP scores of 3–5 are considered consistent with mild pain, 5–8 moderate pain, and >8 is considered severe (4). On average, horses received scores consistent with mild pain at hour 1 following anesthetic recovery before falling below a score of 3. These results suggest that in order to avoid the effects of anesthetic agents on post-anesthetic pain scoring, the first pain scores should be scheduled after 3 h for the CPS and after 2 h for the EQUUS-FAP following anesthetic recovery to standing. With these results in mind, the analgesic plan for each patient should be tailored in accordance with the patient and the procedure, with knowledge that pain scores may not accurately reflect the patient's pain level until the aforementioned time points. Nevertheless, the effects observed here appear to elevate the scores only to the category of mild pain and analgesia should not be withheld from a patient when the clinician believes that it is warranted based on the procedure and clinical signs.

5. Limitations

This study has some limitations which should be considered. It is unclear what level of post-anesthetic discomfort may have been present in the non-surgical group. All subjects were endotracheally intubated following placement of a mouth gag, hoisted onto a padded table, maintained under anesthesia, hoisted again onto a mattress in the recovery stall, and pulled by the halter and tail to assist the recovery phase. It is possible that horses may suffer some persistent discomfort following anesthetic recovery associated with these events. Nevertheless, all subjects received an NSAID and an opioid prior to anesthesia, infusions of analgesic agents during maintenance of anesthesia, and an alpha-2 agonist in recovery, which should have provided analgesia during the post-anesthetic period. Additionally, the students performing the pain scoring were not blinded to the type of procedure and this may have affected their scores. However, there was no effect of reason for anesthesia (surgical vs. non-surgical procedure) on the pain scores observed. The surgical procedures that horses in this study underwent did not result in significant pain in the post-operative period with the analgesic agents that were administered. Therefore, a difference between groups may have been detected if the horses had undergone more painful surgeries. Nevertheless, the aim of this study was not to establish if pain was effectively managed but rather to determine if general anesthesia affected pain scores. Lastly, as this was an observational study, the subjects were varied in signalment, presentation, and procedure that was performed which may have affected the pain scores.

6. Conclusion

Pain scores as measured by CPS and EQUUS-FAP in equine patients recovering from general anesthesia for surgical and non-surgical procedures are elevated above baseline in the hours immediately following general anesthesia. The results presented here question the validity of these scoring systems in the hours immediately following anesthesia. Further research is indicated to determine if other pain scoring systems may be more useful in pain assessment in the hours immediately following anesthetic recovery.

Data availability statement

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

Ethics statement

Ethical approval was waived by the institution's clinical research committee as the study was purely observational in nature and there was no intervention to any subject as a result of the study.

Author contributions

RR: study conception, study design, data collection, data integrity, data analysis, data interpretation, preparation of the manuscript; AK, RR, BH, MMB, and ML: study design, data collection, data integrity, manuscript preparation; MB, JQ, CB, and SD: study conception and design, data collection, manuscript preparation; DS: study conception, study design, data collection, data analysis, manuscript preparation. All authors contributed to the article and approved the submitted version.

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 Pain Research

TYPE Original Research
PUBLISHED 19 January 2024
DOI 10.3389/fpain.2023.1292299

OPEN ACCESS

EDITED BY
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RECEIVED 11 September 2023
ACCEPTED 08 December 2023
PUBLISHED 19 January 2024

CITATION
Auer U, Kelemen Z, Vogl C, von Ritgen S, Haddad R, Torres Borda L, Gabmaier C, Breteler J and Jenner F (2024) Development, refinement, and validation of an equine musculoskeletal pain scale.
Front. Pain Res. 4:1292299.
doi: 10.3389/fpain.2023.1292299

Development, refinement, and validation of an equine musculoskeletal pain scale

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Musculoskeletal disease is a common cause of chronic pain that is often overlooked and inadequately treated, impacting the quality of life of humans and horses alike. Lameness due to musculoskeletal pain is prevalent in horses, but the perception of pain by owners is low compared with veterinary diagnosis. Therefore, this study aims to establish and validate a pain scale for chronic equine orthopaedic pain that is user-friendly for horse owners and veterinarians to facilitate the identification and monitoring of pain in horses. The newly developed musculoskeletal pain scale (MPS) was applied to 154 horses (mean age 20 ± 6.4 years SD) housed at an equine sanctuary, of which 128 (83%) suffered from chronic orthopaedic disease. To complete the MPS, the horses were observed and videotaped from a distance while at rest in their box or enclosure. In addition, they received a complete clinical and orthopaedic exam. The need for veterinary intervention to address pain (assessed and executed by the sanctuary independent from this study) was used as a longitudinal health outcome to determine the MPS's predictive validity. To determine the interrater agreement, the MPS was scored for a randomly selected subset of 30 horses by six additional blinded raters, three equine veterinary practitioners, and three experienced equestrians. An iterative process was used to refine the tool based on improvements in the MPS's correlation with lameness evaluated at the walk and trot, predictive validity for longitudinal health outcomes, and interrater agreement. The intraclass correlation improved from 0.77 of the original MPS to 0.88 of the refined version (95% confidence interval: 0.8–0.94). The refined MPS correlated significantly with lameness at the walk ($r = 0.44$, $p = 0.001$) and trot ($r = 0.5$, $p < 0.0001$). The refined MPS significantly differed between horses that needed veterinary intervention (mean MPS = 8.6) and those that did not (mean MPS = 5.0, $p = 0.0007$). In summary, the MPS showed good interrater repeatability between expert and lay scorers, significant correlation with lameness at the walk and trot, and good predictive validity for longitudinal health outcomes, confirming its ability to identify horses with orthopaedic health problems.

KEYWORDS

chronic pain, pain scale, musculoskeletal pain, discomfort, horse, equine

1 Introduction

Musculoskeletal disease is the leading cause of chronic pain in horses and humans alike (1–8). In equine veterinary practice, lameness due to musculoskeletal pain ranks as the most prevalent diagnosis (1–4, 9–12). Already, in 4- to 5-year-old riding horses, 24% demonstrated moderate to severe orthopaedic clinical findings (12), emphasizing the widespread nature of the problem. The prevalence further increases in older horses, with 51% of horses above 15 years and 77% of horses aged 30 years and older exhibiting lameness, which is strongly associated with pain experienced at rest (3–5, 11, 13).

Despite their high prevalence, musculoskeletal diseases are frequently overlooked as a source of suffering and, as a result, receive inadequate treatment (2–4, 14–16). Indeed, owners reported lameness in only 16% of horses compared with the 77% diagnosed by veterinarians in the same cohort (11). Similarly, in two other groups of horses in training that were perceived to be sound by their owners, 72.5% and 74% showed movement asymmetry during objective lameness evaluation (17, 18). The owners' low perception of musculoskeletal pain compared with the expert diagnoses is concerning from both a veterinary and welfare perspective. It further compounds the undertreatment of pain also observed in older humans that is associated with the erroneous but widespread societal belief that pain is a natural part of ageing and inevitable in later life (15, 19).

Due to the subjective nature of pain, *gold standard* pain assessment tools in human medicine rely on self-reporting, as direct measurement of individual experiences is not feasible (20, 21). For patients unable to communicate in ways easily understood by their caregivers, such as non-verbal human patients and animals, pain assessment depends on physiological and behavioural indicators (21–27). However, physiologic indicators, including changes in heart and respiratory rate, lack the sensitivity and specificity needed for reliable pain detection and discrimination from other sources of distress (28, 29). Although these indicators are commonly used to indicate the presence of pain, little empirical evidence exists to support this practice, as the correlation of vital sign changes with self-reports is weak, and the absence of changes in vital signs does not necessarily mean the absence of pain (28, 29).

By contrast, research has shown a strong correlation between pain behaviours and patients' verbal pain reports, though external observers tend to underestimate pain intensity (30, 31). Consequently, non-verbal pain behaviours, such as facial expressions, lameness, and guarding, have become integral to pain assessments (22, 24, 27–42). Especially facial expressions, which have been demonstrated to encode both the sensory and affective components of pain, are commonly used to recognize and quantify pain in human and veterinary patients who are unable to verbalize (13, 26, 27, 37–50). Postural and gait adaptations that reduce the load on painful tissue to prevent or alleviate pain and protect from further injury (51) are also strongly associated with orthopaedic pain in humans and horses alike (35, 39, 40). However, despite evidence that guarding and posture may be more indicative of musculoskeletal pain than facial expressions (35, 39, 40), body cues are not routinely included in pain assessment.

As some behavioural changes associated with chronic pain may develop gradually and be subtle, making them most easily detected by someone familiar with the animal and its behaviour before and after the onset of pain (26, 52), the inclusion of caretaker assessments can add important cues to facilitate identification of equine pain. Regular pain assessment by caretakers is also essential to optimize treatment, as chronic musculoskeletal conditions typically require prolonged and often life-long palliative treatment and therapy adjustments to address acute flares and fluctuations in pain intensity while minimizing side effects. Hence, there is a clear need for a pain assessment tool that horse owners and veterinarians alike can use to facilitate the identification of pain, communication between veterinarians and clients, and the evaluation of the effectiveness of pain management interventions. This pain scoring system should be based on objective measures that are sensitive and specific to pain and minimize the potential for observer bias and misinterpretation (20, 23). Keeping in mind horses' instinctive tendency to exhibit little indication of pain in the presence of potential predators, such as humans, and to reduce or relieve pain behaviour even during caretaker visits (46), the pain assessment tool should also be applicable remotely using video surveillance or recordings.

Therefore, this study aims to establish, refine, and validate an orthopaedic pain scale that is easy and fast to use by horse caretakers and veterinarians alike, and can also be used to score pain on videos to minimize observer interference with pain behaviour. Based on recent scientific evidence, the newly developed equine musculoskeletal pain scale (MPS) incorporates components of the equine pain face (27, 38), posture, head–neck position, weight-bearing, and weight shifting to assess orthopaedic pain in horses (33, 36, 39, 40, 44, 47, 53).

2 Materials and methods

This prospective, observational cohort study was designed to refine and validate the newly developed MPS, a tool based on components of the equine pain face (38), recent scientific advances demonstrating the importance of posture, weight-bearing, and head position for chronic pain behaviour (5, 13, 33, 36, 40, 44–47, 53–57), and clinical observations in patients suffering from chronic orthopaedic pain. During scale development and refinement, a panel of six experts (three equine veterinarians and three experienced equestrians) assessed content validity and comprehensibility through iterative evaluations of item relevance, comprehensiveness, and clarity (see Sections 2.3 and 2.5) (58–63). The size of the expert panel was based on previous studies establishing a minimum of four to five experts to be adequate for content validation (63, 64). In addition, item relevance was assessed by calculating the correlation between each item and the total MPS and by an item–total correlation (see Sections 2.3 and 2.7). To evaluate the influence of different sources of variability on the MPSs, reliability was determined by calculating the interrater variability in relation to the horses'

variability and the total variability in a mixed model (see Sections 2.3, 2.5, and 2.7) (58–63). The MPS's construct validity was assessed by calculating the correlation of the MPS with subjective lameness scores at the walk and trot and the objective lameness data (see Section 2.4) (58–63). The scale's criterion validity to predict longitudinal health outcomes was evaluated by comparing the MPS score of horses that required veterinary intervention in the subsequent months with those that did not need medical treatment (see Section 2.4) (58–63). Lastly, the scale's responsiveness was assessed by comparing the MPSs of horses that received analgesia before and after treatment (58–63).

2.1 Horses

A total of 154 horses living at an equine sanctuary were included in this study. The horses were maintained in their familiar environment and husbandry conditions, and neither the horses' housing, turn-out, or feeding regime nor any other management factors or veterinary treatments were affected by the study or changed for study purposes.

Before inclusion in the study, all horses underwent an in-depth physical exam. The horses suffering from non-orthopaedic causes of pain or cardiovascular (e.g., ventricular tachycardia) or gastrointestinal (e.g., delayed gastric emptying) disease were excluded from the study.

2.2 Horse examination and assessment parameters

All the horses were examined by the same veterinarian and received a complete clinical and orthopaedic exam in addition to an MPS. To complete the MPS, the horses were discreetly observed and videotaped from a suitable distance (5–10 m), displaying no awareness or curiosity towards the observer, while at rest in their box or enclosure (paddock or pasture). The MPS uses an ordinal scale to measure demeanour (13, 25, 26, 40, 44–47, 49, 65–67), pain face (22, 27, 38, 68, 69), weight shifting, weight-bearing, head–neck posture, limb posture (25, 33, 36, 44, 47, 53, 56, 57), and lameness that is evident while observing the horse from a distance in its enclosure (Supplementary Figure S1). Examinations involving direct interaction with the horses were conducted only after all initial distant observations were finished. This approach was taken to minimize any potential influence on the horses' behaviour and any biasing of the MPS results by the examination process.

The orthopaedic exam included a subjective lameness evaluation grading the lameness at the walk and trot separately on a scale from 0 (sound) to 5 (non-weight-bearing) (70). The horses that were unable to trot because of severe lameness were assigned a score of 5 for the lameness at the trot. Horses with a lameness score >2 at the walk and ≥3 at the trot were considered moderately to severely lame.

In addition, the lameness was assessed objectively in 110 horses (71.4%) using a commercially available multi-sensor inertial gait analysis system (Lameness Locator®, Equinosis, USA) that has been validated to detect and quantify equine lameness (71–76). The horses were considered lame with a Q-score, a metric quantifying movement asymmetry amplitude, >8.5 mm and moderately to severely lame with a Q-score >30 mm (74–80).

2.3 Musculoskeletal pain scale—descriptive statistics

To characterize the MPS and its items, a correlation matrix was calculated among pairs of scores of items and the total using the non-parametric Spearman and the parametric Pearson correlations. In addition, a principal component analysis was calculated to further characterize the relationships among items. Both methods allow evaluation of which variables contribute independently or jointly to the total MPS.

2.4 Musculoskeletal pain scale—validity and predictive performance

The primary measures for assessing the validity of the MPS were the subjective and objective lameness scores. The correlations between the MPS with subjective lameness at the walk and trot and the objective lameness data were calculated using moderate to severe lameness as an indicator of pain.

To assess the MPS's predictive performance for longitudinal health outcomes, the MPSs of horses that required veterinary intervention [analgesia (firocoxib or phenylbutazone) or euthanasia for pain that was unresponsive to treatment] in the months following the exam were compared retrospectively with those that did not need medical treatment. The need for veterinary intervention was determined by the nursing and veterinary staff of the sanctuary based on their independent assessment of the horses' health and pain status, thereby providing an outcome variable independent from the study.

2.5 Musculoskeletal pain scale—interrater agreement and refinement

To ensure the inclusion of horses representing the entire spectrum of pain grades in the interrater agreement analysis, the horses were considered pain-free if their MPS was ≤3 ($n = 64$), mildly painful if their MPS was between 4 and ≤8 ($n = 69$), and moderately to severely painful if their MPS was ≥9 ($n = 20$). A subset of 30 horses, 10 of each pain group, was randomly selected using the GraphPad® random selection tool (https://www.graphpad.com/quickcalcs/randomselect2/). To determine the interrater agreement, six additional raters, three equine veterinary practitioners and three experienced equestrians, completed the MPSs for these 30 horses. The six additional raters were blinded to the horses' medical history, pain group, and exam results and completed the MPS based solely on anonymized videos obtained during the exam. Interrater reliability was assessed using intraclass correlation (ICC) analysis.

Based on the interrater agreement and their feedback regarding the clarity of the item descriptions and the scoring process, the MPS was refined to optimize the discriminative power of the items to ensure unequivocal definitions of each item to limit the potential for misinterpretation and to shorten the time required to complete the MPS to enhance its clinical and research utility. An iterative process was used in tool refinement, considering improvements achieved (content, construct, and criterion validity, comprehensiveness, comprehensibility, reliability, interrater agreement) compared with the original tool when replacing existing items or adding items. Item redundancy was investigated using correlation and principal component analysis. The refined MPS measures seven items on an ordinal scale and can accumulate a maximum score of 26 points, 2 for demeanour, 4 for a pain face, 2 for head–neck posture, 4 for weight shifting, 6 for limb posture, 4 for weight-bearing, and 4 for lameness that is evident while observing the horse from a distance in its enclosure (English version: Figure 1, German version: Supplementary Figure S2). The refined tool was tested in a new randomly selected subset of 30 horses representing the three pain groups ($n = 10$ per group) to assess the interrater agreement using ICC analysis and with a mixed model with rater-ID and horse-ID as random variables (see also Section 2.7). For the ICC, interrater agreement was considered to be very good (for scores 0.81–1.0), good (0.61–0.80), moderate (0.41–0.60), reasonable (0.21–0.4), or poor (<0.2) (81). Based on the excellent interrater agreement for the MPS's lameness item established in the first validation step (ICC score 0.83), only observers one and two rated the lameness item, which had not been changed during the refinement process, as part of the last iteration of the MPS.

2.6 Refined musculoskeletal pain scale—validity and discriminative power

To assess the validity and discriminative power of the refined MPS, the refined MPS was completed for 60 video-recorded behavioural observations that had not been included in the first validation step (see Section 2.4). The construct validity was determined by correlation analysis of the MPS with the subjective lameness at the walk and the trot. The refined MPS's criterion validity to predict longitudinal health outcomes was calculated by comparing the MPSs of the horses that required veterinary intervention with those that did not, using a Mann–Whitney *U* test. The MPS's responsiveness, its ability to discriminate between before and after treatment, was assessed by comparing the MPSs of the horses receiving analgesia before and after intervention.

Receiver operating characteristic (ROC) analysis was utilized to evaluate the global performance of the MPS in discriminating between lame and sound horses and between horses that needed veterinary intervention and those that did not. In addition, the ROC was used to determine cut-off values that minimize misclassification errors (82–84). The optimal cut-off for discriminating between horses suffering from pain and those without any pain was identified as the value where the sum of the sensitivity [=probability of a positive test outcome in a horse that is in pain (true-positive)] and specificity [=probability of a negative test outcome in a pain-free horse (true-negative)] was maximized. If two cut-off values yielded similar sums of sensitivity and specificity, the cut-off with the higher sensitivity was chosen to maximize the likelihood of identifying horses suffering from pain for further diagnostics and therapy if required. Since the MPS item lameness can only be assessed if the horse is moving in its stall or enclosure without being prompted, scoring this item may not always be possible, which could result in a lower maximum MPS. Therefore, we determined the cut-off value for the refined MPS, both including and excluding the lameness item.

2.7 Statistical analyses

Statistical analyses were carried out using GraphPad Prism (version 10.0.2, GraphPad Software LLC, Boston, MA, USA), NCSS 2020 Statistical Software (NCSS, LLC. Kaysville, UT, USA), and the "R" statistical programming language (R Foundation for Statistical Computing, Vienna, Austria, https://www.R-project.org/) (85). The D'Agostino–Pearson, Shapiro–Wilk, Anderson–Darling, and Kolmogorov–Smirnov tests were computed to assess whether data were normally distributed. The *t*-test, ANOVA, and Pearson's correlation test were used for normally distributed parameters, whereas for parameters that were not normally distributed, the Mann–Whitney *U*, Kruskal–Wallis, and the Spearman correlation tests were calculated. A principal component analysis was performed to describe the relationships among the MPS items. For the item–total correlation, the value of the focal item was correlated with the MPS minus the value of the focal item. Furthermore, a mixed model with horse and rater as random effects was computed to evaluate the relative contribution of each to the total variation. The p-values < 0.05 were considered statistically significant. For correlation analysis, correlation coefficients $|r| < 0.3$, $0.3 \leq |r| \leq 0.8$, and $|r| > 0.8$ were considered to indicate weak, significant, and strong correlations, respectively. For ROC analysis, the concordance statistic (c-statistic, equivalent to the area under the ROC curve) represents the probability that a randomly selected patient will have a higher test result than a randomly selected control. It is utilized as a measure of the global accuracy of a diagnostic test and is considered to indicate low, moderate, and high test accuracy at values of $0.5 < c \leq 0.7$, $0.7 < c \leq 0.9$, and $c > 0.9$, respectively (82–84).

3 Results

3.1 Horses

The 154 horses included 67 warmbloods, 25 draft, 25 Arabian, 18 Haflinger horses, and 19 horses of other breeds. The horses were 2–32 years old [mean: 20 years, SD: 6.4 years, median: 21 years, interquartile range (IQR): 16–26 years]. Of the 154 horses, 128 (83%) suffered from chronic orthopaedic disease, such as

10.3389/fpain.2023.1292299

Discomfort / Pain score®

vetmeduni vienna

To be completed while observing the horse (in its stall, paddock,…) from a distance! **Horse:**_____ ___ **Date:**_____

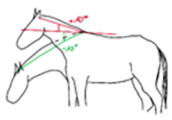
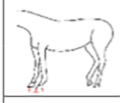
Demeanor (0-2 points)	**Bright, alert and responsive:** interested in and attentive to surroundings, ears move inquisitively, appropriate interactions with and responses to environmental stimuli e.g., ears moving in response to noises		0
	Quiet, alert and responsive: appropriate interactions with and responses to environmental stimuli		0
	Dozing/sleeping: standing quietly, head may be low, eyelids and lips may droop, response to environmental stimuli can be delayed		0
	Depressed/withdrawn: eyes open but dull and unfocused gaze, standing motionless, ears not moving, indifferent and unresponsive to environmental stimuli		2
Pain face (0-4 points)		No pain face: the muzzle, nostrils, chewing muscles and muscles above the eye are relaxed, the nostrils are elongated comma-shaped	0
		Signs of a pain face are present (e.g., dilated nostrils, tense muscles above the eyes, tense chewing muscles, ears flattened, lips pressed together) but disappear in response to the presence of humans or other environmental stimuli (e.g., noise)	2
		Signs of a pain face are present and do not disappear in response to the presence of humans or other environmental stimuli (e.g., noise)	4
Head/neck posture (0-2 points)		Head (using the base of the ears as point of reference) is at the same height as the withers or slightly above/below (angle 0 +/- 10□)	0
		Head is held high, clearly (angle > 10□) above the withers	2
		Head is held low, clearly (angle > 10□) below the withers	2
Limb Posture (add all applicable points, 0-6 points)		Weight bearing on all 4 limbs, the horse stands in a rectangle with its forelimbs and hindlimbs parallel to each other and all 4 cannon bones (MCIII and MTIII) visually appr. vertical to the ground (looking at the horse from the side), may rest/cock a hindlimb (may switch rested hindlimb occasionally)	0
		Forelimbs are not parallel to each other (looking at the horse from the side) but the hooves are placed more than one hoof length (10-15cm) apart	2
		Hindlimbs are not parallel to each other (looking at the horse from the side) but the hooves are placed more than two hoof lengths (20-30cm) apart	2
		Both front cannon bones are not vertical (appr. 90□) to the ground, the forelimbs are camped out (placed in front of the vertical) or camped under (placed behind the vertical)	2
		Both rear cannon bones are not vertical (appr. 90□) to the ground, the hindlimbs are camped under (placed in front of the vertical) or camped out (placed behind the vertical)	2
weight shifting (0-4 points)	No weight shifting		0
	Weight shifting from one limb to another (fore- or hindlimbs) 2- 4 x/ in 2 minutes (weight shifting by moving hooves or lateral movement of head/neck)		2
	Frequent weight shifting from one limb to another (fore- or hindlimbs) > 4 x/ 2min		4
weight bearing (0-4 points)		Weight bearing on all 4 limbs, may rest/cock a hindlimb (may switch rested hindlimb occasionally)	0
		Repeated lifting of a limb for a few seconds	4
		One limb is only minimally weight bearing or non- weight bearing	4
Lameness at the walk (0-4 points, assess only if the horse is moving without being prompted)	Horse does not move → lameness cannot be assessed		NA
	Normal movement, no lameness evident		0
	Stiff gait, slow, decreased range of motion, no clear lameness, (may be lame when turning)		2
	Clearly lame		4

FIGURE 1
Refined musculoskeletal pain scale.

osteoarthritis ($n = 74/154$, 48%), tendinopathy ($n = 29/154$, 19%), or laminitis ($n = 25/154$, 16%) according to their medical records.

3.2 Horse examination and assessment parameters

The 154 horses had a mean original MPS of 4.8 (SD: 3.0, range: 0–14, median: 4.0, IQR: 2.8–7.0). The 11 horses unable to trot were scored 5 for the subjective lameness exam at the trot and assigned a Q-score of 115 (10% higher than the maximum measured Q-score of 104.7). The mean subjective lameness score (maximum of the four limbs, scale of 0–5) at the walk was 1.6 (SD: 1.2, range: 0–5, median: 2.0, IQR: 0.75–2.0) and at the trot, 2.2 (SD: 1.1, range: 0–5, median: 2.0, IQR: 2.0–3.0). The mean Q-score of the objective lameness exam was 28 mm (SD: 35 mm, range: 0.0–104.7 mm, median: 13 mm, IQR: 8.4–23 mm).

The cohort of 60 horses used to validate the refined MPS had a mean MPS of 6.9 (SD: 4.3, range: 0.0–18.0, median: 6.0, IQR: 4.0–10.0) and a mean lameness of 2.1 at the walk (SD: 1.2, range: 0.0–4.0, median: 2.0, IQR: 1.0–3.0) and 2.7 at the trot (SD: 1.6, range: 0.0–5.0, median: 3.0, IQR: 2.0–3.0). One horse was excluded from the lameness exam owing to chronic ataxia.

3.3 Musculoskeletal pain scale—descriptive statistics

The correlations among items and their contribution to the total score were assessed using correlation analysis and principal component analysis. The correlation analysis using the non-parametric Spearman and the parametric Pearson correlations showed negligible or positive correlations of varying strengths among the items of the original MPS (Figure 2). In the original MPS, the item "location in the box/enclosure" correlated little with the other items ($r < 0.25$) and had a correspondingly low item–total correlation of 0.092, while the other items correlated with a correlation coefficient between about 0.3–0.4 and had item–total correlations between 0.18 and 0.445. The correlation of the items with the total original MPS is also shown, from which it is evident that location, demeanour, and head–neck posture have a lower correlation than lameness, weight distribution, and pain face, which show pairwise correlations in the range 0.25–0.75. Using the non-parametric Spearman or the parametric Pearson correlation produced qualitatively and quantitatively similar patterns. As expected from the correlation analysis, all subitems, except demeanour and location, contribute positively to the first principal component, especially lameness and pain face, which load highly (Table 1), while they load with opposite signs on the second principal component. All other items generally dominate one further principal component.

3.4 Musculoskeletal pain scale—validity and predictive performance

The MPS correlated significantly with the subjective lameness score at the walk (Spearman $r = 0.51$, $p < 0.0001$) and the trot (Spearman $r = 0.45$, $p < 0.0001$) and the objective lameness measurements (Spearman $r = 0.37$, $p = 0.0001$, $p \leq 0.0001$).

The MPS was significantly different (difference between means: 2.1 ± 0.5 SEM, $p < 0.0001$) between horses that needed veterinary intervention (mean MPS: 6.2, $n = 49$) and those that did not (mean MPS: 4.1, $n = 105$). The MPS was also significantly

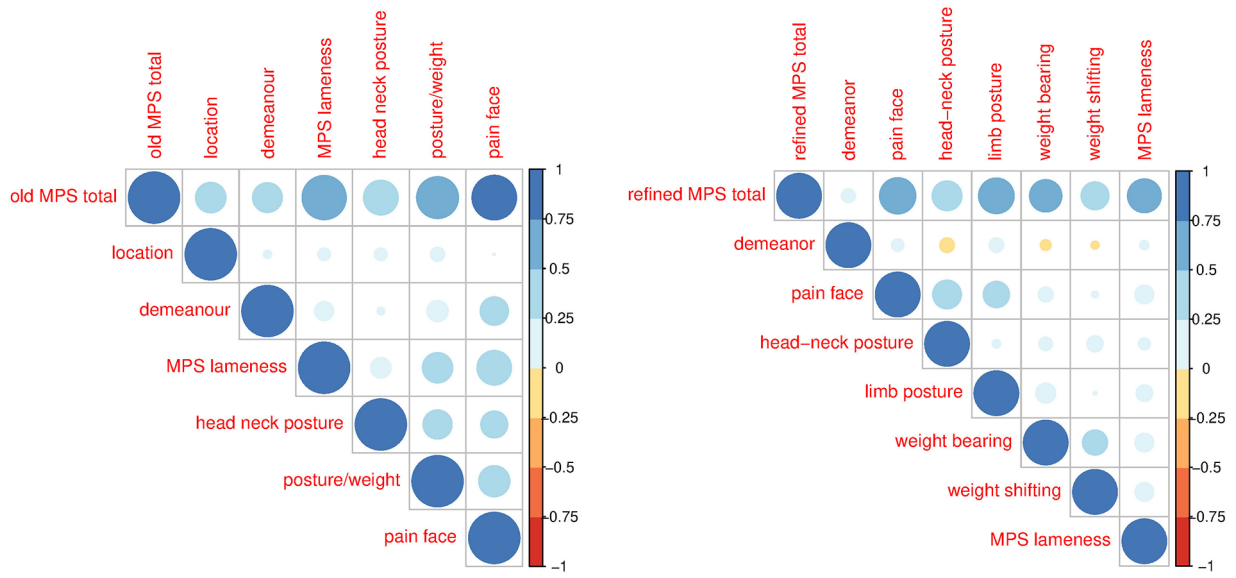


FIGURE 2
Pearson correlations for the original (left) and refined (right) MPS for the total and the various sub-items of the MPS. The colours and sizes of the balls indicate the strength of the correlation.

TABLE 1 The proportion of the principal components indicating their relative importance are given in the first row. The loadings of the items, reflecting their contributions to the principal components, are given in the following for the original (A) and refined (B) MPS. Values below 0.1 are not reported, i.e., left blank.

A							
Component		1	2	3	4	5	6
Location			0.592	0.767	0.243		
Demeanour						0.109	−0.990
Pain face		0.604	−0.605	0.299	0.404		
Head–neck posture		0.112		0.139	−0.164	−0.963	
Limb posture/weight-bearing/shifting		0.331		0.293	−0.864	0.229	
Lameness		0.712	0.521	−0.465			
Proportion of variance		0.483	0.171	0.159	0.119	0.049	0.019
B							
Component	1	2	3	4	5	6	7
Demeanour							0.998
Pain face	0.460		−0.747		0.379	0.281	
Head–neck posture	0.127		−0.479	−0.140	−0.670	−0.529	
Limb posture	0.767	0.467	0.390		−0.176		
Weight-bearing	0.204	−0.133	0.108	−0.845	0.371	−0.276	
Weight shifting		−0.166		−0.416	−0.489	0.746	
Lameness	0.371	−0.856	0.221	0.276			
Proportion of variance	0.369	0.218	0.163	0.124	0.0653	0.0549	0.006

different (difference between means: 4.3 ± 0.5 SEM, $p < 0.0001$) between horses that were clearly lame at the walk (lameness ≥3, mean MPS: 8.3, $n = 29$) and those that were not or only mildly lame (grade 0–2, mean MPS: 4.0, $n = 125$). Similarly, the MPS was significantly different (difference between means: 2.9 ± 0.5 SEM, $p < 0.0001$) between horses that were clearly lame at the trot (lameness ≥3, mean MPS: 6.8, $n = 45$) and those that were not or only mildly lame (grade 0–2, mean MPS: 3.9, $n = 109$). Furthermore, the MPS was significantly different between objectively measured lameness scores, specifically between no lameness (Q-score ≤ 8.5, mean MPS: 3.5) and moderate to severe lameness (Q-score > 30, mean MPS: 7.9, $p < 0.0001$), and between mild (Q-scores 8.5–30, mean MPS: 4.1) and moderate to severe lameness ($p < 0.0001$), but not between mild and no lameness.

3.5 Musculoskeletal pain scale—interrater agreement and refinement

During the refinement process, the item "location in the box/enclosure" was dropped as it was not consistently possible to reliably rate the location on videos, and the MPS was intended to allow for remote scoring to avoid the confounding effect of rater presence on horses' behaviour. The original item, scoring limb posture, weight-bearing, and weight shifting together were divided into three items in the refined tool. The definition of the other items was optimized based on feedback from the raters and interrater agreement to optimize clarity and minimize the potential for misinterpretation. The time required to complete pain assessment was reduced from 7 min with the original MPS to 2 min with the refined MPS, hence enhancing its user-friendliness and corresponding clinical and research utility.

Correlations among the items of the refined MPSs (Figure 2) were lower than for the original MPS, indicating lower item redundancy; demeanour correlated little with the other items ($r < 0.25$); pain face correlated moderately with head–neck and limb posture, and weight-bearing correlated with weight shifting, all with a correlation coefficient between 0.3 and 0.4. The correlation of the items with the total refined MPS is below 0.1 for demeanour and between 0.25 and 0.6 for the other items. The item–total correlation for demeanour was only 0.053, that of the rest of the items between 0.19 (weight shifting) and 0.464 (pain face). Using the non-parametric Spearman or the parametric Pearson correlation produced qualitatively and quantitatively similar patterns. The first principal component has positive loadings for all items of the refined score except demeanour and weight shifting (Table 1). While the item demeanour dominates one principal component of the refined MPS, the loadings of all other items are generally more dispersed over the various other components than for the original MPS. The residual variance can be attributed to the variation in the video quality and hence as technical variance.

The intraclass correlation increased from 0.77 [95% confidence interval (CI): 0.62–0.88] for the original MPS to 0.88 (95% CI: 0.80–0–94) for the refined MPS tool (Supplementary Table S1).

A mixed model analysis evaluated the variance due to variability among horses, among raters, and the residual variance (Table 2). The variability among horses is more than five times higher than that among raters for the total scores as well as

TABLE 2 Mixed model analysis of the total refined MPS and its various subitems.

Variance component	Horse-ID	Rater-ID	Residual
Refined MPS	6.935	0.820	5.313
Demeanour	0.020	0.003	0.158
Pain face	0.555	0.004	1.228
Head–neck posture	0.301	0.003	0.502
Limb posture	1.116	0.482	1.260
Weight-bearing	0.771	0.331	1.429
Weight shifting	0.523	0.000	0.333
Lameness	-	-	-

lameness, head–neck posture, and limb posture, indicating that these variables can be reliably scored by the different raters. The ratio is less favourable for pain face, weight-bearing, and weight shifting. The residual variance can be attributed to the variation in the video quality and hence as technical variance.

3.6 Refined musculoskeletal pain scale—validity and discriminative power

The refined MPS was validated by its significant correlation with the subjective lameness score in walk (Spearman $r = 0.44$, $p = 0.001$) and trot ($r = 0.5$, $p < 0.0001$).

The MPS was significantly different (difference between means: 3.8 ± 1.1 SEM, $p = 0.0009$) between horses that were clearly lame in walk (lameness ≥3, mean MPS: 9.3, $n = 21$) and those that were not or only mildly lame (grade 0–2, mean MPS: 5.6, $n = 38$, Figure 3). Furthermore, the MPS was significantly different (difference between means: 4.2 ± 0.99 SEM, $p < 0.0001$) between horses that were clearly lame at the trot (lameness ≥3, mean MPS: 8.9, $n = 31$) and those that were not or only mildly lame (grade 0–2, mean MPS: 4.7, $n = 28$, Figure 3).

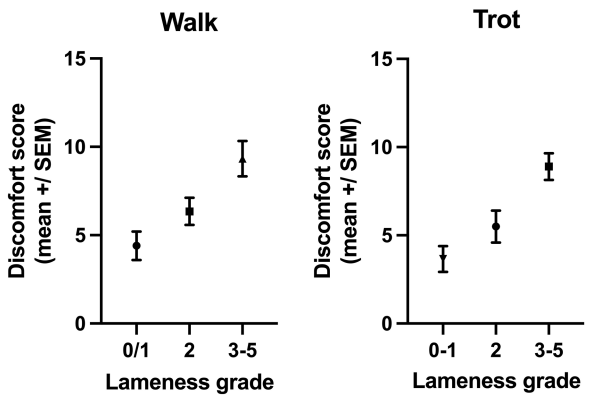


FIGURE 3
The MPSs of horses with a lameness ≥3 at the walk ($p = 0.0009$) or trot ($p < 0.0001$) were significantly higher than the MPSs of horses with no or mild lameness (lameness 0–2).

The refined MPS was also significantly different (difference between means: 3.6 ± 1.0 SEM, $p = 0.0007$) between horses that needed veterinary intervention (mean MPS: 8.6, $n = 31$) and those that did not (mean MPS: 5.0, $n = 29$, Figure 4), hence establishing the predictive performance of the MPS for longitudinal health outcomes.

Lastly, the MPS was significantly different (difference between means: 3.2 ± 1.093 SEM, $p = 0.0168$) in horses receiving analgesia between before and after treatment, confirming its responsiveness (Figure 4).

ROC analysis showed the refined MPS to be moderately accurate in discriminating between horses that needed veterinary intervention and those that did not, with a c-statistic of 0.74 (standard error: 0.064, $p = 0.0002$). The ROC analysis yielded several possible MPS cut-off values with corresponding trade-offs in sensitivity and specificity (Table 3, Figure 4, Supplementary Table S2), with an MPS cut-off >8 providing the best overall combination of sensitivity (51.61%, 95% CI: 34.84%–68.03%) and specificity (89.66%, 95% CI: 73.61%–96.42%) with a Youden index (=specificity plus sensitivity minus one) of 0.41, indicating that horses with an MPS of 8 or greater have a high probability of a painful condition and therefore should be further examined.

Without the item lameness, ROC analysis (*c*-statistic: 0.65, standard error: 0.071, $p = 0.034$) showed that an MPS cut-off of >4 (Youden index of 0.264) yielded the best combination of sensitivity (67.74%, 95% CI: 51.6%–74.2%) and specificity (58.62%, 95% CI: 48.4%–75.5%, Supplementary Figure S2).

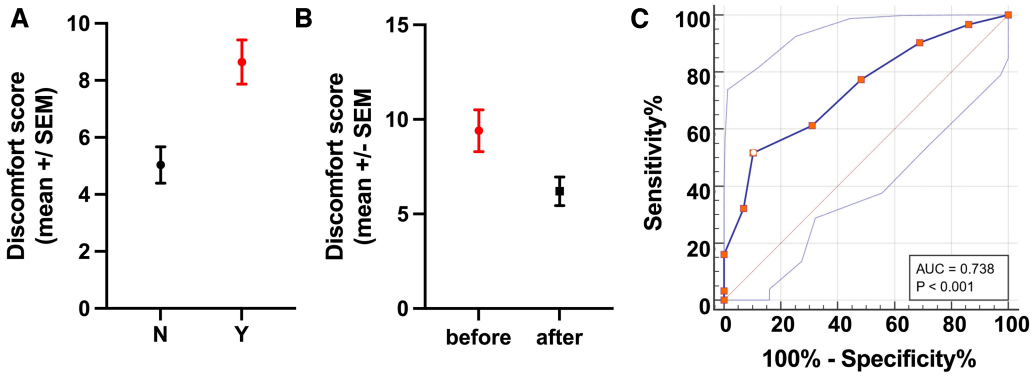


FIGURE 4
Predicative performance and discriminative power for longitudinal health outcomes. (**A**) The refined MPS showed good discriminative power for longitudinal health outcomes as demonstrated by the significant difference ($p = 0.0007$) in the MPSs of horses that needed veterinary interventions (Y, red) compared with those that did not (N, black). (**B**) Evaluation of the MPS responsiveness showed a significant difference ($p = 0.0168$) in the MPSs of horses receiving analgesia before and after treatment. (**C**) The ROC curve plots 100% – specificity% vs. sensitivity% for each MPS value. Using a cut-off for the MPS >8 (shown as a white dot with red border) yields a sensitivity of 51.61% and a specificity of 89.66% (Youden index: 0.41) for identifying horses in need of veterinary intervention (analgesia or euthanasia for unrelenting pain). Thus, horses with an MPS of 8 or above are likely to have a painful condition and should be further examined.

TABLE 3 Sensitivity and specificity plus 95% CI calculated by ROC analysis for each MPS cut-off value for identifying horses needing veterinary intervention.

Cut-off	Sensitivity (%)	95% CI	Specificity (%)	95% CI	Youden index	Likelihood ratio
Differentiation between horses that needed medical intervention and those that did not						
>0	96.77	83.81%–99.83%	13.79	5.5%–30.56%	0.11	1.123
>2.0	90.37	75.10%–96.65%	31.03	17.28%–49.23%	0.21	1.31
>4.0	77.42	60.19%–88.60%	51.72	34.43%–68.61%	0.29	1.6
>6.0	61.29	43.82%–76.27%	68.97	50.77%–82.72%	0.3	1.975
>8.0	51.61	34.84%–68.03%	89.66	73.61%–96.42%	**0.41**	4.989
>10.0	32.26	18.57%–49.86%	93.1	78.04%–98.77%	0.25	4.677
>12.0	16.13	7.093%–32.63%	100.0	88.30%–100.0%	0.16	
>16.0	3.226	0.166%–16.19%	100.0	88.30%–100.0%	0.03	

The best Youden index (the sum of sensitivity and specificity – 1) is indicated in bold.

The difference in the MPSs (difference between means: 2.0 ± 0.9) between horses that needed veterinary intervention (mean: 6.3) and those that did not (mean: 4.3) remained statistically significant also without the lameness item ($p = 0.0331$, Supplementary Figure S2).

4 Discussion

The high prevalence and impact of chronic musculoskeletal conditions and the poor recognition of lameness and the associated pain necessitate the inclusion of pain as a fourth vital sign in the routine evaluation of all horses to facilitate appropriate treatment and improve equine welfare (52, 86, 87). Regular pain assessment using a reliable, valid, and clinically useful tool would enable the identification of pain, timely interventions, monitoring treatment effects, and facilitate communication among veterinarians and caretakers. This study established, refined, and validated a multidimensional MPS and demonstrated its predictive performance for longitudinal health outcomes, discriminative power, and good interrater agreement between veterinary practitioners and equestrian raters. The MPS was validated as a measure for equestrians and veterinarians alike to assess horses for the presence of painful conditions and monitor the efficacy of treatment interventions, not just in a hospital setting but especially also in their home environment. The MPS is based on several pain behaviours, including features of the equine pain face, postural indicators, demeanour, and lameness to accommodate for individual variations in pain behaviour and differences in pain behaviours between acute and chronic pain (13, 26, 40, 41, 88–91).

Pain is a complex, uniquely individual, unpleasant experience associated with actual or potential tissue damage, encompassing both sensory (intensity) and affective (unpleasantness) components (92). The affective dimension of pain is associated with behavioural changes aimed at avoiding pain and minimizing injury (23, 54, 93). While acute pain is a protective response to noxious stimuli, chronic pain persists beyond the expected healing time and may be either a symptom of chronic peripheral disease, maladaptive nervous system dysfunction, or both (89, 90, 94–100). Acute pain tends to respond to anti-inflammatory pain relief. Decreasing the load on the affected area by postural adaptations and guarding during movement and at rest may reduce acute pain (13, 40, 41). By contrast, in chronic pain, due to the central sensitization that is often present, the degree, duration, and spatial extent of pain may be increased and distorted, leading to more widespread pain and multisite hyperalgesia and allodynia (13, 40, 86, 88, 89, 95, 101–103). Therefore, once central sensitization has occurred, pain perception may no longer reflect the presence, intensity, or duration of peripheral noxious inputs (103, 104). Accordingly, behavioural changes associated with chronic pain can vary greatly, necessitating a multidimensional pain assessment tool that includes the effect of pain on demeanour, functional assessments (lameness), and different pain behaviours (24, 41, 86, 89–91, 94–97, 99, 105, 106). Pain behaviours can be categorized into two overlapping groups: protective and communicative pain behaviours (106). Protective pain behaviours, such as postural adjustments and guarding, are often directly associated with the painful area (13, 40). By contrast, communicative pain behaviours, including facial expressions, are universal indicators of pain and mechanisms for communicating pain to conspecifics (13, 40).

Posture, the dynamic alignment and positioning of the body orchestrated by the neuromuscular system, is often mistakenly conflated with conformation, which pertains to the static skeletal architecture and body proportions (13, 33, 40, 45, 47, 48, 107, 108), confounding research into the complex interplay between posture and musculoskeletal pain. Posture facilitates efficient weight distribution across the musculoskeletal system, balancing the centre of gravity over the base of support to minimize energy expenditure and stress on anatomical structures (33, 109, 110). As symmetrical loading of the limbs provides the greatest biomechanical stability and hence requires the least corrective actions and energy to maintain balance (110), sound horses exhibit a symmetrical weight distribution, with approximately 60% of the weight borne by the forelimbs and 40%, by the hindlimbs (33, 111, 112). Conversely, horses afflicted with orthopaedic conditions may attempt to alleviate pain by shifting the weight away from the affected limb, effectively altering their centre of gravity (113). Weight-bearing and stance asymmetry may therefore signal pain relieved by adopting this posture (112). Similarly, an elevated neck posture has been identified as a potential indicator of underlying back disorders in horses (53, 56, 57, 107, 114, 115). Therefore, the MPS includes head–neck posture, limb posture, weight-bearing, and weight shifting as separate items to reflect the postural adaptations commonly

observed in response to orthopaedic pain. We note that due to the complexity of pain, a single item may not correlate highly with other items or show a low item–total correlation, i.e., pain may represent more than one dimension. This may explain why the factor loadings are relatively low and the proportion of the variance explained by the principle components is rather even, especially with the refined MPS. In particular, pain face may integrate many aspects of pain, while other items may reflect a specific condition or individualized reaction.

The horses' interaction with humans may also be variably affected by pain (25, 38, 44). Depending on the intensity of the noxious stimuli and the familiarity of the environment and observer, painful horses may either be reluctant to interact with humans or increase their contact-seeking behaviour (25, 38, 44). Conversely, the horses may reduce or relieve pain behaviour when people approach or interact (46), which can lead to underestimation of the pain and subsequent therapeutic deficits and welfare problems. Therefore, the MPS was designed to be applicable from a distance to avoid disrupting pain behaviours.

This study established the content, criterion, and construct validity of the MPS using an expert panel for content validation, correlation with lameness for criterion validation, and correlation with longitudinal health outcomes for construct validation. As criterion validation assesses how accurately a scale reflects the *gold standard* for measuring the same construct (63), the lacking *gold standard* or other previously validated method for measuring the individual experience of pain is one of the main limitations of this study, which uses lameness as an indicator of orthopaedic pain. Although lameness is a reliable indicator of pain, the absence of overt lameness does not exclude the possibility of pain. This limitation, the lacking gold standard and objective, of quantitative pain measurement also extends to the evaluation of the MPS's construct validity, the assessment of its ability to discriminate between horses in pain and pain-free horses (63). This study uses the need for veterinary intervention, identified by the staff of the sanctuary, to assess the MPS's construct validity. The inherent subjectivity of this assessment is however mitigated by the horses statistically significant reduction in pain in response to analgesia. However, multicentre studies using larger patient cohorts are needed to further evaluate the MPS's utility to identify horses in pain in various husbandry and demographic settings.

While the MPS is a quantitative tool, it is crucial to recognize that pain expression does not directly correlate with the severity of tissue damage but reflects horses' individual experience and personality and that many pain behaviours are part of the communication repertoire of healthy horses as well (26). However, the MPS correlates well with lameness at the walk and trot and showed very good predictive performance for longitudinal health outcomes and discriminative power in identifying lame horses and horses needing veterinary intervention. In this cohort, the maximum MPS was 18 (the horse was 3/5 lame at the walk and too lame to trot), the maximum score of 26 was not reached by any horse, possibly because no horse in this study suffered from severe pain. Based on the ROC analysis and the differences between horses exhibiting obvious lameness (≥3 on a scale of 0–5) or requiring veterinary intervention, as opposed to horses with minimal or no observable health concerns, horses with an MPS exceeding 8 (or 4 if the lameness item cannot be assessed) should undergo further examination to identify the underlying cause and determine if treatment is necessary.

5 Conclusions

In summary, the MPS showed good interrater repeatability between expert and lay scorers, significant correlation with lameness at the walk and trot, and good predictive validity for longitudinal health outcomes, confirming its ability to identify horses with musculoskeletal pain. Given the prevalence of chronic musculoskeletal conditions, the poor recognition of lameness, and the suffering caused by unrelieved pain, pain assessment should be included in all veterinary examinations, and caretakers should regularly evaluate their horse's pain status to facilitate timely therapeutic interventions. Routine pain assessment using a reliable and validated tool may help address the widespread problem of unrelieved pain already voiced by the philosopher Michel de Montaigne in 1589: "For heaven's sake, let medicine someday give me some good and perceptible relief and you will see how I shall cry out in good earnest: At last I yield to an efficient science."

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 requirement of ethical approval was waived by the Institutional Ethics Committee of the University of Veterinary Medicine, Vienna, for the studies involving animals because this study was non-invasive and entailed only monitoring the horses under their current conditions of life. No specific veterinary treatments or interventions were carried out for the purpose of this study. The studies were conducted in accordance with the local legislation and institutional requirements.

Author contributions

UA: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. ZK: Data curation, Investigation, Project administration, Writing – review & editing. CV: Formal analysis, Methodology, Software, Validation, Visualization, Writing – review & editing. SR: Investigation, Writing – review & editing. RH: Investigation, Writing – review & editing. LT: Investigation, Writing – review & editing. CG: Investigation, Writing – review & editing. JB: Investigation,

Writing – review & editing. FJ: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing.

Funding

The authors 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

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpain.2023.1292299/full#supplementary-material

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frontiers | Frontiers in Pain Research

TYPE Original Research
PUBLISHED 19 February 2024
DOI 10.3389/fpain.2024.1347548

OPEN ACCESS

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RECEIVED 01 December 2023
ACCEPTED 08 February 2024
PUBLISHED 19 February 2024

CITATION
Haralambus R, Juri M, Mokry A and Jenner F (2024) The impact of opioid administration on the incidence of postanaesthetic colic in horses.
Front. Pain Res. 5:1347548.
doi: 10.3389/fpain.2024.1347548

The impact of opioid administration on the incidence of postanaesthetic colic in horses

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Effective management of postoperative pain is essential to ensure patient welfare, reduce morbidity and optimize recovery. Opioids are effective in managing moderate to severe pain in horses but concerns over their adverse effects on gastrointestinal (GI) motility and associated increased colic risk limit their widespread use. Studies investigating the impact of systemic opioids on both GI motility and colic incidence in horses have yielded inconclusive outcomes. Therefore, this retrospective study aims to assess the influence of systemic administration of butorphanol, morphine, and methadone on post-anaesthetic colic (PAC) incidence. Horses undergoing general anaesthesia for non-gastrointestinal procedures that were hospitalized for at least 72 h post-anaesthesia were included in this study. Anaesthetised horses were stratified by procedure type into horses undergoing diagnostic imaging without surgical intervention, emergency or elective surgery. In addition, patients were grouped by opioid treatment regime into horses receiving no opioids, intraanaesthetic, short- (<24 h) or long-term (>24 h) postoperative opioids. Administered opioids encompassed butorphanol, morphine and methadone. The number of horses showing signs of colic in the 72 h after anaesthesia was assessed for each group. A total of 782 horses were included, comprising 659 undergoing surgical procedures and 123 undergoing diagnostic imaging. The overall PAC incidence was 15.1%. Notably, horses undergoing diagnostic imaging without surgery had a significantly lower PAC rate of 6.5% compared to those undergoing surgery (16.7%, $p = 0.0146$). Emergency surgeries had a significantly lower PAC rate of 5.8% compared to elective procedures (18%, $p = 0.0113$). Of the 782 horses, 740 received intraoperative opioids and 204 postoperative opioids, 102 of which long-term (≥24 h). Neither intraoperative ($p = 0.4243$) nor short-term postoperative opioids ($p = 0.5744$) increased PAC rates. Notably, only the long-term (≥24 h) administration of morphine significantly increased PAC incidence to 34% ($p = 0.0038$). In contrast, long-term butorphanol (5.3% PAC, $p = 0.8482$) and methadone (18.4% PAC, $p = 0.6161$) did not affect PAC rates. In summary, extended morphine administration was the only opioid treatment associated with a significantly increased risk of PAC.

KEYWORDS

opioids, morphine, butorphanol, methadone, horse, equine, colic

1 Introduction

Postoperative pain is prevalent among the majority of patients undergoing surgical procedures. Effective pain management is crucial to mitigate suffering, reduce morbidity, and facilitate recovery and rehabilitation. Although the analgesic properties of opioids and opiates, such as butorphanol, buprenorphine, methadone, and morphine,

are well-established, their use in managing perioperative and post-traumatic pain in equine patients is limited by concerns about potential adverse gastrointestinal side effects (1–9). Constipation is a widely recognized side effect of opioid treatment in all species, affecting up to 95% of human patients, attributed to diminished coordinated motility, prolonged transit time, enhanced fluid absorption from intestinal contents and decreased secretion of fluids and electrolytes into the intestinal lumen (10–20). While opioids provide analgesia by stimulating μ-, κ- and δ-opioid receptors in the brain, the dorsal horn of the spinal cord and peripheral tissues, the activation of opioid receptors within the gastrointestinal tract can decrease motility and induce alterations in the secretion, absorption and transport of electrolytes and fluids (9, 11–17, 21–24).

Post-anaesthetic colic (PAC) represents a common complication in equine patients, with reported incidence rates reaching up to 21.1% (5, 6, 25–28). Research into risk factors for the development of PAC and the influence of systemic opioids on gastrointestinal (GI) motility has produced equivocal results, ranging from decreased risk (25), no elevated risk (6, 26, 27, 29–32) to a fourfold rise in colic cases following perioperative opioid administration (5). The inconsistency in these outcomes could be attributed to differences in the GI side effects associated with various opioid agonists and variations in the dosage, frequency, administration method, and duration of opioid use across these studies.

Butorphanol, morphine, methadone and hydromorphone are commonly used in equine analgesia and their pharmacokinetics and pharmacodynamics have been thoroughly investigated (2, 24, 29, 30, 32, 33). Butorphanol, a synthetic strong κ-opioid receptor agonist and weak μ-opioid receptor antagonist (2, 34–38), has been observed to induce a transient reduction in gastrointestinal motility in anesthetized horses when used as CRI at a dosage of 13 µg/kg/h (34). In contrast, pre- or intraoperative butorphanol administration at a mean dosage of 0.007 mg/kg and 0.05 mg/kg respectively has been reported to reduce the risk of PAC (25).

Similarly, morphine, a μ-opioid agonist, has been shown to decrease gastrointestinal propulsive motility and to increase PAC rates after orthopaedic surgery four-fold when administered intravenously at a dosage of 0.08–0.3 mg/kg compared to no opioid or butorphanol (5, 9, 35, 39, 40). Conversely, two other studies found no association between peri-anaesthetic intravenous morphine administration at a dosage of 0.1–0.17 mg/kg and increased PAC risk (6, 41). Notably, the administration of epidural morphine after laparoscopy has been demonstrated to provide effective pain relief without compromising gastrointestinal motility (42).

Methadone, a synthetic μ-and δ-agonist with *N*-methyl-d-aspartate (NMDA) antagonist activity and the ability to inhibit serotonin and noradrenaline uptake, is not extensively approved for equine use and consequently, its utilization is less prevalent compared to morphine or butorphanol. Although it has also been shown to reduce borborygmi (2), the potential influence of perianaesthetic methadone administration on the occurrence of post-anaesthetic colic (PAC) remains unexplored.

Therefore, this study aims to assess and compare the impact of intra- and postoperative administration of the commonly used opioids—butorphanol, morphine and methadone—on the incidence of PAC in equine patients.

2 Materials and methods

2.1 Horses

This retrospective chart review includes data from all horses over the age of one year that underwent general anaesthesia at the University of Veterinary Medicine Vienna's Equine Hospital in the 5-year period from October 2013 to December 2018 and remained hospitalized at the clinic for a minimum of 72 h post-anaesthesia. Patients undergoing abdominal surgery, presenting with gastrointestinal disorders, or requiring multiple surgeries such as repeated arthroscopic lavages within the initial 72-h window, were excluded from the study (Figure 1).

The cases were stratified into an elective and an emergency group. Patients in the emergency category were not fasted due to the immediate need for surgical intervention. In contrast, horses in the elective group were fasted for a minimum of 6 h before anaesthesia.

Furthermore, both elective and emergency surgeries were categorized based on the type of procedure, distinguishing between orthopaedic and soft tissue surgeries. Horses subjected to general anaesthesia for computer tomography or magnetic resonance imaging, without concurrent surgical intervention, constituted the control group.

In addition to the horses' age, sex, breed and weight, details of intra- and post-anaesthetic opioid regimen, including drug type, dosage, route and duration of administration, duration of anaesthesia and intraoperative blood pressure and all physical exam parameters in the 72 h following the anaesthesia were extracted from the records. Opioids were categorized into intra- and post-anaesthetic administration, with further subdivision into short-term (<24 h) and long-term (≥24 h) treatment.

Patients were classified as experiencing post-anaesthetic colic if they displayed clinical symptoms such as depression, pawing, reduced appetite, or rolling, necessitating a comprehensive colic assessment in the 72 h following anaesthesia.

2.2 Statistics

Data were summarized using standard descriptive statistics including mean, standard deviation, and range for continuous variables and for categorical variables frequency and proportion. Associations between categorical variables were examined by the Chi-Square test. The effect of age, sex, procedure type (anaesthesia only or surgery), emergency vs. elective surgery, anaesthesia duration, intra- and postoperative opioid administration and opioid type on PAC rate was assessed using multiple logistic regression with PAC as the outcome variable, anaesthesia without surgery as the reference level for procedure type, elective surgery as the reference level for surgery and

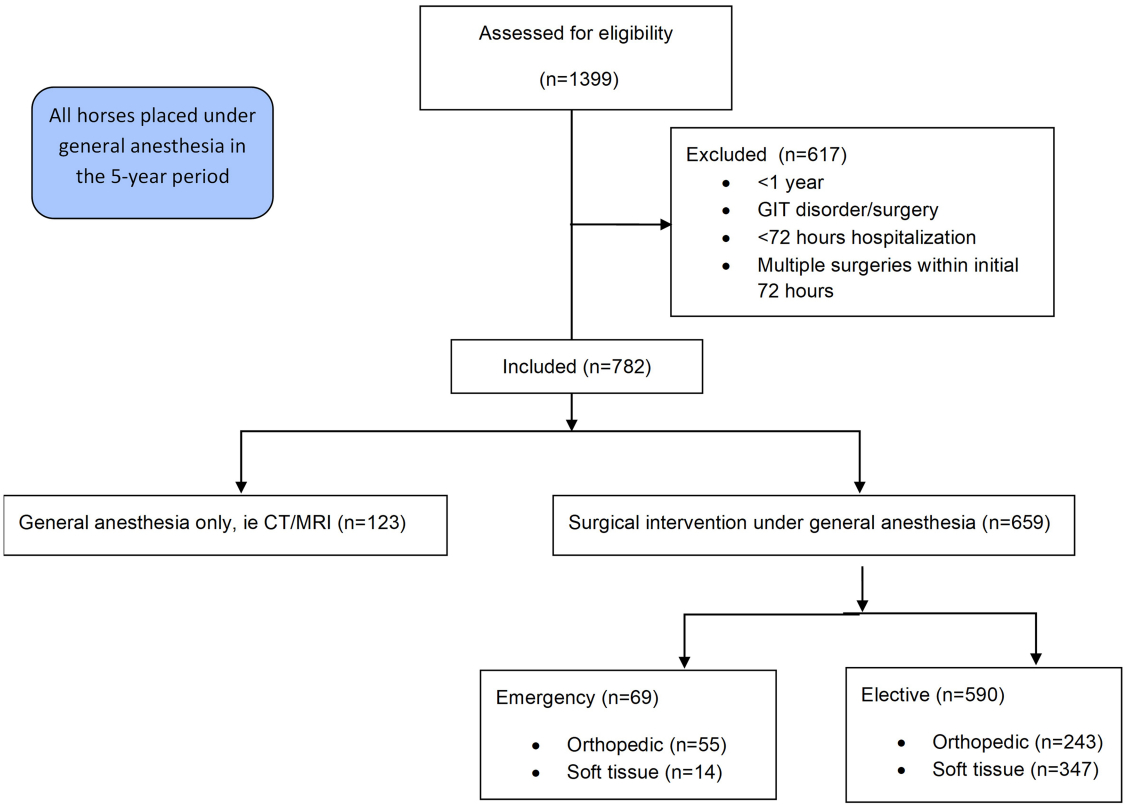

FIGURE 1
Consort flow illustrating inclusion and exclusion criteria.

gelding as the reference level for sex. All computations were carried out in Graphpad Prism (Version 10.0.2).

3 Results

A total of 782 cases, 246 (31.5%) mares, 313 (40%) geldings and 223 (28.5%) stallions met the inclusion criteria. Horses' age ranged from 1 to 30 years (mean: 9.1 years, s.d.: 6 years).

Of the 782 cases, 659 (84.3%) horses were surgical patients, the remaining 123 (15.7%) were anesthetized for diagnostic imaging purposes without concurrent surgical intervention (Table 1). The 659 surgical patients were divided into 69 (10.5%) emergency and 590 (89.5%) elective cases. The 69 emergencies included 55 (79.7%) orthopaedic and 14 (20.3%) soft tissue emergencies. In contrast, the elective cases comprised 243 (41.2%) orthopaedic and 347 (58.8%) were soft tissue surgeries. Anaesthesia duration (overall mean: 107 min, s.d.: 65 min) was significantly different between horses undergoing anaesthesia for diagnostic imaging (mean: 42 min, s.d.: 37 min), elective (mean: 115 min, s.d.: 61 min) or emergency (mean: 145 min, s.d.: 67 min) surgery ($p < 0.0001$).

The overall incidence of post-anaesthetic colic (PAC) was 15.1% (118/782, Table 1). Age, sex and anaesthesia duration had no statistically significant effect on PAC rates (Table 2).

However, horses anesthetized for diagnostic imaging purposes without concurrent surgical intervention had a significantly lower PAC rate of 6.5% ($n = 8/123$) compared to horses undergoing surgery with a PAC rate of 16.7% ($n = 110/659$, $p = 0.0146$, Table 2). Furthermore, horses anesthetized for emergency surgery had a significantly lower PAC rate of 5.8% ($n = 4/69$), than horses undergoing elective procedures with a PAC rate of 18% ($n = 106/590$) ($p = 0.0113$; Table 2). The type of surgery (soft tissue vs. orthopaedic) did not have a significant effect on PAC rate with 15.1% (45/298) orthopaedic and 18% (65/361) soft tissue surgery patients suffering from PAC ($p = 0.9$).

Overall, 34 horses (4.35%) received no opioids during their hospital stay. Of the 748 horses (95.65%) that were administered opioids, 196 (25.1%) received opioids intra- and postoperatively, 544 (69.6%) only intraoperatively and 8 (1%) only postoperatively, accumulating to 740 horses (94.6%9 with intraoperative and 204 (26.1%) with postoperative opioid treatment. Postoperative opioid therapy lasted for <24 h in 119 (58.3%) patients and long-term (≥24 h) in 85 (41.7%; Table 1).

Butorphanol dosage ranged from 0.01 mg/kg to 0.03 mg/kg intravenously as single (i.e., sedation) or repeated intravenous bolus injections. Morphine was administered at 0.1 mg/kg intramuscularly every 4–6 h. Methadone was utilized at 0.1 mg/kg intramuscularly every 4–6 h as a bolus ($n = 43$) or as part of a continuous rate infusion (CRI, $n = 1$) at a dosage of 0.017 mg/kg/h, combined with lidocaine 2% (1.2 mg/kg/h) and ketamine 10% (0.3 mg/kg/h) for post-anaesthetic pain management. No horse received methadone during anaesthesia (Table 1).

TABLE 1 Number of horses receiving opioids and developing PAC by overall, intraoperative and postoperative short-term (<24 h) and long-term (>24 h) opioid administration. As horses received multiple opioids as well as intra- and postoperative opioids, cumulative percentages can exceed 100%.

		Overall		Anesthesia only		Elective surgery		Emergency surgery	
		n (%)	PAC *n* (%)	*n* (%)	PAC *n* (%)	*n* (%)	PAC *n* (%)	*n* (%)	PAC *n* (%)
Overall horses		782	118 (15.1)	123 (15.7)	8 (6.5)	590 (89.4)	106 (18)	69 (10.5)	4 (5.8)
Overall opioids	No opioid	34 (4.3)	3 (8.8)	2 (1.6)	0	30 (5.1)	3 (10)	2 (2.9)	0
	Opioids total	748 (95.7)	115 (15.4)	121 (98.4)	8 (6.6)	560 (94.9)	103 (18.4)	67 (97.1)	4 (6)
	Butorphanol	624	93	118	8	455	81	51	4
	Morphine	292	55	9	2	234	49	49	4
	Methadone	44	8	5	1	32	6	7	1
Intraop. opioids	No opioid	42 (5.4)	5 (11.9)	4 (3.3)	0	35 (5.9)	5 (14.3)	3 (4.3)	0
	Opioids total	740 (94.6)	113 (15.3)	119 (96.7)	8 (6.7)	555 (94.1)	101 (18.2)	66 (95.7)	4 (6.1)
	Butorphanol	589	85	116	8	437	76	36	1
	Morphine	265	46	4	0	216	42	45	4
	Methadone	4	0	0	0	2	0	2	0
Opioids <24 h	No opioid	680 (87)	103 (15.1)	110 (89.4)	6 (5.5)	527 (89.3)	94 (17.8)	43 (62.3)	3 (7)
	Opioids total	102 (13)	15 (14.7)	13 (10.6)	2 (15.4)	63 (10.7)	12 (19)	26 (37.7)	1 (3.8)
	Butorphanol	91	11	11	1	56	9	24	1
	Morphine	14	3	2	1	8	2	4	0
	Methadone	2	1	0	0	2	1	0	0
Opioids >24 h	No opioid	680 (87)	95 (14)	98 (79.7)	6 (6.1)	525 (89)	87 (16.6)	57 (82.6)	2 (3.5)
	Opioids total	102 (13)	23 (22.5)	25 (20.3)	2 (8)	65 (11)	19 (29.2)	12 (17.4)	2 (16.7)
	Butorphanol	19	1 (5.3)	19	1 (5.3)	0	0	0	0
	Morphine	47	16 (34)	3	1 (33.3)	37	14 (37.8)	7	1 (14.3)
	Methadone	38	7 (18.4)	5	1 (20)	28	5 (17.9)	5	1 (20)

TABLE 2 Odds ratio estimates and 95% confidence intervals (CI, *Z* and *p*-values calculated using multiple logistic regression with PAC as the outcome variable.

Variable	Odds ratio estimate	Odds ratio 95% CI	*Z*	*p*-value
Sex: mare vs. gelding	0.9334	0.5634–1.557	0.2667	0.7897
Sex: stallion vs. gelding	0.6985	0.4102–1.185	1.329	0.1839
Age	0.9822	0.9478–1.019	0.9728	0.3306
Diagnostic imaging vs. Surgery	0.3248	0.1192–0.7461	2.443	0.0146
Orthopaedic vs. soft tissue surgery	1.032	0.6265–1.703	0.1248	0.9007
Emergency versus elective surgery	4.007	1.536–13.80	2.539	0.0111
Anaesthesia duration	0.9995	0.9958–1.003	0.2443	0.8070
IntraOP opioids overall	0.6695	0.2211–1.646	0.7990	0.4243
Short-term postOP opioids overall	0.4128	0.009769–7.663	0.5616	0.5744
Short-term postOP butorphanol	2.036	0.1141–85.27	0.4559	0.6485
Short-term postOP morphine	1.322	0.1424–42.94	0.2083	0.8350
Short-term postOP methadone	0.4180	0.009406–20.28	0.4883	0.6253
Long-term postOP butorphanol	1.240	0.1896–24.46	0.1914	0.8482
Long-term postOP morphine	0.3263	0.1543–0.7086	2.897	0.0038
Long-term postOP methadone	0.7798	0.3123–2.247	0.5014	0.6161

Neither opioids administered during anaesthesia ($p = 0.4243$) nor short-term opioid administration after anaesthesia ($p = 0.5744$) increased the incidence of PAC (Tables 1, 2). Notably, only the long-term administration of morphine resulted in a statistically significant increase of PAC ($p = 0.0038$) with 34% (16/47) horses that received morphine for ≥24 h developing PAC (Tables 1, 2, Figure 2). In contrast, long-term butorphanol [5.3% (1/19) PAC, $p = 0.8482$] and methadone [18.4% (7/38) PAC, $p = 0.6161$] had no significant effect on PAC rate, compared to horses receiving no long-term postoperative opioids [14% (95/680) PAC; Tables 1, 2, Figure 2].

4 Discussion

Morphine administration for longer than 24 h postoperatively emerged as the sole opioid significantly associated with increased PAC incidence in this study, in contrast to both butorphanol and

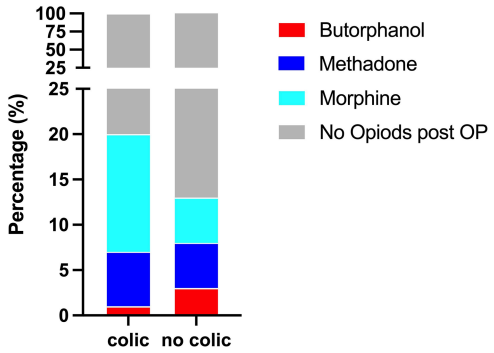

FIGURE 2
Percentage of horses receiving long-term (≥24 h) post-anaesthetic opioids with and without colic symptoms.

methadone. This finding aligns with numerous human studies consistently ranking morphine highest in the opioid potency profile order for inducing constipation (30, 43–45). Notably, the dosage necessary for morphine's analgesic effect significantly surpasses that needed for its gastrointestinal side effects, approximately fifty-fold in humans and fourfold in experimental animals (46, 47). In horses, morphine administered intravenously at dosages as low as 0.05 mg/kg, half the dosage typically used clinically to provide analgesia, decreased propulsive gastrointestinal motility for up to 6 h and significantly reduced defecation frequency, faecal matter weight and moisture content (9, 39, 48–50).

Opioids affect gastrointestinal motility by activating the μ-, κ- and δ-opioid receptors of the myenteric and submucosal plexus neurons in the enteric nervous system, interstitial cells of Cajal and immune cells (13–15, 22, 51–58). Broadly, opioids induce delayed gastric emptying and constipation by disrupting neurotransmission between enteric neurons and their targets, namely, smooth muscles and epithelial cells (15). The inhibition of excitatory neurons in the myenteric plexus decreases propulsive peristalsis while the concomitant suppression of inhibitory neuromuscular transmission increases intestinal muscle tones and non-propulsive tonic contractions, which may cause abdominal cramps (13–15, 22, 51–58). Additionally, opioid-induced inhibition of submucosal secretomotor neurons diminishes epithelial secretion of Cl^- and osmotic water movement, exacerbating constipation (13–15, 22, 51–58).

While all three classical opioid receptor types are found in the gastrointestinal tract and contribute to analgesic and adverse effects, mechanistic studies using μ-selective drugs and μ-receptor-knockout mice indicate that opioid-induced gastrointestinal tract dysfunction is primarily mediated by the μ-receptor (13–15, 22, 51–58). Strong μ-agonists, such as morphine and methadone are thus more likely to induce adverse gastrointestinal side effects. Correspondingly, butorphanol, a mixed agonist-antagonist opioid analgesic with an affinity ratio for the μ-, δ-, and κ-opioid receptor of 1:4:25 and a a 3-, 10-, and 30-fold lower half maximal inhibitory concentration (IC50) for these receptors than morphine (38, 59), had the lowest PAC rate in this study. The lower PAC incidence following methadone compared to morphine administration observed in this study, is consistent with reported constipation relief in 80% of human patients after switch from morphine to methadone (19, 31, 60, 61). The extraopioid analgesic effects caused by methadone's non-competitive antagonist activity at the *N*-methyl-D-aspartate receptor and its function as a serotonin re-uptake inhibitor, may contribute to the lower rate of gastrointestinal effects observed with methadone compared to morphine therapy (19, 31, 60, 61). Serotonin exerts a variety of effects on intrinsic enteric neurons, extrinsic afferents, enterocytes and smooth muscle cells and its agonists are used as promotility agents to promote gastric emptying and to alleviate constipation (62–65).

Interestingly, although anaesthesia duration had no influence on PAC rate, horses anesthetized for diagnostic imaging purposes had a significantly lower PAC rate (6.5%) than horses undergoing surgery (16.7% PAC) and horses receiving no opioids postoperatively had a higher PAC (14%) rate than horses receiving butorphanol (5.3%), underscoring the importance of postoperative pain management. Pain initiates a spinal reflex arc, increases sympathetic activity and cortisol and endogenous opioid release, thus inhibiting propulsive gastrointestinal motility (66–69). Effective pain management is thus crucial to ensure patients' welfare and minimize postoperative morbidity and complications.

Notably, emergency surgeries were associated with a significantly lower PAC rate than elective surgeries in our study, which is in contrast to previous studies in which out-of-hours surgeries and horses that were not fasted before anaesthesia carried an increased risk of PAC (5, 26). The exclusion of horses with gastrointestinal problems, the more intensive supportive care and monitoring provided to emergency cases may contribute to the lower PAC rate in emergency surgeries in our study. However, horses undergoing emergency surgery, in contrast to elective procedures, also were not fasted prior to anaesthesia. Therefore, the lower PAC rate may also be attributable to the ongoing provision of food rather than the horse being presented as an emergency. Although this interpretation is more plausible from a clinical perspective, the simultaneous occurrence of these two factors does not allow statistical analysis to test this hypothesis.

The relatively high overall PAC rate in this study (15.1%), which is at the high end of the reported range of 2.8–21.1% (5, 6, 25–28), may be due to the inclusion criteria necessitating hospitalisation for 72 h post anaesthesia, which may bias toward a patient population with more severe problems and the stringent definition of colic symptoms combined with the close monitoring in our clinic.

The study's limitations are inherent in its retrospective design, the non-randomized allocation of patients to the different opioid treatment groups, the absence of both standardized pain assessment and stratification based on pain severity, and the relatively low number of patients in the diverse treatment subgroups. Hence, the selection of opioid type, administration route, and duration was determined by the anticipated or subjectively perceived level of pain, potentially leading to both over- and undertreatment of pain. Moreover, given the absence of comprehensive data regarding equipotent dosages of these opioids in equines, the employed drug dosages likely lack equipotency, thereby hindering direct comparison of their analgesic properties. Studies assessing the equianalgesic potencies of different opioids in horses and associated side effects are required. Additionally, while all three classical opioid receptor types are found in the mammalian gastrointestinal tract, their distribution patterns exhibit inter-species variability (9, 11–17, 21–23, 43, 58). For the horse only the opioid receptor distribution pattern in the small intestine has been studied (70). Given the pivotal role of colonic dysfunction in opioid-induced gastrointestinal complications across species, further investigations to determine the opioid receptor distribution in the equine colon are needed.

5 Conclusions

Intraoperative opioids and postoperative pain management with butorphanol and morphine did not increase the incidence of PAC in our study. Long-term (>24 h) morphine was the only opioid increasing PAC rate. In addition, patients undergoing surgery had a significantly higher PAC incidence than horses anaesthetized for diagnostic imaging and horses receiving no postoperative opioids had a higher PAC rate than those receiving butorphanol, underscoring the role of pain and pain management in PAC.

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.

Author contributions

RH: Conceptualization, Data curation, Formal Analysis, Investigation, Writing – review & editing. MJ: Data curation, Investigation, Writing – review & editing. AM: Data curation, Investigation, Writing – review & editing. FJ: Conceptualization, Formal Analysis, Investigation, Methodology, 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 Pain Research

TYPE Original Research
PUBLISHED 11 March 2024
DOI 10.3389/fpain.2024.1373555

OPEN ACCESS

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RECEIVED 20 January 2024
ACCEPTED 29 February 2024
PUBLISHED 11 March 2024

CITATION
Paranjape VV, Knych HK, Berghaus LJ, Cathcart J, Giancola S, Craig H, James C, Saksena S and Reed RA (2024) Evaluation of physical variables, thermal nociceptive threshold testing and pharmacokinetics during placement of transdermal buprenorphine matrix-type patch in healthy adult horses. Front. Pain Res. 5:1373555. doi: 10.3389/fpain.2024.1373555

Evaluation of physical variables, thermal nociceptive threshold testing and pharmacokinetics during placement of transdermal buprenorphine matrix-type patch in healthy adult horses

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Background: Matrix type transdermal buprenorphine patches have not been investigated in horses and may provide an effective means of providing continuous pain control for extended period and eliminating venous catheterization.
Objective: Assessment of the physiological variables (heart rate, respiratory rate, body temperature) and thermal nociceptive threshold testing, and describing the pharmacokinetic profile of transdermal buprenorphine matrix-type patch (20 μg h^{-1} and 40 μg h^{-1} dosing) in healthy adult horses.
Study design: Randomised experimental study with a Latin-square design.
Methods: Six adult healthy horses received each of the three treatments with a minimum 10 day washout period. BUP0 horses did not receive a patch (control). BUP20 horses received one patch (20 μg h^{-1}) applied on the ventral aspect of the tail base resulting in a dose of 0.03–0.04 μg kg^{-1} h^{-1}. BUP40 horses received two patches placed alongside each other (40 μg h^{-1}) on the tail base resulting in a dose of 0.07–0.09 μg kg^{-1} h^{-1}. Whole blood samples (for determination of buprenorphine concentration), physiological variables and thermal threshold testing were performed before (0 h) and at 2, 4, 8, 12, 16, 24, 32, 40, 48, 56, 64, 72, and 96 h after patch application. The patches were removed 72 h following placement and were analyzed for residual buprenorphine content.
Results: Between the three groups, there was no change in physiological variables across timepoints as compared to baseline ($p > 0.1$). With the higher dose, there was a significant increase in thermal thresholds from baseline values from 2 h until 48 h and these values were significantly higher than the group receiving the lower patch dose for multiple timepoints up to 40 h. 40 μg h^{-1} patch led to consistent measurable plasma concentrations starting at 2 h up to 96 h, with the mean plasma concentrations of > 0.1 ng/ml from 4 h to 40 h.

Conclusions: 20 μg h^{-1} and 40 μg h^{-1} patch doses were well tolerated by all horses. At higher dose, plasma buprenorphine concentrations were more consistently measurable and blunted thermal thresholds for 48 h vs. 32 h with 20 μg h^{-1} dosing as compared to control.

KEYWORDS

opioids, equine, analgesia, pain, pharmacology, thermal antinociception, pain-free

1 Introduction

In recent years, there have been significant advances in pain management for horses, with several researchers investigating pharmacology of opioid analgesics. The clinical implication of these studies is to improve the wellbeing and welfare of this species by including multimodal analgesic regimes for treatment of acute and chronic conditions. Opioids form an integral part of analgesic protocols due to their high potency and efficacy in treating different types of pain in human and veterinary medicine. Injectable μ-receptor opioid agonists such as morphine, hydromorphone and methadone are commonly used to treat perioperative pain in horses. However, clinicians hesitate to use this drug class in horses due to the apparent narrow margin between analgesia and excitation or arousal, gastrointestinal hypomotility, and challenges posed in quantifying consistent analgesic effects (1, 2).

The transdermal therapeutic system has also been assessed in horses for synthetic μ-opioid agonists such as fentanyl due to the advantage of: (i) providing noninvasive, continuous pain control for extended periods; (ii) preventing wide variations in serum drug concentrations; (iii) reducing severity of adverse effects associated with repeated post-dose peaks in plasma concentration as seen with injectable route; (iv) avoiding end of dose breakthrough pain; and (v) preventing first-pass metabolism occurring commonly with an oral route of administration (3, 4). Buprenorphine is another opioid that is available for transdermal drug delivery via patch application. This drug is a semi-synthetic, highly lipophilic oripavine derivative that is classified as a high affinity partial μ-receptor agonist and a κ -receptor antagonist. It's affinity for the opiate receptor is double and its potency is approximately 30 times higher than morphine. Its therapeutic response lasts much longer than other opioids and it has a wider safety profile (5–7). A transdermal matrix patch buprenorphine formulation which was initially developed for human use, has been successfully investigated in dogs (8–11), cats (12), pigs (13, 14), sheep (15, 16) and primates (17). It is evident that with transdermal buprenorphine patch, there exists a discrepancy between species with regards to nociceptive threshold testing, analgesic effects and detectable plasma concentrations. Several equine studies report the clinical utility of injectable buprenorphine (i.e., intravenous, intramuscular, subcutaneous and sub-lingual) to treat mild to moderate pain (18–24), increase nociceptive threshold (21–23), and offer superior-long lasting antinociception in comparison to butorphanol (24). To the authors' knowledge, administration of buprenorphine via transdermal patch has not been described in horses to date.

The objectives of the present study which investigated the placement of transdermal buprenorphine matrix-type patch (20 μg h^{-1} and 40 μg h^{-1} dosing) in healthy adult horses were to: (i) assess the physiological variables (heart rate, respiratory rate, body temperature) and thermal nociceptive threshold testing; and (ii) describe the pharmacokinetic profile and correlate the plasma concentrations with the level of thermal antinociception. We hypothesized that buprenorphine when delivered via the transdermal patch will: (i) minimally affect the physical examination and provide anti-nociception as detected by higher thermal threshold; and (ii) achieve quantifiable and clinically relevant plasma concentrations which will be dose dependent and correlate with the duration of thermal anticonception.

2 Material and methods

2.1 Ethics statement

This study was approved by the University of Georgia Institutional Animal Care and Use Committee (animal use protocol: A2021 03-010-Y1-A3).

2.2 Study animals

Six, university-owned adult, healthy horses (4 mares and 2 geldings) aged 17 ± 8 years and weighing 524 ± 44 kg were enrolled in this masked, prospective, Latin square study design. The animals were deemed healthy based on clinical history, thorough physical examination and a normal complete blood count and biochemistry profile. The entire study and all procedures took place in a temperature-controlled facility. The horses were transferred from the farm to the 3.65 m × 4.26 m stall in this facility for acclimatization 16–20 h prior to treatment administration on each occasion. During the entire duration of the study when the horses were housed in this research environment, they were provided with 0.7 kg of senior feed (Senior formula; Seminole Feed, Ocala, FL, USA) and 2–3 flakes of timothy hay twice daily with *ad libitum* access to water. On the same day, i.e., day of arrival at the facility, a 14-gauge, 13 cm intravenous catheter (DayCath; MILA International, Florence, Kentucky, USA) was placed aseptically in the cranial region of the jugular vein on the selected side for the purpose of blood collection for pharmacokinetic analysis. The horses were then weighed and a physical examination was performed to record the baseline heart rate (HR), respiratory rate (RR) and rectal

temperature (Temp). The catheter was periodically flushed with heparinized saline and was monitored closely for blood clots and catheter patency.

2.3 Treatment groups and transdermal buprenorphine patch application

All enrolled study horses were administered all of the following three treatments and the randomization by application of Latin square was predetermined (www.randomizer.org). The washout period between treatments was a minimum of ten days. The ventral aspect of the tail base was wiped clean with a dry 10. 16 cm × 10.16 cm gauze pad to remove dirt and skin debris. It was ensured that this area would allow sufficient skin to patch contact such that two patches could be situated next to each other without overlap. A transdermal patch which contained 20 mg total buprenorphine (20 μg h^{-1}; TEVA Pharmaceuticals Inc., Parsippany, NJ, USA) with dimensions 7.4 cm × 7.4 cm was applied in this location and was further secured with a 7.62 cm porous elastic adhesive tape covering (Elastikon; Johnson & Johnson, New Brunswick, NJ, USA) as shown in Figure 1.

- **BUP0:** horses did not receive a patch, instead only the elastic adhesive tape was wrapped around the tail base
- **BUP20:** horses received one patch (20 μg h^{-1}) resulting in a dose of 0.03–0.04 μg kg^{-1} h^{-1} based on their bodyweights on the day of treatment
- **BUP40:** horses received two patches placed alongside each other (40 μg h^{-1}) resulting in a dose of 0.07–0.09 μg kg^{-1} h^{-1} based on their bodyweights on the day of treatment

FIGURE 1
Placement of a transdermal matrix patch system containing 20 mg total buprenorphine (20 μg h^{-1}; TEVA pharmaceuticals Inc., parsippany, NJ, USA) with dimensions 10.16 cm × 10.16 cm and further secured with a 3-inch porous elastic adhesive tape covering (Elastikon; Johnson & Johnson, New Brunswick, NJ, USA) on the ventral aspect of the tail base. BUP0 horses did not receive a patch, instead only the elastic adhesive tape was wrapped around the tail base. BUP20 horses received one patch (20 μg h^{-1}) resulting in a dose of 0.03–0.04 μg kg^{-1} h^{-1} based on their bodyweights on the day of treatment. BUP40 horses received two patches placed alongside each other (40 μg h^{-1}) resulting in a dose of 0.07–0.09 μg kg^{-1} h^{-1} based on their bodyweights on the day of treatment.

2.4 Nociceptive thermal threshold testing

A coin toss aided in randomization of the metacarpus (right or left), and the skin area over the dorsal aspect was clipped with a #50 blade on the day before the treatments were administered. Cutaneous thermal threshold testing was carried out using a validated commercial wireless device (WTT2; TopCat Metrology, UK), consisting of a display control unit mounted on the horse's withers via a surcingle wrapped around the thorax with a buckle attachment (Figure 2A). The thermal element comprised of a heating and temperature sensing element was placed directly on the shaved area over the metacarpus and secured around the leg via a nylon Velcro strap (Figure 2B). Prior to each reading, the ambient temperature and skin temperature at the site of the thermal probe was documented. The masked operator standing outside the stall (VP, RR) controlled the temperature of the thermal probe via an infrared remote. The thermal element was activated by a button and heated at a rate of 0.8 °C s^{-1} until the horse reacted to the thermal stimulus by displaying avoidance behavior, i.e., pawing, stomping, lifting or rubbing their nose on the stimulated front leg (Supplementary Materials: Videos). Upon observation of an avoidance behavior following thermal stimulation, the threshold temperature for that timepoint was recorded. The unit would not heat above 55 °C and would automatically discontinue the stimulus if this temperature was reached in order to avoid thermal injury to the tissue. When the unit reached 55 °C, this was recorded as the threshold temperature for that timepoint (25–27). The Velcro strap was removed between data points and the area underneath the thermal element was examined carefully for redness and tissue damage, and the location of the thermal element on the metacarpus was varied in order to prevent tissue injury. Horses enrolled in the study had been included in previous studies utilizing thermal threshold and were accustomed to use of the device. Baseline measurements were taken in triplicate prior to patch application, with a 10-min interval between each stimulus.

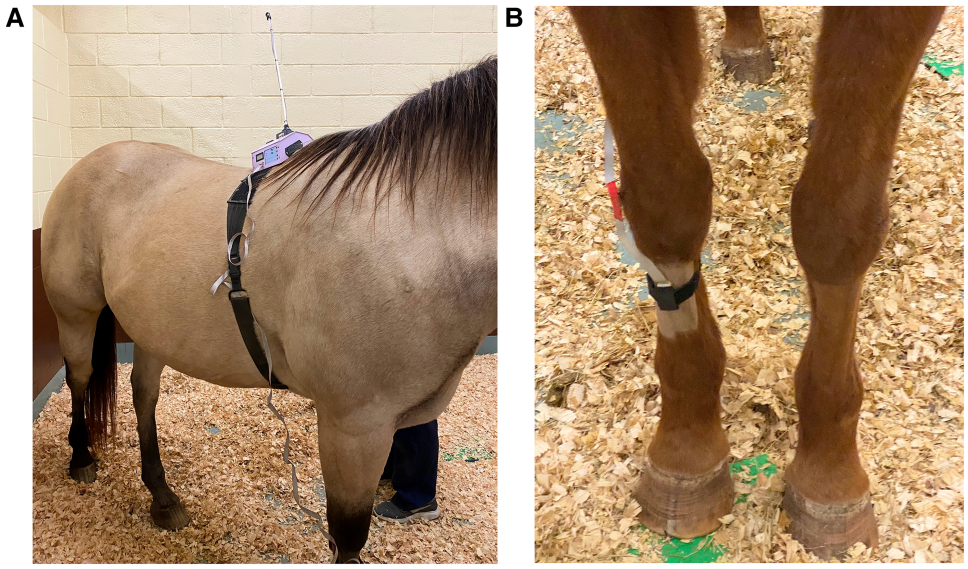

FIGURE 2
((A) left) Attachment of the wireless thermal threshold testing system to the horse. Cutaneous thermal threshold testing was carried out using a validated commercial wireless device (WTT2; TopCat Metrology, UK), consisting of a display control unit and heating block mounted on the horse's withers via a surcingle wrapped around the thorax with a buckle attachment. ((B) right) The thermal probe comprising of a heating and temperature sensing element was placed directly on the shaved area over the metacarpus and secured around the leg with the help of a nylon Velcro strap.

Thermal threshold data was then obtained at 2, 4, 8, 12, 16, 24, 32, 40, 48, 56, 64, 72, and 96 h after patch application.

2.5 Study timeline and data collection

The entire timeline of the study during administration of a treatment is depicted in Figure 3. Following instrumentation, baseline data was acquired. The treatment allocated to the horse (BUP0, BUP20 or BUP40) was then applied. The patch and/or elastic adhesive tape was removed 72 h following placement. Last data collection for pharmacodynamic variables (HR, RR, rectal Temp and thermal thresholds) and blood sampling was performed at 96 h, which marked the end of data collection for that treatment. Upon completion of all three treatments, the horses were transferred back to their farm from the research

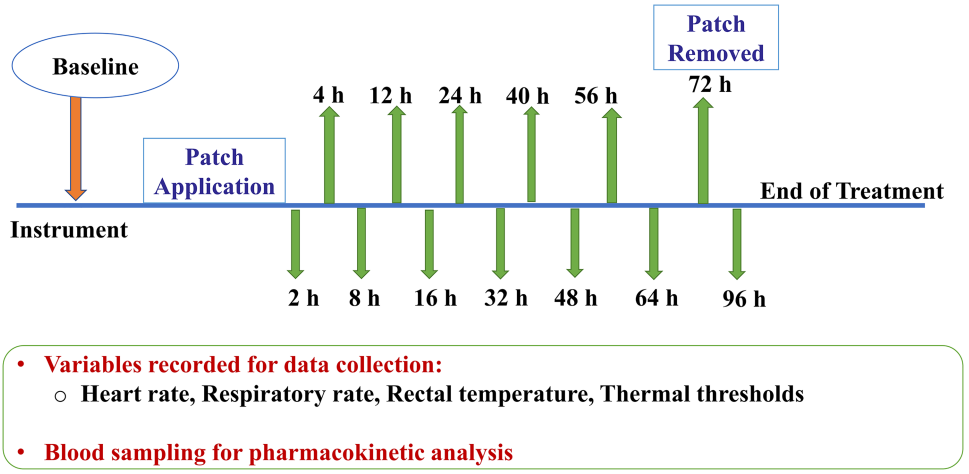

FIGURE 3
Following instrumentation, baseline data (0 h) was acquired. Dependent on the group allocated to the horse (no patch, 20 μg h^{-1}, 40 μg h^{-1}), the treatment was initiated. The patch and/or elastic adhesive tape was removed 72 h after treatment began. Last data collection for variables (heart rate, respiratory rate, rectal temperature and thermal thresholds) and blood sampling was performed at 96 h timepoint, which marked the end of treatment. For pharmacokinetic data acquisition, 6 ml of venous blood was drawn before (baseline or 0 h) and at 2, 4, 8, 12, 16, 24, 32, 40, 48, 56, 64, 72, and 96 h after patch application. The sampling jugular catheter and patch was removed at 72 h timepoint.

facility. Once the patches were removed, they were collected in sterile bags and stored at −80 °C until later analysis of residual buprenorphine content. Whole blood was obtained for determination of buprenorphine concentration before (baseline or 0 h) and at 2, 4, 8, 12, 16, 24, 32, 40, 48, 56, 64, 72, and 96 h after patch application. A 10-ml waste sample was procured from the jugular catheter before drawing 6 ml of venous blood for buprenorphine plasma concentrations. The sampling jugular catheter was removed after 72 h, and the following blood samples were obtained by direct jugular venipuncture. Blood samples were collected in lithium heparin tubes (Green BD Hemogard™; Becton-Dickinson, Franklin Lakes, NJ, USA) and immediately underwent centrifugation at 1,300*g* for 10 min. The resultant supernatant plasma was aspirated via a 1-ml disposable pipette (Thermo Fisher Scientific, Waltham, MA, USA) and transferred to cryogenic vials (Labcon™ 1.5 ml SuperSpin™; Thermo Fisher Scientific) that were then stored at −80 °C until analysis (within 2 months of sample collection).

2.6 Determination of plasma concentration and pharmacokinetics

Plasma calibrators were prepared by dilution of the buprenorphine working standard solutions (Cerilliant, Round Rock, TX) with drug free equine plasma to concentrations ranging from 0.01 to 70 ng ml^{-1}. Calibration curves and negative control samples were prepared fresh for each quantitative assay. Quality control samples (equine drug free plasma spiked with buprenorphine at three concentrations within the standard curve) were included with each sample.

Prior to analysis, 0.5 ml of the plasma samples were diluted with 2.0 ml 0.1 M pH 6 phosphate buffer and 0.1 ml water containing d4-buprenorphine internal standard (Cerilliant, Round Rock, TX; 40 ng ml^{-1}). All samples were vortexed gently to mix, and subjected to solid phase extraction using C18UC columns 200 mg $3ml^{-1}$ (UCT Bristol, PA). Briefly, columns were conditioned with 2.5 ml of methanol and 3 ml of water. The samples were loaded onto the column and a minimum of 2 min was allowed for samples to pass through the column. The columns were subsequently rinsed with 2 ml 50% methanol in water, prior to eluting with 2.5 ml methanol. Samples were dried under nitrogen, dissolved in 120 µl of 10% acetonitrile (ACN) in water, with 0.2% formic acid and 40 µl injected into the liquid chromatography tandem mass spectrometry (LC-MS/MS) system.

The analyte concentrations were measured using positive heated electrospray ionization HESI(+). Quantitative analysis was performed on a TSQ Altis triple quadrupole mass spectrometer coupled with a Vanquish liquid chromatography system (Thermo Scientific, San Jose, CA). The spray voltage was 3,500 V, the vaporizer temperature was 400 °C, and the sheath and auxiliary gas were 40 and 15 respectively (arbitrary units). To optimize product masses and collision energies of each analyte, the analytes were infused into the mass spectrometer. Chromatography employed an ACE 3 C18 10 cm × 0.21 cm 3 µm column (Mac-Mod Analytical, Chadds Ford, PA) and a linear gradient of ACN in water with a constant 0.2% formic acid at a flow rate of 0.4 ml min^{-1}. The initial ACN concentration was held at 10% for 0.3 min, ramped to 95% over 4.6 min and held at that concentration for 0.3 min, before re-equilibrating for 2.8 min at initial conditions.

Detection and quantification was conducted using selective reaction monitoring (SRM) of initial precursor ion for buprenorphine [mass to charge ratio (*m*/*z*) 468.3] and the internal standard d4-buprenorphine [(*m*/*z*) 472.3]. The response for the product ions for buprenorphine (*m*/*z* 101.0, 186.9, 243.0, 396.2, 414.2) and the internal standard (*m*/*z* 100.9, 186.9) were plotted, and peaks at the proper retention time integrated, using Quanbrowser software (Thermo Scientific). Quanbrowser software was used to generate calibration curves and quantitate analyte in all samples by linear regression analysis. A weighting factor of 1/X was used for all calibration curves.

2.6.1 Patches analysis

For analysis, the patches were cut into 1 square cm portions and divided into two 50 ml plastic tubes. Each tube was extracted three times with 30 ml methanol by rotating for 30 min and sonicating for 5 min. The extracts were combined and brought to a final volume of 200 ml with methanol, 200 µl was then diluted to 2 ml with methanol and 100 µl of this was subjected to solid phase extraction. A volume of 20 µl was injected into the LC-MS/MS system with the analytical conditions described previously for plasma.

The response for buprenorphine was linear and gave correlation coefficients of 0.99 or better. Accuracy was reported as percent nominal concentration and precision was reported as percent relative standard deviation. Accuracy was 100% for 0.075 ng ml^{-1}, 104% for 4 ng ml^{-1} and 101% for 40 ng ml^{-1}. Precision was 5% for 0.075 ng ml^{-1}, 3% for 4 ng ml^{-1} and 2% for 40 ng ml^{-1}. The technique was optimized to provide a limit of quantitation of 0.01 ng/ml and a limit of detection of approximately 0.005 ng ml^{-1} for buprenorphine.

2.7 Data analysis

Numerical data such as HR, RR, rectal Temp and thermal thresholds were assessed for normality using the Shapiro-Wilk test and by observing histograms and normal Q–Q residual plots. Mixed-effects two factor analysis of variance was used to interpret the effects of time and treatment (fixed nominal effects) and the association of horse-time and horse-treatment were added as random effects. Ambient temperature, a fixed continuous effect, was also included in the model analysis for thermal threshold. To adjust for lack of sphericity, the Greenhouse–Geissner correction was applied. For making multiple comparisons with baseline measurements, the *post hoc* Tukey Honest Significant Difference test and Dunnett's test was conducted. For all analyses (SAS 9.4; SAS Institute Inc., Cary, NC, USA), $p < 0.05$ was considered statistically significant.

The peak concentration (C_{max}) and time to peak plasma concentration (T_{max}) were determined by visual inspection of the

concentration-time data. Non-compartmental analysis and a commercially available computer software program (Phoenix Winnonlin v8.3, Certara, Princeton, NJ) were used for determination of pharmacokinetic parameters. The slope of the terminal portion of the curve, lambda z (λ_z) was used for calculation of the half-life (HL λ_z) using the equation $0.693/\lambda$. The area under curve from time 0 to infinity ($AUC_{0\rightarrow\infty}$) was determined by using the linear up log down trapezoidal rule and dividing the last measured plasma concentration by the terminal slope extrapolated to infinity.

3 Results

All horses successfully completed the study and patch application was well tolerated on the ventral aspect of the tail base. This site was observed to keep the patches intact in good contact with the skin and no missing data was reported due to patch dislodgement. Upon patch removal, there was no evidence of skin inflammation, papules, skin irritation or redness. All horses remained clinically healthy throughout the study and no clinically apparent adverse effects were noted at any buprenorphine dose during entire study period. Based on the subjective data during physical examination, no horses showed signs of colic with either dose or appeared excited. Overall, the horses cooperated well and stood quietly using a halter with lead rope restraint while the physical examination was being conducted.

3.1 Physical examination and thermal thresholds

The variables followed normal distribution and hence the values are represented as mean ± standard deviation. The HR at the baseline timepoint for BUP0, BUP20 and BUP40 was 41 ± 3, 40 ± 4 and 39 ± 4 beats/min, respectively. The RR at baseline timepoint for BUP0, BUP20 and BUP40 was 21 ± 4, 20 ± 3 and 20 ± 4 breaths/min, respectively. The rectal Temp at baseline timepoint for BUP0, BUP20 and BUP40 was 99.2 ± 0.97, 99.7 ± 1.2 and 98.9 ± 1.02 °F, respectively. Between the three groups, there was no change in HR (Figure 4), RR (Figure 5) and rectal Temp (Figure 6) across timepoints as compared to baseline and when compared to each other in a single horse as well as between horses ($p > 0.1$). There was no effect of treatment ($p > 0.4$) or time ($p > 0.2$), and absence of interaction between treatment and time on HR, RR and rectal Temp.

During the entire experiment, ambient temperature ranged between 11.3 °C and 23.8 °C (17.3 ± 3.4 °C). The skin temperature was not different between horses undergoing each of the three treatments ($p > 0.3$) and was 29.6 ± 3.9 °C for BUP0, 29.3 ± 2.2 °C for BUP20 and 29.4 ± 2.7 °C for BUP40. There was a significant effect of treatment ($p < 0.001$) and time ($p < 0.001$), and a significant interaction between treatment and time on thermal threshold readings. With BUP40 treatment, there was a significant increase in thermal thresholds from baseline values as well as in comparison with BUP0 treatment from 2 h until 48 h

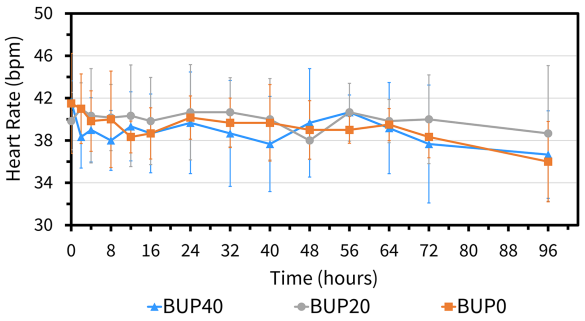

FIGURE 4
Mean ± standard deviation of heart rate values (beats per min; bpm) in six horses at various timepoints, from baseline (0 h) which coincides with before patch application to 2, 4, 8, 12, 16, 24, 32, 40, 48, 56, 64, 72, and 96 h after patch application. The three treatment groups were BUP0 (orange line with solid squares; horses did not receive a patch, instead only the elastic adhesive tape was wrapped around the tail base), BUP20 (grey line with solid circles; horses received one 20 μg h^{-1} patch resulting in a dose of 0.03–0.04 μg kg^{-1} h^{-1} based on their bodyweights on the day of treatment) and BUP40 (blue line with solid triangles; horses received two 20 μg h^{-1}patches placed alongside each other resulting in a dose of 0.07–0.09 μg kg^{-1} h^{-1} based on their bodyweights on the day of treatment). The patch was removed at the 72 h timepoint.

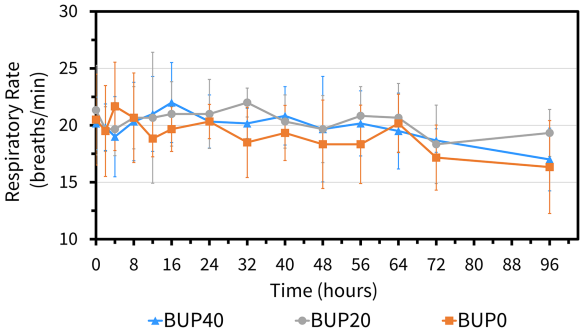

FIGURE 5
Mean ± standard deviation of respiratory rate values (breaths per min) in six horses at various timepoints, from baseline (0 h) which coincides with before patch application to 2, 4, 8, 12, 16, 24, 32, 40, 48, 56, 64, 72, and 96 h after patch application. The three treatment groups were BUP0 (orange line with solid squares; horses did not receive a patch, instead only the elastic adhesive tape was wrapped around the tail base), BUP20 (grey line with solid circles; horses received one 20 μg h^{-1} patch resulting in a dose of 0.03–0.04 μg kg^{-1} h^{-1} based on their bodyweights on the day of treatment) and BUP40 (blue line with solid triangles; horses received two 20 μg h^{-1} patches placed alongside each other resulting in a dose of 0.07–0.09 μg kg^{-1} h^{-1} based on their bodyweights on the day of treatment). The patch was removed at the 72 h timepoint.

(Figure 7). After this timepoint, the thermal thresholds were observed to reach the baseline values. Additionally, when horses receiving 20 μg h^{-1} and 40 μg h^{-1}were compared, BUP40 thermal thresholds were significantly higher than BUP20 for

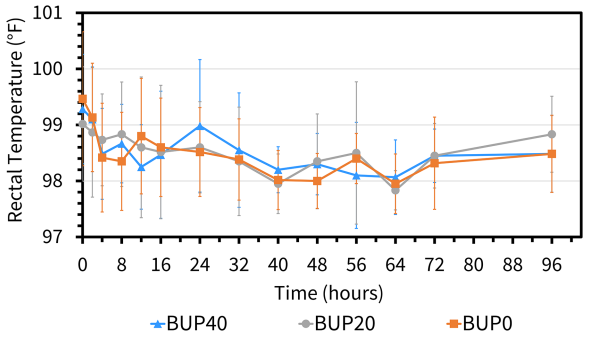

FIGURE 6
Mean ± standard deviation of rectal temperature values (degree fahrenheit; °F) in six horses at various timepoints, from baseline (0 h) which coincides with before patch application to 2, 4, 8, 12, 16, 24, 32, 40, 48, 56, 64, 72, and 96 h after patch application. The three treatment groups were BUP0 (orange line with solid squares; horses did not receive a patch, instead only the elastic adhesive tape was wrapped around the tail base), BUP20 (grey line with solid circles; horses received one 20 μg h^{-1} patch resulting in a dose of 0.03–0.04 μg kg^{-1} h^{-1} based on their bodyweights on the day of treatment) and BUP40 (blue line with solid triangles; horses received two 20 μg h^{-1} patches placed alongside each other resulting in a dose of 0.07–0.09 μg kg^{-1} h^{-1} based on their bodyweights on the day of treatment). The patch was removed at the 72 h timepoint.

multiple timepoints up to the 40 h timepoint, after which they were similar across horses. In BUP20 treatment, thermal thresholds were significantly increased as compared to BUP0 up to 32 h timepoint.

3.2 Pharmacokinetics

Buprenorphine concentrations in horses were detected as early as 2 h and as long as 96 h in both groups (Figure 8). All six horses in BUP40 treatment showed measurable plasma concentrations starting at 2 h and persisting through the last sampling point, with the mean plasma concentrations of >0.1 ng/ml from 4 h to 40 h. The plasma concentration noted as a group mean was 0.18 ng ml^{-1} from 8 h to 16 h. In BUP20 horses, the measurable plasma concentrations were detected up to 96 h (> 0.02 ng ml^{-1} and <0.09 ng ml^{-1}), and plasma concentration recorded was a group mean of 0.09 ng ml^{-1} from 8 h to 16 h timepoints. Norbuprenorphine was not detected in any horse at concentrations above the limits of detection at any time point.

The pharmacokinetic parameters generated for BUP20 and BUP40 treatments using noncompartmental analysis are presented in Table 1. The area under the curve percent extrapolation was well below 25% for both groups. When the patches were removed and submitted for analysis, the amount of buprenorphine extracted from the patch/patches was 33.5 ± 0.54 mg (83.7 ± 0.01%) in the BUP40 treatment and 17.70 ± 0.81 mg (86.5 ± 0.04%) in the BUP20 treatment.

4 Discussion

The present study indicated that the mean plasma concentrations in horses receiving the high-dose transdermal

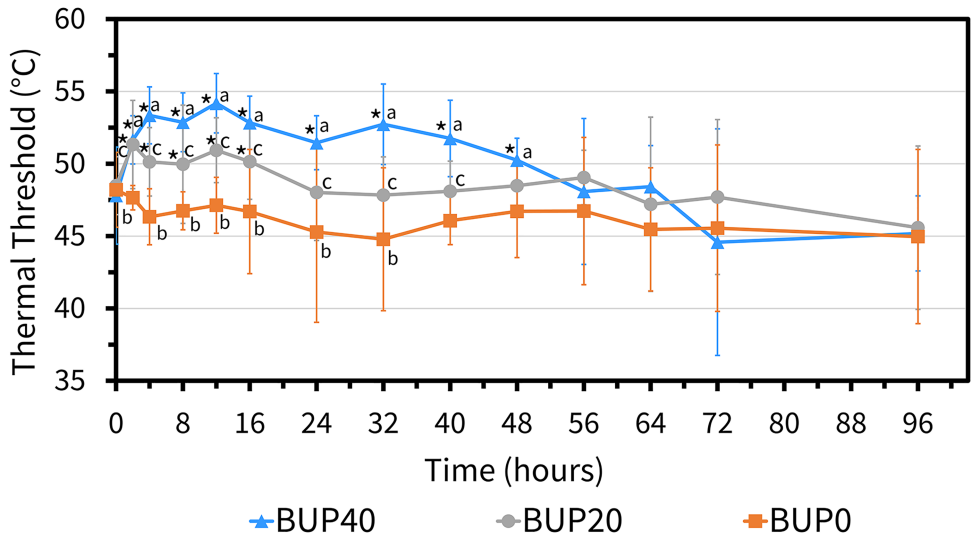

FIGURE 7
Mean ± standard deviation of thermal threshold values (degree celsius; °C) in six horses at various timepoints, from baseline (0 h) which coincides with before patch application to 2, 4, 8, 12, 16, 24, 32, 40, 48, 56, 64, 72, and 96 h after patch application. The three treatment groups were BUP0 (orange line with solid squares; horses did not receive a patch, instead only the elastic adhesive tape was wrapped around the tail base), BUP20 (grey line with solid circles; horses received one 20 μg h^{-1} patch resulting in a dose of 0.03–0.04 μg kg^{-1} h^{-1} based on their bodyweights on the day of treatment) and BUP40 (blue line with solid triangles; horses received two 20 μg h^{-1} patches placed alongside each other resulting in a dose of 0.07–0.09 μg kg^{-1} h^{-1} based on their bodyweights on the day of treatment). The patch was removed at the 72 h timepoint. *Significant difference from baseline (0 h) i.e., before patch application; [a]Significant difference between BUP0 and BUP40; [b]Significant difference between BUP0 and BUP20; [c]Significant difference between BUP20 and BUP40.

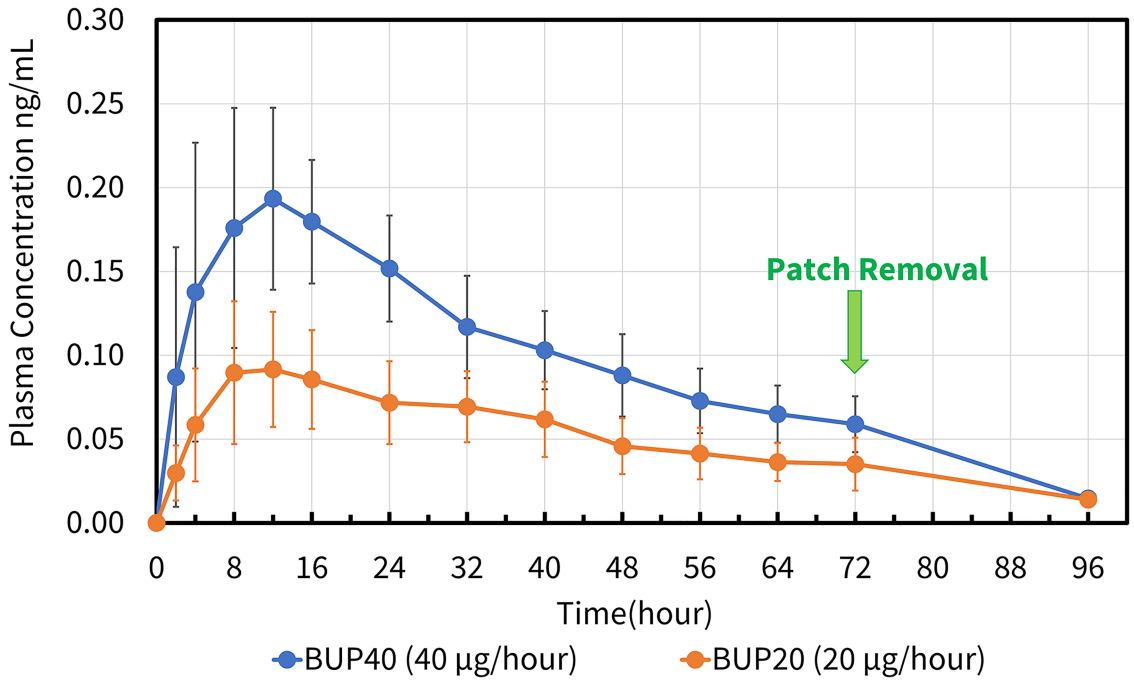

FIGURE 8
Mean ± standard deviation of plasma concentrations of buprenorphine overtime in six horses from baseline (0 h) which coincides with before patch application to 2, 4, 8, 12, 16, 24, 32, 40, 48, 56, 64, 72, and 96 h after patch application. The three treatment groups were BUP0 (horses did not receive a patch, instead only the elastic adhesive tape was wrapped around the tail base), BUP20 (orange line with solid circles; horses received one 20 µg h^{-1} patch resulting in a dose of 0.03–0.04 µg kg^{-1} h^{-1} based on their bodyweights on the day of treatment) and BUP40 (blue line with solid circles; horses received two 20 µg h^{-1} patches placed alongside each other resulting in a dose of 0.07–0.09 µg kg^{-1} h^{-1} based on their bodyweights on the day of treatment). The patch was removed at the 72 h timepoint.

TABLE 1 Mean ± standard deviation of pharmacokinetic parameters generated for two transdermal buprenorphine patch doses in six, adult healthy horses using noncompartmental analysis.

Pharmacokinetic Parameter	BUP20 horses receiving one patch (20 µg h^{-1})	BUP40 horses receiving two patches (40 µg h^{-1})
Tmax (h)	12.70 ± 3.93	11.33 ± 4.68
Cmax (ng ml^{-1})	0.10 ± 0.04	0.21 ± 0.06
AUC_% Extrap_Obs (%)	14 ± 14.2	3.75 ± 1.09
AUCINF_Obs (h × ng ml^{-1})	5.34 ± 1.44	9.23 ± 2.19
AUClast (h × ng ml^{-1})	4.69 ± 1.56	8.89 ± 2.15

Tmax, time of the maximum measured plasma concentration; Cmax, maximum measured plasma concentration; AUC_% Extrap_Obs, percentage of the area under the curve that has been derived after extrapolation; AUCINF_Obs, area under the curve from time of dosing to infinity; AUClast, area under the curve from the time of dosing to the last measurable concentration.

formulations of buprenorphine (40 µg h^{-1}) were >0.1 ng ml^{-1} that lasted from 4 h to 40 h of patch application and coincided with increased thermal thresholds during that entire duration. This highlights a correlation between the plasma concentrations and anti-nociceptive effect for thermal nociception in the study horses. The pattern of plasma concentrations was similar in dogs (9), cats (12), mice and rabbits (28), but significantly varied from findings in pigs (13, 14) and Cynomolgus Macaques (17). The therapeutic threshold for buprenorphine in horses has not been identified, however it has been stipulated in humans and dogs and seems to be relatively similar within species. Typically, the analgesic drug doses are based on correlation between the plasma concentration and observable anti-nociceptive effect in the face of a nociceptive stimulus that can aid in determining the therapeutic plasma concentration. By extrapolating pharmacokinetic data and evaluation of response to pain, the therapeutic plasma buprenorphine concentration threshold is 0.1–0.5 ng ml^{-1} in humans (29, 30) and 0.1–0.6 ng ml^{-1} in dogs (31, 32). While BUP40 treatment demonstrated plasma concentrations >0.1 ng ml^{-1}, this was not the case in BUP20 treatment. Even though with 20 µg h^{-1} patch, measurable plasma concentrations were seen up to 96 h, the group mean was never >0.1 ng ml^{-1}. Also, even though the thermal thresholds were higher than BUP0 treatment, they were significantly lower than BUP40. Once the patches were removed at the 72 h timepoint, the residual plasma concentrations dropped significantly in both groups and the thermal thresholds were similar between groups and reached baseline values. Since there were no differences found in the physical examination among BUP0, BUP20 and BUP40 treatments, both doses appeared safe and well tolerated in all horses.

Previous exploratory studies with buprenorphine in horses utilized average doses of 5–10 µg/kg via intravenous (18, 20, 21, 33–41), intramuscular (23, 24, 38, 42) and sub-lingual (40, 42, 43) routes. A common observation in most of these studies irrespective of the route used was its potential for inducing excitement, increasing spontaneous locomotory activity,

decreasing gut sounds and elevating HR in healthy pain-free horses. In spite of opting for the subcutaneous route for buprenorphine administration in a few equine studies, the gastrointestinal side effects, compulsive behavior and restlessness persisted (22, 44). The doses in the present study were selected carefully on the basis of the behavioral and physiologic responses reported in these studies. We anticipated that 0.03–0.04 μg kg^{-1} h^{-1} (20 μg h^{-1}) and 0.07–0.09 μg kg^{-1} h^{-1} (40 μg h^{-1}) would be a safe, well tolerable dosage regime for our horses which would prevent systemic complications and excitement. Moreover, currently, the highest concentration of transdermal system available for buprenorphine in the United States is 20 μg h^{-1} and since the selected location was the ventral aspect of the tail base, placement of only two patches next to each was possible without overlap to administer 40 μg h^{-1}. Future studies are imperative to evaluate whether a higher transdermal patch dose can lead to plasma concentrations lasting more than 48 h, coinciding with therapeutic drug concentrations yielding adequate analgesia, but still devoid of any systemic complications. It is also important to acknowledge that our study evaluated a one-time transdermal application of buprenorphine to combat thermal nociception, unlike some of the above-mentioned studies that investigated synergism of buprenorphine in conjugation with alpha$_2$ adrenergic agonists.

During the present study, in BUP40 treatment, Cmax was 0.21 ± 0.06 ng ml^{-1}, while in BUP20 treatment Cmax was 0.10 ± 0.04 ng ml^{-1}. The time of the maximum measured plasma concentration was 11.33 ± 4.68 h and 12.70 ± 3.93 h in BUP40 and BUP20 horses, respectively. A single buprenorphine transdermal (hydrogel matrix technology) application in rabbits at 8.4 mg patch resulted in Cmax of 0.97 ± 0.49 ng ml^{-1} and Tmax of 3–24 h. The same type of patch in mice at 0.8 mg patch caused Cmax of 9.3 ± 1.4 ng ml^{-1} and Tmax of 1–24 h (31). Upon application of a 35 μg h^{-1} buprenorphine matrix patch in cats, plasma concentrations were 0.83 ± 0.61 ng ml^{-1} at 12 h, 1.49 ± 0.93 ng ml^{-1} at 22 h, and once the patch was removed after 72 h, they were 4.24 ± 1.31 ng ml^{-1}. The Cmax was 10 ± 0.81 ng ml^{-1} and this peak occurred at times ranging from 34 h after the patch was applied to 6 h after it was removed (12). Variation in first detectable plasma buprenorphine concentrations ranging from 8 to 24 h was reported in Göttingen minipigs that underwent transdermal matrix patch application (30 μg h^{-1}). Plasma buprenorphine concentrations reached a Cmax of 0.6 ± 0.1 ng ml^{-1} at a Tmax of 63.0 ± 3 h. The mean plasma buprenorphine concentration was still above 0.1 ng ml^{-1} at the last time point, i.e., 72 h (13). Another swine study did not support the use of transdermal buprenorphine patch due to large variability in drug plasma concentrations, distribution and magnitude with 35 μg h^{-1} and 70 μg h^{-1} dosing. Serum concentrations of buprenorphine were not detected in any of the animals administered the lower dose. For the high dose, Cmax was attained 12 h after application, and concentrations decreased rapidly after 18 h, while Tmax varied from 18 to 42 h with average 0.3 ± 0.2 ng ml^{-1} at 33 h (14). After a 70 μg h^{-1} patch application in dogs, buprenorphine plasma concentrations increased during the first 36 h and then remained around the 0.7–1.0 ng ml^{-1} range for the remainder of the study. A decrease in plasma buprenorphine concentration was not observed during the period of time studied. Plasma buprenorphine concentrations remained under 0.02 ng ml^{-1} in one dog (9). Evaluation of 10 μg h^{-1} and 20 μg h^{-1} transdermal buprenorphine patched in Cynomolgus Macaques indicated Cmax of 3.43 ± 1.18 ng ml^{-1} and Tmax of 57.00 ± 15.10 h for low dose and Cmax of 8.07 ± 3.85 ng ml^{-1} and Tmax of 45 ± 6 h for high dose. For 20 μg h^{-1}, 0.1 ng ml^{-1} within 8 h after application for 3 of the 4 macaques was observed which remained above this plasma level for 144 h in all 4 animals. In all cases, plasma concentrations fell below the minimum by 24 h after the patch was removed (17). In pregnant sheep, maternal plasma buprenorphine concentrations expressed as median (minimum-maximum) about 26 h after a transdermal patch administration of 40 μg h^{-1} were 0.25 (0.11–1.26) μg L^{-1}, while the fetal buprenorphine concentrations were 0.04 (0.01–0.07) μg L^{-1} (15).

The primary metabolite of buprenorphine is norbuprenorphine, which was undetectable following transdermal administration in the study. This analysis was in accordance with previous studies where norbuprenorphine was unmeasurable following either intravenous or sublingual route (38, 40, 42). Considering norbuprenorphine has only 25% of the intrinsic analgesic activity of buprenorphine and a low permeability into the brain, it may have minimal clinical significance (45). There is no available literature highlighting anti-nociceptive effect of norbuprenorphine in horses and hence it is uncertain whether this metabolite contributes to antinociception. It is possible that the high stability of molecular ions of norbuprenorphine may present a challenge to be detected by tandem mass spectrometry. The assay may not have the sensitivity for measuring this metabolite and this lack of optimization could affect this finding. Also, the limited metabolism of buprenorphine to norbuprenorphine may play a role. Interestingly, when the residual amount on the patches in BUP20 and BUP40 treatments was analyzed in our study, 83%–86% of the buprenorphine was still in the patch. This could be due to the patch not being in firm contact with the skin and the patch formulation altering the diffusivity of the skin lipids, sweat induced changes in skin pH or variation in skin temperature, or skin response to the pre-patch skin preparation. Skin hydration which varies in response to ambient humidity and temperature can affect integrity and barrier properties of the skin resulting in variations in the amount of drug absorbed (46, 47). The majority of buprenorphine left over in the patch explains why we saw lower plasma concentrations and hence, did not observe any significant behavioral effects, differences in the physical examination or gastrointestinal symptoms. However, it also signifies that in spite of partial drug uptake (around 14%–17%), the plasma concentrations obtained in the BUP40 treatment were >0.1 ng ml^{-1} and blunted thermal nociception for a significant period. Dependent on the literature in other species (9, 12–15, 17), once the patch is applied, there is a larger gradient between plasma and the central nervous system that is the cause of delayed increase in plasma concentrations that lead to slow transfer of buprenorphine into the central nervous system and occupying few opioid receptors. This was in contrast to our study, which saw an

increase in plasma concentrations enough to counteract thermal nociception as early as 2 h and lasting up to 48 h in BUP40 horses. At <0.1 ng ml^{-1} concentration, the thermal thresholds began to return to baseline and once the patch was removed at 72 h, plasma concentrations significantly declined in the next 24 h.

In recent years, transdermal opioid delivery systems have become popular across different species and has contributed to significant advances for effective pain management via maintenance of steady blood drug concentrations over longer periods. The established transdermal opioid delivery systems are drug-in-adhesive, reservoir and matrix-type. In the present study, buprenorphine was administered via a matrix patch that includes an adhesive polymer matrix containing the drug homogenously embedded in the center. On the top of this matrix is the backing layer made up of elastomers that protects the patch from the outer environment and is impermeable to the drug. On the bottom of this matrix is the lining layer that protects the patch during storage and is peeled off before use (3, 4, 46, 47). The absence of a drug reservoir can help lower drug abuse and detrimental impact of accidental consumption. Special features that ease the crossing of buprenorphine through the skin are lower molecular weight, compact molecular structure, high lipophilicity, adequate degree of ionization, sufficient water solubility, high efficacy to restitute for limited absorption, reduced melting temperature, relatively shorter half–life, low daily dosage regime, dosing enabling absorption from a relatively small area, and matrix patches in which a total amount of a drug is localized homogenously in an adhesion layer (3). This technology ensures the release of the opioid is regulated due to the gradient concentration between the patch and the skin. Several factors can account for species-specific differences and inter-patient variability with respect to drug uptake from the patch and buprenorphine absorption via the skin such as: (i) thickness of stratum corneum and epidermis, (ii) hair density, (iii) regional blood flow, (iv) drug molecular kinetics, (v) genetics, (vi) underlying skin disease or injury, (vii) formulation of the drug-polymer matrix, (viii) skin temperature, and (ix) skin preparation (razor shaving, alcohol). The Fick's law of diffusion controls the rate of drug input from the transdermal system into the systemic circulation through skin penetration barriers, where the drug delivery is directly proportional to the drug concentration in the matrix and coefficient of drug diffusion. It is vital to note that the drug penetration into the skin is not constant and is dependent on the duration of patch application and overtime variations in cutaneous properties, available drug in the matrix and depletion of enhancers required for drug delivery (3, 48). The site chosen for patch application in the current study was the ventral aspect of the tail base, that has been previously described in horses along with the influence of patch location on uptake of fentanyl from the transdermal patch (48, 49). This is a site with limited access to the horse, thus lowering the potential for accidental removal, yet the area can easily accommodate two patches as shown in the BUP40 group. However, considering there was significant drug left behind on the patch after removal, investigating the influence of other sites such as metacarpus, gaskin, antebrachium, interscapular area is imperative to ensure the poor buprenorphine uptake from the patch is not related to the site of application.

Nociceptive threshold tests are a standard approach for assessing an anti-nociceptive effect in laboratory settings, where mechanical and/or thermal stimuli are employed to assess the efficacy of analgesics. A thermode based system was initially designed and validated in cats, however after technological advances the use of this thermal threshold testing is adapted and has gained popularity in equine studies (25–27). This well described model of thermal nociception was incorporated in our study to determine the analgesic response of different doses of transdermal buprenorphine patch on thermal thresholds. We observed a dose dependent influence on the thermal thresholds in our study horses, which is a similar occurrence previously reported with injectable opioids. This increment in the thermal thresholds was shorter lived and lasted from 2 h to 48 h timepoint in BUP40 treatment. This pattern seemed to follow trends of plasma buprenorphine concentration of >0.1 ng ml^{-1}. The behavioral endpoints such as pawing, stomping, lifting or rubbing their nose on the stimulated front leg have been considered reliable for nociceptive threshold testing (25–27) and hence were carefully followed to ensure reflex related mannerisms could be differentiated from conscious perception of pain arising from the stimulated area. The horses were housed in individual stalls so that housing them close to each other wouldn't impact the results of this testing. Since there is minimal information regarding how ambient temperatures or skin temperatures can cause variation in thermal thresholds in horses, the skin temperature was not different between horses in the three treatment groups and hence doesn't seem to be a factor affecting the thermal threshold readings. The area proximal to the coronary band i.e., the mid cannon bone was chosen since it is less affected by variations in ambient and skin temperatures and blood flow that allows successful detection of behavioral endpoints (27). Gender predisposition to nociceptive sensitivity cannot be ruled out in our study.

This study has some limitations. An intravenous treatment was not included in the study design, and therefore, the bioavailability of the matrix buprenorphine was not calculated. Only a small sample size consisting of healthy, pain-free adult horses was utilized. The physiologic and behavioral effects of opioid administration can differ significantly in painful vs. non-painful animals, hence future studies in clinical patients exhibiting signs of pain are warranted. The genetic involvement for transdermal drug uptake is defined in humans, however its impact cannot be rule out in our study horses. Noxious thermal stimuli have been previously used in experimental pain models to produce superficial acute short-lasting pain, which does not best reflect visceral and somatic pain processes commonly encountered in clinical patients. Behavioral analysis and gastrointestinal function were not assessed using standards published in the literature (e.g., video footage, pedometer data, gastrointestinal motility scores, fecal and urine output, visual analog scoring, ataxia grading, sedation scores). We were not able to perform objective scoring for parameters due to the longer duration of the treatments and less available staff making it impractical. Since

the undesirable effects can be of lesser magnitude in painful horses, future clinical studies are required to objectively quantify these effects and determine their association with transdermal buprenorphine patch in painful vs. non-painful horses.

5 Conclusion

The therapeutic approach of applying transdermal opioid patch provides various advantages such as decreasing animal stress and handling, reducing frequency of dosing, providing continuous drug delivery over an extended period, lowering the peaks and troughs in analgesic effect and the potential for less risk for systemic side effects. If undesirable events occur, the patch can be removed quickly. Following extensive literature review, this appears to be the first report of transdermal buprenorphine patch in horses. In the present study, 20 μg h^{-1} and 40 μg h^{-1} patch doses were safe and well tolerated by all horses as assessed by a physical examination. With the higher dose, there was a significant increase in thermal thresholds from baseline values from 2 h until 48 h and these values were significantly higher than the group receiving the lower patch dose for multiple timepoints up to 40 h. With the 20 μg h^{-1} patch, thermal thresholds were significantly increased as compared to baseline up to 32 h timepoint. However, the 40 μg h^{-1} patch led to consistent measurable plasma concentrations starting at 2 h up to 96 h, with the mean plasma concentrations of >0.1 ng/ml from 4 h to 40 h. Further research should aim at: (i) investigating the effect of higher dosages of the transdermal buprenorphine patch on duration of analgesia and measurable plasma concentrations, (ii) replacing the patch periodically to assess whether the plasma concentrations and analgesia are better maintained, (iii) and comparing analgesic and systemic effects in painful and non-painful horses.

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 study was approved by University of Georgia Institutional Animal Care and Use Committee (animal use protocol: A2021 03-010-Y1-A3). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

VP: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. HK: Conceptualization, Formal Analysis, Methodology, Software, Validation, Writing – review & editing. LB: Data curation, Investigation, Project administration, Resources, Supervision, Writing – review & editing. JC: Data curation, Investigation, Writing – review & editing. SG: Data curation, Investigation, Writing – review & editing. HC: Data curation, Investigation, Writing – review & editing. CJ: Data curation, Investigation, Writing – review & editing. SS: Formal Analysis, Software, Validation, Writing – review & editing. RR: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – review & editing.

Funding

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

This study was funded by the Virginia-Maryland College of Veterinary Medicine-Equine Research Competition Grants Program (source: Equine Racing Funds; grant fund no: 179767)

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.

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The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpain.2024.1373555/full#supplementary-material

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frontiers | Frontiers in Pain Research

TYPE Original Research
PUBLISHED 20 March 2024
DOI 10.3389/fpain.2024.1373759

OPEN ACCESS

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RECEIVED 20 January 2024
ACCEPTED 11 March 2024
PUBLISHED 20 March 2024

CITATION
Reed RA, Berghaus LJ, Reynolds RM, Holmes BT, Krikorian AM, Sakai DM, Ishikawa Y and Knych HK (2024) The pharmacokinetics and pharmacodynamics of fentanyl administered via transdermal patch in horses. Front. Pain Res. 5:1373759. doi: 10.3389/fpain.2024.1373759

The pharmacokinetics and pharmacodynamics of fentanyl administered via transdermal patch in horses

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Introduction: Understanding the pharmacokinetics and pharmacodynamics of fentanyl in horses is crucial for optimizing pain management strategies in veterinary medicine.
Methods: Six adult horses were enrolled in a randomized crossover design. Treatments included: placebo, two 100 mcg/h patches (LDF), four 100 mcg/h patches (MDF), and six 100 mcg/h patches (HDF). Patches were in place for 72 h. Blood was obtained for fentanyl plasma concentration determination, thermal threshold, mechanical threshold, heart rate, respiratory rate, and rectal temperature were obtained prior patch placement and at multiple time points following patch placement for the following 96 h. Fentanyl plasma concentration was determined using LC-MS/MS. Data were analyzed using a generalized mixed effects model.
Results: Mean (range) maximum plasma concentration (Cmax), time to Cmax, and area under the curve extrapolated to infinity were 1.39 (0.82–1.82), 2.64 (1.21–4.42), 4.11 (2.78–7.12) ng/ml, 12.7 (8.0–16.0), 12.7 (8.0–16.0), 12 (8.0–16.0) h, 42.37 (27.59–55.56), 77.24 (45.62–115.06), 120.34 (100.66–150.55) h ng/ml for LDF, MDF, and HDF, respectively. There was no significant effect of treatment or time on thermal threshold, mechanical threshold, respiratory rate, or temperature ($p > 0.063$). There was no significant effect of treatment on heart rate ($p = 0.364$). There was a significant effect of time ($p = 0.003$) on heart rate with overall heart rates being less than baseline at 64 h.
Conclusions: Fentanyl administered via transdermal patch is well absorbed and well tolerated but does not result in an anti-nociceptive effect as measured by thermal and mechanical threshold at the doses studied.

KEYWORDS

horse, fentanyl, pharmacokinetics, pharmacodynamics, transdermal, opioid, pain

1 Introduction

Pain management in horses is currently largely limited to the use of opioids and non-steroidal anti-inflammatory agents (NSAIDs). Indeed, opioids are a mainstay in the management of acute pain in horses (1–3). Unfortunately, most opioids are available only as injectable formulations that require intravenous or intramuscular injections with

the need for frequent administration. This treatment approach creates peaks in plasma concentration that may result in adverse effects and nadirs in plasma concentration during which horses may experience pain.

Fentanyl, a pure mu agonist opioid, is available for use in injectable and transdermal patch formulations (4–11). Fentanyl is a highly lipophilic opioid with a rapid onset and brief duration of action (12, 13). In horses, a single intravenous bolus provides an anti-nociceptive effect lasting only 10–30 min. For this reason, the injectable formulation of fentanyl must be administered as a continuous intravenous infusion to maintain the plasma concentrations needed for long-term analgesia (5).

There are two studies in the literature finding an anti-nociceptive effect of fentanyl whose findings differ considerably with respect to the plasma concentration required to achieve this effect. One of these studies (5) was performed using an intravenous bolus dose and an antinociceptive effect was measured via thermal and mechanical threshold in non-painful horses. The other was a clinical study in which fentanyl patches were applied to horses with pain refractory to NSAID therapy (11). In the first study, plasma concentrations of fentanyl measured when an anti-nociceptive effect occurred shortly after the bolus dose (6.1–6.8 ng/ml) (5) were much higher than those achieved with the fentanyl patch in the second study (1.1 ng/ml) (11). This suggests that analgesia may be achieved at lower plasma concentrations when administered transdermally than are required with intravenous administration. Furthermore, fentanyl patches have provided analgesia at concentrations below 1.1 ng/ml in people (0.63 ng/ml) (14) and dogs (0.6 ng/ml) (15), further supporting additional study of the fentanyl patch in horses. Thus, transdermal fentanyl administration represents a potential means of providing continuous opioid mediated analgesia without the need for maintenance of an intravenous catheter or a frequent dosing schedule.

The aims of the study presented here were twofold: (1) to describe the pharmacokinetics of fentanyl administered via transdermal matrix patch at three different doses, and (2) describe the associated pharmacodynamics with regard to anti-nociceptive effect as measured by thermal and mechanical threshold and effect on physical exam parameters. The authors hypothesized that fentanyl would be well absorbed, exhibiting dose dependent pharmacokinetics, and provide a dose dependent anti-nociceptive effect with minimal adverse effects on physical exam variables.

2 Materials and methods

2.1 Study design

This study was a prospective, randomized, masked, cross-over design. This study was approved by the University of Georgia Institutional Animal Care and Use Committee (Animal Use Protocol A2020 05-010).

2.2 Methods

Six horses weighing 528 ± 49 kg (4 males, 2 females) with a mean age of 14 (range 7–23) years were enrolled. Normal health status was confirmed based on physical exam prior to enrollment.

Based on previous pharmacokinetic studies published by the investigators and others in veterinary medicine, data from 6 animals is sufficient to evaluate the interindividual variability of the pharmacokinetic parameters. A sample size calculation with a one-tailed paired t-test found that, on each treatment, four horses would provide sufficient power to detect an effect of 3°C in thermal threshold with a standard deviation of 1.4°C compared to baseline, power of 0.9 and alpha of 0.05 (G*Power 3.1.9.7, Heinrich-Heine-Universität, Düsseldorf, Germany). The additional two horses allowed for some variation in observed values from the hypothetical values used for sample size calculation.

Horses were housed in $12' \times 12'$ (3.7 m × 3.7 m) stalls in a temperature-controlled facility for the duration of the study and were acclimatized to the stalls for at least 12 h prior to treatment. Each horse received 1 lb (0.45 kg) of feed and 2–3 flakes of Timothy hay twice daily throughout the study period. All procedures were designed to minimize stress and discomfort to the horses, with continuous monitoring for any signs of adverse effects.

The study was completed in two phases. A fentanyl patch phase for collection of pharmacodynamic and pharmacokinetic data and an intravenous bolus phase to aid in determination of bioavailability of fentanyl from the patch.

2.2.1 Phase I

In the first phase, horses received each of four treatments in a randomly assigned (www.randomizer.org) order with a minimum of a 7-day washout between treatments. These treatments included two 100 mcg/h patches (LDF) (Mylan N.V., Canonsburg, PA) for 72 h, four 100 mcg/h patches (MDF) for 72 h, six 100 mcg/h patches (HDF) for 72 h and placebo (no patches placed). All patches were placed on the metatarsi with half of the patches on each leg. Prior to patch placement, the dorsal aspect of the metatarsi was clipped with a #50 clipper blade and dry gauze was used to wipe away gross debris. All patches were covered with opaque elastic tape (Elastikon, Johnson & Johnson, New Brunswick, NJ). For the placebo treatment, the metatarsi were similarly wrapped to facilitate masking. These fentanyl matrix patch dosages were chosen based on the plasma concentrations achieved by the investigators in a previous study (16), the published range of analgesic plasma concentrations reported for people and dogs (~0.6 ng/ml) (14, 15), and the analgesic concentrations achieved in horses after intravenous bolus administration (6.1–6.8 ng/ml) with the aim of achieving fentanyl plasma concentrations similar to those reported in both studies (5).

2.2.1.1 Blood collection for pharmacokinetic analysis in first phase

Each horse was weighed and a baseline physical exam was performed prior to each treatment. Horses were instrumented with a 14 gauge intravenous catheters (Mila International, Inc.,

KY, USA) in one jugular vein for blood collection during the first 24 h of treatment. Subsequent samples were collected via direct venipuncture. Blood samples were obtained at baseline and again at 2, 4, 8, 12, 16, 24, 32, 40, 48, 56, 64, 72, and 96 h after placement. Following removal of a 10 ml waste sample, a total of 6 ml of whole blood was obtained at each timepoint, and stored in lithium heparin tubes (Becton, Dickinson, and Company, NJ, USA) for no longer than 1 h prior to processing. Blood was centrifuged at 1,300 g for 10 min and plasma was harvested and placed in cryovials (VWR, International, PA, USA) prior to storage at −80°C until analysis. All fentanyl patches were labeled and stored at −80°C until analysis to determine the amount of residual fentanyl in the patches.

2.2.1.2 Pharmacodynamic data collection in first phase

Pharmacodynamic data (thermal/mechanical threshold, heart rate, respiratory rate, body temperature, and borborygmi score) were recorded at baseline and the same time points following treatment outlined above for blood sampling. The anti-nociceptive effect of treatment was determined using thermal and mechanical threshold testing (Topcat Metrology Ltd, United Kingdom) over the metacarpus. A coin toss was used to randomize the assigned leg for each unit at each time point. The dorsal aspect of both metacarpi were clipped using a #50 clipper blade to ensure good contact. For the thermal threshold device, a heating element was applied over the shaved area and connected to a control unit secured to the horse's withers via a surcingle. A masked operator using a wireless remote increased the temperature by 0.8°C/s until the horse exhibited an avoidance behavior (stomping, kicking, sniffing of the stimulated forelimb). The temperature at which this avoidance behavior occurred was the thermal threshold for that time point. A maximum temperature of 55°C was not exceeded in order to avoid tissue injury. For the mechanical threshold device, an actuator with a 1 × 1 mm pin was attached to the shaved metacarpus opposite that of the thermal threshold device. This device was controlled by a masked operator increasing the pressure exerted by the pin until an avoidance behavior was observed (stomping, kicking, sniffing of the stimulated limb). The pressure in Newtons (N) at which this behavior occurred was the mechanical threshold for that time point. A maximum pressure of 20 N was not exceeded in order to avoid tissue injury. Baseline thermal and mechanical thresholds were obtained in triplicate prior to treatment administration. Single measurements were obtained for each subsequent measurement following treatment. Physical exam variables were recorded at each timepoint prior to measurement of thermal and mechanical threshold. Borborygmi was scored using a previously published scoring system (17) assigning a score to each quadrant following 1 min of auscultation with the sum of these values being the total score for that timepoint.

2.2.2 Phase II

At least 30 days following the first phase, all horses underwent the second phase of treatment which included a single 2 mg fentanyl bolus administered intravenously to aid in determination of fentanyl bioavailability from the patch. For this treatment, horses were instrumented with 14-gauge intravenous catheters in both jugular veins prior to treatment. One catheter was used for administration of treatment and immediately removed following treatment. The catheter in the opposite jugular vein was used for sampling. Following collection of a 10 ml waste sample, a total of 6 ml of whole blood was obtained prior to treatment and again at 1, 3, 5, 7, 10, 20, 30, 40, 50, 60, 90, 120, 150, 180, 240, 300, 360, and 420 min following fentanyl administration. The sampling catheter was removed following collection of the final sample.

2.3 Fentanyl concentration determination

Plasma calibrators were prepared by dilution of the fentanyl working standard solutions (Cerilliant, Round Rock, TX) with drug free equine plasma to concentrations from 0.005 to 50 ng/ml. Calibration curves and negative control samples were prepared fresh for each quantitative assay. Quality control samples were included with each sample set as an additional check of accuracy.

Prior to analysis, 400 µl of plasma samples were diluted with 100 µl of water containing 4 ng/ml of d5-fentanyl internal standard (Cerilliant, Round Rock, TX), and 2 ml 0.1M phosphate buffer at pH 7 and vortexed briefly to mix. The samples were subjected to solid phase extraction using Cerex PolyChrom Clin II (35 mg/ 3cc) columns (Tecan SPE Inc., Baldwin Park, CA). Plasma samples were loaded on and passed through the columns using a CEREX system 48 Processor with positive pressure SPE manifold (SPE Ware, Baldwin Park, CA). A minimum of 2 min was allowed for samples to pass through the column. Subsequently, the columns were rinsed consecutively with 3 ml of water, 2 ml 1M acetic acid, and 3 ml methanol prior to elution with 2 ml of methanol:ammonium hydroxide (97:3, v:v). Samples were dried under nitrogen, reconstituted in 120 µl of redissolve solution, 10% ACN in water with 0.2% formic acid and 30 µl injected into the liquid chromatography tandem mass spectrometry (LC-MS/MS) system.

Quantitative analysis was performed on a TSQ Altis triple quadrupole mass spectrometer coupled with a Vanquish liquid chromatography system (Thermo Scientific, San Jose, CA). The spray voltage was 3,500 V, the vaporizer temperature was 350°C, and the sheath and auxiliary gas were 50 and 10 respectively (arbitrary units). The standards were infused into the instrument to optimize product masses and collision energies of the analytes. Chromatography employed an ACE 3 C18 5 cm × 2.1 mm column (Mac-Mod Analytical, Chadds Ford, PA) and a linear gradient of acetonitrile (ACN) in water containing 0.2% formic acid, at a flow rate of 0.35 ml/min. The initial ACN concentration was held at 5% for 0.2 min, ramped to 95% over 3.8 min and held at that concentration for 0.2 min, before re-equilibrating for 2.9 min at initial conditions.

Detection and quantification was conducted using selective reaction monitoring (SRM) of initial precursor ion for fentanyl [mass to charge ratio *(m/z)* 337.2] and the internal standard d5-fentanyl (*(m/z)* 342.2). The response for the product ions for

fentanyl (*m/z* 105.2, 132.2, 188.2) and the internal standard d5-fentanyl (*m/z* 102.9, 104.9, 188.0) were plotted and peaks at the proper retention time integrated using Quanbrowser software (Thermo Scientific). Quanbrowser software was used to generate calibration curves and quantitate fentanyl in all samples by linear regression analysis. A weighting factor of 1/X was used for all calibration curves.

Patch Analysis: Fentanyl patches were cut into 1 cm portions and mixed in 100 ml of methanol and subsequently serially diluted tenfold in redissolve solution three times. The samples were quantitated with a calibration curve prepared in the redissolve solution. Ten microliters of the sample was injected in the LC-MS/MS system utilizing the analytical conditions described for plasma samples. Total dose absorbed from the patch was determined by subtracting the residual amount of fentanyl in the patch from the total fentanyl in the patch formulation (10 mg per patch).

2.4 Pharmacokinetic analysis

The peak concentration (C_{max}) and time to peak plasma concentration (T_{max}) were determined by visual inspection of the concentration-time data. For determination of initial estimates for subsequent model fitting, non-compartmental analysis (NCA), using a commercially available computer software program (Phoenix Winnonlin v8.3, Certara, Princeton, NJ) was used. Subsequent to NCA, a nonlinear mixed effects modeling (NLME) approach with the Phoenix NLME software program was used to fit a compartmental model to the data. The first-order conditional estimation method with interaction (FOCE-ELS) was used in the model-building process. Both two and three compartment models were evaluated. For residual error models, additive, multiplicative, and Poisson error models were all considered. Random effects were included for all structural variables and were modeled with log linear functions. A diagonal variance-covariance matrix was used for the random effects. In assessing which model provided the best fit, visual analysis of the observed vs. predicted concentration graphs, residual plots, Akaike Information Criterion, %CV, and -2LL were considered. A simultaneous fit of the intravenous and transdermal data was attempted but the fit was poor. Transdermal data were too variable and had inadequate frequency of sampling to fully determine the elimination phase using a population PK approach. For transdermal administration, pharmacokinetic parameters from NCA are reported.

Bioavailability was calculated for individual horses using the formula:

$$(AUC_{transdermal}/Dose_{transdermal})/(AUC_{IV}/Dose_{IV})$$

where the transdermal dose is the total amount absorbed determined by analysis of the patches. The bioavailability was calculated for each horse within a dose group and the individual values averaged and reported.

2.5 Statistical analysis

All analyses were performed using JMP Statistical Discovery (Cary, NC). Normality of the data was assessed by examination of histograms and normal Q-Q plots of residuals and Shapiro Wilk tests. Pharmacokinetic parameters were analyzed with generalized linear mixed model with treatment as a fixed effect and horse as a random effect. Where a significant effect treatment was found, a Tukey test was used for multiple comparisons between treatments. Heart rate, respiratory rate, temperature, and borborygmi scores were analyzed using a generalized linear mixed effects model with treatment, time, and the interaction of treatment and time as fixed effects. Horse was included as a random effect. A Dunnett's test for multiple comparisons to baseline was utilized for variables in which the mixed effects model found a significant effect of time. Generalized linear mixed models were selected due to their ability to handle the correlated data structure inherent in crossover study designs and to account for inter-individual variability. Figures were generated with GraphPad Prism. For all analyses, $p < 0.05$ was considered statistically significant.

3 Results

3.1 Fentanyl concentration determination

The response was linear and gave correlation coefficients of 0.99 or better. Accuracy was reported as percent nominal concentration and precision as percent relative standard deviation. Accuracy was 97% for 0.0075 ng/ml, 112% for 2 ng/ml and 102% for 25 ng/ml. Precision was 12% for 0.0075 ng/ml, 3% for 2 ng/ml and 3% for 25 ng/ml. The technique was optimized to provide a limit of quantitation (LOQ) of 0.005 ng/ml and a limit of detection (LOD) of approximately 0.0025 ng/ml in plasma and an LOQ of 0.01 ng/ul and an LOD of approximately 0.005 ng/ul for the patches.

3.2 Pharmacokinetics

Plasma concentrations of fentanyl for HDF, MDF, and LDF are depicted in Figure 1. Pharmacokinetic variables following non-compartmental analysis (NCA) for all patch treatments are presented in Table 1. The maximum plasma concentration (Cmax) of HDF was significantly higher than MDF ($p = 0.048$) and LDF ($p = 0.001$). There was no difference in Cmax between MDF and LDF ($p = 0.089$). The area under the curve extrapolated to infinity (AUCinf) of HDF was significantly greater than MDF ($p = 0.002$) and LDF ($p = <0.001$); and MDF was significantly greater than LDF ($p = 0.008$). There was no difference between groups in time to maximum plasma concentration (Tmax) ($p > 0.89$), terminal half-life (HL λ_Z) ($p > 0.586$), clearance ($p > 0.304$) for all, or terminal slope of the plasma concentration time curve (λ_Z) ($p > 0.686$).

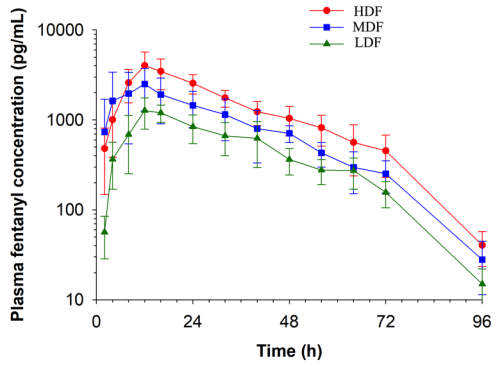

FIGURE 1
Fentanyl plasma concentration (mean ± SD) over time following application of two (LDF), four (MDF) and six (HDF) 100 mcg/h (16.8 mg/patch) matrix patches to healthy adult horses ($n = 6$).

A 3-compartment model and a multiplicative residual error gave the best fit to the plasma concentration data from the intravenous administration. The diagnostic plots, used to assess the fit for the NLME model are provided as Supplementary Figures 1, 2. The pharmacokinetic parameters (estimate and % coefficient of variation for the fixed and random effects) for the NLME model are shown in Table 2.

Plasma concentrations from the intravenous treatment group are presented in Figure 2. Total dose absorbed from the patch ($Dose_{patch}$) and calculated bioavailability are presented in Table 3. There was no significant difference between groups in bioavailability of the fraction absorbed from the patch ($p > 0.346$ for all).

3.3 Pharmacodynamics

All treatments were well tolerated. There were no adverse effects of treatment noted and no horse showed signs of colic at any time. All patches remained well adhered to the skin until the time of patch of removal. There was no evidence of irritation of the skin at the treatment site following patch removal.

3.3.1 Thermal and mechanical threshold

Ambient temperature during the study period ranged between 19.9°C and 22.1°C (mean ± standard deviation, 20.6 ± 0.4°C). Mean thermal and mechanical threshold over time are presented in Figures 3, 4, respectively. For thermal threshold, there was no significant effect of treatment ($p = 0.418$), time ($p = 0.063$), or their interaction ($p = 0.457$). For mechanical threshold, there was a significant effect of time ($p < 0.001$). However, a *post-hoc* Dunnett's test for multiple comparisons revealed no significant difference from baseline at any timepoint. There was no effect of treatment ($p = 0.437$) or the interaction of treatment and time ($p = 0.698$).

3.3.2 Physical exam variables

In regard to heart rate, least squares mean (LSM) and 95% confidence interval [CI (LL, UL)] were 40 [30, 43], 37 [34, 41], 35 [32, 39], and 38 [34, 42] beats per minute for LDF, MDF, HDF, and P, respectively. There was a significant effect of time ($p = 0.003$). *Post hoc* Dunnett's test for multiple comparisons revealed heart was significantly lower than baseline at 64 h ($p = 0.006$) only. There was no significant effect of treatment ($p = 0.3644$) or the interaction of treatment and time ($p = 0.579$).

LSM and 95% CI respiratory rate was 14 [12, 17], 12, [9, 14], 11 [8, 13], and 13 [11, 15] breaths per minute for LDF, MDF, HDF, and P, respectively. There was a significant effect of time ($p < 0.001$). However, *post-hoc* Dunnett's test for multiple comparisons revealed no significant difference from baseline at any timepoint. There was no significant effect of treatment ($p = 0.072$) or the interaction of treatment and time ($p = 0.385$).

LSM and 95% CI rectal temperature was 99.0 [98.5, 99.6], 99.3 [98.7, 99.8], 99.5 [98.9, 100.0], and 99.4 [98.8, 99.9]°F for LDF, MDF, HDF, and P, respectively. There was a significant effect of time ($p < 0.001$). *Post hoc* Dunnett's test revealed no significant difference from baseline at any timepoint. There was no effect of treatment ($p = 0.560$) or the interaction of treatment and time ($p = 0.532$).

LSM and 95% CI borborygmi scores were 13 [12, 14], 12 [12, 13], 12 [12, 13], and 13 [12, 14] for LDF, MDF, HDF, and P, respectively. There was no effect of treatment ($p = 0.155$), time ($p = 0.230$), or the interaction of treatment and time ($p = 0.744$).

TABLE 1 Pharmacokinetic parameters (mean and range) generated by non-compartmental analysis for fentanyl following placement of two (LDF), four (MDF), and six (HDF) 100 mcg/h matrix patches in healthy adult horses ($n = 6$).

	LDF	MDF	HDF
C_{max} (ng/ml)	1.39 (0.82–1.82)	2.64 (1.21–4.42)	4.11 (2.78–7.12)[a]
T_{max} (h)	12.7 (8.0–16.0)	12.7 (8.0–16.0)	12 (8.0–16.0)
AUC_{inf} (h ng/ml)	42.37 (27.59–55.56)	77.24 (45.62–115.06)[a]	120.34 (100.66–150.55)[a,b]
AUC % extrap	0.435 (0.170–0.821)	0.805 (0.088–2.27)	0.448 (0.200–0.846)
HL λ_Z (h)	7.94 (6.37–11.4)	9.73 (6.50–15.9)	9.18 (6.71–12.2)
λ_Z (1/h)	0.091 (0.061–0.109)	0.080 (0.044–0.107)	0.079 (0.057–0.103)
Cl/F (ml/min/kg)	15.57 (11.36–22.88)	9,929.3 (5,794.0–14,608.8)	16.01 (12.58–18.81)
Vd/F (L)	5,558.9 (3,535.0–7,202.2)	9,098.9 (3,260.4–16,768.9)	6,583.3 (4,706.2–7,694.1)

C_{max}, maximum plasma drug concentration; T_{max}, time of maximum plasma drug concentration; AUC_{inf}, area under the curve extrapolated to infinity; AUC % extrap, percent of area under the curve extrapolated; HL λ_Z, terminal half-life; λ_Z, terminal slope of the plasma concentration time curve; Cl/F, systemic clearance per fraction absorbed for patch treatments, total systemic clearance for IV treatment; Vd/F, volume of distribution per fraction absorbed for patch treatments and volume of distribution at steady state for intravenous treatment.
[a]Significantly greater than LDF ($p < 0.008$, for both).
[b]Significantly greater than MDF ($p = 0.002$).

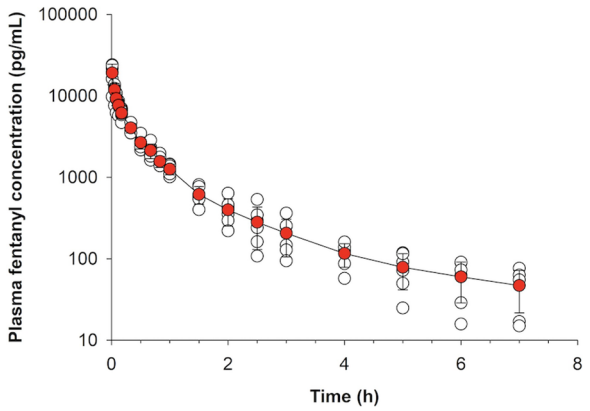

FIGURE 2
Fentanyl plasma concentration (mean ± SD) over time following intravenous administration of 2 mg fentanyl to healthy adult horses (n = 6).

TABLE 2 Model typical values (tv) for fentanyl following a single intravenous (2 mg) administration to horses (n = 6).

Parameter	Estimate	CV (%)
tvA (pg/ml)	12,661.7	19.4
tvB (pg/ml)	6,613.1	23.0
tvC (pg/ml)	528.6	27.1
tvAlpha (1/h)	17.8	34.8
tvBeta (1/h)	2.12	15.4
tvGamma (1/h)	0.375	12.5
$t_{1/2\alpha}$ (h)	0.039	34.8
$t_{1/2\beta}$ (h)	0.327	15.4
$t_{1/2\gamma}$ (h)	1.85	12.5
AUC_{last} (h*pg/ml)	5,243.4	5.39
Cl (ml/h/kg)	722.4	5.39
V1 (L/kg)	0.191	17.1
V2 (L/kg)	0.185	10.1
V3 (L/kg)	0.351	21.3
stdev0	0.150	10.9
Between subject variability (%CV)		
A	0.104	33.1
B	0.059	24.5
C	0.209	48.2
Alpha	1.48 × 10−6	0.12
Beta	0.004	5.92
Gamma	0.035	18.9

tvA, tvB and tvC, intercepts at t = 0 for the model equation; tvalpha, tvbeta and tvgamma, slopes for the modeled equation; V_1, V_2, V_3, volumes of the central, second and third compartments, respectively; Vss, volume of distribution at steady state (calculated as MRT * Cl); $t1_{/2\alpha}$, phase 1 half-life; $t_{1/2\beta}$, phase 2 half-life; $t_{1/2\gamma}$, phase 3 half-life; AUC_{last}, area under the curve until the last time point; Cl, total serum clearance. stdev0 = the estimated residual standard deviation for plasma data.

TABLE 3 Mean ± SD dose of fentanyl absorbed and associated bioavailability after application of two (LDF), four (MDF) and six (HDF) 100 mcg/h (16.8 mg/patch) matrix patches to healthy adult horses (n = 6).

Treatment	Target dose (mg)	Dose absorbed (mg)	Fractional bioavailability (%)
LDF	33.6	28.8 ± 4.8	57.8 ± 5.7
MDF	67.2	57.9 ± 7.1	49.9 ± 14.0
HDF	100.8	86.0 ± 6.6	54.3 ± 8.6

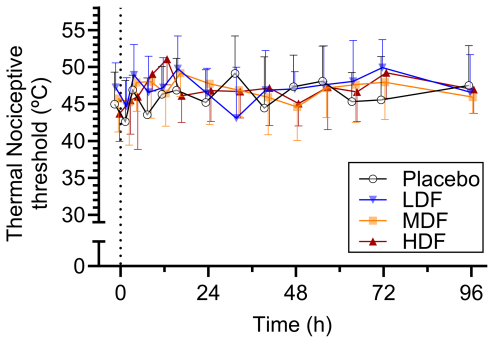

FIGURE 3
Thermal nociceptive thresholds (°C) (mean ± SD) at from 0 to 96 h following application of zero (placebo), two (LDF), four (MDF) and six (HDF) 100 mcg/h (16.8 mg/patch) matrix patches to healthy adult horses (n = 6).

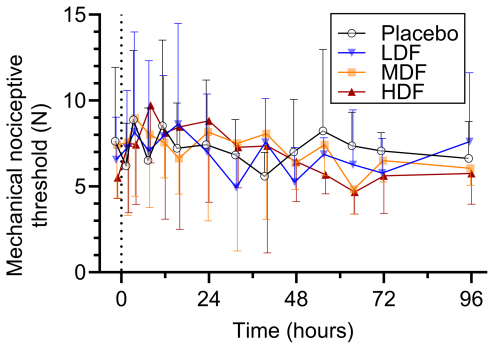

FIGURE 4
Mechanical nociceptive thresholds (°C) (mean ± SD) at from 0 to 96 h following application of zero (placebo), two (LDF), four (MDF) and six (HDF) 100 mcg/h (16.8 mg/patch) matrix patches to healthy adult horses (n = 6).

4 Discussion

4.1 Pharmacokinetics

In the current study, we chose to model the intravenous data using a population pharmacokinetic model, which differs from previous reports of fentanyl pharmacokinetics in the horse. This modeling approach allowed for reporting of inter-individual variability for the pharmacokinetic parameters as well providing an overall estimate of residual variability. While the model fit in the current study was good, it is important to note that that the number of horses included in the current study is low and the model could be strengthened by the addition of more animals.

Although modeling of transdermal data with a population model was attempted, the fit was poor and therefore, NCA was used for this data set.

In the present study, fentanyl administered via a transdermal matrix patch exhibited a dose dependent Cmax and AUCinf with similar terminal half-lives and clearance rates across all treatments. The pharmacokinetics of a single dose of fentanyl administered via a matrix patch have been previously described in a study investigating the effect of patch location by Skrzypczak et al. (16). In that study, fentanyl from two 100 mcg/h patches was well absorbed at the metacarpus, tail, and inguinal abdominal region. The Cmax reported in that study ranged from 1.55 to 2.07 ng/ml which is higher than the 1.378 ng/ml achieved with placement of two 100 mcg/h patches in the present study. Indeed, the decision to apply two, four, and six 100 mcg/h patches was based on the results of this previous study with the hope that six patches would result in plasma concentrations in excess of the 6.1–6.8 ng/ml reported to provide an antinociceptive effect (5). The lower plasma concentrations reported here could be due to placement of the patch on the metatarsus, as opposed to one of the previously studied locations, suggesting there may be site-dependent absorption variability associated with the metatarsus in comparison to the previously studied sites. However, there is not enough room to place six patches on the ventral tail base, ensuring continuous good contact of six patches in the inguinal region for 72 h would have been difficult to achieve while maintaining masking of observers, and the metacarpus was occupied by the thermal and mechanical threshold units. Therefore, these locations were not chosen for patch placement. The Tmax reported here was 12 h for all groups, which is consistent with the previous study reporting a Tmax of 10–14 h.

The AUCinf of 42.374 ng h/ml was slightly lower in the present study than the previous study that described 44.6–46.6 ng h/ml with placement of two patches (16). This is most likely attributed to differences in absorbance of fentanyl from the patch at the metatarsal location. The previous study did not determine how much fentanyl remained in the patch following removal, and therefore it is impossible to say if the horses received the same fraction of the dose incorporated into the patch.

Comparing matrix patches to reservoir patches, it appears that reservoir patches may be superior in regard to exposure to fentanyl. Indeed, placement of two 100 mcg/h reservoir patches resulted in a Cmax of 2.6 ng/ml and AUCinf of 80–92 ng h/ml (8). However, differences in study design could account for this difference. This superior absorption from the reservoir patches may be attributed to the fact that the skin was clipped and shaved prior to patch application which could have resulted in damage or removal of the stratum corneum, the rate limiting barrier in the absorption of transdermal fentanyl (18). Additionally, reservoir patches that became dislodged before 24 h of application were replaced with a new patch which may have contributed to the higher Cmax and AUCinf in that study compared to that reported for fentanyl matrix patches.

Compared to intravenous administration, the terminal half-life following transdermal administration is prolonged. Although this interpretation should be made with caution, as the minimum number of data points (3–4) were used in the calculation of the terminal slope, this suggests that flip-flop kinetics occurs with transdermal administration of fentanyl matrix patches in horses.

4.2 Pharmacodynamics

Previous studies examining the effect of fentanyl on thermal threshold in horses have yielded conflicting results. Intravenous bolus administration of a 2.5, 5, and 10 mcg/kg fentanyl resulted in a dose dependent increase in thermal threshold using a radiant thermal stimuli, although fentanyl plasma concentrations were not reported in that study (6). A later study, using the same intravenous fentanyl dosages as the previous study described an increase in thermal threshold measured at the withers following administration of 10 mcg/kg, corresponding to a plasma concentration of 6.1–6.8 ng/ml (5). Conversely, stepped infusions of fentanyl did not result in a significant difference in thermal threshold from placebo at plasma concentrations as high as 7.82 ng/ml (9). These conflicting results may be due to differences in study design. In the latter study, the thermal threshold stimulus had an automatic cutout at 45°C as opposed to 56°C in the former study utilizing the same thermal stimulus model. The lower cutout temperature may have resulted in the inability of the investigators to accurately report the thermal threshold of the horses with higher fentanyl plasma concentrations due to blunting of the data by the low cut out temperature. In the study reported here, an automatic cutout of 55°C was applied and therefore any effect of fentanyl on thermal threshold should have been captured. Moreover, the lack of effect on thermal threshold observed here is likely due to the lower-than-expected plasma concentration achieved in the HDF group (4.11 ng/ml).

Only a single other study has examined the effect of fentanyl on mechanical threshold in horses. In that study, there was a significant increase in mechanical threshold 10 min following administration of 10 mcg/kg. However, the effect was not observed at 30 min following administration (5). No effect of fentanyl on mechanical threshold was found in the present study and this is likely due to the lower fentanyl plasma concentration. The absence of a significant anti-nociceptive effect, despite well-tolerated treatments, underscores the need for further investigation into the dose-response relationship of transdermal fentanyl in horses.

Fentanyl was generally well tolerated in all horses in the present study. There was no effect of fentanyl treatment on any physiologic variable studied, with the exception of a decreased heart rate overall at 64 h following treatment. This time point was at midnight so this effect may simply be due to a circadian effect. Previous studies of fentanyl in horses administered either intravenously or via transdermal patch have either found no difference in physiologic variables (5, 8, 16) or increases in heart rate, respiratory rate (9), and rectal temperature (8) only at high plasma concentrations.

Pure mu agonist opioids are known to decrease gastrointestinal motility via activity at opioid receptors in the myenteric plexus of the gastrointestinal tract. An *in vitro* study of the effect of different opioids on motility of equine intestine revealed that this effect is

mediated by activity at kappa and not mu receptors (19). However, in that study, fentanyl decreased motility but this effect was not reversed by administration of mu or kappa antagonists, leading the authors to conclude that the effect of fentanyl on gut motility is more likely attributed to the antimuscarinic effects of this drug. Nevertheless, in the present study, all horses continued to defecate, showed no signs of colic, and borborygmi scales were unaffected by treatment. These findings are in agreement with other studies of fentanyl in horses (5, 8, 9, 16).

This study does suffer from limitations. The small sample size may affect the generalizability of the findings reported here. Additionally, as with all studies utilizing a thermal and/or mechanical threshold technique, it is possible that this model does not accurately reflect the type of pain that horses experience clinically associated with surgery and inflammation. Further studies utilizing clinical models of pain are requisite to fully describe the potential of fentanyl as an analgesic in horses. Additionally, the plasma concentrations achieved were far lower than were targeted in the study design. The number of patches applied for each treatment were based on plasma concentrations achieved in previous studies using the same type of fentanyl matrix patch (16) with the aim of achieving the plasma concentration that was previously described as anti-nociceptive (5). If an additional eight or ten patch treatment group were included, then that plasma concentration may have been achieved and an anti-nociceptive effect observed.

5 Conclusion

Our study demonstrates that while fentanyl patches are well-tolerated in horses, the doses studied did not achieve an anti-nociceptive effect. These findings highlight the need for further research to identify effective analgesic strategies using transdermal fentanyl. Future studies should explore higher doses of fentanyl, alternative patch locations, or clinical pain models that more closely resemble post-operative pain in horses.

Data availability statement

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

Ethics statement

The animal study was approved by University of Georgia College of Veterinary Medicine Institutional Animal Care and Use Committee (IACUC). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

RR: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. LB: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Writing – original draft, Writing – review & editing. RR: Data curation, Investigation, Methodology, Project administration, Writing – original draft, Writing – review & editing. BH: Data curation, Investigation, Methodology, Project administration, Writing – original draft, Writing – review & editing. AK: Data curation, Investigation, Methodology, Project administration, Writing – original draft, Writing – review & editing. DS: Data curation, Investigation, Methodology, Project administration, Validation, Visualization, Writing – original draft, Writing – review & editing. YI: Data curation, Investigation, Methodology, Project administration, Writing – original draft, Writing – review & editing. HK: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, 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.

This project was fully funded by Grayson Jockey Club Research Foundation, Project #: RGJCR0001484205.

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/fpain.2024.1373759/full#supplementary-material

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frontiers | Frontiers in Pain Research

TYPE Original Research
PUBLISHED 25 April 2024
DOI 10.3389/fpain.2024.1342954

OPEN ACCESS

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RECEIVED 22 November 2023
ACCEPTED 10 April 2024
PUBLISHED 25 April 2024

CITATION
Gisler J, Chiavaccini L, Blum S, Montavon S and Spadavecchia C (2024) Pressure pain mapping of equine distal joints: feasibility and reliability.
Front. Pain Res. 5:1342954.
doi: 10.3389/fpain.2024.1342954

Pressure pain mapping of equine distal joints: feasibility and reliability

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Background: Osteoarthritis is a prevalent degenerative joint disease initiating chronic pain and lameness in horses. While several objective gait analysis systems have been developed and validated to quantify lameness severity in horses, methods to evaluate whether peripheral sensitization contributes to the pain experienced are missing.
Objectives: To evaluate whether periarticular pressure pain mapping could be proposed as an auxiliary assessment tool in horses. Specific aims were to evaluate the feasibility and intra- and inter-rater reliability of pressure pain thresholds (PPT) determination at sites overlying the distal thoracic limb joints of clinically healthy horses.
Study design: Prospective, randomized validation study.
Methods: For feasibility assessment, PPT were measured with a hand-held digital algometer at six periarticular landmarks (2 sites per joint, 3 joints) bilaterally on the distal thoracic limb of 40 healthy horses (20 warmblood and 20 Freiberger). The joints tested were the metacarpophalangeal, on the latero-palmar and dorsal aspects (L-MCP and D-MCP), the proximal interphalangeal, on the dorsal and palmar aspect (D-PIP and P-PIP) and the distal interphalangeal, on the dorsal and lateral aspect (D-DIP and L-DIP). A feasibility score, ranging from 0 to 5, was attributed to each testing session. For intra- and inter-rater reliability assessment, L-MCP and D-MCP were selected to be tested again at 2 weeks intervals in 20 out of the 40 horses. Data were analyzed using a mixed-effect linear model to test differences in threshold per site and limb. Intra- and inter-rater correlation was calculated. Bland-Altman plots were performed to evaluate the variability of the measures.
Results: The procedure was considered feasible (score <2) in 95% of horses (95% CI 88%–100%). Overall, median [interquartile range (IQR)] PPT was 9.4 (7.5–11.3) *N*. No significant side differences were found. P-PIP and D-DIP recorded significantly lower PPT ($p < 0.001$ and $p = 0.002$, respectively) than L-MCP. Median (IQR) were 9.9 (7.3–12.4) *N*, 8.4 (6.1–10.5) *N* and 9.0 (7.4–10.6) *N* for L-MCP, P-PIP and D-DIP, respectively. The intra-rater agreement was 0.68 (95% CI 0.35–0.86) for L-MCP, and 0.50 (95% CI 0.08–0.76) for D-MCP. Inter-rater agreement was 0.85 (95% CI 0.66–0.94) for L-MCP and 0.81 (0.57, 0.92) for D-MCP.
Main limitations: Evaluation of feasibility was performed only for distal thoracic limbs joints; no data are provided for hind limbs or proximal joints. Only warmblood and Freiberger horses were included. Intra- and inter-rater reliability assessments were performed exclusively on data collected at the MCP joint.

Conclusion: Pressure pain mapping of distal thoracic limb joints was feasible in horses. Local sensitivity differed among sites and no side differences were noticed. Data collected from the MCP joint suggest highly variable, subject dependent intra-rater reliability, ranging from poor to good, and good to excellent inter-rater reliability. Further studies evaluating pathologic vs. healthy joints are needed before recommendations can be made about clinical usability and diagnostic validity.

KEYWORDS

pain, equine, osteoarthritis, pressure algometry, complementary diagnostic

1 Introduction

Osteoarthritis (OA) is a degenerative joint disease prevalent in horses as well as in many other species, including dogs, cats and humans. The predominant symptoms are chronic pain and lameness. Although the latter is the chief reason for impaired athletic performance and quality of life of affected horses, the mechanisms behind joint pain in OA are still poorly understood. Osteophytes and periosteal elevation, cartilage abnormalities, subchondral cysts, increased intraosseous pressure in the subchondral bone, bone marrow lesions and inflammation of the synovial membrane have all been reported to be potential local contributors to the generation of chronic pain accompanying OA (1, 2). However, strikingly, typical OA radiographic changes are only weak risk factors for the occurrence of pain and the severity of structural changes is not necessarily associated with pain intensity in both horses (3) and humans (4, 5). In contrast, the impairment of autonomic joint innervation, the plasticity of nociceptive fibers supplying periarticular structures, as well as the sensitization and dysfunction of descending pain inhibition mechanisms might be crucial promoters of pain development and maintenance in OA (2, 6). Which factor or factor combination predominates in individual patients remains to be determined.

While several objective gait analysis systems have been developed and validated to quantify lameness severity in horses (7–9), methods to evaluate whether simultaneous peripheral and central sensitization mechanisms are contributing to the pain experienced are missing. In humans, recently developed advanced and thorough sensory testing methods allow the characterization of the patient-specific OA pain phenotype (10, 11). For example, pressure pain sensitivity maps projected on tridimensional contour models can be constructed for individual patients and specific joints to obtain a visual impression of sensitivity distribution; similarly, using computer controlled mechanical stimulation, temporal summation can be assessed by repeating subthreshold stimulations and determining the extent of facilitation, which might reflect the presence of central sensitization. Such a personalized approach promotes mechanism-based therapy and, thus, better pain relief with fewer adverse effects (12). A validated, reliable and quantitative method to assess periarticular sensitivity in OA-affected horses is essential to establish the extent of peripheral sensitization involvement and, thus, to provide adequate and individualized treatment.

In humans and dogs, the non-invasive technique of pressure pain mapping, also known as pressure algometry or mechanical nociceptive threshold testing, has been used to quantify sensitization in several musculoskeletal conditions, including OA (13–16). So far, pressure pain mapping has not been used to quantify naturally occurring OA-associated pain in horses. At the same time, it has been described to assess limb sensitization in a model of experimentally induced carpal OA (17). Several trials explored the usability of pressure algometry to evaluate back pain (18, 19) or alteration of limb sensitivity (20, 21) in equines, and a summarizing review of results obtained with this method in horses has been recently published (22). On the other hand, the feasibility and reliability of periarticular pressure pain mapping have never been evaluated in horses.

The study objectives were to evaluate the feasibility and intra- and inter-rater reliability of PPT measured bilaterally over the distal thoracic limb joints in healthy horses. It was hypothesized that: (1) pressure pain mapping of the distal equine joints would be feasible; (2) repeatability and reliability of PPT mapping would be overall acceptable.

2 Materials and methods

The study was approved by the Committee for Animal Experimentation of the Canton of Bern, Switzerland (license number BE81/2022). The trial was carried out at the National Equine Center in Bern from October 2022 to January 2023.

2.1 Horses

Forty healthy Swiss warmblood ($n = 20$) and Freiberger ($n = 20$) horses, mare and geldings, aged >3 years and belonging to the Swiss Armed Forces were included. Horses were kept in single stalls in large stables under standard housing conditions and were regularly ridden or driven. Prior to study inclusion, a complete physical examination was performed by army veterinarians (JG, SB) supervised by an experienced equine specialist (SM) (Supplementary Material Appendix S1 for details). Lameness was assessed using the Americal Association of Equine Practitioners (AAEP) scale (0–5, with 0 indicating a normal gait and 5 non-weight bearing lameness) on a hard, straight surface at walk and trot. Distal thoracic limb joints were

visually observed and palpated. The degree of joint effusion, reaction to palpation and reaction to flexion were subjectively scored using a numerical rating scale (0 = absent; 1 = mild; 2 = moderate; 3 = severe).

To be included, horses had to be free of clinically detectable orthopedic, neurologic or systemic diseases and have no evidence of pain or mobility impairment. Lameness, joint effusion, reaction to palpation and to flexion had to be ≤1. Horses were excluded if they received any anti-inflammatory or analgesic drugs in the 2 weeks prior to the study. Physical and orthopedic examinations were repeated before each experimental session to ensure that no changes had occurred between appointments. If lameness or other symptoms appeared, horses were excluded from further testing, and the noticed clinical issue was recorded. Skinfold thickness was measured with a caliper (Universal Vernier Caliper, Tesa, Switzerland) over the scapula and cranial to it at the neck basis, anticipating that this factor might influence potential breed differences in PPT. Time from the last shoeing was recorded; no experiment was performed during the first week following shoeing. All horses were tested in the afternoon, in their own stall, manually held by the halter and lead rope by an assistant not performing the measurements. At least 1 h had to elapse between feeding or daily training exercise and testing.

2.2 Study design

Three consecutive study phases were designed to reach the set goals.

2.2.1 Phase 1

This phase aimed at assessing feasibility, site sensitivity and left-right differences of distal limb joints PPT mapping.

Horses were acclimated with the stimulation method with an initial short training period just preceding the beginning of the experiment. During this period, the algometer was applied to at least three undefined sites distal to the metacarpophalangeal joint, not corresponding to the test sites, until the horse showed a response. Thereafter, the actual experiment began.

The same observer (JG) performed all the PPT measurements in this phase. All 40 horses were tested bilaterally at six sites, two per joints (Table 1 for anatomical details). The sites were the L-MCP and D-MCP, on the latero-proximal and dorsal aspect of the metacarpophalangeal joint, the D-PIP and P-PIP, on the dorsal and palmar aspect of the proximal interphalangeal joint, and the D-DIP and L-DIP, on the dorsal and lateral aspect of the distal interphalangeal joint (Figure 1). These sites correspond to those described to perform arthrocentesis in the same joints (23).

2.2.2 Phase 2

This phase aimed to assess intra-rater reliability for a single observer (JG). Twenty horses randomly selected from the initial cohort were tested at least 2 weeks after Phase 1. Horses who recorded a feasibility score >2, were removed from the pool before randomization. The two sites on the metacarpophalangeal joint, L-MCP and D-MCP, were evaluated on the left and right thoracic limb. Data collected during Phase 2 were then compared to those collected in Phase 3 by the same observer.

TABLE 1 Periarticular stimulation sites.

Sites	Description
L-MCP	Metacarpophalangeal joint, latero-proximal-palmar aspect, midway between the distal end of the fourth metacarpal bone and the lateral proximal sesamoid bone
D-MCP	Metacarpophalangeal joint, dorsal aspect, lateral to the common digital extensor tendon at the level of the palpable joint space
D-PIP	Proximal interphalangeal joint, dorsal aspect, distal to the lateral bony eminence of the proximal phalanx and lateral to the common digital extensor tendon
P-PIP	Proximal interphalangeal joint, palmar aspect, proximal and central to the transverse bony prominence of the middle phalanx
D-DIP	Distal interphalangeal joint, dorsal aspect, central above the coronary band
L-DIP	Distal interphalangeal joint, lateral aspect, proximal to the lateral collateral cartilage, midway between the dorsal and palmar sides of the middle phalanx

2.2.3 Phase 3

This last phase aimed to assess intra-rater reliability and generate the data set to evaluate inter-rater reliability. Two observers (JG and SB) performed PPT testing during this session.

The same horses included in Phase 2 were included in Phase 3. At least 2 weeks elapsed between Phases 2 and 3. Both observers evaluated the horses once; they tested the two metacarpophalangeal joint sites (L-MCP and D-MCP) on both thoracic limbs in the same order, the first observer being randomized for each horse with the flip of a coin. At least 60 min elapsed between observers.

2.3 Pressure pain threshold determination

For PPT determination, a hand-held digital algometer, equipped with a flat 2 mm diameter tip (ProdPro, Top Cat Metrology Ltd, UK), was applied perpendicular to the skin at predefined periarticular sites (Table 1 for anatomical details) until a behavioral reaction was elicited. A constant force rate increase of 2 N/s was kept with the guidance of warning LED lights on the instrument. During stimulation, the operator was not aware of the applied force. Stimulation was stopped when a weight shifting to the contralateral limb, a voluntary limb lifting and/or stamping occurred, or when the cut-off force of 25 N was reached. At this point, the operator withdrew the probe, and the peak force (N) displayed on the device was recorded as the threshold.

The limb (left or right) and the order of sites to be tested were randomized for each horse with the flip of a coin and a random number generator (https://www.matheretter.de/rechner/zufallsgenerator), respectively. Each site was tested three times and the two closest values were averaged for further analysis. The same order of site testing was kept for each repetition during a session. Minimal inter-stimulation interval was 20 s,

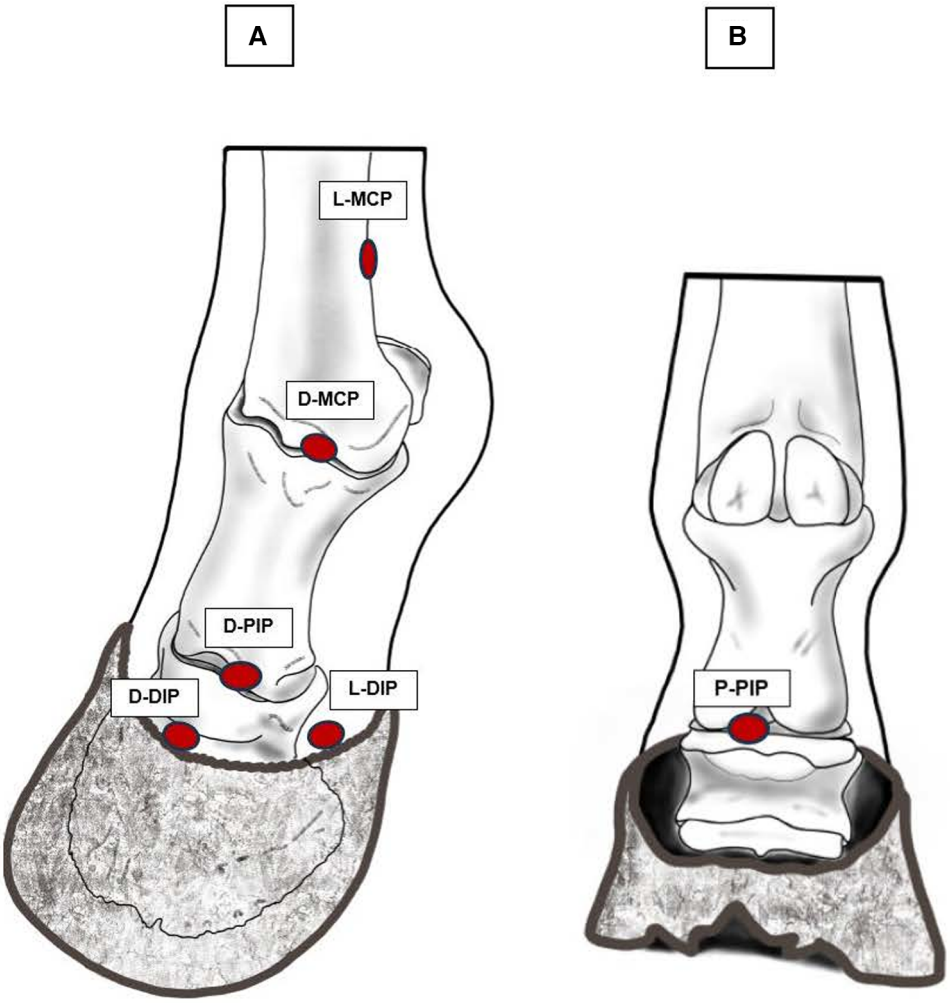

FIGURE 1
The 6 periarticular sites tested with algometry. (**A**) Dorso-lateral view of the distal thoracic limb, L-MCP and D-MCP on the latero-proximal-palmar and dorsal aspect of the metacarpophalangeal joint, respectively, D-PIP on the dorsal aspect of the proximal interphalangeal joint, D-DIP and L-DIP on the dorsal and lateral aspect of the distal interphalangeal joint, respectively. (**B**) Palmar view of the distal thoracic limb, P-PIP, on the palmar aspect of the proximal interphalangeal joint. Original drawings inspired by images available in reference (23).

with 60 s between tests on the same site. If the horse moved or the algometer jerked or slipped, the measurement was repeated.

During the whole stimulation session, particular care was dedicated to ensuring a quiet environment and keeping the general behavior of the horse under strict observation. If the horse appeared distracted from noises or other external conditions during threshold assessment, the stimulation was interrupted and repeated once the horse returned to a calm, concentrated attitude.

Complete physical and orthopedic examinations were repeated 1 day and 1 week after each experimental session for safety monitoring. Furthermore, tested joints and periarticular skin areas were regularly inspected for the presence of abnormal sensitivity to touch, temperature, swelling, lesions, or any other abnormality that could be noticed.

2.4 Feasibility assessment

At the end of each session, each horse was assigned a feasibility score that described the ease with which data could be collected (Table 2), adapting a scoring system previously described for dogs (24). Feasibility was scored on a scale ranging from 0 to 5, where scores ≤ 2 represented "easy data collection" and those >2 "difficult data collection". Horses scoring >2 were excluded from subsequent testing to avoid unnecessary discomfort.

2.5 Sample size

Sample size was calculated using a web-based tool based on previously described methods (25, 26). Assuming an ICC $\rho = 0.6$,

TABLE 2 Feasibility scores for evaluation or the ease with which pressure pain thresholds data were collected.

Score	Description
0	No problem. Minimum restraint needed; excellent cooperation; clear reaction to stimuli
1	Mild difficulty. Mild restraint needed; good cooperation; clear reaction to stimuli
2	Moderate difficulty. Moderate restraint needed; good cooperation >50% of the time; mild sensitivity of extremities being touched; mild variation in reaction to stimuli
3	Significant difficulty. Significant restraint needed; good cooperation <25% of the time; moderate sensitivity of extremities being touched; moderate variation in reaction to stimuli
4	Extreme difficulty. Constant restraint required; not cooperative; unclear reaction to stimuli, not confident in data collected
5	Impossible. Could not collect data due to the horse's disposition and/or lack of confidence in the reactions seen being due to the stimulus

Modified from (24).

with an expected width of the 95% confidence interval (CI) of 0.4, two PPT values per subject and a drop-out rate of 10%, 13 horses were considered necessary to assess reliability. As for reliability testing a group of 20 subjects was suggested to be a clinically representative sample (27), the number of horses included in the present study was increased to 20 per breed in Phase 1 and to 20 in total in Phases 2 and 3.

2.6 Statistical analysis

Data elaboration and analysis were performed with Stata/BE 17.0 for Mac (StataCorp LLC, College Station, TX) and SigmaPlot 14 (Systat Software, Palo Alto, CA). Descriptive statistics was used for demographic data. Continuous data were checked for normality of distribution using the Shapiro-Wilk normality test and graphically with histogram and the normal quantile plot function in Stata. Since the normality assumption was not met, data were reported as median [interquartile range (IQR)]. Categorical data were presented as proportions or percentages. The Mann-Whitney Rank Sum test was used to compare skinfold thickness between breeds.

In order to consider PPT mapping suitable for clinical use, the horse needed to score ≤2 in the feasibility score. A one-sample proportion *z*-test was used to compare the observed proportion of the sample to the 70% cutoff proposed in clinical feasibility studies in other species (24).

In Phase 1, to check the effect of breed, testing site and side on PPT, a mixed effect linear model was used, with the horse as the random effect, breeds, the six sites tested and left and right sides as fixed effects. Due to a lack of normality distribution, PPT was transformed using the Box-Cox transformation prior to analysis. For Phases 2 and 3, the intra- and inter-class correlation coefficients (ICC) were calculated, and the 95% CI was assessed to identify the precision of the estimate. The ICC values were classified as follows: <0.20 indicated poor agreement; 0.21–0.40 fair agreement; 0.41–0.60 moderate agreement; 0.61–0.80 good agreement and >0.80 excellent agreement (28). Systematic error between sessions and between raters was estimated using the Wilcoxon signed-rank test. *P*-values ≤0.05 were considered statistically significant. Bland-Altman plots were performed to graphically represent differences between two consecutive PPT measurements and between the two raters.

3 Results

A total of 40 healthy horses were included in the study: 20 Warmblood and 20 Freiberger. The median (IQR) age of horses was 4 (3, 7) year-old and they weighed 540 (495, 570) kg. The sample included 14 (35%) mares and 26 (65%) geldings. There were 5 (25%) mares and 15 (75%) geldings in the Warmblood group, and 9 (45%) mares and 11 (55%) geldings in the Freiberger group ($p = 0.19$). Skinfold thickness was significantly different between breeds at both tested sites, being 4.5 (4.0–4.8) mm in the Warmblood group and 5 (4.5–5.5) mm in the Freiberger group at the scapula ($p = 0.023$), and 4 (4–4.5) mm in the Warmblood group and 4.8 (4–5) mm in the Freiberger group at the neck basis ($p = 0.004$).

3.1 Feasibility and site specificity

In Phase 1, only two horses received a feasibility score >2, because they were moving too much their thoracic limbs and were then excluded from Phases 2 and 3. Consequentially, the procedure was then deemed "feasible" in 95% of the animals (38 out of 40 horses), which was statistically significantly superior to the hypothesized 70% (95% CI 88%–100%, $p < 0.001$).

A total of 480 PPT observations were collected in Phase 1, ranging between 1.5 and 25 N [the median (IQR) PPT obtained was 9.4 (7.5, 11.3) N] (Table 3). The final multivariable regression model was significant and predicted the data well (Wald chi2 = 67.28, $p < 0.001$). When accounting for breed differences and for the side (left or right), P-PIP ($p < 0.001$) and D-DIP ($p = 0.002$) recorded a significantly lower mechanical threshold compared to L-MCP. Regardless of the side, the median (IQR) mechanical threshold was 8.4 (6.1, 10.5) N for P-PIP and 9.0 (7.4, 10.6) N for D-DIP compared to 9.9 (7.3, 12.4) N for L-MCP. There was no difference in mechanical thresholds between breeds ($p = 0.14$) or sides ($p = 0.33$). Given the lack of statistically significant difference of PPT obtained

TABLE 3 Median (IQR) pressure pain thresholds in 40 healthy horses.

Site	PPT left limb (N)	PPT right limb (N)
L-MCP	9.9 (7.7, 11.3)	9.8 (7.3, 14.9)
D-MCP	9.6 (7.6, 12.2)	9.5 (7.4, 11.5)
D-PIP	10.3 (7.8, 12.1)	9.9 (8.1, 10.9)
P-PIP	9.0 (6.4, 12.3)	7.9 (6.0, 10.1)
D-DIP	9.0 (6.7, 10.9)	9.1 (7.4, 10.0)
L-DIP	9.3 (8.3, 12.3)	9.8 (8.2, 11.9)

Data at each site were obtained averaging the closest two of three consecutive measurements. All measurements were performed by one observer.

from the left and right sides at each site, the PPT values of the left and right sides were averaged for further analysis.

3.2 Intra-rater reliability

For L-MCP, the median (IQR) PPT obtained by the first observer (JG) in the first measurement was 10.4 (9.4, 11.8) N and in the second one, 2 weeks later, 9.8 (8.8, 12.9) N. For D-MCP, the median (IQR) PPT obtained was 10.1 (8.8, 12.6) N in the first measurement and 11.1 (9.2, 12.8) N in the second one. The intra-rater agreement was 0.68 (95% CI 0.35–0.86) for L-MCP, and 0.50 (95% CI 0.079–0.76) for D-MCP, indicating moderate-to-good and poor-to-good repeatability, respectively (Table 4). The median difference between the first and second measurements was 0.55 N (95% CI −0.98, 1. 85; $p = 0.62$) for L-MCP and −0.35 N (95% CI −1.97, 1.30; $p = 0.34$) for D-MCP, indicating lack of systematic error in measurements.

3.3 Inter-rater reliability

For L-MCP, the median (IQR) PPT recorded by rater 1 (JG) and 2 (SB) were 9.8 (8.8, 12.9) N and 11.2 (9.0, 13.0) N, respectively. For D-MCP, the median (IQR) PPT recorded by rater 1 and 2 were 11.1 (9.2, 12.8) N and 11.3 (9.6, 11.7) N, respectively. The agreement was good-to-excellent between raters for both sites, being 0.8 (0.7–0.9) for L-MCP and 0.8 (0.6–0.9) for D-MCP (Table 5). Median difference between raters was −0.61 N (95% CI −2.52, 1.42; $p = 0.59$) for L-MCP and 0.14 (95% CI −1.40, 1.45, $p = 0.83$) for D-MCP, indicating lack of systematic error in measurements.

The Bland-Altman plots for all the evaluations are included in Figure 2 illustrating the distribution of the PPT values difference when plotted against the mean and estimate an agreement interval within which 95% of the differences lied.

No adverse events possibly related to the testing procedures were observed throughout the study.

TABLE 4 Intra-rater agreement (ICC) and 95% confidence interval of pressure pain thresholds measurements made 2 weeks apart on 20 horses by one rater.

Intra-rater	ICC (95% CI)	Systematic error		
		Median difference	95% CI	*p*-value
L-MCP	0.68 (0.35–0.86)	0.55 N	−0.98, 1.85 N	0.62
D-MCP	0.50 (0.08–0.76)	−0.35 N	−1.97, 1.30 N	0.34

TABLE 5 Inter-rater agreement (ICC) and 95% confidence interval of pressure pain thresholds measurements made on 20 horses by two raters, on the same day at 1 h interval.

Inter-rater	ICC (95% CI)	Systematic error		
		Median difference	95% CI	*p*-value
L-MCP	0.85 (0.66–0.94)	−0.61 N	−2.52, 1.42 N	0.59
D-MCP	0.81 (0.57–0.92)	0.14 N	−1.40, 1.45 N	0.83

4 Discussion

The main finding of this study was that periarticular PPT showed excellent feasibility when 6 periarticular sites were tested bilaterally on the distal thoracic limbs of clinically healthy horses. Tested side and breed did not affect thresholds. Reliability, evaluated on a single joint, was quite variable, being better when 2 different raters performed the tests on the same day than when the same sites were tested by the same observer 2 weeks apart. The overarching aim was to verify whether this quantitative sensory testing method, already used in humans and dogs affected by chronic joint pain (10, 14, 16), could be proposed to assess periarticular sensitivity in horses affected by OA. Indeed, the crucial role of peripheral sensitization and thus enhanced local sensitivity to the overall pain experience in OA has often been highlighted (1, 2, 13). A tool to quantify its extent could contribute to characterizing the individual pain phenotype, establishing an appropriate therapy, and following up on the disease's progress over time.

In the present study, we evaluated the feasibility of pressure pain testing in horses not exposed to this method before. Feasibility was assessed using a scoring system previously described for dogs (24) and adapted for the current experimental setup in horses. To maximize tolerability, horses were tested in their home stall under standard conditions. In 95% of subjects, it was easy to perform pressure testing and good to excellent cooperation was observed. This finding suggests better tolerability than initially expected. Indeed, we assumed that if 70% of the horses had tolerated the testing well, the method could have been considered clinically feasible. While this is an encouraging result, it might at least partially be linked to the particular population of horses included, as they had all been selected for military duties at the age of 3 years. These animals must be willing to work, agreeable and cooperative; therefore, they might not truly reflect the characteristics of a mixed population of sport and leisure horses commonly encountered in veterinary practice. For comparison, mechanical nociceptive testing using pressure algometry was feasible in 83% of dogs in the previously mentioned study (24) and in 83% of horses undergoing repeated testing over a period of 10 weeks (17).

In the present study, PPT was site-specific. In particular, P-PIP and D-DIP, on the proximal and distal interphalangeal joints, respectively, had lower thresholds than L-MCP, situated on the proximo-lateral aspect of the metacarpophalangeal joint. These results confirm previous observations, as different sensitivity to nociceptive mechanical stimulation for different anatomical areas has been previously reported in humans and horses. In humans, pressure sensitivity maps indicating distinctive sensitivity areas have been drawn for several body regions, such as the knee and the shoulder (14). In horses, higher thresholds were found in

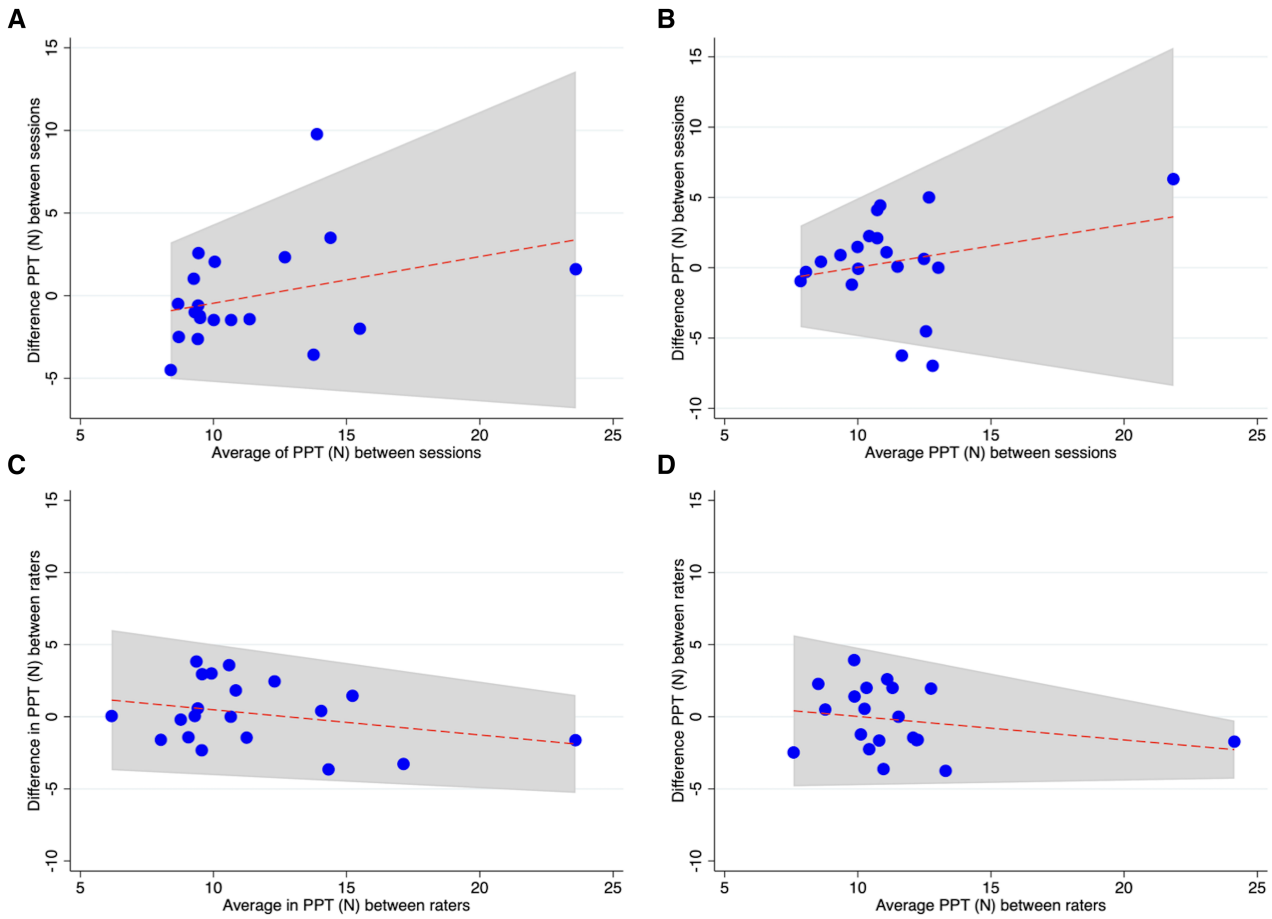

FIGURE 2
Bland-Altman plots shows distribution of PPT for each site and rater: (A, B) PPT recorded between two sessions from rater 1 at L-MCP and D-MCP, respectively. (C, D) PPT recorded between raters at L-MCP and D-MCP, respectively.

distal compared to proximal regions of the thoracic limb (17), in lumbar vs. thoracic back areas (19) and in specific sites of the face (29). As highlighted in previous literature, a direct comparison of PPT values among species and studies is only meaningful if the same instrument tip size, configuration and force application rate are applied (22, 30). Our thresholds are grossly comparable to those previously reported for distal equine thoracic limbs (average 5.2 N) (31) and donkeys (6.2 ± 2.1 N) (32), when, as in the present study, an algometer with a 2 mm diameter tip was applied. On the other side, they are largely different from those reported using a flat rubber tip of 1 cm^2 (200 ± 40 N) (20). These differences highlight the importance of carefully considering technical details when comparing studies and results. Thus, for future clinical applications, in the absence of specific reference values for a specific equipment, stimulating tip and force rate increase, it seems reasonable to directly compare only threshold values found at the same joint and location bilaterally, rather than multiple sites on the same limb.

Our results concerning side differences confirm those of previous studies in equines indicating that no significant differences are present between PPTs measured on the left and right limbs for a given site (17, 20). This is an important finding, as consistent side differences in affected joints can be interpreted as possibly disease-related in clinical settings. In OA, both enhanced and decreased peripheral sensitization has been reported in other species, depending on the disease stage and age (13–16, 33). In humans, lower thresholds are typically reported for the affected joints compared to healthy controls (11) and similar results have been reported for horses undergoing experimentally induced carpal osteoarthritis (17).

As no significant differences were detected between sides, left and right threshold values for specific sites were averaged for further analysis. This approach has often been applied when establishing reference values for quantitative sensory testing methods in healthy subjects (17, 24). Similarly, it is common to perform multiple testing at specific sites and exclude extreme values from the averaging (16). As these tests are based on well-defined, objectively measurable inputs (i.e., the applied pressure) but on a merely behavioral output (i.e., the limb lifting or weight shifting), it is quite common to get outliers, as responses are unspecific. Keeping the two closest measured values and excluding one or two extremes enhance the probability of

correctly defining thresholds (16). Thus, we followed this approach in the current study and recommend doing the same while applying the method in the clinical context.

In the current study, Freiberger horses, a traditional cold-type Swiss breed, and warmblood horses were enrolled. Anticipating that skin thickness differences might account for differences in thresholds, skinfolds at the scapula and neck basis were measured with a caliper. While no significant differences in pressure thresholds were found between breeds, thicker skin was found for Freiberger horses compared to warmblood. We assessed skinfold thickness using a caliper as commonly done in nutritional studies in humans. As the skin thickness was evaluated at proximal sites, it might reflect the presence of higher amount of subcutaneous fat in these body regions and thus not be adequate to estimate skin thickness at distal sites. For this purpose, ultrasound imaging at the site of interest would have been more adequate. To the best of our knowledge, this is the first study specifically assessing breed differences in pain sensitivity in horses. Future studies could investigate other modalities, such as thermal and tactile thresholds and other breeds, to substantiate this preliminary finding.

If a certain method is intended for use in clinical practice, it has to provide comparable results over time when applied to healthy individuals and when different observers perform the testing. Nevertheless, due to the semiquantitative nature of PPT, a perfect agreement between sessions and raters cannot be expected. To provide some examples from other species, when test-retest repeatability was evaluated, ICC ranging from 0.6 to 0.9 were reported in humans (11) and from 0.46 to 0.78 in horses, when the axial skeleton was evaluated with algometry, depending on the examiner (22, 34). Such ICC values were interpreted as showing adequate agreement in those studies. As it has been pointed out, several factors can influence repeatability, such as the body region to be tested, the experience of the examiner and the duration between sessions (22). In the present study, intra-rater reliability was evaluated by comparing thresholds obtained by a single observer in two testing sessions 2 weeks apart, measured at two sites on the same joint bilaterally. This testing paradigm was established to mimic a clinical situation in which a target joint would be tested on both limbs for internal comparison and retested at intermediate time intervals to verify the effect of treatment or disease progression. When averaged values per site were compared between sessions for the same observer, an agreement ranging from poor to good was found. For L-MCP, on the lateral aspect of the metacarpophalangeal joint, reliability was higher than for D-MCP, reflecting location-specific ease of testing in a repeatable way. This site-specificity is interesting to notice, as it might also be true for other joints. Sites located on the dorsal aspect of the limb might tend to hinder withdrawal more than lateral or palmar/plantar sites and thus originate higher variability in the results. This has been previously pointed out by Haussler in his review on algometry in horses (22), but further data would be necessary to confirm that a dorsal approach should rather be avoided when testing periarticular sensitivity. Furthermore, looking at threshold differences between sessions in the Bland-Altman plots it is evident that some individuals, mostly those having higher thresholds, were showing larger differences, reflected by the large range found for the 95% confidence interval of the intraclass correlation coefficient. Thus, horses having high initial thresholds might not be adequate candidate for a follow-up with algometry. Additionally, interesting to notice, is the absence of a clear direction of change over time, as some horses showed higher and other lower thresholds at the second appointment. In contrast, a previous study in horses found higher thresholds at the second appointment, with the extent of increase depending on the interval between sessions. A short interval of 1–3 days led to higher changes than 5–7 days (17). In dogs, opposite results were found, with mechanical thresholds decreasing at the second testing session (16, 35). This was interpreted as potential result of stress-induced analgesia at the first occasion (16, 35, 36), but it could also be explained by a learning process. Indeed, animals can learn that if they react earlier (i.e., at lower pressure), they might interrupt stimulation and thus avoid unpleasant feelings. In the author's experience, there are rather clear differences in the "true" evoked reactions and the learned early avoidance behavior, and this is at least partially reflected in the feasibility of testing. Animals that learn to react at minimal contact and do not "concentrate" on the testing should be excluded from this diagnostic modality. Thus, the number of repetitions, tested sites and the testing frequency might affect the results of mechanical nociceptive testing in animals. In Phases 2 and 3, we tested only two sites per limb, and the testing interval was 2 weeks. Shorter intervals and multiple sites or repetitions within a session have been shown to reduce tolerability (36).

When two raters performed the measurements, the agreement was good to excellent for both sites when tested on the same day at 1-h interval. This finding suggests that comparing pressure thresholds collected by different practitioners from the same horse over time would be acceptable.

Several devices have been described in the literature to measure mechanical nociceptive thresholds in horses. Most commonly, simple and cheap hand-held algometers with a 1 cm^2 stimulating tip are used (22). The major drawback with these instruments is that the force or pressure application rate is not monitored, making repeatability inherently tricky. Indeed, in nociceptive testing, the force application rate strongly modulates the outcome. In the present study, a Prod Plus was used. This instrument, already described in other equine studies (21), was purposely developed for veterinary testing. It allows monitoring the force application rate through a practical LED light system and exchanging stimulating tips based on the species and site to be tested. As unpleasant pressure is finally responsible for evoking nocifensive responses, it is fundamental to apply force at a constant rate in a reliable way. The examiner manually exerts increasing force through the instrument, the applied pressure depending on the stimulating surface as clearly demonstrated for threshold determination in horses (30). Hence, the possibility of exchanging tips strongly increases testing reliability as the force necessary to evoke a response should remain acceptable for the operator. The cut-off force of 25 N suggested for this instrument

guarantees the feasibility of testing for most operators and, on the other hand safety for the animals, as the risk of physical damage is minimized. Thus, the overall acceptable intra-rater and inter-rater reliability found in the present study is mainly linked to the adequacy of the instrument selected and to the stimulation protocol adopted.

We tested periarticular algometry in healthy horses to verify whether this method could become a complementary diagnostic tool for horses affected by OA. Importantly, great care should always be taken to avoid damage to the delicate anatomical structures that could arise from improper use of the testing instrument, including too fast force application rate, oblique positioning of the stimulating tip, or not respecting the recommended safety cut-off values. This careful approach should also and in particular apply whenever new bone formation and osteophytes are present or suspected, potentially modifying underlying anatomical structures and landmarks.

4.1 Limitations

The horses included in the present study were considered clinically healthy and free of lameness based on a thorough clinical examination, but not including imaging or laboratory testing. Only warmblood and Freiberger horses were included, thus the data presented here can only be considered representative for these two breeds. Evaluation of feasibility was performed merely for distal thoracic limb joints; no inferences can be made for pelvic limbs or proximal joints. Intra- and inter-rater reliability assessments were performed exclusively on data collected from two sites overlying the MCP joint and results might be different for other joints and sites.

5 Conclusion

Pressure pain mapping of distal thoracic limb joints was feasible in horses using the approach and equipment applied in the current study. Local sensitivity was site-specific and no side or breed differences were noticed. Data collected from the MCP joint suggest highly variable subject-dependent intra-rater reliability, ranging from poor to good, and good to excellent inter-rater reliability. Studies evaluating pathologic vs. healthy joints are needed before recommendations can be made about clinical usability and diagnostic validity.

Data availability statement

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

Ethics statement

The animal study was approved by Committee for Animal Experimentation of the Canton of Bern, Switzerland (license number BE81/2022). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

JG: Writing – original draft, Writing – review & editing, Investigation, Methodology, Data curation. LC: Writing – original draft, Writing – review & editing, Data curation, Formal Analysis, Methodology. SB: Writing – review & editing, Investigation, Methodology. SM: Recourses, Writing – review & editing, Conceptualization, Methodology. CS: Writing – original draft, Writing – review & editing, Conceptualization, Investigation, Methodology, Supervision, Data curation, Project administration, Resources.

Funding

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

Acknowledgments

We thank Manuela Hofer from the NPZ for her support during the experimental sessions and Ekaterina Gamez Maidanskaia for the anatomical illustration of the stimulation sites.

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.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpain.2024.1342954/full#supplementary-material

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frontiers | Frontiers in Pain Research

TYPE Original Research
PUBLISHED 19 June 2024
DOI 10.3389/fpain.2024.1390322

OPEN ACCESS

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RECEIVED 23 February 2024
ACCEPTED 06 June 2024
PUBLISHED 19 June 2024

CITATION
Paranjape VV, Knych HK, Berghaus LJ, Giancola S, Cathcart J and Reed RA (2024) Plasma concentrations of buprenorphine administered via matrix-type transdermal patches applied at three different anatomical locations in healthy adult horses. Front. Pain Res. 5:1390322. doi: 10.3389/fpain.2024.1390322

Plasma concentrations of buprenorphine administered via matrix-type transdermal patches applied at three different anatomical locations in healthy adult horses

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Background: Anatomical location-dependent differences in transdermal opioid penetration are well described in human patients. Although this has been investigated in horses with fentanyl, there is no literature available on location-dependent plasma buprenorphine concentrations when administered as a transdermal matrix-type patch.

Objective: This study aims to compare the plasma concentrations achieved from the matrix-type transdermal buprenorphine patches placed at different anatomical sites (metacarpus, gaskin, and ventral tail base) in healthy adult horses.

Study design: This is a randomized experimental study with a Latin square design.

Methods: Six adult horses were given each of three treatments with a minimum 10-day washout period. For each treatment, two 20 μg h^{-1} matrix-type buprenorphine patches were applied to the ventral aspect of the tail base ($Tail_{TDP}$), metacarpus region ($Metacarpus_{TDP}$), or gaskin region ($Gaskin_{TDP}$). Whole blood samples (for determination of buprenorphine concentration) and physiological variables were collected before (0 h) and at 0.5, 2, 4, 6, 8, 10, 12, 16, 24, 32, 48, 56, 72, 96 and 120 h after patches were applied. The patches were removed 96 h following placement and were analyzed for residual buprenorphine content. Buprenorphine concentrations were measured in plasma by LC-MS/MS. A mixed-effects model was used to analyze the physiological variables.

Results: Between the three treatment groups, there was no change in physiological variables across timepoints as compared to baseline and when compared to each other in a single horse and between horses ($p > 0.3$). When comparing all three locations, the buprenorphine uptake was observed to be more consistent with respect to measurable plasma concentrations >0.1 ng ml^{-1} when applied to the ventral aspect of the tail base. In the $Tail_{TDP}$ group, the mean plasma buprenorphine concentrations were >0.1 ng ml^{-1} from 2 to 32 h. The highest group mean was 0.25 ng ml^{-1} noted at 4 h.

Conclusions: The metacarpal and gaskin regions presented more erratic and inconsistent buprenorphine uptake and plasma concentrations as compared to the ventral aspect of the tail base. Further research must be directed at investigating the optimal dose, achievable duration of analgesia, change in measurable plasma concentrations, and behavioral and systemic effects.

KEYWORDS

opioids, equine, analgesia, pain, pharmacology, gaskin, tail base, metacarpus

1 Introduction

In the past decade, effective pain management in horses has become feasible thanks to research involving various analgesic drugs along with the development of pain scales allowing recognition of overt pain behaviors, changes in facial expressions and head position, and patients' response to palpation and human interaction. The clinical impact of these studies is to enhance the well-being and welfare of this species by optimizing treatment strategies for pain based on severity and chronicity and utilizing multimodal analgesic regimes. Equine clinicians use various pharmaceutical classes to treat pain but the drug selection and route of administration is limited by some considerations specific to horses. Opioids are the most effective analgesics and are the mainstay of perioperative analgesia for treating pain in human and veterinary medicine. Injectable pure μ-receptor opioid agonists such as morphine, hydromorphone, and methadone are routine choices to treat perioperative pain in horses. However, clinicians hesitate to use this drug class in horses due to the apparent narrow margin between analgesia and excitation or arousal, gastrointestinal hypomotility, and challenges posed in quantifying consistent analgesic effects (1, 2).

The transdermal therapeutic system has also been assessed in horses for synthetic μ-opioid agonists such as fentanyl due to the advantage of (i) providing non-invasive, continuous pain control for extended periods; (ii) preventing wide variations in serum drug concentrations; (iii) reducing severity of adverse effects associated with repeated post-dose peaks in plasma concentration as seen with an injectable route; (iv) avoiding end-of-dose breakthrough pain; and (v) preventing first-pass metabolism occurring commonly with an oral route of administration (3, 4). Buprenorphine is another opioid that is available for transdermal drug delivery via patch application.

Buprenorphine is a semi-synthetic, highly lipophilic oripavine derivative that is classified as a high-affinity partial μ-receptor agonist and a κ-receptor antagonist that displays slow-dissociation kinetics. Its affinity for the opiate receptor is double, and its potency is approximately 30 times higher than morphine. Its therapeutic response lasts much longer than other opioids, and it has a wider safety profile. The partial agonism at the μ-receptor is a unique feature of buprenorphine and is attributed to its many distinctive properties, specifically that its analgesic effects plateau at higher doses, and ceiling effects on respiratory depression occur, which makes it safer than pure agonists of the μ-receptor (5–7). A transdermal matrix patch buprenorphine formulation, which was initially developed for human use, has been investigated for extra-label purposes in dogs (8–11), cats (12), pigs (13, 14), sheep (15, 16), and primates (17). Several equine studies report the clinical utility of injectable buprenorphine (i.e., intravenous, intramuscular, subcutaneous, and sublingual) to treat mild to moderate pain (18–24), increase nociceptive threshold (21–23), and offer superior-long lasting antinociception in comparison to butorphanol (24). However, there is minimal literature available on the use of buprenorphine via transdermal patch in horses (25, 26).

In horses, the ventral aspect of the tail is a common location for a transdermal patch system since the location is easily accessible, the application is easy, the patches can be secured, and contact with the skin can be maintained by covering the patch with an adhesive tape (27). It is crucial to understand that not only is the ease of application an important factor but so are the onset and duration of action and achievable plasma concentrations. The prediction of plasma concentrations is difficult with a transdermal route of administration due to the variability in drug absorption and systemic availability across species that can be influenced by the location of the patch (27–32). The objective of the present study was to compare the plasma concentrations achieved from the matrix-type transdermal buprenorphine patches placed at different anatomical locations (metacarpus, gaskin, and ventral tail base) in healthy adult horses. We hypothesized that the absorption of buprenorphine from the ventral tail base would be most reliable and yield consistent, quantifiable, and clinically relevant plasma concentrations.

2 Material and methods

2.1 Ethics statement

This study was approved by the University of Georgia Institutional Animal Care and Use Committee (animal use protocol: A2021 06-011).

2.2 Study animals

Six, university-owned adult, healthy horses (four mares and two geldings) aged 19 ± 7 years and weighing 559 ± 58 kg were enrolled in this prospective, Latin square study design. The animals were deemed healthy based on clinical history, thorough physical examination, and a normal complete blood count and biochemistry profile. The horses were housed in 3.65 m × 4.26 m

stalls for acclimatization 16–20 h prior to treatment administration on each occasion. During the entire duration of the study when the horses were housed in this research environment, they were provided with 0.7 kg of senior feed (senior formula; Seminole Feed, Ocala, FL, USA) and 2–3 flakes of timothy hay twice daily with *ad libitum* access to water. On the same day, i.e., the day of arrival at the facility, a 14-gauge, 13 cm intravenous catheter (DayCath; MILA International, Florence, KY, USA) was placed aseptically in the cranial region of the jugular vein on the selected side for blood collection for pharmacokinetic analysis. The horses were then weighed, and a physical examination was performed to record the baseline heart rate (HR), respiratory rate (RR), and rectal temperature ($Temp_{rectal}$). The catheter was periodically flushed with saline (0.9% sodium chloride; Baxter International Inc., Deerfield, IL, USA) and was monitored closely for blood clots and patency.

2.3 Treatment groups and transdermal buprenorphine patch application

All horses in our study were administered to each of the following three treatment groups, and the randomization by application of Latin square was predetermined (www.randomizer.org). The washout period between treatments was a minimum of 10 days. The hair was clipped over the location of interest using a #50 clipper blade as required to allow enough area for two patches placed alongside each other in a vertical arrangement without overlap and adequate patch-to-skin contact was ensured. The clipped area was then wiped clean with a dry 10.16 cm × 10.16 cm gauze pad to remove dirt and skin debris. Two transdermal patches, each containing 20 mg total buprenorphine (20 µg h^{-1}; Amneal Pharmaceuticals LLC., Piscataway, NJ, USA) with dimensions 74 mm × 74 mm, were applied to the assigned location using their adhesive surface and were further secured with a 7.62 cm porous elastic adhesive tape covering (Elastikon; Johnson & Johnson, New Brunswick, NJ, USA) as shown in Figure 1. Hence, the dose received was 0.07–0.09 µg kg^{-1} h^{-1} based on their body weights. The firm adherence of the patch at the location was confirmed by visual inspection at each data and blood collection timepoint. The three selected locations were as follows:

1. **$Tail_{TDP}$:** patch application to the ventral aspect of the tail base (Figure 1A)
2. **$Metacarpus_{TDP}$:** patch application to the dorsal surface of the metacarpus (Figure 1B)
3. **$Gaskin_{TDP}$:** patch application to the gaskin region located between stifle and hock joints (Figure 1C)

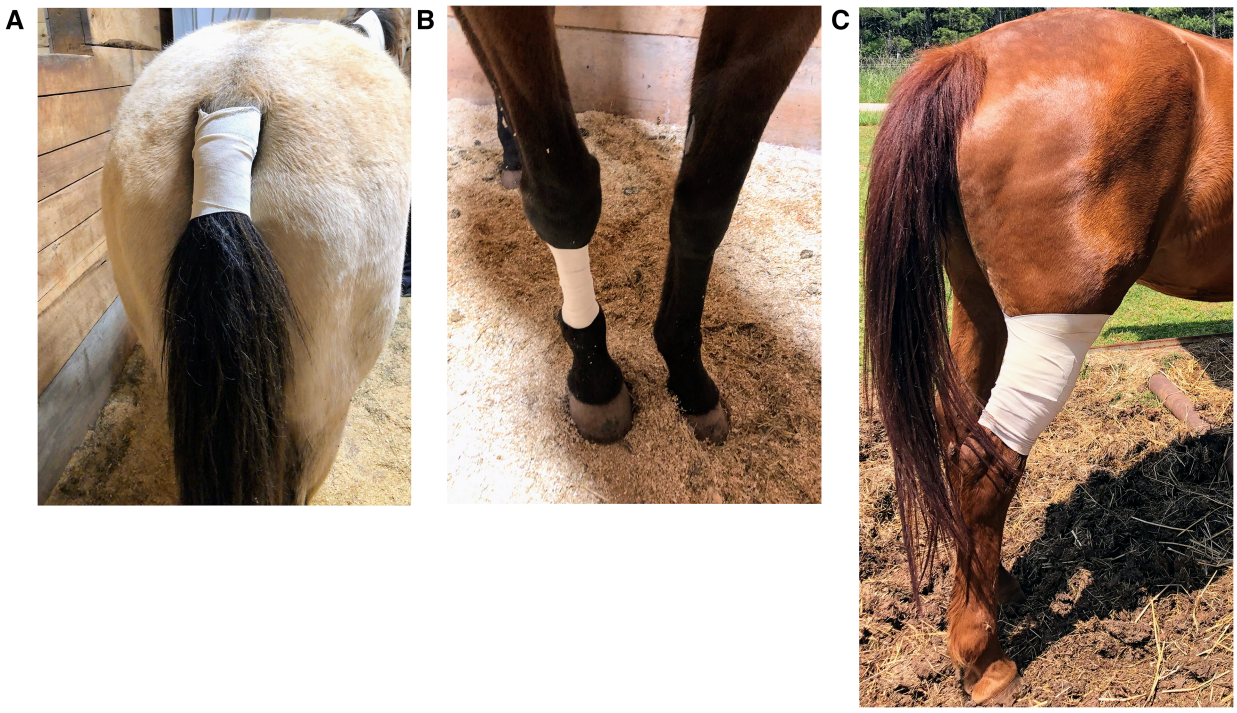

FIGURE 1
Placement of two transdermal matrix-type patch systems each containing 20 mg total buprenorphine (20 µg h^{-1}; Amneal Pharmaceuticals LLC., Piscataway, NJ, USA) with dimensions 74 mm × 74 mm and further secured with a 7.62 cm porous elastic adhesive tape covering (Elastikon; Johnson & Johnson, New Brunswick, NJ, USA) on the ventral aspect of the tail base. BUP0 horses did not receive a patch, instead only the elastic adhesive tape was wrapped around the tail base. Hence, the total content was 40 mg, and the dose received was 0.07–0.09 µg kg^{-1} h^{-1} based on their body weights on the day of treatment. The three selected patch locations were as follows: (**A**) $Tail_{TDP}$, patch application to the ventral aspect of the tail base; (**B**) $Metacarpus_{TDP}$, patch application to the dorsal surface of the metacarpus; and (**C**) $Gaskin_{TDP}$, patch application to the gaskin region located between stifle and hock joints.

2.4 Study timeline and data collection

The entire timeline of the study during administration of a treatment is depicted in Figure 2. Following instrumentation for the IV catheter, baseline data (0 h) consisting of HR, RR, and $Temp_{rectal}$ was acquired along with a collection of 6 ml whole blood from the jugular catheter. On the treatment day, each horse underwent patch application in the location designated by the randomization. Following application, additional whole blood samples were obtained for determination of buprenorphine plasma concentration at 0.5, 2, 4, 6, 8, 10, 12, 16, 24, 32, 48, 56, 72, 96, and 120 h after the patches were applied. A 10 ml waste sample was procured from the jugular catheter before drawing the 6 ml sample of venous blood for buprenorphine plasma concentrations. The sampling jugular catheter was removed after 72 h, and the following blood samples were obtained by direct jugular venipuncture. The transdermal patches were also removed at the 96 h timepoint. They were collected in sterile bags and stored at −80°C until later analysis of residual buprenorphine content. The last data collection for physiologic variables and blood sampling was performed at 120 h, which marked the end of data collection for that treatment. Blood samples were collected in lithium heparin tubes (Green BD Hemogard; Becton-Dickinson, Franklin Lakes, NJ, USA) and immediately underwent centrifugation at 1,300×*g* for 10 min. The resultant supernatant plasma was aspirated via a 1 ml disposable pipette (Thermo Fisher Scientific, Waltham, MA, USA) and transferred to cryogenic vials (Labcon 1.5 ml SuperSpin; Thermo Fisher Scientific), which were then stored at −80°C until analysis (within 2 months of sample collection).

2.5 Determination of buprenorphine concentrations

Plasma calibrators were prepared by dilution of the buprenorphine working standard solution (Cerilliant, Round Rock, TX, USA) with drug-free equine plasma to concentrations ranging from 0.01 to 70 ng ml^{-1}. Calibration curves, negative control samples, and quality control samples were freshly prepared for each assay. Quality control samples (drug-free equine plasma fortified with buprenorphine) were prepared at 0.15, 4.0, and 40 ng ml^{-1} and were included with each sample set.

For drug extraction, 0.5 ml of plasma samples were diluted with 2.0 ml 0.1M pH 6 phosphate buffer and 0.1 ml water containing d4-buprenorphine as the internal standard (40 ng ml^{-1}; Cerilliant, Round Rock, TX). All samples were vortexed gently to mix and subjected to solid phase extraction using C18UC columns 200 mg 3 ml^{-1} (UCT, Bristol, PA, USA). Prior to the addition of the samples, the columns were conditioned with 2.5 ml of methanol and 3 ml of water. Samples were loaded onto the column, and a minimum of 2 min was allowed for samples to pass through the column. The columns were rinsed with 2 ml 50% methanol in water, prior to eluting with 2.5 ml methanol. Samples were then dried under nitrogen, dissolved in 120 µl of 10% acetonitrile (ACN) in water with 0.2% formic acid, and 40 µl injected into the liquid chromatography-tandem mass spectrometry (LC-MS/MS) system.

Buprenorphine concentrations were measured in plasma by LC-MS/MS using positive heated electrospray ionization HESI (+). A TSQ Altis triple quadrupole mass spectrometer coupled with a Vanquish liquid chromatography system (Thermo Scientific, San Jose, CA, USA) was used for quantitative

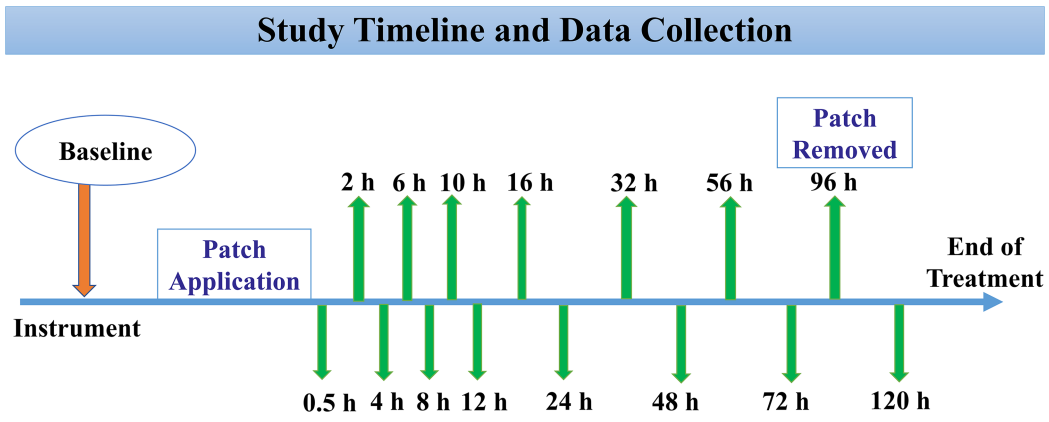

FIGURE 2
Following instrumentation, baseline data (0 h) was acquired consisting of physical examination and collection of jugular blood samples. Depending on the patch location (ventral aspect of the tail base, metacarpal, and gaskin region), the treatment was initiated by placing two transdermal matrix-type patch systems each containing 20 mg total buprenorphine (20 µg h^{-1}; Amneal Pharmaceuticals LLC., Piscataway, NJ, USA). Hence, the total content was 40 mg, and the dose received was 0.07–0.09 µg kg^{-1} h^{-1} based on their body weights on the day of treatment. Following this, 6 ml whole blood samples were obtained for determination of buprenorphine plasma concentration at 0.5, 2, 4, 6, 8, 10, 12, 16, 24, 32, 48, 56, 72, 96, and 120 h after the patches were applied. The transdermal patches were removed at the 96 h timepoint. The last data collection for physiologic variables and blood sampling was performed at 120 h, which marked the end of data collection for that treatment.

analysis. Product masses and collision energies were optimized by infusing the analytes into the mass spectrometer. Chromatography employed an ACE 3 C18 10 cm × 2.1 mm 3 µm column (Mac-Mod Analytical, Chadds Ford, PA, USA) and a linear gradient of ACN in water with a constant 0.2% formic acid at a flow rate of 0.4 ml min^{-1}. The initial ACN concentration was held at 10% for 0.3 min, ramped to 95% over 4.6 min, and held at that concentration for 0.3 min, before re-equilibrating for 2.8 min at initial conditions.

Detection and quantification were conducted using selective reaction monitoring (SRM) of the initial precursor ion for buprenorphine [mass to charge ratio (*m/z*) 468.3] and the internal standard d4-buprenorphine [(*m/z*) 472.3]. The response for the product ions for buprenorphine (*m/z* 101.0, 186.9, 243.0, 396.2, 414.2) and the internal standard (*m/z* 100.9, 186.9) were plotted, and peaks at the proper retention time-integrated, using Quan Browser software (Thermo Fisher Scientific). Quan Browser software was used to generate calibration curves and quantify the analyte in all samples by linear regression analysis. A weighting factor of 1/X was used for all calibration curves.

The patches were cut into 1 cm^2 portions and divided into two 50 ml plastic tubes. Tubes were extracted three times with 30 ml methanol by rotating for 30 min and sonicating for 5 min. The extracts were combined, brought to a final volume of 200 ml with methanol, and 200 µl was subsequently diluted to 2 ml with methanol. An aliquot (100 µl) was subjected to solid phase extraction as described for the plasma samples, and 20 µl was injected into the LC-MS system using the analytical conditions described previously.

The response for buprenorphine was linear and gave correlation coefficients of 0.99, or better accuracy was reported as percent nominal concentration and precision were reported as percent relative standard deviation. Accuracy was 98% for 0.15 ng ml^{-1}, 99% for 4 ng ml^{-1}%, and 104% for 40 ng ml^{-1}. Precision was 5% for 0.15 ng ml^{-1}, 2% for 4 ng ml^{-1}, and 2% for 40 ng ml^{-1}. The technique was optimized to provide a limit of quantitation of 0.01 ng ml^{-1} and a limit of detection of approximately 0.005 ng ml^{-1} for buprenorphine.

2.6 Data analysis

Numerical data such as HR, RR, and $Temp_{rectal}$ were assessed for normality using the Shapiro–Wilk test and by observing histograms and normal Q-Q residual plots. Mixed-effects two-factor analysis of variance was used to interpret the effects of time and treatment (fixed nominal effects) and the association of horse-time and horse-treatment was added as random effects. To adjust for the lack of sphericity, the Greenhouse–Geissner correction was applied. For making multiple comparisons with baseline measurements, the *post hoc* Tukey honest significant difference test and Dunnett's test were conducted. For all analyses (SAS 9.4; SAS Institute Inc., Cary, NC, USA), $p < 0.05$ was considered statistically significant.

3 Results

All horses successfully completed the study, and patch application was well tolerated in all three locations. Application sites were observed at each timepoint to ensure the patches were intact and in good contact with the skin. In one horse, the patches did not adhere well at the gaskin region, resulting in missing data from the 48 h timepoint until the last timepoint. Upon patch removal, there was no evidence of skin inflammation, papules, skin irritation, or redness. All horses remained clinically healthy throughout the study, and no clinically apparent adverse effects were noted with the buprenorphine dose during the entire study period. Based on the subjective data during physical examination, no horse showed signs of colic or central nervous system excitation with the dose used. Overall, the horses cooperated well and stood quietly using a halter with lead rope restraint while the physical examination was being conducted.

3.1 Physical examination

The physical examination variables followed a normal distribution, and hence the values are represented as mean ± standard deviation. The HR at the baseline timepoint for $Tail_{TDP}$, $Metacarpus_{TDP}$, and $Gaskin_{TDP}$ was 38 ± 4, 39 ± 3 and 41 ± 3 beats/min, respectively. The RR at the baseline timepoint for $Tail_{TDP}$, $Metacarpus_{TDP}$, and $Gaskin_{TDP}$ was 22 ± 3, 19 ± 4, and 21 ± 3 breaths/min, respectively. The $Temp_{rectal}$ at baseline timepoint for $Tail_{TDP}$, $Metacarpus_{TDP}$, and $Gaskin_{TDP}$ was 98.9 ± 0.84, 99.9 ± 0.93, and 99.5 ± 0.98°F, respectively. Between the three treatment groups, there was no change in HR, RR, and $Temp_{rectal}$ across timepoints as compared to baseline and when compared to each other in a single horse as well as between horses ($p > 0.3$). There was no effect of treatment ($p > 0.2$) or time ($p > 0.1$) and no significant interaction between treatment and time on HR, RR, and $Temp_{rectal}$.

3.2 Plasma buprenorphine concentrations

In the $Tail_{TDP}$ group, the mean plasma buprenorphine concentrations were >0.1 ng ml^{-1} from 2 h to 32 h. The highest group mean was 0.25 ng ml^{-1} noted at 4 h. In the $Metacarpus_{TDP}$ group, the mean plasma buprenorphine concentrations were >0.1 ng ml^{-1} from 32 to 56 h. The highest group mean was 0.15 ng ml^{-1} noted at 32 h. In the $Gaskin_{TDP}$ group, the mean plasma buprenorphine concentrations were >0.1 ng ml^{-1} from 10 to 32 h. The highest group mean was 0.13 ng ml^{-1} noted at 32 h. Out of the total six horses, one horse in the $Tail_{TDP}$ group, five horses in the $Metacarpus_{TDP}$ group, and four horses in the $Gaskin_{TDP}$ group had detectable plasma buprenorphine concentrations at the 120 h timepoint. Norbuprenorphine was not detected in any horse at concentrations above the limits of detection at any time point.

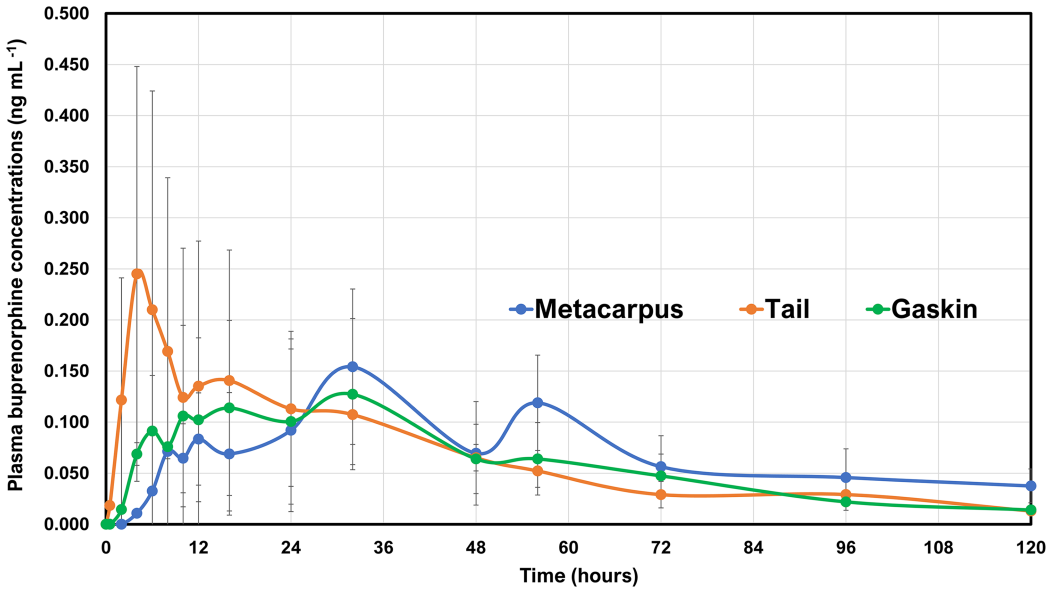

FIGURE 3
Mean ± standard deviation of plasma concentrations of buprenorphine overtime in six horses from baseline (0 h), which coincides with before patch application to 0.5, 2, 4, 6, 8, 10, 12, 16, 24, 32, 48, 56, 72, 96, and 120 h after the patches were applied. Two transdermal matrix-type patch systems each containing 20 mg total buprenorphine (20 μg h^{-1}; Amneal Pharmaceuticals LLC., Piscataway, NJ, USA) were placed in three different locations. Tail, patch application to the ventral aspect of the tail base (orange lines with orange circles); Metacarpus, patch application to the dorsal surface of the metacarpus (blue line with blue circles); and Gaskin, patch application to the gaskin region located between stifle and hock joints (green line with green circles). The total content was 40 mg, and the dose received was 0.07–0.09 μg kg^{-1} h^{-1} based on their body weights on the day of treatment. The transdermal patches were removed at the 96 h timepoint.

When comparing all three locations, the buprenorphine uptake was observed to be more consistent with respect to measurable plasma concentrations >0.1 ng ml^{-1} when applied to the ventral aspect of the tail base (Figure 3).

When the patches were removed and submitted for analysis, the amount of buprenorphine extracted from patches was 23 ± 1.5 mg (54.5 ± 4.3% left) in the $Tail_{TDP}$ group, 21.1 ± 2.3 mg (55.6 ± 6.5% left) in the $Metacarpus_{TDP}$ group, and 23.4 ± 2.5 mg (58 ± 5.3% left) in the $Gaskin_{TDP}$ group.

4 Discussion

For the present study, the aim was to determine buprenorphine plasma concentrations in healthy horses from transdermal patches applied at three different locations i.e., the ventral aspect of the tail base, the metacarpal region, and the gaskin area. Skin preparation and the process of patch application were followed as per the standard published in other equine studies to maintain uniformity in the technique (26, 27, 33–36). The plasma buprenorphine concentrations were consistently >0.1 ng ml^{-1} as quickly as 2 h and lasted up to 32 h for the $Tail_{TDP}$ group. Although the other two locations yielded measurable plasma concentrations, they were >0.1 ng ml^{-1} at fewer timepoints. The drug was detected faster in the plasma and a higher peak was observed in the $Tail_{TDP}$ group. In our horses, the desired level for plasma buprenorphine concentration was set at a minimum of 0.1 ng ml^{-1} and was based on a recent study (26) that showed the placement of two transdermal buprenorphine patches (each containing 20 mg total buprenorphine) on ventral tail base resulted in a consistent increase in thermal thresholds that coincided with ≥0.1 ng ml^{-1} in healthy horses. To the author's knowledge, the present study is the first to report buprenorphine plasma concentrations in horses following patch application at different locations.

Transdermal opioid delivery systems have gained immense popularity across different species, which has contributed to significant advances in effective pain management via the maintenance of steady blood drug concentrations over longer periods. The established transdermal opioid delivery systems are drug-in-adhesive, reservoir, and matrix-type. In the present study, buprenorphine was administered via a matrix patch that includes an adhesive polymer matrix containing the drug homogeneously embedded in the center. On the top of this matrix is the backing layer made up of elastomers that protect the patch from the outer environment and it is impermeable to the drug. On the bottom of this matrix is the lining layer that protects the patch during storage and is peeled off before use (3, 4, 37, 38). The matrix-type patch is relatively thinner, lighter, and flexible, which benefits skin conformability and adherence. The thickness of the adhesive polymer matrix layer indicates that some of the drug will diffuse through the layer before reaching the skin. This design enables the drug to get across the dermis to the cutaneous blood vessels for absorption into circulation where it becomes available systemically. If the active form of the drug remains largely in the periphery, there is limited penetration into

the systemic circulation, which reduces the incidence of adverse effects (3, 4). Adherence of the patch with the skin is crucial for the efficacy of this transdermal delivery system. Skin and body movement, rubbing the patch subject the patch to sheer stress impacting adhesion. Moreover, environmental factors such as sweating, moisture, and ambient temperature have a direct effect on patch-to-skin contact. It is possible that these mechanisms may have played a role in the present study and contributed to the inconsistent or lower plasma buprenorphine concentrations for patches placed on the gaskin and metacarpal regions.

Skrzypczak et al. (27) applied matrix-type fentanyl patches to the inguinal abdominal region (lateral to udder or prepuce), dorsal metacarpus, and ventral aspect of the tail base in healthy horses. They observed that the maximum fentanyl concentration and the time taken to reach this drug concentration were similar between locations. The patches were well tolerated at these sites and no treatment was affected by the loss of patch via dislodgement. The other locations that have been studied to evaluate reservoir-type fentanyl patches in horses are the proximal lateral antebrachium (33), medial or lateral antebrachium and gaskin region (34), and mid-dorsal thorax (35). There is a significant location-dependent difference in transdermal fentanyl penetration in horses (27, 32), sheep (28), and rabbits (29), with less drug available for the systemic activity for patches applied to the dorsal carpal region in horses, whereas the groin and thorax skin have a similar pattern (32). Several factors can account for species-specific differences and inter-patient variability with respect to drug uptake from the patch and absorption via the skin such as (i) thickness of stratum corneum and epidermis, (ii) density of hair follicles and sweat glands, (iii) regional cutaneous blood flow, (iv) drug molecular kinetics, (v) genetics, (vi) underlying skin disease or injury, (vii) formulation of the drug–polymer matrix, (viii) skin temperature, and (ix) skin preparation (razor shaving, alcohol). Fick's law of diffusion controls the rate of drug input from the transdermal system into the systemic circulation through skin penetration barriers, where the drug delivery is directly proportional to the drug concentration in the matrix and coefficient of drug diffusion. It is vital to note that the drug penetration into the skin is not constant and is dependent on the duration of patch application and overtime variations in cutaneous properties, available drugs in the matrix, and depletion of enhancers required for drug delivery (3, 32). In the present study, cleaning the application site could have disrupted the stratum corneum, and not all drug from the patch was delivered while in contact with the skin. Erratic drug uptake between locations could have been a consequence of altered diffusion capacity of the skin lipids, differences in skin thickness, and variations in skin pH due to sweat, moisture, and altering body temperature. In one horse belonging to the $Gaskin_{TDP}$ group, the patches were seen to not firmly adhere due to sweat, moisture, and leg movement and the bandage tended to slip down in that area. This finding is clinically relevant and should be taken into consideration when using this patch location.

Special features that ease the crossing of buprenorphine through the skin are lower molecular weight, compact molecular structure, high lipophilicity, an adequate degree of ionization, sufficient water solubility, high efficacy to restitute for limited absorption, reduced melting temperature, relatively shorter half-life, low daily dosage regime, dosing enabling absorption from a relatively small area, and matrix patches in which a total amount of a drug is localized homogenously in an adhesion layer (3, 5–7). This technology ensures the release of the opioid is regulated due to the gradient concentration between the patch and the skin. Patch delivery systems are designed particularly to contain more amount of drugs than the patch actually can deliver. In the event the patch is not removed from the location, increased dose administration and prolonged pharmacological effects can occur. We removed the patch from the horses at the 96 h timepoint, and the residual drug was determined. The buprenorphine residue on the patch was 21–23 mg (54%–58%) of the total amount (40 mg). The residual amount can be influenced by the type of patch, drug load and concentration, the thickness of the adhesive layer, and the composition and thickness of the backing layer. Although this can be a safety concern with the potential for abuse, the excess amount of the drug remaining in the patch after use is necessary to ensure a saturated concentration of the drug is maintained and drug delivery occurs at a clinically effective rate. The development of metered-dose pumps or active diffusion systems may prove beneficial to increase drug efficiency and improve safety or abuse liability profiles. Poor patch-to-skin contact and variable skin hydration can occur in response to ambient humidity and temperature and affect the integrity and barrier properties of the skin resulting in variations in the amount of drug absorbed (37, 38). The 54%–58% buprenorphine left over in the patch explains why we saw lower plasma concentrations and, hence, did not observe any significant behavioral effects and differences in the physical examination. However, it also signifies that despite partial drug uptake, the plasma concentrations obtained were >0.1 ng ml^{-1} for multiple timepoints in the $Tail_{TDP}$ group and relatively fewer timepoints for the $Metacarpus_{TDP}$ and $Gaskin_{TDP}$ groups.

The primary metabolite of buprenorphine is norbuprenorphine, which was undetectable following transdermal administration in the present study. This analysis was in accordance with previous studies where norbuprenorphine was unmeasurable following either intravenous or sublingual route (39–41). Considering norbuprenorphine has only 25% of the intrinsic analgesic activity of buprenorphine and a low permeability into the brain, it may have minimal clinical significance (42). There is no available literature highlighting the antinociceptive effect of norbuprenorphine in horses and hence it is uncertain whether this metabolite contributes to antinociception. It is possible that the high stability of molecular ions of norbuprenorphine may present a challenge to be detected by tandem mass spectrometry. The assay may not have the sensitivity for measuring this metabolite and this lack of optimization could affect this finding.

Previous exploratory studies with buprenorphine in horses utilized average doses of 5–10 µg/kg via intravenous (18, 20, 21, 40, 42–48), intramuscular (23, 24, 39, 49), and sublingual (40, 42, 50) routes. A common observation in most of these studies irrespective of the route used was its potential for inducing excitement,

increasing spontaneous locomotory activity, decreasing gut sounds, and elevating HR in healthy pain-free horses. Despite opting for the subcutaneous route for buprenorphine administration in a few equine studies, the gastrointestinal side effects, compulsive behavior, and restlessness persisted (22, 51). The dose in the present study was selected carefully based on the behavioral and physiologic responses reported in these studies. We anticipated that 0.07–0.09 μg kg^{-1} h^{-1} (40 μg h^{-1}) would be a safe, well-tolerated dosage regime for our horses, which would prevent systemic complications and excitement as confirmed in the previous equine studies (25, 26). Moreover, currently, the highest concentration of transdermal system available for buprenorphine in the USA is 20 μg h^{-1}, and since the selected locations were the ventral aspect of the tail base, metacarpal and gaskin regions, placement of only two patches next to each was possible without overlap to administer 40 μg h^{-1}. Future studies are imperative to evaluate whether a higher transdermal patch dose can lead to plasma concentrations lasting for a longer duration coinciding with therapeutic drug concentrations yielding adequate analgesia but still devoid of any systemic complications. In addition, even though mild, diffuse erythema with a small number of papules has been reported with buprenorphine transdermal system in pigs (13), no adverse effects were noted locally near or at the area of patch location in our study horses.

This study presented a few limitations. An intravenous treatment was not included in the study design, and therefore, the bioavailability of the matrix buprenorphine patch was not calculated. Only a small sample size consisting of healthy, pain-free adult horses was utilized. The physiologic and behavioral effects of opioid administration can differ significantly in painful vs. non-painful animals; hence, future studies in clinical patients exhibiting signs of pain are warranted. A genetic involvement for transdermal drug uptake has been defined in humans; however, its impact cannot be ruled out in our study of horses. Aging induces structural and functional variations in the skin layers and changes in hydration and lipidic structure may affect the barrier function of the stratum corneum specially for hydrophilic compounds. Hence, potential alterations affecting the transdermal opioid diffusion in younger vs. older horses need further investigation. Noxious thermal stimuli to evaluate the analgesic effect of transdermal buprenorphine patches at various locations for superficial acute short-lasting pain were not included. The minimum therapeutic levels for buprenorphine via this route remain unknown. Behavioral analysis and gastrointestinal function were not assessed using standards published in the literature (e.g., video footage, pedometer data, gastrointestinal motility scores, fecal and urine output, visual analog scoring, ataxia grading, and sedation scores). Since the undesirable effects can be of lesser magnitude in painful horses, future clinical studies are required that objectively quantify these effects and determine their association with transdermal buprenorphine patch administration in painful vs. non-painful horses.

5 Conclusion

Following extensive literature review, this appears to be one of the earlier reports of transdermal buprenorphine patch administration in horses. In the present study, 40 μg h^{-1} buprenorphine transdermal patches applied at the ventral aspect of the tail base, metacarpal, and gaskin region were well tolerated by all horses as assessed by a physical examination. In the $Tail_{TDP}$ group, the mean plasma buprenorphine concentrations were >0.1 ng ml^{-1} from 2 to 32 h. The highest group mean was 0.25 ng ml^{-1} noted at 4 h. In the $Metacarpus_{TDP}$ group, the mean plasma buprenorphine concentrations were >0.1 ng ml^{-1} from 32 to 56 h. The highest group mean was 0.15 ng ml^{-1} noted at 32 h. In the $Gaskin_{TDP}$ group, the mean plasma buprenorphine concentrations were >0.1 ng ml^{-1} from 10 to 32 h. The highest group mean was 0.13 ng ml^{-1} noted at 32 h. Norbuprenorphine was not detected in any horse at concentrations above the limits of detection at any time point. When comparing all three locations, the buprenorphine uptake was observed to be more consistent with respect to measurable plasma concentrations >0.1 ng ml^{-1} when applied to the ventral aspect of the tail base. The other two locations presented more erratic and inconsistent buprenorphine uptake and plasma concentrations. Further research must be directed at investigating the effect of higher dosages of the transdermal buprenorphine patch on the duration of analgesia, measurable plasma concentrations, and behavioral and systemic effects. It is imperative that clinicians can compare analgesic and systemic effects in painful and non-painful horses.

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 study was approved by the University of Georgia Institutional Animal Care and Use Committee (animal use protocol: A2021 06-011). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

VP: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. HK: Conceptualization, Data curation, Formal Analysis, Software, Validation, Writing – review & editing. LB: Investigation, Resources, Writing – review & editing. SG: Investigation, Resources, Writing – review & editing. JC: Investigation, Resources, Writing – review & editing. RR: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Project administration, Resources, Supervision, Visualization, Writing – review & editing.

Funding

The authors declare financial support was received for the research, authorship, and/or publication of this article.

This study was funded by the Virginia-Maryland College of Veterinary Medicine-Equine Research Competition Grants Program (source: Equine Racing Funds; grant fund no.: 179767)

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 authors 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 Pain Research

TYPE Original Research
PUBLISHED 23 July 2024
DOI 10.3389/fpain.2024.1410302

OPEN ACCESS

EDITED BY
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RECEIVED 31 March 2024
ACCEPTED 05 July 2024
PUBLISHED 23 July 2024

CITATION
Nowak M, Martin-Cirera A, Jenner F and Auer U (2024) Time budgets and weight shifting as indicators of pain in hospitalized horses.
Front. Pain Res. 5:1410302.
doi: 10.3389/fpain.2024.1410302

Time budgets and weight shifting as indicators of pain in hospitalized horses

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Introduction: Pain assessment in horses presents a significant challenge due to their nonverbal nature and their tendency to conceal signs of discomfort in the presence of potential threats, including humans. Therefore, this study aimed to identify pain-associated behaviors amenable to automated AI-based detection in video recordings. Additionally, it sought to determine correlations between pain intensity and behavioral and postural parameters by analyzing factors such as time budgets, weight shifting, and unstable resting. The ultimate goal is to facilitate the development of AI-based quantitative tools for pain assessment in horses.
Materials and methods: A cohort of 20 horses (mean age 15 ± 8) admitted to a university equine hospital underwent 24-h video recording. Behaviors were manually scored and retrospectively analyzed using Loopy® software. Three pain groups were established based on the Pain Score Vetmeduni Vienna : pain-free (P0), mild to moderate pain (P1), and severe pain (P2).
Results: Weight shifting emerged as a reliable indicator for discriminating between painful and pain-free horses, with significant differences observed between pain groups ($p < 0.001$) and before and after administration of analgesia. Additionally, severely painful horses (P2 group) exhibited lower frequencies of feeding and resting standing per hour compared to pain-free horses, while displaying a higher frequency of unstable resting per hour.
Discussion: The significant differences observed in these parameters between pain groups offer promising prospects for AI-based analysis and automated pain assessment in equine medicine. Further investigation is imperative to establish precise thresholds. Leveraging such technology has the potential to enable more effective pain detection and management in horses, ultimately enhancing welfare and informing clinical decision-making in equine medicine.

KEYWORDS

equine pain, equine discomfort, pain score, posture, time budgets, weight shifting

1 Introduction

Pain is a critical determinant of patient welfare and plays a crucial role in guiding clinical decisions. In horses, a nonverbal prey species inherently inclined to display minimal signs of pain in the presence of potential threats including humans (1–3), the assessment of pain poses a notorious challenge. Particularly, mild to moderate pain, whether acute or chronic, may lead to falsely low scores, resulting in an underestimation of pain intensity (4). Physiologic parameters like heart and respiratory rate lack the requisite sensitivity and specificity for reliable pain detection and

differentiation from other sources of distress (5–7). Consequently, the focus has shifted toward investigating pain behaviors, such as facial expressions and alterations in activity patterns or mental status, as indicators of pain.

As healthy, stress-free horses adhere to highly repetitive, individual daily routines with specific time allocations for different activities (time budgets), deviations from these established time budgets can serve as signals of discomfort, pain, or potential disease (2, 3, 7–9). However, accurate time budget analysis requires continuous observation over extended periods, limiting its practicality for pain evaluation in clinical settings. Automated video analysis emerges as a promising solution, eliminating the need for continuous human observation and facilitating the use of time budgets for early pain and health issue detection.

Healthy horses evenly distribute their weight, with 60% of the weight on the forelimbs and 40% on the hind limbs (8). Although horses may occasionally rest one hind limb at a time, their overall weight distribution remains balanced (8). Horses suffering from orthopedic pain may reduce the load on the affected limb by positioning it away from the center of gravity e.g., by pointing the affected limb (9–11). Notably, postural adjustments, aimed at minimizing the load on painful tissues to prevent or alleviate pain and safeguard against further injury, exhibit a strong association with orthopaedic pain in both humans and horses (11). These postural adjustments lend themselves to automated video analysis, thus opening avenues for the development of a real-time, continuous, and objective quantification of pain. Despite these advancements, no study has yet established a definitive link between the degree of weight shifting and equine discomfort or pain.

Therefore, this study aims to identify pain-associated behaviors amenable to automated AI-based detection in video recordings and determine correlations between pain intensity and behavioral and postural parameters. We hypothesized that, time budgets, weight shifting, and unstable resting are potentially good parameters to identify equine pain.

2 Material and methods

2.1 Horses and video recording

Horses admitted to the University Equine Hospital of the University of Veterinary Medicine Vienna are allocated randomly to 4 × 4 m box stalls based on availability, with the stables being bedded with shavings and cleaned twice daily.

This study recruited a cohort of 200 horses assigned to one of four stables equipped with video surveillance cameras during the period spanning from April to November 2021, with the owner's consent. Inclusion criteria mandated hospitalization for a minimum of three consecutive days to allow for a period of acclimatization lasting at least 24 h post-admission before the onset of video recording. Horses were video recorded for 24-h employing either a GoPro® action camera or an Acaris webcam (Horse Protector®) camera. These cameras were strategically positioned at a height of 2.5 m in a corner at the front of the box stall, affording a panoramic view of the entire enclosure. The recordings were made in time lapse mode with two picture per second. Only horses that received full rations of food, comprising hay dispensed four times daily, were eligible for inclusion in the study. All horses had unlimited access to water.

From the initial pool of 200 horses, a subset of 20 animals was randomly chosen for analysis, irrespective of the cause for hospitalization and the pain status of the horses (refer to Table 1).

The recording period commenced no earlier than the second day of admission when the first set of 24 videos was available after admission and no surgery. In the case of surgery, the full set of 24-h videos was available the day after surgery when the horses were on a full food ratio.

2.2 Pain medication, examination and pain score

During their hospital stay, all horses were examined at least twice daily, at 8:00 am and 8:00 pm, which included a comprehensive physical exam and determination of pain, using the Pain Score Vetmeduni Vienna (12), (Supplementary Files, Figure 1). Treatments, including the administration of pain medication as deemed necessary, were provided according to each horse's specific medical condition, determined solely by the clinician's discretion, and were not influenced by the study (Table 1). Exam and treatment data were collected retrospectively from the digital medical history.

Based on the mean pain score from two pain assessments conducted over 24 h, the horses were stratified *post hoc* into three groups: the pain-free group (P0, score ≤ 2), the mildly to moderately painful group (P1, $2 < \text{score} \leq 8$) or the severely painful group (P2 score > 8).

2.3 Behaviour scoring

Behaviors were systematically assessed using Loopy® (Loopbio, Vienna, Austria), a video coding interface that supports the coding of a wide range of behaviors for multiple individuals and provides corresponding plotting and analysis tools. Surveillance videos were uploaded into the software and time corresponding to the presence of veterinary professionals, nurses, technicians, or students in the stall during activities such as feeding, medication administration, or examination, was deducted from the total video duration. After an ethogram was defined in the program (Table 2), videos underwent manual behaviour scoring by a veterinarian (M.N.), who was blinded to the medical history and treatment of the patients and consistently adhered to the behavior-scoring guidelines adapted from V Boy and others (13) (Table 2).

Initially, the assessment focused on resting standing, feeding, lying, and movement behaviors as well as weight shifting. The resting phase, defined by a lack of ambulation or eating, was later subdivided into unstable resting and resting standing, collectively referred to as total resting time. Unstable resting behavior was counted during the total resting phase if it persisted for more than 10 frames (=5 s).

10.3389/fpain.2024.1410302

TABLE 1 Horses' diagnosis, medication, and pain scores post-admission (postadm) and post-surgery (postsurg).

Horse	Breed	Sex	Age	Weight	Diagnosis	Day of video recording after surgery/admission	Pain medication	Pain group	Mean pain score
A	Miniature Warmblood	M	30	275	Chronic laminitis	No surgery, d2 postadm	Firocoxib 1x daily PO	P1	4
B	Warmblood	F	6	560	Tremor of unknown origin	No surgery, d2 postadm	Phenylbutazone 2× daily PO	P0	2
C	Coldblood	F	21	618	Abscess on lower breast	d3 postsurg	Phenylbutazone 2× daily PO	P1	3
D	Warmblood	M	13	636	Septic tarsocrural joint	d3 postsurg	Flunixin Meglumine 2× daily IV	P1	4
E	Warmblood	F	23	615	Lameness front limb grade 2/5	No surgery d2 postadm	Firocoxib 1× daily PO	P0	2
F	Warmblood	M	6	500	Old wound front limb	d3 postadm	Flunixin Meglumine 2× daily IV	P0	2
G	Warmblood	M	23	480	Equine asthma, cough	No surgery d2 postadm.	No therapy	P0	0
H	Warmblood	M	8	558	Osteoarthritis tarsus	No surgery d2 postadm	No therapy	P0	2
I	Warmblood	M	14	604	Septic nuchal bursa	d4 postsurg	No therapy	P1	7
J	Warmblood	M	2	340	Colic	d3 postsurg	Flunixin Meglumine 2× daily IV	P0	1
K	Warmblood	F	14	555	Colic	d6 postsurg	Flunixin Meglumine 1× daily PO	P1	4
L	Warmblood	M	19	451	Colic	d3 postsurg	Flunixin Meglumine 2× daily IV	P1	5
M	Coldblood	M	25	636	Lameness stifle	No surgery d2 postadm	No therapy	P1	4
N	Warmblood	F	10	550	Olecranon fracture	d19 postsurg	Phenylbutazone 2× daily PO	P1	3
O	Warmblood	F	13	530	Osteosynthesis P1 fracture	d3 postsurg	Phenylbutazone 2× daily PO	P1	5
P	Coldblood	F	15	770	Dental problem	No surgery d2 postadm	No therapy	P1	2
Q	Warmblood	M	26	480	Septic arthritis	d2 postsurg	Flunixin Meglumine 2× daily IV	P2	10
R	Warmblood	M	12	519	Choke	No surgery, d2 postadm	No therapy	P0	0
S	Warmblood	F	7	598	Epileptic episode	No surgery, d2 postadm	Flunixin Meglumine 2× daily IV	P0	0
T	Warmblood	F	3	348	Septic arthritis	d3 postsurg	Flunixin Meglumine 2× daily IV	P2	11

d, day; IV, intravenous, PO, per os. P0, pain free group; P1, group with mild to moderate pain; P2, group with severe pain.

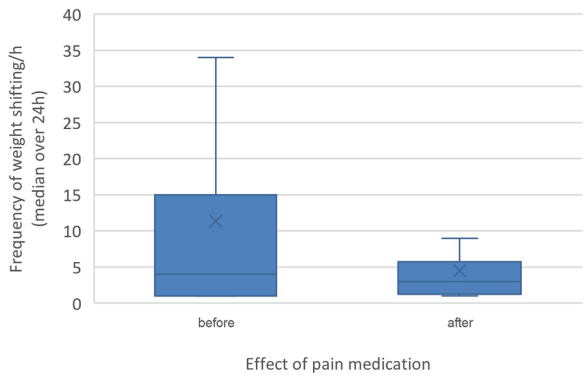

FIGURE 1
Effect of pain medication (before pain medication versus after) on weight shifting. The difference in weight shifting was statistically significant ($p < 0.001$).

2.4 Time budgets

Data from Loopy® were extracted as a CSV (Comma-Separated Values) file for subsequent analysis. The duration of five behavioral categories—feeding, resting standing, unstable resting, locomotion, and lying—was quantified per hour. The mean duration of each behavior episode within its respective category was computed per hour and labeled as duration of feeding (D_feed), resting standing (D_RS), and unstable resting (D_UR). Additionally, the total activity count, representing the number of behavior switches (not including weight shifting) per hour, and the frequency of occurrences of feeding (C_feed), resting (C_RS), and unstable resting (C_UR) were documented as means per hour over a 24-h period. The total resting time (TRT) was obtained by summing the durations of resting standing (RS) and unstable resting. Subsequently, time budgets were calculated as a percentage of time each horse spent on

TABLE 2 Ethogram used to manually score horses' behavior.

Lying	The horse is lying in lateral or sternal recumbency; the duration of lying is measured from the moment the horse lies down to when it resumes a standing position.
Resting standing (RS)	The horse is motionless (not eating), asleep or drowsy, allowing the ears and tail to move, with the head held motionless at height of the withers or slightly above or below.
Unstable resting (UR)	The horse remains stationary (taking fewer than 3 steps in any direction, not eating), displaying small, restless movements or behaviors that are often repetitive and can include actions like shifting weight with or without lifting the limbs, swaying, nodding the head, or other subtle gestures, described as fidgeting by Torcivia and McDonell (3), either in a state of drowsiness or alertness while observing the surroundings.
Feeding and foraging (feed)	Activities such as eating, foraging, nibbling or sniffing food either on the ground or in a feeder, or actively searching for food. The onset of feeding behavior was marked from the moment the horse lowered its head and started to eat or forage until it raised the head again.
Locomotion	Forward or backward movement of more than one limb for more than three steps resulting in a new position within the stable.
Weight shifting (WS)	Scored as an event; Frequent shifting of the primary weight-bearing limb or limbs with or without lifting the hooves.

each behavior divided by the observation (video) time and then we obtained a mean per hour.

2.5 Weight shifting

Weight shifting (WS) was scored as an event and reported as number of events per hour. Additionally, the ratio of the number of weight shifts to the total resting time (WS/TRT) per hour was calculated to provide a relation to the resting time.

2.6 Statistical analysis

NCSS Statistical Software® [NCSS 2023 Statistical Software (2023). NCSS, LLC. Kaysville, Utah, USA, ncss.com/software/ncss.] was used for data analysis. Kolmogorov-Smirnov tests were performed on the data to assess normality. Data are presented as median and range (min-max). The impact of pain medication (yes/no), as well as before and after treatment, and the pain group on time budgets and weight shifting was assessed using Kruskal-Wallis tests. Subsequently, a *post hoc* analysis was conducted with the Kruskal-Wallis Multiple Comparison *Z*-Value Test (Dunn's Test), with statistical significance set at a *p*-value less than 0.05.

3 Results

3.1 Horses

The horses' mean age was 15 ± 8 years, and their mean weight was 534 ± 139 kg. Further details about the population, including medication specifics and reasons for admission, are provided in Table 2.

3.2 Time budget

The median time budget over 24 h for feeding was 46% (range: 0–97), for resting it was 16% (range: 0–82), and for unstable resting it was 19% (range: 2–96). Total resting comprised 47% (range: 2–96), while locomotion accounted for 0.5% (range: 0–36). Fourteen horses were observed lying during the 24-hour period, with a mean time budget of 4 ± 15%. The median frequency of weight shifts per hour was 3 (range: 1–106), while the total activity count was recorded as 24/h (range: 1–269). Detailed time budgets per horse are provided in (Supplementary Table S1).

3.3 Medication

Six (30%) horses received no pain medication (Table 1), two of which (K, V) were assigned to P0 and the other to P1 (J, L, P, S). Three horses (15%, one in P0, two in P1) received phenylbutazone (2 mg/kg, PO or IV BID), six horses (30%, two in P0, three in P1 and two in P2) flunixin meglumine (1,1 mg/kg, IV, BID) and two horses (10%, one in P0, one in P1) firocoxib (0,1 mg/kg, PO, SID).

Pain medication had no significant influence on the time budgets. The frequency of weight shifting per hour was significant lower ($p < 0.001$, median weight shifts/h = 2, range: 1–43) in horses without pain medication compared to the horses that received pain medication (median weight shifts/h = 5, range: 1–106). Although pain medication was not adjusted based on horses' pain score but rather administered based on clinician preference, horses showed significantly ($p < 0.001$) less weight shifting (median weight shifts/h = 3, range: 1–106) after receiving pain medication than before (median weight shifts/h = 4, range 1–56, Figures 1, 2). However, the response to treatment was individually variable.

3.4 Pain groups

Based on the Pain Score Vetmeduni Vienna, eight horses were allocated to P0, ten horses to P1 and two horses to P2.

The time budgets for lying ($p < 0.001$) and total rest ($p = 0.011$) were significantly different between pain groups (Table 3, Figure 3). The time budget for feeding was lower ($p = 0.247$), but the time budgets for resting standing ($p = 0.141$) and unstable resting ($p = 0.44$) were higher in P2 compared to P0 and P1.

The frequency of feeding ($p = 0.003$) and resting standing ($p < 0.001$) per hour was significantly lower in the P2 group compared to P1, while the frequency of unstable resting ($p = 0.001$) was significantly higher in the P2 group compared to P1. Additionally, the mean duration of resting standing was significantly longer ($p < 0.001$), and the mean duration of unstable resting sessions was significantly shorter ($p < 0.001$) in the P2 group. The duration for feeding was significantly longer

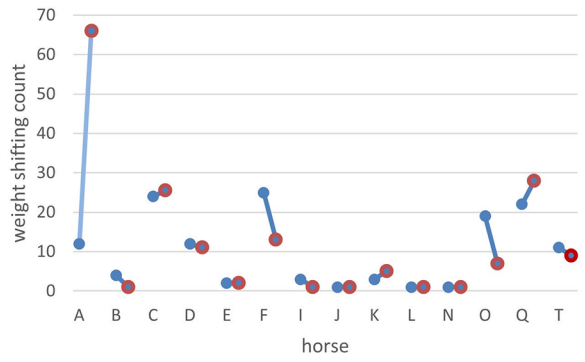

FIGURE 2
Effect of pain medication on weight shifting comparing the three hours before medication (blue dots) to the three hours after medication (red dots). On the x-axis are the horses (indicated by their ID), and on the y-axis is the count of weight shifting before and after treatment. Horse A was in the pain group P1, with the diagnosis chronic laminitis and received Firocoxib orally once per day.

($p = 0.014$) in P1 compared to P0. Total activity per hour was significantly lower in P2 compared to P0 ($p = 0.032$) (Table 3).

The frequency of weight shifting per hour ($p < 0.001$) and the ratio of weight shifting/total rest were significantly ($p < 0.001$ for WS/TRT) lower in P0 compared to P1 and P2 (see Figure 4, and Table 3).

4 Discussion

Evaluating equine pain in is a complex but indispensable aspect of effective clinical decision-making. The inherent subjectivity of pain behavior evaluation hinders objective and quantitative assessment. Thus, the integration of artificial intelligence (AI)-based analysis of video or sensor data emerges as a promising avenue. This technology offers the potential of continuous pain assessment over extended durations, minimizing observer bias and interference, thereby enhancing the precision and objectivity of pain evaluation in equine patients. However, successful implementation of AI-based analysis hinges on the identification and definition of robust, quantifiable parameters that can be readily analyzed based on video data and can accurately distinguish between painful and pain-free animals.

In this study, weight shifting, and unstable resting emerged as promising indicators for distinguishing between horses experiencing pain and those that are pain-free. In addition, severely painful horses (P2 group) exhibited lower frequencies of feeding and resting standing per hour compared to pain-free horses, while displaying a higher frequency of unstable resting per hour.

These findings align with previous research emphasizing postural behavior as a reliable indicator of pain, particularly in orthopedic conditions (14). Horses often redistribute weight away from a painful limb in search of relief, a behavior documented in various painful conditions such as laminitis (15). While healthy horses typically alternate weight-bearing on their hindlimbs during periods of rest, those experiencing pain may exhibit weight-shifting or adopt a three-legged body support (16–19).

Although previous studies have suggested thresholds for weight shifting indicative of physical fatigue, a definitive cut-off distinguishing physiological weight shifting from pain-related weight shifting remains elusive. While a frequency exceeding 7 weight shifts per 5 min has been linked to physical fatigue (20), horses with laminitis have been observed to shift weight between contralateral limbs up to 46 times per 10 min before analgesic intervention (21). In our study, we found a significantly higher incidence of weight shifting in painful horses (median: 13/h, range: 1–67) compared to pain-free counterparts (median: 2/h, range: 1–86). Notably, weight shifting was not only associated with orthopedic pain but was also observed in horses recovering from colic surgery that showed no clinical signs of laminitis. None of these horses underwent an orthopedic examination so we cannot exclude and subclinical orthopedic problem which was not mentioned by the owner or in the medical history. This unexpected finding invites further studies looking into the occurrence of weight shifting in a larger group of horses suffering from non-orthopaedic pain.

TABLE 3 Discomfort indices and time budgets per pain group.

Parameter	P0	P1	P2	*p*-value
Weight shifting (per h)	2 (1–86)*	3 (1–106)	13 (1–67)	<0.001
Weight shifting/total resting time (WS/TRT)	0.05 (0–1.6)*	0.15 (0–3.4)	0.34 (0–1.8)	<0.001
Feeding (%)	48 (1–100)	46 (0–100)	34 (0–100)	0.247
Resting standing (%)	17 (1–89)	12 (0–99)	24 (0.89)	0.141
Unstable resting (%)	18 (1–94)	21 (0–100)	17 (0–100)	0.44
Locomotion (%)	0.5 (0–16)	0,3 (0–20)	0 (0–36)	0.05
Lying (%)	0 (0–100)	0 (0–99)	0 (0–79)	<0.001
Total resting (%)	43 (1–100)	49 (0–100)	64 (0–100)*	0.011
Total activity/h	23 (1–203)	25 (1–188)	8 (2–99)*	0.032
Frequency of feeding/h	5 (1–34)	5 (1–70)	2 (1–22)*	<0.003
Frequency of resting standing/h	4 (1–68)	4 (0,43–73)	2 (1–15)*	<0.001
Frequency of unstable resting/h	9 (1–50)	8 (1–47)	10,5 (1–48)*	0.001
Duration of feeding (min)/h	3 (9–47)	6 (0–107)*	4 (0–43)	0.014
Duration of unstable resting (min)/h	1 (0–28)	2 (0–32)	0.5 (0–8)*	<0.001
Duration of resting standing (min)/h	1.5 (0–21)	2 (0–57)	9 (0–87)*	<0.001

Values are provided as median and range by pain group based on the Pain Score Vetmeduni Vienna. P0 – pain free group; P1 – group with mild to moderate pain; P2 – group with severe pain. (*)- indicates statistical significance from other groups, with a *p*-value lower than 0.05.

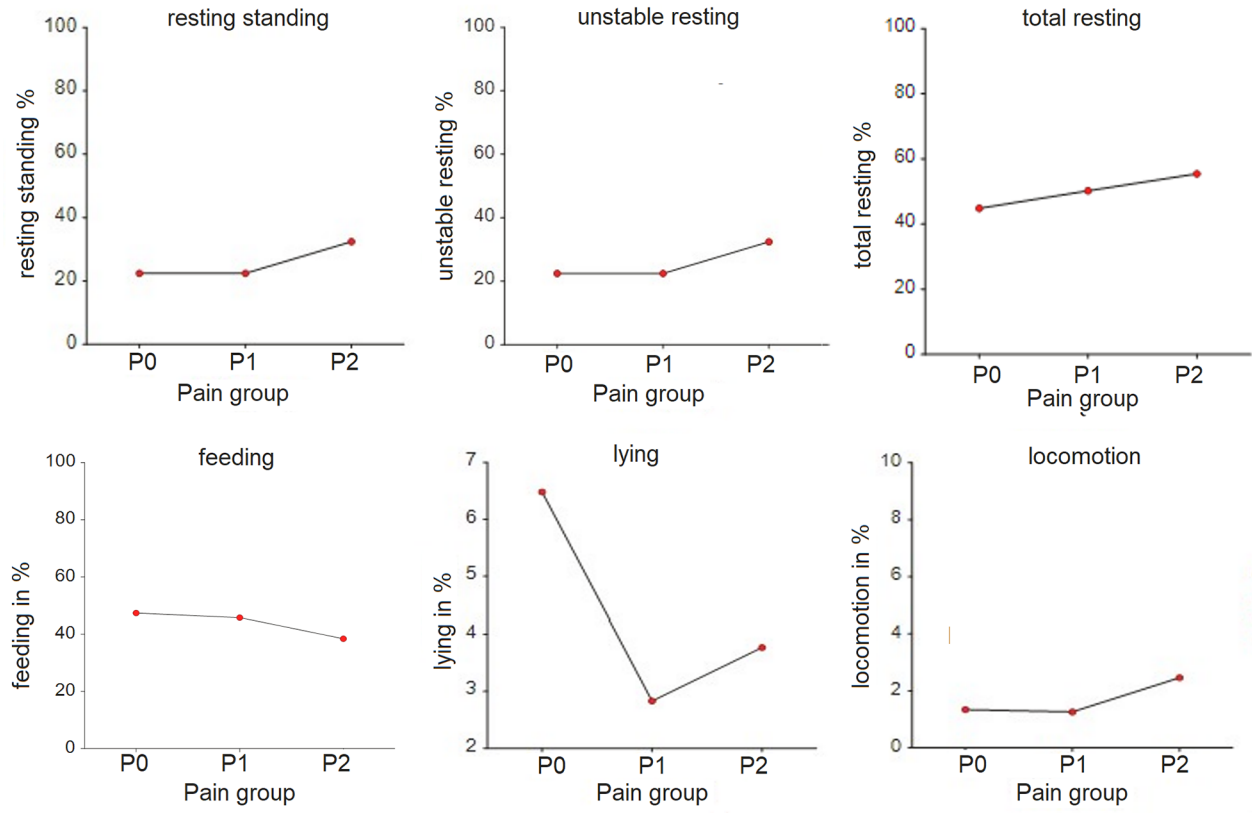

FIGURE 3
Time budgets (in %) for lying, feeding, locomotion, unstable resting, resting standing and total resting by pain group allocation of the horses. P0 – pain free group, *n* = 8; P1 – group with mild to moderate pain, *n* = 10; P2 – group with severe pain, *n* = 2. The grouping is done based on the Pain Score Vetmeduni Vienna.

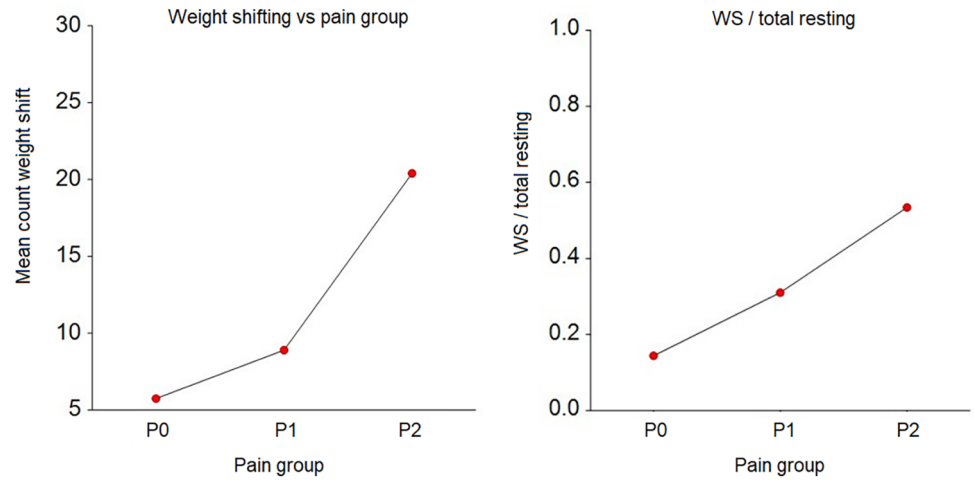

FIGURE 4
Frequency of weight shifting and weight shifting (WS)/total rest by pain group. P0 – pain free group, *n* = 8; P1 – group with mild to moderate pain, *n* = 10; P2 – group with severe pain, *n* = 2. The grouping is done based on the Pain Score Vetmeduni Vienna.

The significant difference in weight shifting frequency between horses before (median: 4/h, range: 1–56) and after (median: 3/h, range: 1–106) pain medication and the immediate decrease in weight shifting after administration of analgesia support the utility of weight shifting as an indicator for discomfort and pain assessment. Since horses primarily shift weight during rest, we

calculated the weight-bearing ratio not only per hour but also relative to total rest time. This analysis revealed similarly significant differences between pain free horses (P0) and horses suffering from moderate to severe pain (P1, P2).

Based on the observation that some stationary horses exhibited movements beyond weight-shifting, including head and whole-body adjustments, we categorized stationary non-feeding time periods into two distinct behaviors: resting standing and unstable resting. Restlessness or fidgeting in horses has been suggested in previous research as a possible sign of discomfort (3). In this study, horses in pain tended to exhibit longer total resting periods disrupted frequently by short periods of unstable resting. However, a limitation of this study is the lack of differentiation between unstable resting and standing alert during environmental observation. These behaviors can resemble each other visually, potentially leading to misclassification. Therefore, while unstable resting shows promise as an indicator, further data are required for complete validation.

Animals experiencing pain or stress might exhibit behaviors such as avoiding stimuli, withdrawing, or becoming inactive (22). Behavioral variability, which refers to how frequently an animal transitions between different behaviors, has been recognized as an adaptive strategy indicative of exploratory behavior and overall good health. Various studies have explored behavior switching in the context of equine stereotypies, knowledge acquisition, anxiety, and food-related behaviors (23). The frequency of behavior switches provides insight into the responsiveness of an animal's disposition to internal and external stimuli. A reduction in behavior switching was linked to higher levels of pain due to joint inflammation (6). Our study showed similar results, with a significant reduction in activity in horses suffering from severe pain (P2). While the frequency of unstable resting per hour was higher in in P2 horses compared to pain-free (P0) horses, the number of resting standing episodes per hour was lower. Horses experiencing severe pain also exhibited a decrease in both feeding time and feeding attempts. However, the duration of individual feeding phases was significantly longer compared to pain-free horses. Nevertheless, further studies with larger sample sizes are needed to assess the utility of the time budget for feeding as an indicator of the severity of pain or discomfort.

The study has several limitations. The time-intensive nature of analyzing 24-h behavior restricted us to manual labeling of specific behaviors by a single observer during video observation. This approach introduces subjectivity and potential bias based on human perception.

Another limitation is the uneven distribution of data due to random horse selection regardless of existing problems. For ground truth data collection for the development of an AI model, horses were video recorded irrespective of their clinical condition. Analysis occurred retrospectively, after the horses were already discharged from the hospital, by observers blinded to the horses' medical history and treatments. Clinical decisions and treatments, including the administration of pain medication as deemed necessary, were provided according to each horse's specific medical condition, determined solely by the clinician's discretion, and were not influenced by the study. Other limitations of the study include the low number of horses experiencing severe pain, and the high individual variability among horses.

5 Conclusion

In conclusion, our study suggests that weight shifting and unstable resting, alongside the time budgets for feeding and total resting, seem to be promising indicators for distinguishing pain in horses. Weight shifting was significantly different between pain groups and could differentiate between mild-moderate pain (P1, P2) and pain-free (P0) conditions. It also showed significant differences in horses before and after receiving pain medication, indicating its potential utility in evaluating analgesic efficacy. Additionally, the duration and frequency of specific behavior sequences, like feeding, resting standing and unstable resting emerged as novel markers of equine pain that warrant further investigation. These parameters exhibited significant differences between pain groups (P2 group to P0 for all abovementioned, except for mean duration for feeding, where P1 was significantly different to P0, P2), indicating potential opportunities for AI-based analysis and automated pain assessment in equine medicine. However, while these indicators could differentiate between pain free and painful horses, they could not distinguish between different levels of pain experienced by the horses. Therefore, further studies with a larger study population, focusing on specific types of pain and pain intensities, with or without treatment, are needed to refine these findings. Leveraging (AI)-based analysis of video or sensor data based on the quantifiable indicators identified in this study may ultimately enhance pain assessment and management in horses, leading to improved welfare and clinical decision-making in equine medicine.

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 manuscript presents research on animals that do not require ethical approval for their study.

Author contributions

MN: Conceptualization, Data curation, Investigation, Methodology, Writing – original draft, Writing – review & editing. AM-C: Conceptualization, Data curation, Investigation, Writing – review & editing. FJ: Conceptualization, Methodology, Supervision, Validation, Writing – original draft, Writing – review & editing. UA: Conceptualization, Data curation, Formal

Analysis, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Validation, 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

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpain.2024.1410302/full#supplementary-material

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frontiers | Frontiers in Veterinary Science

TYPE Original Research
PUBLISHED 23 August 2024
DOI 10.3389/fvets.2024.1437989

OPEN ACCESS

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RECEIVED 24 May 2024
ACCEPTED 26 July 2024
PUBLISHED 23 August 2024

CITATION
Amari M, Rabbogliatti V, Ravasio G, Auletta L, Brioschi FA, Riccaboni P, Dell'Aere S and Roccabianca P (2024) Development of an ultrasound-guided radiofrequency ablation technique in the equine cadaveric distal limb: histological findings and potential for treating chronic lameness.
Front. Vet. Sci. 11:1437989.
doi: 10.3389/fvets.2024.1437989

Development of an ultrasound-guided radiofrequency ablation technique in the equine cadaveric distal limb: histological findings and potential for treating chronic lameness

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Introduction: Radiofrequency (RF) relieves chronic pain in humans, but it is unexplored in horses affected by chronic lameness. This study aims to describe the technique and the histological effects of ultrasound (US)-guided radiofrequency ablation (RFA) of palmar digital nerves (PDNs) in horse's fetlock and pastern, *ex vivo*.

Methods: After assessing the US anatomy of lateral and medial PDNs in fetlock and pastern *in vivo* ($n = 10$ horses; 20 forelimbs), US-guided RFA was performed on these sites in cadaveric forelimbs ($n = 10$) applying four different settings with increasing invasiveness ($n = 40$ total treatments): 60°C, 6 min (GROUP LOW); 70°C, 4 min (GROUP MEDIUM); 90°C, 2 min (GROUP HIGH); 80°C, 8 min (GROUP VERY HIGH). Needle-tip-to-nerve proximity was assessed with US and methylene blue, injected through the port of the RF needle. Nerves were collected for microscopical assessment.

Results: Transverse palmaro-lateral and palmaro-medial US images of fetlock and pastern detected PDNs consistently, close to the palmar digital artery. With in-plane US technique, RFA was performed at target in 31/40 cases, with significantly higher number of failures in fetlock ($p = 0.008$). PDNs histology identified thermal injury/coagulation with axonal degeneration and collagen homogenation. Nuclear smearing of arterial leyomyocytes was also observed. Nerve coagulation was significantly associated with treatment ($p = 0.03$) and needle-tip-to-nerve proximity (US distance: $p = 0.009$; blue distance: $p = 0.04$).

Discussion: The PDNs were easily visualized and reached with the RF needle by US in-plane-guided technique. RFA produced axonal thermal damage and intensity-related coagulation effectiveness. To ensure effective nerve coagulation, it is crucial that the needle is accurately positioned in close proximity to the target nerve. Based on the histopathological findings, HIGH and VERY HIGH RFA treatments might be worth of being tested *in vivo* in clinical studies aimed at treating chronic lameness of the distal forelimb in horses.

KEYWORDS

axonal thermal damage, chronic pain, horses, palmar digital nerves, thermal radiofrequency, histopathological nerve lesion

1 Introduction

Chronic lameness is a major cause of reduced life quality in horses (1). Limited analgesic options and prolonged confinement during convalescence may lead to consider euthanasia (2, 3). The use of systemic non-steroidal anti-inflammatory drugs provides some degree of relief but long-term administration is often required (4). Moreover, several adverse effects including gastrointestinal tract ulceration, right dorsal colitis, and/or acute nephrotoxicity are reported when non-steroidal anti-inflammatory drugs are administered for prolonged time (5). In addition, horses suffering from neuropathic pain may not respond favorably to non-steroidal anti-inflammatory drugs (6) due to maladaptive sensitization of pain pathways, resulting in ongoing pain, hyperalgesia and allodynia (1). Palmar and plantar digital surgical neurectomy may also be an option when other alternatives are not available, but it may lead to complications such as painful neuroma formation (7, 8) and an increased risks of injury to the lower limb (9).

Radiofrequency (RF) is a non-pharmacological interventional technique that has been applied to treat neuropathic chronic pain unresponsive to other pharmacological and non-pharmacological techniques in humans (10–12). The duration of pain relief after RF varies from 3 to 24 months, depending on the treated site, the technique used, and the individualized response (13–16). This therapy and its efficacy seem not to have been evaluated in horses. The RF entails placing an insulated needle with a conductive tip close to the target nerve. A high-frequency electric current generator, to which the needle is connected, produces a small electric field at the tip, generating thermal energy that creates a small lesion near the affected target site. Radiofrequency ablation (RFA) employs temperature ranges of 60–90°C to induce thermal neurodestructive lesions (17) that result in Wallerian degeneration developing over the next 2–3 weeks. These changes cause subsequent alteration of the transmission and conduction of nociceptive impulses and interruption of pain signals (18, 19). Since the application of RFA induces, even if temporary (17), denervation, it is generally limited to nerves composed by sensory fibers only, as a motor deficit could ensue (19). The equine distal forelimb is innervated by the medial and lateral palmar nerves, which become the medial and lateral palmar digital nerves (PDNs) proximal to the metacarpophalangeal joint (20). At this level, nerves do not contain myelinated motor fibers and are mainly composed of myelinated sensory fibers, unmyelinated sympathetic and unmyelinated peptidergic sensory axons (21). Therefore, RFA may represent an attractive therapeutic option in horses with chronic lameness unresponsive to other treatments. However, the occurrence of complications similar to those described following surgical neurectomy cannot be excluded. The aims of the present study were to: (1) Identify the ultrasonographic anatomical landmarks of the lateral and medial PDNs in the fetlock and pastern regions in living horses; (2) Evaluate the feasibility of close RF needle-to-nerve positioning in these two regions using an ultrasound (US)-guided technique, *ex vivo*; (3) Describe the histopathological nerve lesions produced by RFA and evaluate the frequency of nerve coagulation at different settings with increasing invasiveness. The hypotheses were that the nerves could be easily visualized and reached with the needle by US-guided technique, and that the coagulative effectiveness would increase as the intensity of the RFA treatment performed increased.

2 Materials and methods

The present study complied with ethical standards, and it was conducted under the approval of the Institutional Ethical Committee for Animal Care at the University of Milan (OPBA_51_2023). Informed written consent was obtained by the owners of all live horses enrolled in the study. No Ethical Committee for Animal Care oversight was required in the *ex vivo* phases, conducted using material collected during *post-mortem* examination after collection of owners' written consent. The study was divided into three phases: phase I, concerning the assessment and description of the US anatomy of the lateral and medial PDNs in the region of the pastern and fetlock of live horses, with a specific focus on the US landmarks for effective US-guided RF needle positioning; phase II, consisting in the application of the US-guided RFA treatment and the injection of a small volume of methylene blue solution into isolated equine distal forelimbs, and the subsequent anatomical dissection to assess needle tip-to-nerve proximity; phase III, evaluating histological nerve lesions produced by four RFA treatment protocols on the forelimbs from phase II.

2.1 Phase I. Ultrasonographic landmarks of PDNs in fetlock and pastern regions: *in vivo* study

Ten horses referred to the Veterinary Teaching Hospital of the University of Milan undergoing diagnostic or surgical standing procedures not related to the current study were enrolled in phase I. Horses weighing less than 200 kg, less than 2 years of age or with current or previous episodes of forelimb lameness were excluded from the study. All other horses were considered eligible. Horses were restrained with the halter in a horse stock, in a quiet and clean area, in quadrupedal standing on a flat surface. The skin of the pastern and fetlock of both forelimbs was shaved, washed, and rinsed with alcohol. An acoustic standoff (Standoff pad; Esaote; Genova; Italy) was placed between the skin and probe to enhance visualization of superficial structures. To improve further acoustic coupling, a large amount of ultrasound gel (Ultrasound Gel; GIMA S.p.A.; Gessate; Italy) was applied to the probe. Ultrasonographic images were obtained using a portable US system (Sonosite M-Turbo) mounting a high-frequency 6–13 MHz linear array transducer (HLF38x; Sonosite Inc., WA, United States). The scanning depth was set at 2.2–2.7 cm and frequency

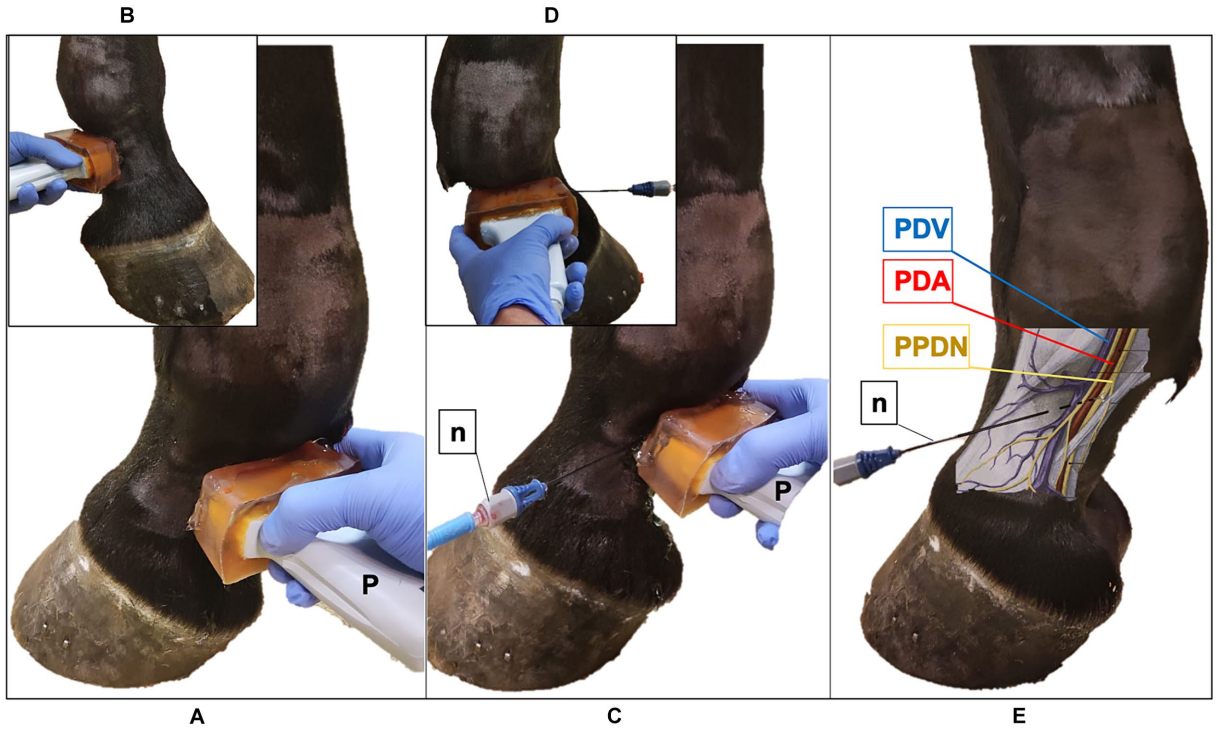

FIGURE 1
Ultrasound (US) probe and the radiofrequency (RF) needle positioning in the pastern region of the equine forelimb. **(A)** The US probe (P) is positioned transversal to the middle third of the proximal phalanx, with the marker pointing dorsally; palmaro-medial approach. **(B)** Palmaro-lateral approach. **(C)** US-guided in-plane technique, palmaro-medial approach: the RF needle (n) is inserted with a dorso-palmar direction to reach the proper palmar digital nerve (PPDN). **(D)** Palmaro-lateral approach. **(E)** Anatomical representation of the neurovascular bundle: RF needle reaches the PPDN in the subcutaneous tissue. PDV, palmar digital vein; PDA, palmar digital artery.

was adjusted to obtain the clearer images as possible with less artifacts for each subject. An anesthetist experienced in US-guided loco-regional anesthesia collected the US images from both forelimbs. To identify the lateral and the medial PDNs in the pastern region, the US probe was positioned transversal to the middle third of the proximal phalanx, with the marker pointing dorsally and using a palmaro-lateral and a palmaro-medial approach, respectively (Figures 1A,B). Subsequently, the fetlock region was scanned with the same purpose; the US probe was applied transversal to the metacarpophalangeal joint with the marker pointing dorsally, and the US images were acquired using a palmaro-medial and palmaro-lateral approach, at the level of the proximal portion of the medial and lateral proximal sesamoid bone, respectively (Figures 2A,B). In the four examined regions, the anatomical structures and their echogenicity were identified, and a color flow Doppler was applied for a better identification of blood vessels and their relationship with adjacent anatomical structures. The US landmarks, target points and the best access point for an in-plane needle visualization were determined; the four sites studied in this phase were selected for RFA treatment in the next phases.

2.2 Phase II. US-guided RFA treatment and assessment of RF needle tip-to-nerve proximity: *ex vivo* study

Phase II was performed on 10 fresh cadaver forelimbs: five right and five left forelimbs. Forelimbs were obtained from five horses euthanized for reasons not related to the present study. Limbs from horses weighing less 200 kg, less than 2 years old, or with a recent medical history of forelimb disorders were excluded. All other horses undergoing euthanasia were deemed suitable as donor. All forelimbs were separated at the carpus immediately after death and treatments were performed within 1 h from collection in a temperature-controlled examination room (21 ± 2°C). Each forelimb was divided into two regions, the pastern and the fetlock, further divided into lateral and medial sites. The skin of the pastern and fetlock was shaved and cleaned, and a dispersive return path electrode (GD-pad Corded; Diros Technology Inc., Ontario, Canada) was applied on the dorsal aspect of the proximal metacarpus and was connected to a RF generator (OWL URF-3AP RF Generator; Diros Technology Inc., Ontario, Canada). With the US technique described in phase I, the four sites selected for RFA treatment were examined and US landmarks were identified. An 18-gauge, three-tined, 5-mm active tip, 100-mm RF (TRIDENT) needle (RF Trident™ Cannulae; Diros Technology Inc., Ontario, Canada; Figure 3) was inserted using an in-plane US technique (Figures 1C,D, 2C,D). For the lateral pastern and fetlock, the RF needle was introduced in a dorso-lateral to palmaro-medial direction, while for the medial pastern and fetlock, in a dorso-medial to palmaro-lateral direction. The RF needle was inserted with an approaching angle of 0 to 30° to the sagittal plane until the needle tip reached the target PDN, thus maintaining a 90° needle inclination relative to the nerve axis (Figures 1E, 2E). The corresponding images were recorded and stored. Hence, RF needle tines were deployed and the RFA treatment performed. Four RFA

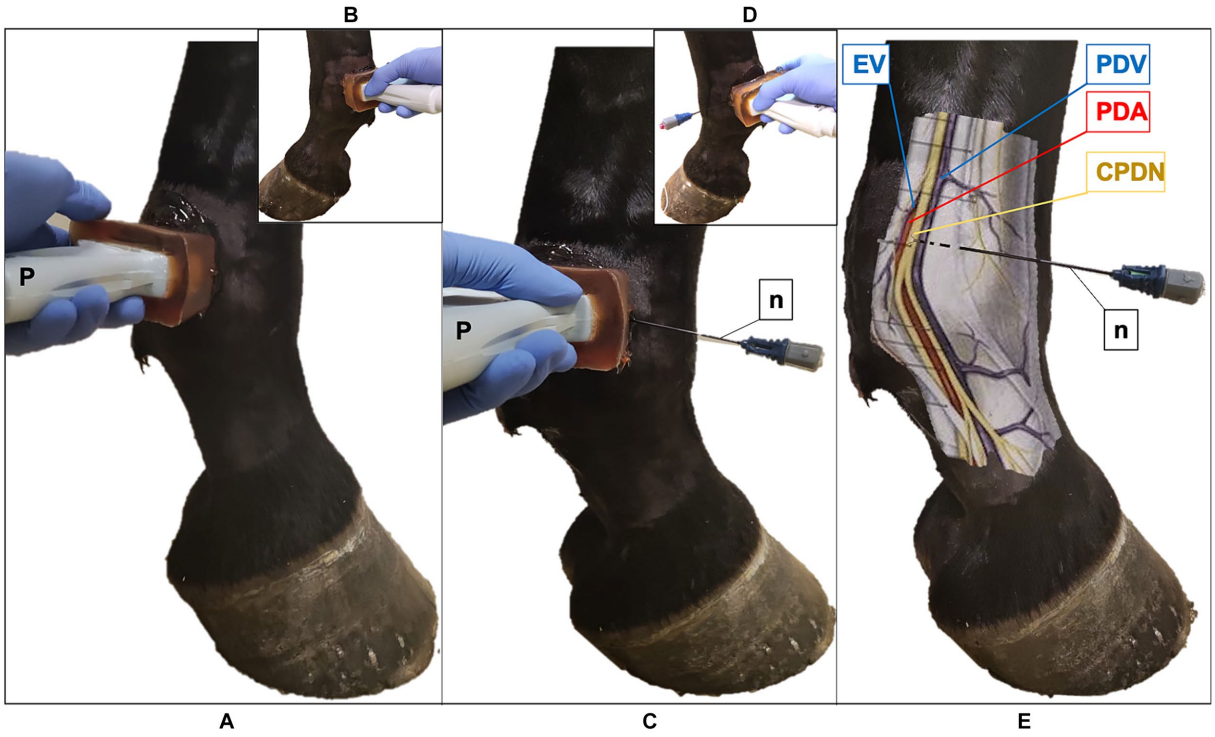

FIGURE 2
Ultrasound (US) probe and the radiofrequency (RF) needle positioning in the fetlock region of the equine forelimb **(A)** The US probe (P) is positioned transversal to the metacarpophalangeal joint at the level of the proximal portion of the proximal sesamoid bone, with the marker pointing dorsally; palmaro-medial approach **(B)** Palmaro-lateral approach. **(C)** US-guided in-plane technique, palmaro-medial approach: the RF needle (n) is inserted with a dorso-palmar direction to reach the common palmar digital nerve (CPDN). **(D)** Palmaro-lateral approach. **(E)** Anatomical representation of the medial neurovascular bundle: RF needle reaches the CPDN in the subcutaneous tissue. Note the presence of the ergot vein (EV) in the palmaro-medial approach, that is absent in the palmaro-lateral approach. PDV, palmar digital vein; PDA, palmar digital artery.

treatments were tested: 60°C for 6 min (Group LOW), 70°C for 4 min (Group MEDIUM), 90°C for 2 min (Group HIGH) and 80°C for 8 min (Group VERY HIGH). The RFA treatment to be applied to each site was randomly selected,[1] in order to obtain five trials for each treatment group in the pastern region, and five trials in the fetlock region. Both the region and the side from which to start, as well as the sequence of sites to be treated were randomly selected. Then, 0.1 mL of methylene blue was injected through the injection port of the RF cannula under real-time US guidance (22). After completing the described procedures, an experienced anatomist performed the cadaveric dissection to identify anatomical structures and tissues stained with methylene blue and to evaluate its proximity to PDNs (tip-to-nerve blue distance). For this purpose, the distance from the center of the colored tissues, which was assumed to have been the position of the active tip, and the target nerve, as well as the length of the stained nerve and its thickness were measured with a ruler (22). Any staining of blood vessels and non-target structures was also recorded. Moreover, the distance between the tip of the needle and the nerve was retrospectively measured on the stored US images by an anesthetist experienced in US-guided loco-regional anesthesia with the dedicated US software (tip-to-nerve US distance). All the described measurements were performed by the two independent authors, blinded to each other results, who performed each measurement once. The RF treatment was considered to have been carried out "at target" if at least 2 of the 3 following criteria were achieved: tip-to-nerve US distance less than 2 mm; tip-to-nerve blue distance less than 2 mm; length of stained nerve greater than 5 mm.

2.3 Phase III. Histological evaluation of nerve lesions induced by four RFA treatment protocols

For microscopical evaluation, all blue and grossly coagulated tissues, and a minimum of 2 cm^3 of tissue including the treated PDNs and the corresponding artery and vein, were removed, laid onto a rigid surface and fixed with pins to avoid nerve contraction artifacts, and fixed in a 1/10 volume of 10% neutral buffered formalin for 48 h. Tissues were trimmed longitudinally and transversally and were routinely processed for 12 h, embedded in paraffin, cut in 5 µm sections and routinely stained with hematoxylin and eosin to assess thermal damage to the nerve and surrounding tissues. Negative controls consisted in nerves and adjacent tissues from the same forelimbs, collected 5 cm proximally from the RFA site. Negative controls were fixed and processed together with the coagulated nerves and were utilized for comparison. Histopathological alterations were

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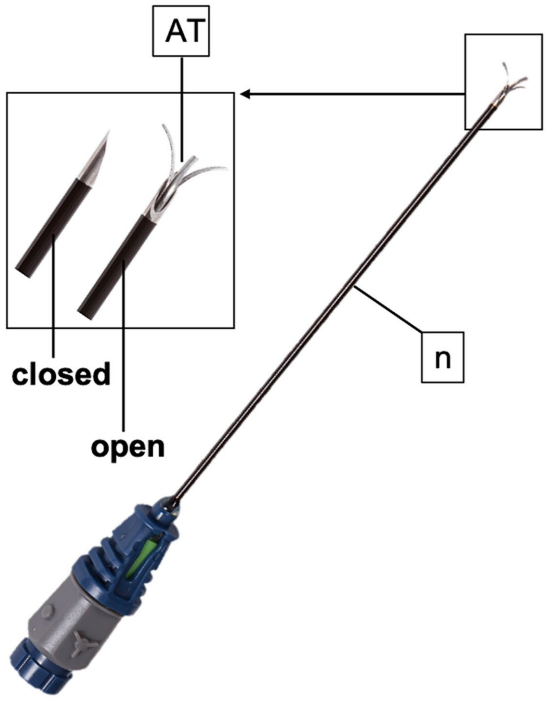

FIGURE 3
TRIDENT radiofrequency needle (n) used in the present study. The active tip (AT) possesses three tines that deploy at 10, 2 and 6 o'clock.

evaluated twice by a ECVP diplomate veterinary pathologist who was blinded to the treatment group. The efficacy of RFA was evaluated microscopically by qualitative assessment of presence or absence of tissue coagulation and degeneration. Presence of axonal swelling and degeneration, collagen homogenation, nuclear smearing, or basophilia/amphophilia were considered as evidence of thermal injury, i.e., nerve coagulation when determined in live animals, on the basis of previously described morphologic features (12, 23). If partial coagulation of the nerve was observed, i.e., coagulation affecting part of the nerve but not its entirety, the case was considered as successful coagulation for statistical purposes.

2.4 Statistical analysis

Dedicated software for statistical analysis was used to perform all the evaluation described hereafter (JMP Pro, v. 17.0, SAS Institute, Cary, NC, United States; MedCalc version 19.2.6, MedCalc Software Ltd., Acacialaan 228,400 Ostend, Belgium). Data were tested for normality with the Shapiro–Wilk's *W* test. Data were reported as mean ± standard deviation, median (range) or as number of samples (% of the total), where appropriate. To assess the statistical power of the study, we performed a *post hoc* power analysis for the chi-squared test using the contingency table which describe the distribution of nerve coagulation outcomes within each treatment group. With our observed effect size (1.70), an alpha error of 0.05% and 3 degrees of freedom, the analysis yielded a power estimate of 99%. The nerve thickness was compared between the four RFA treatment groups with the Kruskal Wallis test. The number of treatments "at target" was compared between the four RFA treatment groups with a contingency table and chi-square test. The association between nerve coagulation and axonal degeneration, nuclear smearing, and the coagulation of other structures (i.e., artery and collagen) was explored with a contingency table, and the chi-square or Fisher's *F* test. A nominal logistic univariate analysis was performed to evaluate which factor influenced the presence of nerve coagulation at the histological evaluation, as the dependent variable. Moreover, the presence of other stained structures and the presence of other coagulated structures were separately evaluated as dependent variables. The following variables were evaluated as independent: treatment group (group LOW; group MEDIUM; group HIGH; group VERY HIGH), treated region (pastern; fetlock), nerve thickness (mm), tip-to-nerve US distance (mm), tip-to-nerve blue distance (mm), length of stained nerve (mm), target (yes; no), presence of other stained structures (artery; vein). *Post hoc* analysis was applied between significantly associated variables with the chi-square test for categorical variables and with the Mann–Whitney's *U* test. Moreover, the association between the RFA treatment "at target" and the treatment region was evaluated with contingency table, and the chi-square or Fisher's *F* test, as well. Nerve thickness was compared between the treatment regions with the Mann–Whitney's *U* test. A Spearman's rank correlation (r_s) test was used to evaluate the strength of association between the tip-to-nerve US and blue distance measurements, and between these distances and the length of stained nerve. The overall ability of the US guided technique was evaluated by calculating the accuracy, sensitivity, specificity, positive and negative predicting values by comparing being "at target" or not with the presence/absence of nerve coagulation. The 95% confidence intervals (CI) were calculated as well. The analysis was repeated within each treatment group.

3 Results

In the phase I, a total of 20 forelimbs from 10 horses were examined, obtaining the evaluation of 20 lateral and 20 medial pasterns, and 20 lateral and 20 medial fetlock regions. The position of the different anatomical structures and their relationship with the neurovascular bundles were considered consistent in all the limbs evaluated. Transverse palmaro-lateral and palmaro-medial US images of pastern (Figure 4) and fetlock (Figure 5) regions allowed easy identification of the neurovascular bundle close to the skin surface in all horses. In the pastern region, the neurovascular bundles were superficial to the distal branch of the superficial digital flexor tendon and the deep digital flexor tendon. More deeply, the proximal phalanx, the oblique and the straight sesamoidean ligament were visualized (24). In the fetlock region, the neurovascular bundles were superficial compared to the third metacarpal bone, the lateral or medial branch of the suspensory ligament and the corresponding proximal sesamoid bone (25). In both regions, the palmar digital veins (PDVs) showed an anechoic vascular bed with a thin wall easily compressible, whereas the palmar digital arteries (PDAs) were smaller, anechoic, round, pulsatile structures with a thicker wall. In addition, in the transverse palmaro-medial scan of the fetlock, the ergot vein, after branching from the medial PDV, was always visible, palmar to the medial PDN. The PDN was always observed palmar to the PDV both on the medial and lateral sides, with a coarse grainy appearance and a clear distinction from the subcutaneous tissue. In the pastern region, the

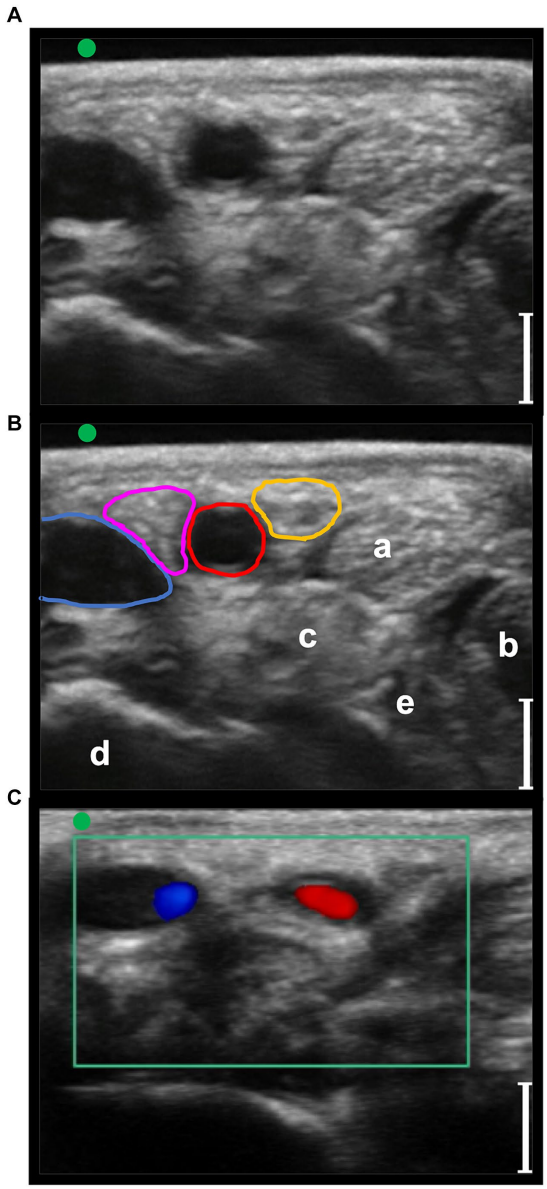

FIGURE 4
Transverse palmaro-lateral ultrasonographic images of the equine pastern at the level of the middle third of the proximal phalanx with the marker (green dot) pointing dorsally, *in-vivo*. **(A,B)** *in-vivo* scan. **(C)** *in-vivo* scan with color flow doppler. Blue line, lateral palmar digital vein; Red line, lateral palmar digital artery; yellow line, lateral proper palmar digital nerve; purple line, lateral dorsal palmar digital nerve branch of the middle phalanx; a, distal branch of the superficial digital flexor tendon; b, deep digital flexor tendon; c, oblique sesamoidean ligament; d, proximal phalanx; e, straight sesamoidean ligament; bars equal to 5 mm.

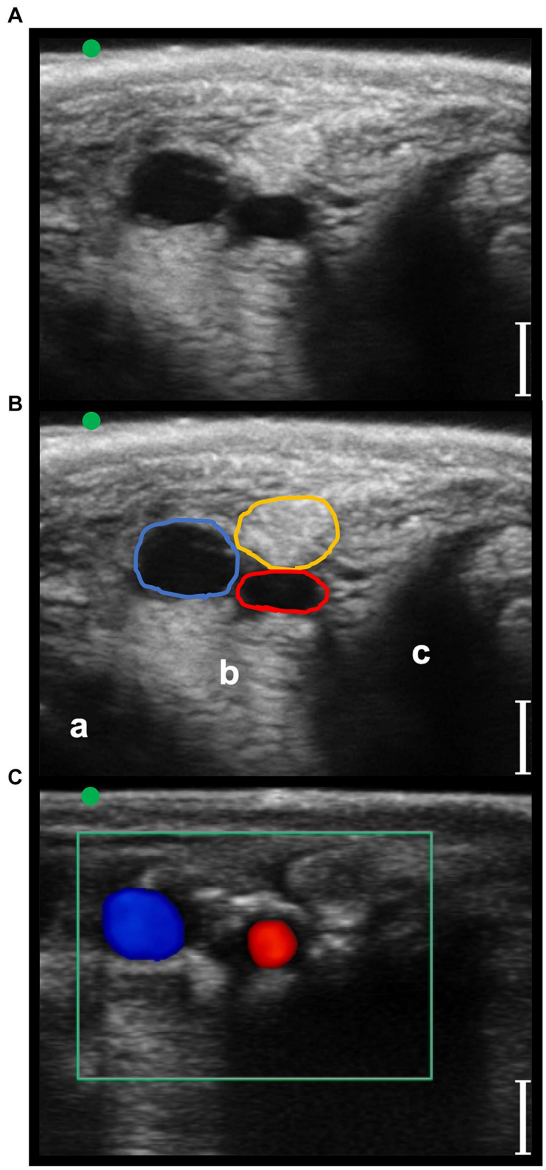

FIGURE 5
Transverse palmaro-lateral ultrasonographic images of the equine fetlock at the level of the proximal portion of the lateral proximal sesamoid bone, with the marker (green dot) pointing dorsally, *in-vivo*. **(A,B)** *in-vivo* scan. **(C)** *in-vivo* scan with color flow doppler. Blue line, lateral palmar digital vein; Red line, lateral palmar digital artery; yellow line, lateral common palmar digital nerve; a, third metacarpal bone; b, lateral branch of the suspensory ligament; c, lateral proximal sesamoid bone; bars equal to 5 mm.

proper PDNs and their intermediate *rami*, i.e., the dorsal PDN branch of the middle phalanx, were always identified palmar and dorsal to the PDA, respectively (26). In the fetlock region, the lateral and medial common PDNs were superficial to the lateral and medial PDAs, before branching into the lateral and medial proper PDNs and their corresponding dorsal *rami*, i.e., dorsal PDN branch of the proximal phalanx, which were observed running palmar and dorsal to the PDA, respectively (26).

In phase II and III, a total of 10 forelimbs, resulting in 10 lateral and 10 medial fetlocks and 10 lateral and 10 medial pasterns, were included. All the anatomic structures of interest studied during the *in vivo* phase I were detectable in the phase II *ex vivo* experiment. The RF needle was clearly ultrasonographically identifiable, as well (Figures 6, 7).

No significant differences were observed between the four RFA treatment groups regarding nerve thickness and number of treatments performed "at target." Results are summarized in Table 1. The number of treatments "at target" were 31 (77.5%); of which four did not display all the three criteria to be categorized as "at target." In particular, three

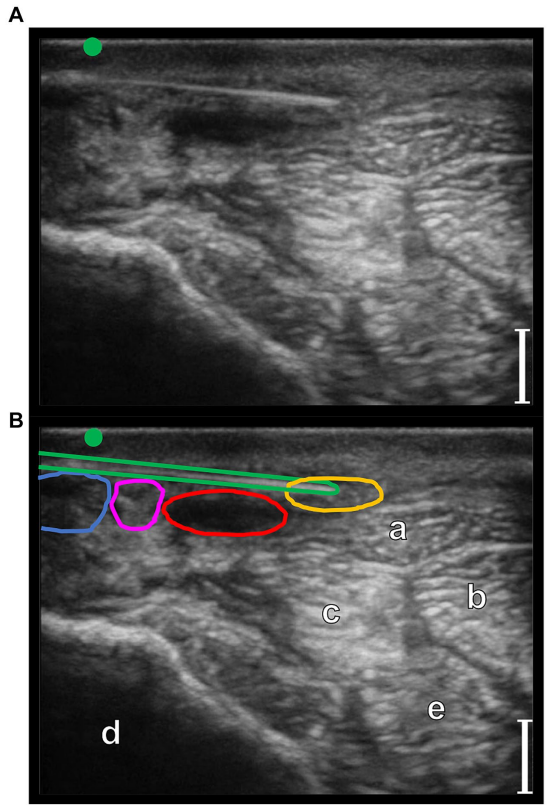

FIGURE 6
Transverse palmaro-lateral ultrasonographic image of the equine pastern at the level of the middle third of the proximal phalanx with the marker (green dot) pointing dorsally, *ex-vivo*. **(A,B)** *ex-vivo* scan. Radiofrequency needle (green line) positioning on the target before opening the tines. Blue line, lateral palmar digital vein; Red line, lateral palmar digital artery; yellow line, lateral proper palmar digital nerve; purple line, lateral dorsal palmar digital nerve branch of the middle phalanx; a, distal branch of the superficial digital flexor tendon; b, deep digital flexor tendon; c, oblique sesamoidean ligament; d, proximal phalanx; e, straight sesamoidean ligament; bars equal to 5 mm.

had a higher tip-to-nerve blue distance, and one displayed a shorter length of stained nerve.

In the four treatment groups, histological examination of the PDNs was consistent with thermal injury. Lesion comprised oedema, increased intracytoplasmic clear empty axonal vacuoles (hydropic degeneration), degeneration of nerve sheaths and hypereosinophilia of axons. Also, collagen homogenation that appeared intensely eosinophilic with loss of distinct borders, loss of fibrillar pattern interpreted as coagulation (27), and areas of increased basophilia/amphophilia ascribe to electrical impulse tissue damage (Figures 8, 9). In the arterial walls of the coagulated areas variably severe nuclear changes including pyknotic, fusiform nuclei (nuclear smearing) were consistently present (Figure 9F). Lesions were readily visible in longitudinal sections comprising the entire length of the nerves examined, while transverse sections often did not include the specific area of nerve/tissue damage.

Presence of nerve coagulation was significantly associated with axonal degeneration, nuclear smearing and presence of other coagulated structures. The specific results are reported in Table 2. No association was observed between coagulated non-target structures and treatment groups ($p = 0.32$), even when considering if the RF was performed "at target" ($p = 0.16$) or not ($p = 0.53$).

Treatment group significantly influenced the presence of nerve coagulation, with the VERY HIGH group showing the highest and the LOW group the lowest frequency of coagulated nerves, even when only treatments performed "at target" were considered. In contrast, when RF did not result "at target," no nerve coagulation was observed in any group. The specific results are reported in Table 3.

The treated site did not show any influence on the presence of nerve coagulation ($p = 0.53$); nonetheless, the treated site was significantly associated with the number of treatments "at target," with a higher number of failures in the fetlock region (Table 4).

The nerve thickness did not show any influence on the presence of nerve coagulation ($p = 0.25$); nevertheless, the nerve thickness resulted significantly higher ($p < 0.0001$) in the fetlock region (6; 4–9 mm) compared to the pastern region (4; 3–6 mm).

The tip-to-nerve US and blue distances and the length of stained nerve resulted significantly associated with the presence of nerve coagulation, and all resulted significantly correlated to each other. The tip-to-nerve US and blue distances resulted associated with staining and coagulation of non-target structures. No association was observed between stained and coagulated non-target structures ($p = 0.36$). The specific results are summarized in the Supplementary Tables 1, 2.

Overall, the US-guided technique showed an accuracy of 67.5%, with a sensitivity of 100% and specificity of 41%. The specific results in the whole sample and within each treatment group are reported in Table 5.

4 Discussion

In human medicine, RF has been employed for many years, and numerous studies have reported its use to treat hand and foot neuropathic pain (28–30). To the authors' knowledge, only one preclinical experimental study investigating histological and electrophysiological effects of RF technique on the canine sciatic and saphenous nerves is currently available (18), whereas no reports are available for horses. Distal forelimbs are one of the most common sites of lameness in horses (31–33). Since chronic lameness is often difficult to treat, manage, and resolve (4), and represents a major cause of reduced quality of life in horses (1), additional options to control lameness induced by chronic pain are desirable. Hence, this study was designed to identify US anatomical landmarks in horses with the perspective of applying this technique in clinical cases.

Ultrasonography was used to identify the optimal sites of treatment for its confirmed ability to easily identify anatomical landmarks, since it provides optimal visualization of soft tissues compared to other techniques, such as fluoroscopy (23). For this reason, the US technique ensures accurate positioning of the needle close to the target and increases the procedure's safety (34). The equine pastern and fetlock regions have been studied with US (24, 25), but without focusing on the identification of a specific US window for the recognition of the PDNs. Indeed, in clinical practice, diagnostic analgesia is performed using a blind technique, and the nerve is identified only by palpation (35, 36). In the present study, transverse palmaro-lateral and palmaro-medial US images allowed for straightforward detection of the neurovascular bundle close to the skin surface in all horses. In addition, the use

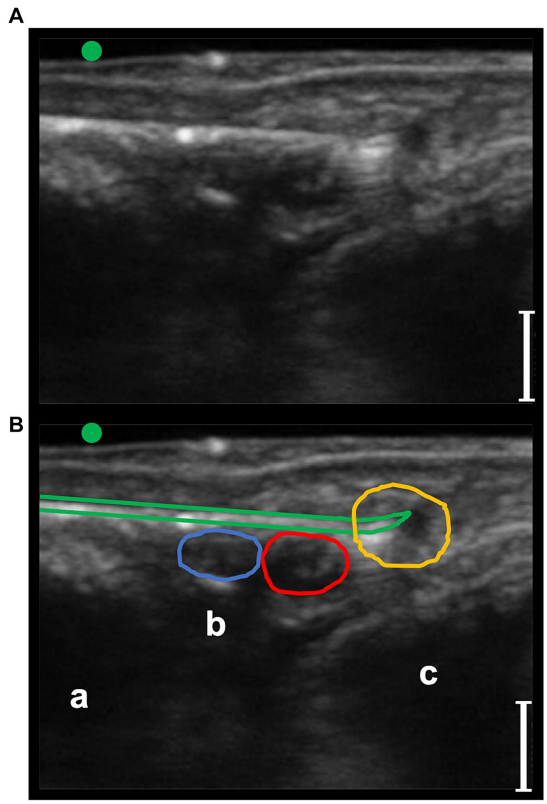

FIGURE 7
Transverse palmaro-lateral ultrasonographic image of the equine fetlock at the level of the proximal portion of the lateral proximal sesamoid bone, with the marker (green dot) pointing dorsally, *ex-vivo*. **(A,B)** *ex-vivo* scan. Radiofrequency needle (green line) positioning on the target after opening the tines in the cadaver forelimb. Blue line, lateral palmar digital vein; Red line, lateral palmar digital artery; yellow line, lateral common palmar digital nerve; a, third metacarpal bone; b, lateral branch of the suspensory ligament; c, lateral proximal sesamoid bone; bars equal to 5 mm.

TABLE 1 Mean ± standard deviation or median (range) of the nerve thickness (in mm) and proportion of radiofrequency ablation (RFA) treatments "at target" in the four groups ($n = 10$ per group).

Treatment group	Nerve thickness (mm)	RFA "at target"
Low	4.5 (3.0–8.0)	8/10
Medium	4.8 ± 1.1	8/10
High	4.9 ± 1.8	7/10
Very High	5.0 (3.0–9.0)	8/10
	Kruskal-Wallis $p = 0.88$	Chi-square $p = 0.93$

The p values represented the difference between the four treatment groups.

of color flow Doppler further facilitated the identification of blood vessels and their distinction from the nerve *in vivo*. The transverse section of the PDN was easily distinguishable from the subcutaneous tissue and it was always visualized adjacent to the PDA, confirming the first hypothesis of this study. Hence, according to the primary aim, the PDA should be considered the best US landmark for the identification of PDNs in both the pastern and fetlock regions.

The second aim of this study was to evaluate the feasibility of an US-guided RFA treatment in horses using an *ex vivo* model. Due to the nerve proximity to critical anatomical structures and the requirement for precise targeting with a RF needle, phase II of the study employed the in-plane US technique for its intuitiveness and safety (34, 37). The RF needle was carefully advanced through the subcutaneous tissue in a dorso-lateral to palmaro-medial direction for lateral fetlock and pastern and in a dorso-medial to palmaro-lateral direction for medial fetlock and pastern and with an approaching angle of 0 to 30° to the sagittal plane until it reached the nerve, thus maintaining a 90° needle inclination relative to the nerve axis. Finlanson and colleagues (2017) proved that the standard RF needle, whose lesion is ellipsoidal around the active tip, requires a parallel approach to the target and loses effectiveness as the angle of inclination to the nerve increases (38). In contrast, the caudal deployment mechanism of the three tines of the TRIDENT needle which deploy at 10, 2 and 6 o'clock (38) allows the formation of a 3-dimensional "pear-shaped" lesion, thus increasing the distal width of the lesion, but varying minimally its length (39). This pyramid-like conformation is less sensitive to the actual inclination of the needle with respect to the nerve, with similar morphologies at angles of 0 and 90 degrees, and the development of a larger lesion surface area at 90 degrees compared with other RF needles (38). For these reasons, this particular RF needle was chosen for the in-plane US approach applied in the present study. Moreover, the tines are flexible and, once the active needle tip is in contact with the target nerve, they are deployed and advanced 2 mm to easily surround the nerve, further increasing the surface area involved by the RFA. Based on manufacturer unpublished data, in a standard model, the estimated lesion size generated by RFA with the TRIDENT needle ranges from 0.5×0.5 mm to 10×10 mm, based on the temperature/min settings of the RF generator. The US technique applied in the present study enabled the needle to be correctly positioned on the target nerves with a success rate of 77.5%. The altered echogenicity of the tissues, the absence of blood flow through the vessels, and the impossibility of using color flow Doppler, made accurate identification of the anatomical landmarks and the nerve more difficult in the phase II *ex vivo* study, likely further increasing the failure rate (40). The higher incidence of failures observed in the fetlock region is possibly attributable to a larger amount of subcutaneous tissue compared with the pastern region, which could have increased nerve mobility allowing for its caudal shifting upon needle insertion. Furthermore, the proximal sesamoid bone curved surface likely exacerbated challenges in maintaining optimal contact between the probe and the skin, thus increasing the difficulty of accurately positioning the needle close to the nerve. Considering the purpose of the present study, the positioning of the needle tip had to be assessed very precisely. To this end, the volume of methylene blue injected was very small, according to a previous human cadaveric study (22). In contrast, previous studies evaluating the accuracy of US-guided peripheral nerve blocks in equine cadavers used considerably larger volumes of dye (41). However, the dispersion of dye within cadavers may not accurately reflect the spread of the injectate in live animals (42). Indeed, methylene blue evaluation is limited by the bias of color spread in the tissues, and by its potential drainage in lymphatic vessels (43), and it cannot be evaluated *in vivo*. For this reason, the needle tip-to-nerve US distance was also assessed. Nonetheless, the tip-to-nerve blue distance also proved to be reliable in identifying correct needle positioning and strongly correlated with

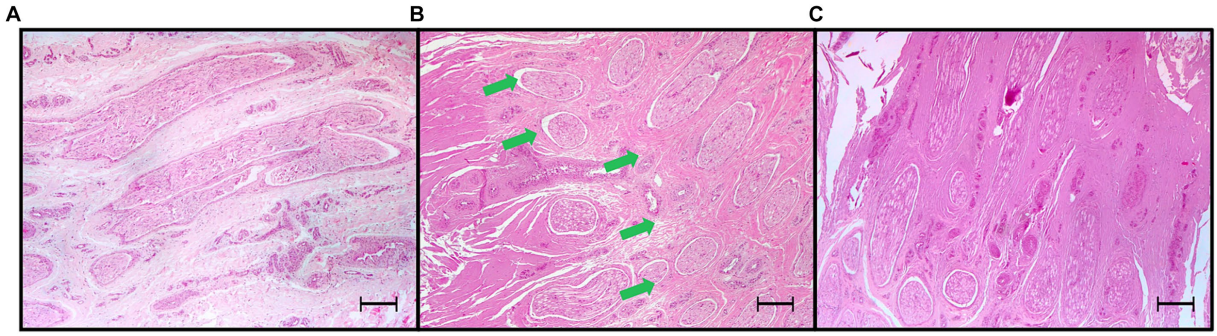

FIGURE 8
Light microscopic images (4x) of hematoxylin and eosin-stained sections of palmar digital nerves harvested from equine cadaveric forelimbs after radiofrequency ablation treatment. **(A)** Negative control: normal microscopical features. **(B)** Radiofrequency ablation treatment (Group MEDIUM: 70°C, 4 min): partial coagulation of the nerve. The radiofrequency treatment caused coagulation of tissues characterized by homogenation, loss of fibrillary patterns, increased eosinophilia and basophilia, leaving a portion of the palmar digital nerve undamaged (green arrows). **(C)** Total coagulation of the nerve tissue (Group VERY HIGH: 80°C, 8 min). Note the increased basophilia/amphohilia of the collagen in correspondence of the coagulated areas. Bars equal to 250 μm.

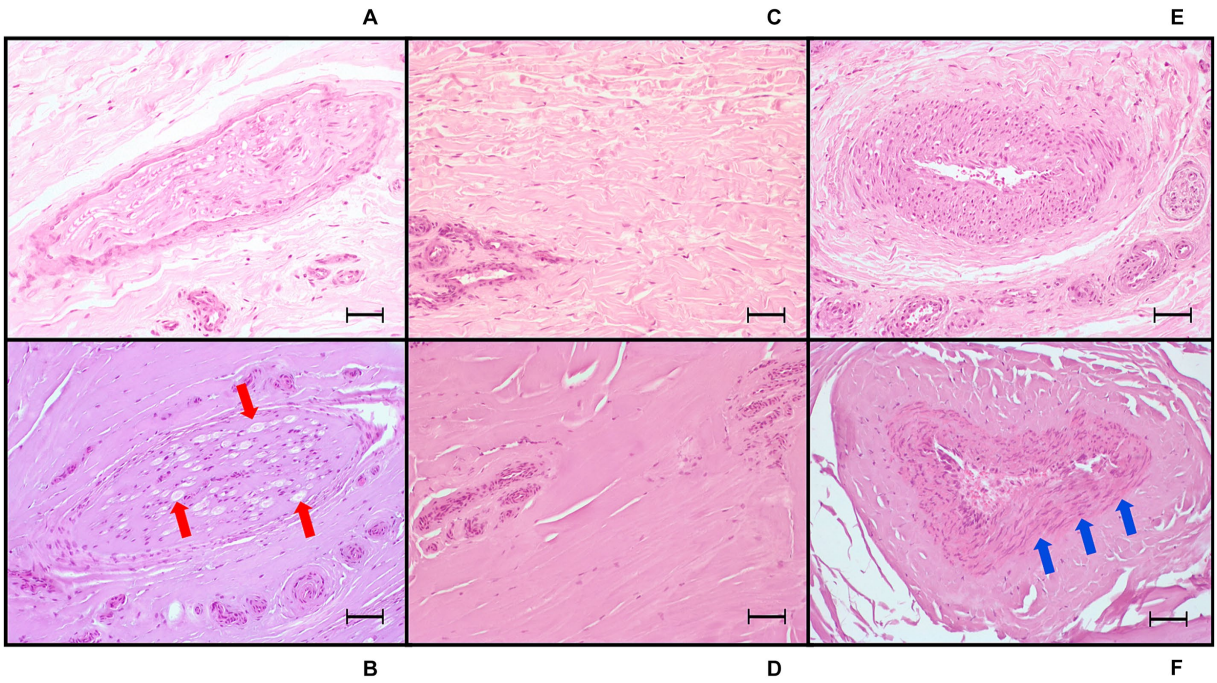

FIGURE 9
Light microscopic images (20x) of hematoxylin and eosin-stained sections of tissue treated with radiofrequency ablation, harvested from equine cadaver forelimbs. **(A,C,E)** Negative controls of nerve, collagen, and arterial tissue, respectively. **(B)** Radiofrequency ablation treatment (Group VERY HIGH: 80°C, 8 min): nerve coagulation, with axonal swelling and degeneration, increased intracytoplasmic vacuoles (red arrows) and cytoplasmic eosinophilia, perineurial sheath homogenation (coagulation) and swelling. **(D)** Extensive collagen coagulation with homogenation and loss of the fibrillar pattern. **(F)** Arterial wall coagulation: presence of severe nuclear smearing (blue arrows). Bars equal to 50 μm.

the US distance. Indeed, it was observed that both US and blue needle tip-to-nerve distances were significantly associated with the presence of coagulation, highlighting the importance of an extremely precise RFA needle positioning. Thus, despite the use of strict evaluation criteria that integrate two different methodologies to delineate the precise positioning of the RF needle, i.e., methylene blue and US, and the procedure was deemed successful when two out of the three proposed criteria were fulfilled, the US-guided RFA treatment has yet demonstrated a high success rate.

The third aim of this study was to assess the microscopical changes produced on PDNs, which are responsible for the transmission of pain signal from the distal limb. In the present study, histopathological lesions observed in PDNs were always consistent with axonal thermal damage, aligning with the existing literature (12, 23, 44). The

TABLE 2 Number of axonal degeneration, nuclear smearing, and coagulation of other structures in the presence or absence of nerve coagulation.

		Nerve coagulation		
		Yes	No	
Axonal degeneration	Yes	18	0	Fisher's F $p < 0.001$
	No	0	22	
Nuclear smearing	Yes	17	10	Fisher's F $p = 0.002$
	No	1	12	
Other coagulated structures	Artery	0	3	Chi-square $p < 0.001$
	Artery and collagen	17	7	
	Collagen	1	0	
	No	0	12	

These tests were performed on the overall sample ($n = 40$).

TABLE 3 Number of presence/absence of nerve coagulation in radiofrequency ablation (RFA) treatment groups and considering only RFA treatments performed "at target" ($n = 10$ per group).

Treatment group	Nerve coagulation		Nerve coagulation in RFA "at target"	
	Yes	No	Yes	No
Low	1	9	1	7
Medium	4	6	4	4
High	5	5	5	2
Very High	8	2	8	0
	Chi-square $p = 0.03$		Chi-square $p < 0.001$	

The p values represented the difference between the four treatment groups.

TABLE 4 Number of radiofrequency ablation (RFA) treatments performed "at target" in fetlock and pastern region ($n = 20$ per region).

	RFA treatment "at target"	
	Yes	No
Fetlock	12	8
Pastern	19	1
	Fisher's F $p = 0.008$	

application of RFA heat above 60°C led to rapid protein denaturation and extensive tissue coagulation (45, 46). According to Seddon's classification (47), axonotmesis occurred, with disruption of axons, myelin, and supporting connective tissue except for the epineurium (17). Following Sunderland's more detailed classification (48), a third-or fourth-degree peripheral nerve injury was generated, thus involving also the endoneurium or even the perineurium, respectively (46). When considering the *in vivo* effects, the thermal neurolysis produced by RFA has been reported to produce shortly thereafter Wallerian degeneration, initially developing at the target site, microscopically characterized by axonal swelling, fragmentation, and secondary demyelination (18); thereafter, the remaining distal portions of the axon and its myelin sheath also degenerate (46). This process causes transitory interference with nerve transmission, resulting in temporary denervation and pain relief (17). With an

TABLE 5 Sensitivity, specificity, PPV and NPV of the US-guided technique compared to the histologic confirmation of nerve coagulation (overall = 40; $n = 10$ per group).

Treatment group	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Overall	100 (81.5–100)	41 (21–64)	58 (49–66)	100 (66–100)
Low	100 (2.5–100)	22 (3–60)	12.5 (9–17)	100 (16–100)
Medium	100 (40–100)	33 (4–78)	50 (36–64)	100 (16–100)
High	100 (48–100)	60 (15–95)	71 (46–88)	100 (29–100)
Very High	100 (63–100)	100 (16–100)	100 (63–100)	100 (16–100)

PPV, positive predictive values; NPV, negative predictive values; US, ultrasound.

increasing degree of severity of axonal injury, the ability and rate of axonal regeneration diminishes with a longer duration of pain relief that can last months to years (46), but with a potential for neuroma-in-continuity formation in fourth-degree injuries (49). Indeed, in humans, post-procedural complications are considered extremely rare, and the most reported is neuropathic pain (50). Unfortunately, due to the *ex vivo* nature of this study, the clinical grade of the peripheral nerve lesions and their progression could not be evaluated as it was not possible to assess either the duration of pain relief or the possible occurrence of post-treatment complications. Currently, unresponsive chronic pain in the horse is finally dealt with surgical neurectomy, which has been associated with an increased risk of distal limb injuries, such as lesions at the deep digital flexor tendon, luxation of the distal interphalangeal joint, and sub-solar injuries (9). It cannot be anticipated if these complications may arise also after the RFA treatment. Future clinical studies are needed to elucidate these aspects. Finally, we could confirm that increasing the RFA treatment intensity determined a higher PDNs coagulation effectiveness. Indeed, beyond the already discussed needle inclination, size, active tip length, and the number of tines, it should be bore in mind that according to the literature, the size of lesions induced by RF is influenced by the temperature and the treatment duration (51). In the current study, four RFA treatments employing varying temperature/min settings underwent evaluation: the LOW and VERY HIGH treatments were selected to assess the effect of extreme settings. The MEDIUM treatment served as an intermediary setting positioned between the aforementioned groups, while the HIGH treatment applied settings often employed in human clinical studies (52, 53). Although it has been reported that larger lesions can improve treatment outcomes and increase the duration of pain relief in humans (54), the proximity of the nerve trunk to other important structures such as the arteries that need to be preserved (17) must be considered. Furthermore, in humans, nerve tissue is the least resistant to electric current passage and it is therefore the most vulnerable to electric-thermal damage (44). On the contrary, blood vessels would be less sensitive due to the blood flow dissipating hyperthermia, the heat sink effect (45). Therefore, it might be hypothesized that a precise, small, and localized RF-induced lesion would be sufficient to involve the nerves without damage to adjacent relevant anatomical structures. However, it has been reported that thermal injury caused by RFA might be too small, thus failing to induce complete denervation (17). Partial denervation may lead to diminished extent and duration of pain relief, potentially resulting in early recurrence of pain or lack of pain elimination (12). In the present study, the success rate of nerve coagulation was significantly higher as the RFA intensity settings increased, with the

highest frequency of coagulated nerve in VERY HIGH group but did not differ between the treatment sites. Also, partial nerve coagulation was observed in LOW and MEDIUM groups. Specifically, within the LOW group, the single case displaying nerve lesions exhibited only partial coagulation. In addition, in the MEDIUM treatment group, 3 out of 4 coagulated nerves were characterized by partial coagulation at the histologic evaluation. Due to these findings, the effectiveness of these two treatments in providing pain relief *in vivo* is at least unlikely for the LOW treatment group and remains to be explored at the clinical level. Applying the LOW and MEDIUM treatments in the effort of minimizing damage to non-target tissue should be avoided, since no significant differences in coagulation of non-target structures were observed between treatment groups. This was likely due to the size of lesions that was always enough extensive to involve the PDA, since the artery is very close to the PDNs. In fact, the correct needle position and the finding of coagulation were associated with nuclear smearing of the arterial leyomyocytes. These microscopical findings are in agreement with previous descriptions (12, 23, 44). Similar lesions have been also reported in humans, but in contrast to the findings from this work, they have been more commonly associated with the application of high-intensity electrical or high-power laser devices (55). Regarding PDVs, as the distance increased, a significant increase in vein blue dye staining was observed, but the PDV was never coagulated. This result may likely derive from the altered dye spread in cadaveric tissues and the greater distance of the PDVs with the PDNs. These findings are important to consider for the *in vivo* application of this technique, as the use of RFA in the fetlock and pastern regions could result in the development of collateral damage on non-target anatomical structures, especially the PDA. Nonetheless, both clinical and experimental PDAs damage did not provoke clinically relevant consequences as collateral circulation developed rapidly and tissue necrosis did not develop (56–59). Noteworthy, it has been previously described that in cadaveric limbs the absence of vascular heat sink effect may be a possible cause of overestimation of the collateral thermal injury compared to the *in vivo* effects of RFA treatment mitigated by adequate blood flow (45). Hence, potential RFA induced damage of the PDA should be explored in future studies to assess its clinical relevance.

This study retains some limitations, in addition to those already mentioned, all possibly related to the use of cadaveric limbs. The US window assessment in phase I was performed on living horses in standing position, while RF needle placement in phase II was not performed with a weight-bearing limb. This may have altered the relationships between anatomical structures and affected the success rate of both the US technique and the RFA treatments. A cut-off value for the US distance between the nerve and the active needle tip, beyond which coagulation did not occur was not evaluated. It would be interesting to ascertain such a value to be applied in the clinical setting. Furthermore, the extension of the lesions generated by the RFA treatments may differ from those generated in live animals due to different electrothermal properties and temperature of the tissue. Similarly, histological findings might not be comparable to those *in vivo* because of the absence of both vascular and cellular reactions in dead tissues and of the thermal dissipating ability of the blood flow. Finally, further clinical research is deemed necessary to determine the efficacy and duration of pain relief, to assess the emergence of potential complications, and to allow the definition of the optimal procedural parameters *in vivo*.

5 Conclusion

In conclusion, with transverse palmaro-lateral and palmaro-medial US images of fetlock and pastern regions the palmar digital neurovascular bundles in horses were easily detected. *In vivo*, color flow Doppler imaging allowed a better distinction of blood vessels from nerves, and visualized the PDN consistently adjacent to the PDA, which was deemed as the best US landmark. Despite the need of extremely precise RF needle positioning, the use of the in-plane US technique for this purpose proved to be successful, with a 77.5% of success rate of placing the needle "at target," albeit with some greater difficulty in the fetlock region. The use of a TRIDENT needle, which creates a "pear-shaped" lesion with a larger distal ablative area that is less sensitive to angulation changes, probably improved the efficacy of the procedure, despite a 90 degrees needle-to-nerve angle. Furthermore, the tip-to-nerve distance significantly affected the presence of nerve coagulation, highlighting the importance of positioning the RF needle as close to the target nerve as possible. In all groups, the histopathological findings revealed consistent axonal degeneration induced by RFA on PDNs of horses, aligning with the existing literature. The temperature/min setting in the treatment groups was significantly associated with the success rate of nerve coagulation, but not with coagulation of non-target structures. On the basis of the obtained results, HIGH and VERY HIGH RFA treatments might be worth of being applied in future *in vivo* clinical studies, focusing on treating chronic lameness of the distal forelimb in horses, since these protocols could potentially display an effective and long-lasting pain relief. Nonetheless, it is important to consider that potential complications, if any, might occur after the RFA treatment.

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 Institutional Ethical Committee for Animal Care at the University of Milan (OPBA) n° 51_2023. 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

MA: Conceptualization, Data curation, Investigation, Methodology, Project administration, Writing – original draft. VR: Data curation, Investigation, Methodology, Resources, Writing – original draft. GR: Conceptualization, Investigation, Methodology, Project administration,

Supervision, Validation, Writing – review & editing. LA: Formal analysis, Visualization, Writing – review & editing. FB: Investigation, Validation, Writing – review & editing. PiR: Resources, Visualization, Writing – review & editing. SD'A: Data curation, Validation, Writing – review & editing. PaR: Conceptualization, Data curation, Formal analysis, Methodology, Resources, Supervision, Writing – review & editing.

Funding

The author(s) declare that financial support was received for the research, authorship, and/or publication of this article. The authors acknowledge the support of the APC central fund of the University of Milan.

Acknowledgments

Authors would like to thank Bruna Consonni for her technical support and availability and Lidia Trianti, for her invaluable help in removing the shoes from the horses.

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.2024.1437989/full#supplementary-material

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frontiers | Frontiers in Pain Research

TYPE Original Research
PUBLISHED 05 September 2024
DOI 10.3389/fpain.2024.1429849

OPEN ACCESS

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RECEIVED 08 May 2024
ACCEPTED 19 August 2024
PUBLISHED 05 September 2024

CITATION
Laleye BOF-XV, Seye M and Chiavaccini L (2024) Early recognition of pain: improving colic outcomes in horses in Senegal. Front. Pain Res. 5:1429849. doi: 10.3389/fpain.2024.1429849

Early recognition of pain: improving colic outcomes in horses in Senegal

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Background: Limited knowledge exists on recognition and treatment of equine abdominal pain in low- and middle-income countries. This study aimed at finding indicators for recognizing abdominal pain, evaluating responses to clinical and behavioral changes, and assessing the impact of timely referral on colic outcomes in a suburban region of Senegal. The final goal was to identify factors that may be leveraged to improve the outcome of horses presented for abdominal pain in Senegal.
Study design: Retrospective, observational cohort study.
Methods: Data from 26 foals and 40 adult horses referred for acute abdomen between 2013 and 2014 and the first semester of 2023 were reviewed. Signs of abdominal pain were grouped into behavioral, posture modification and animal interactions with the environment. Time to referral was defined as the time between the recognition of abdominal pain and referral. The association of time to referral and the outcome was calculated for each subpopulation and compared using logistic regression analysis as appropriate.
Results: A significant proportion of owners (47%) and veterinarians (77.8%) relied on behavioral changes to detect abdominal pain in foals. Most owners referred foals within 24 h, while veterinarians referred within 12 h. Mortality in foals exceeded 50% when referral was delayed by 12 h or more. In adult horses, groomers often were the first noticing behavioral changes (79%), and they referred the horse within three hours, whereas owners typically delayed referral for 24 h or longer, leading to increased hospitalization expenses.
Limitations: The study considered a limited cohort in an suburban area of Senegal. Sourcing complete data was challenging. Additionally, accurately assessing owner experience was difficult due to the participant group's heterogeneity. Absence of a reliable system to measure daily horse-owner interaction time and logistical challenges in the abdominal pain symptom alert chain were also limiting factors.
Conclusions: Early detection is critical for positive colic outcomes in both foals and adult horses. Therefore, raising awareness and providing training to horse owners for prompt recognition of symptoms and referral is essential. This proactive approach aims to improve overall outcomes and reduce the financial burden of equine hospitalization in Senegal.

KEYWORDS

acute abdomen, abdominal pain, colic management, horse health, low-income countries, referral time, Senegal

1 Introduction

In Senegal, horses play a pivotal role in various aspects of the country's life, influencing sectors like the military, economy, sports, and leisure (1, 2). The equine industry holds significant importance in the daily lives of many Senegalese households today (1). Similar to the rest of the world (3–5), colic colic is considered a leading cause of mortality among foals and adult horses in Senegal. Colic refers to abdominal pain and can result from various disease processes beyond gastrointestinal issues. While many colic cases in horses can be resolved through medical treatment, some require intensive care or surgical intervention. The colic outcome is closely tied to the time of referral, emphasizing the importance of early identification of abdominal pain signs (6–9). Owners, bearing the primary responsibility for identifying symptoms and determining when to seek veterinary assistance, play a crucial role in this process (8, 10). Despite high confidence among horse owners in the UK and US in recognizing abdominal pain, a study revealed a discrepancy between their knowledge and clinical scenario responses, attributed to a lack of understanding and difficulty in recognizing subtle signs of abdominal pain (10). Little is known about the status of equine abdominal pain recognition and treatment in low- and middle-income countries (11), where factors such as limited resources, inadequate training, cultural diversity, and language barriers often result in animals not receiving basic pain treatment. A pragmatic way to change this situation is to look at the country environment in a critical way and identify areas that need prioritization.

This study aimed to explore the criteria considered by horse owners and handlers when assessing signs of abdominal pain in both adult and neonatal horses in Senegal. The objective of the study were: (a) To recognize indicators used by owners or caregivers to recognize abdominal pain; (b) To evaluate their response to changes in clinical and behavioral parameters; (c) To identify the impact of timely referral on colic outcomes in a suburban region of Senegal.

2 Materials and methods

This was a retrospective, observational cohort study. Institutional Animal Care and Use Committee approval was not required due to the retrospective nature of the study. The owner consented to the anonymized use of data with the signed consent to treat.

2.1 Data collection

The sample included 66 medical records, 40 from adult horses (>5 years old) and 26 from newborn foals (≤4 weeks old) collected from two distinct databases. The dataset comprised the medical records from 24 adult horses and 22 newborn foals referred to the hospital of the National Stud Farm in the Kébemer department, within the Louga region, between 2013 and 2014. At the national stud, all horses showing abdominal pain belonging to the stud herds are supported financially. For horses with colic that are boarding or hospitalized, a flat fee is charged to the owner. Additionally, records from 16 adult horses and four newborn foals collected between January and June 2023 were included from the database of the SahelVet ambulatory practice located in Ngaye Mékhé, within the Tivaouane department, in the Thiès region of Senegal. This practice offers standardized treatment bundle for colic encompassing a clinical examination, transrectal palpation, nasogastric catheterization, and administration of sedatives or antispasmodics. If the horse's general condition requires more intensive management, such as parenteral rehydration or multiple additional visits, the owner is charged accordingly. At the National Stud Farm hospital, all information retrieved from admitted veterinary patients are documented daily in a Microsoft Word Document (Microsoft Corporation, Redmond, WA) saved on a local computer. While at the private equine practice, data are recorded daily in an official paper register. For analysis purposes, only cases with complete and accurate records were retained.

2.2 Referring personnel

The referring personnel was categorized into three groups: horse owners (regardless of ownership duration, experience, or practiced sport), veterinarians responsible for routine evaluations of newborn foals at the Kébemer National Stud, and horse caregivers (such as professional groomers). In the Kébemer department, all foals are required to undergo evaluation by a veterinarian shortly after birth for registration. This assessment involves providing a detailed description of the foal, conducting a comprehensive clinical examination, and administering a tetanus injection. Additionally, the health of the mare is assessed during this visit. At the time of data collection, three veterinarians employed by the Kébemer National Stud were responsible for initially identifying any births from stallions within the national stud. This examination typically occurred within 12 h of a reported birth, although delayed notifications were possible due to owner negligence or if the foal was born outside the National Stud system. The group of horse caregivers consisted of 16 groomers employed by the Kébemer National Stud between 2012 and 2014. These caregivers were evenly divided into two groups: one responsible for monitoring mares and the other for stallions. The brood groom group attended to the care of both stud-owned mares and those in boarding for follicular monitoring, insemination, and pregnancy diagnosis. The groom group in charge of stallions ensured the well-being of stallions in the stud. The groomers' professional experience ranged from 2 to 5 years, with no formal professional training in the field; their expertise was primarily acquired on the job.

2.3 Assessment and understanding of abdominal pain in the horse

Signs of abdominal pain were grouped into three categories for the purpose of the analysis: behavior modification (BM); posture modification (PM) and change in interaction with the environment (IE). In foals BM included: agitation; tenesmus (Figure 1); attempts to defecate and assuming an "abnormal body position" as described by the referring person, and based on the interpretation of the person collecting the history. For adult horses, the BM included: pawing the ground; flank-watching; rolling for an extended period/multiple times; attempt to urinate or defecate, kicking the abdomen and assuming an "abnormal body position". Change in posture (PM) was defined as weight shifting, fence or box walking, lying down or getting up restlessly or multiple times in both adult and newborn horses (Figure 2). Change in horse IE was defined as an adult or newborn horse appearing abnormally quiet or dull or reported inappetence.

2.4 Mortality

Due to the absence of veterinary structures offering equine colic surgery in Senegal, three possible outcomes were identified. The horse condition resolved with medical management, the horse died of spontaneous causes or it was euthanized as a consequence of the condition.

2.5 Statistical analysis

All data were entered in a Microsoft Excel worksheet (Microsoft Excel for Mac Version 16.65; Microsoft Corporation, WA, USA). Data elaboration and analysis were conducted with IBM SPSS Statistics and Stata/BE 17.00 (StataLLC, TX, USA). Continuous data were checked for normality using the Shapiro Wilk normality test and graphically with normality quantile plot

FIGURE 1
A foal with severe tenesmus, as an example of behavior modification (BM).

FIGURE 2
Examples of posture modifications (PM) of foals in colic. **(A)** Lying down and rolling **(B)** lying down motionless **(C)** weight shifting.

and histogram distribution. Continuous data were presented as mean ± standard deviation (SD) or median [first and third interquartile (IQR)] if normally or not normally distributed, respectively. Categorical data were presented as proportions and percentage. The association between the referring personnel and the understanding of abdominal pain signs and between time to referral and mortality was compared for each subpopulation using Pearson's chi-square and Fisher's exact tests as appropriate, and using logistic regression. Statistical significance was set at $P \leq 0.05$.

3 Results

The findings are presented separately for newborn foals and adult horses, with each category undergoing individual analysis to underscore pertinent results and trends.

3.1 Demographics

All 26 foals evaluated in the study were less than a week old, with 38.4% (10/26) being females. These foals exhibited signs of abdominal discomfort within the first hours after birth, primarily attributed to meconium retention (65.4%; 17/26) and other undiagnosed causes of colic (34,6%; 9/26). Among the 40 reported cases of abdominal pain in adult horses, 52.5% (21/40) were stallions and 47.5% (19/40) were mares. The majority of cases involved indigenous breeds (45%; 18/40), alongside Thoroughbreds (37.5%; 15/40), Arabian Horses (10%; 4/40), and Warmbloods (7.5%; 3/40), reflecting the equine population in the area. The mean ± SD age of adult horses referred for abdominal pain was 11.9 years ± 4.5.

3.2 Referring personnel

In cases involving foals, the initial identification of abdominal pain symptoms predominantly stemmed from reports by either owners (65.4%; 17/26) or stud veterinarians (34.6%; 9/26). Owners detected abdominal pain in 11.8% (2/17) of instances within the first 12 h of life, leading to a unanimous diagnosis of meconium retention by attending veterinarians When concerns arose between 12 and 24 h (47%; 8/17), causes of abdominal pain varied, encompassing meconium retention (50%; 4/8), generalized weakness (25%; 2/8), or unspecified gastrointestinal issues (25%; 2/8). In cases where concerns emerged between 24 and 48 h after initial signs (41.2%; 7/17), veterinarians diagnosed meconium retention (57.4%; 4/7), unspecified causes (28.5%; 2/7), and generalized weakness (14.2%; 1/7). Veterinarians identified abdominal pain within the first 12 h of life in 77.8% (7/9) of cases, predominantly addressing meconium retention (85.7%; 6/7) and weak foals (14.3%; 1/7). In the remaining instances (22.2%; 2/9), intervention occurred 24 h after symptom onset, with one case diagnosed as meconium retention and the other as generalized weakness.

In adult horses, the responsibility for identifying symptoms of abdominal pain rested primarily with owners in 52.5% of cases (21/40), while groomers were accountable for 47.5% (19/40) of recognitions. Among owners, 47.6% (10/21) promptly informed the veterinarian within 24 h of symptom recognition. However, a notable proportion (33.4%; 7/21) delayed referral until 48 h after the initial symptom recognition, while a few (9.5%; 2/21) waited even longer, up to 72 h or 120 h (5 days) before seeking veterinary assistance. Owner-initiated therapy was reported in 4/17 (23.5%) abdominal pain cases referred to the veterinarian. In contrast, groomers consistently alerted the referring veterinarian in less than 3 h from the initial recognition of abdominal pain symptoms, ensuring swift intervention.

3.3 Assessment and understanding of abdominal pain in the horse

Encompassing both foals and adult horses, owners and veterinarians predominantly relied on BM (only), PM (only) and BM with PM, to identify abdominal pain symptoms (27/47; 57.44%). In contrast, most groomers were prompted by observing changes in the horse's behavior associated with alterations in IE, such as eating habits (16/19; 84.2%), alone or in association with BM (17/19; 89.4%). Only in two instances they relied on PM only. Further details regarding how various demographic groups assessed and responded to signs of abdominal pain within the population are provided in Tables 1, 2.

3.4 Mortality

In cases where foals were referred by owners within 12 h, no mortality was observed. However, when owners referred the foal to the veterinarian within 24 h of initial abdominal pain signs, the mortality rate increased to 57.2% (10/17). If the referral was delayed to 48 h, the mortality rate was 42.8% (3/7). When veterinarians identified a foal suspected of abdominal pain during the registration visit, the mortality rate was 14.2% for interventions within the first 12 h and rose to 50% for those conducted within the first 12–24 h (Table 2).

TABLE 1 Assessment and understanding of colic indicators by referring personnel.

Horses (66)	Referring personnel (66)	Indicators of colic		
		BM *n* (%)	IE *n* (%)	PM *n* (%)
Foals (26)	Owners (17)	8 (47%)	5 (29%)	4 (17%)
	Veterinarians (9)	9 (100%)	–	2 (22%)
Adults (40)	Owners (21)	19 (90.4%)	7 (33%)	2 (9.5%)
	Groomers (19)	17 (89.4%)	16 (84.2%)	2 (10.5%)

BM, behavior modification; IE, interaction with environment; PM, posture modification; n, total number. Some horses were identified has having abdominal pain using two or more combinations of indicators.

10.3389/fpain.2024.1429849

TABLE 2 Detection time in case of colic declared by owners, veterinarians and groomers and the relation with mortality.

Horse (66)	Referring personnel		*n* (%)	Mortality *n* (%)	*P*-value
	Personnel (*n*)	Time			
Foals (26)	Owners (17)	<12 h	2 (11.8%)	0	0.44
		12–24 h	8 (47%)	5 (62.5%)	
		24–48 h	7 (41.2%)	3 (42.8%)	
	Veterinarians (9)	<12 h	7 (77.8%)	1 (14.2%)	0.31
		12–24 h	2 (22.2%)	1 (50%)	
Adults (40)	Owners (21)	12–24 h	10 (52.6%)	2 (20%)	0.55
		48 h	8 (36.8%)	0	
		72 h	2 (10.6%)	0	
		>72 h	1 (9.5%)	0	
	Groomers (19)	<12 h	19 (100%)	3 (15.7%)	

n, total number.

For adult horses where abdominal pain symptoms were reported by the owner, a mortality rate of 20% (2/10) was observed only if the signs of abdominal pain occurred within 24 h prior to referral. No mortality was observed in other colic cases, regardless of the delay (Table 2). At the Kébemer National Stud, the mortality rate observed when the groomer notified of abdominal pain (always within less than 3 h) was 15.7%. There was no association of mortality with referring personnel, time to referral, or their interaction ($p = 0.2$).

Regarding adult horse abdominal pain cases, the final treatment expenses increased by 33.4% if the horse was referred within 48 h, 66.7% if referred within 72 h, and 80% if referred 120 h or later. No invoice was reported for foals, so a comparison was not possible.

4 Discussion

Foals, renowned for their fragility in their early hours, demand careful attention from owners (3). However, owners often struggled to detect the initial signs of abdominal pain in foals, resulting in variable response times. Unlike veterinarians, who swiftly identified abdominal pain based on behavioral changes, owners' detection capabilities were not as rapid, leading to delayed responses. This observation aligns with findings from a study conducted in Brazil, which aimed to assess horse owners' experience, recognition, and attitudes towards equine abdominal pain in Rio Grande do Norte (11). Horse owners encountered challenges in identifying and interpreting early abdominal pain warning signs. Our study further suggested that the origin of abdominal pain significantly influenced owners' detection abilities, as they readily identify overt signs but struggle with insidious conditions with a progressive evolution, such as meconium retention. These conditions would also have the best outcome if properly and promptly addressed (12). On the other hand, situations where owners quickly detected foal distress typically indicated poor prognoses, regardless of management timing.

In cases of adult equine colic, a similar pattern emerged, characterized by disparities in recognized warning signs and highly variable response times. This discrepancy arose from the comparatively lesser attention given to working horses compared to newborn foals (1). Consequently, owners may have overlooked certain early warning signs. Moreover, typically, owners observed their horses only during working hours and meals, with limited contact outside these periods. The location of the equine enclosure may further exacerbate this issue; the farther it is from the owner's home, the less frequently the horse is monitored. However, studies have shown that even when owners spent considerable time with their equine companions, they only provided timely alerts with proper awareness of early colic signs (10). In contrast, professionals like veterinarians and groomers are better trained to assess a horse's condition. In developing countries like Senegal, accessibility to healthcare and limited health education contribute to widespread where owner-initiated medication of horses among the population (13). This habit is also reflected in veterinary medicine, where owners often take the initiative to provide medical care to their animals without direct oversight or intervention from a veterinarian. When owners notice signs of colic, it is not uncommon that they resort to self-initiated therapy, using traditional remedies, before contacting a veterinarian. This practice is prevalent among horse owners, who commonly employ conventional medicinal practices to manage various equine pathologies. In cases of colic, the most frequently used treatment methods include administering decoctions of plants, such as Prosopis Juliflora, through the nostrils or the oral cavity. Some owners opt for alcohol-based solutions, such as beer or wine, using the same routes of administration. In those instances, owners typically seek veterinary assistance only when their self-initiated therapy efforts prove ineffective. Our study found that owner-initiated therapy was reported in 23.5% of colic cases referred to veterinarians. However, this figure may need reassessment, considering that some owners may withhold information about self-administered treatments from the veterinarian. Suspicion may arise during examination upon observing colored (yellow or green) single or bilateral nasal discharge, indicative of nasopharyngeal administration. Respiratory issues, often stemming from aspiration due to improper use of the nasopharyngeal route, are the most common complications in such cases. In all the cases reported in this study, Prosopis Juliflora decoctions were administered orally.

When examining the implications of delayed alerts, it is suggested that in the case of foals, tardy notifications may correlate with reduced survival probabilities, although we were unable to establish a statistically significant association with the small number of subjects. Extensive research underscores the pivotal role of timely referral for newborn foals (14). Conversely, late referrals in cases of adult horse colic did not exhibit a corresponding increase in mortality. Even if the referring personnel were professional and the referral was made promptly, a horse with a surgical reason for colic could still succumb to the condition. Conversely, if the referring personnel were owners and delayed the referral, a horse with a medical reason for abdominal pain could still survive. Therefore, mortality alone did not provide a comprehensive assessment of abdominal pain management in our study. Other factors, such as the nature and severity of the colic and the timeliness and appropriateness of interventions, had to be considered to evaluate the effectiveness of abdominal pain management practices fully. This disparity could also be attributed to the prevalent etiologies of abdominal pain in Senegal, primarily stemming from food impactions or stasis in the large intestine, a consequence of suboptimal feeding practices and inadequate deworming protocols. The survival rates for such colic surpass those observed for colic affecting the small intestine (15). However, it is imperative to acknowledge that delayed notifications necessitate a heightened allocation of resources for horse management. Severe climatic conditions exacerbate dehydration rapidly, necessitating fluid therapy and adjunctive therapeutic measures, thereby magnifying the financial burden associated with medical management. Analogous to observations in foal abdominal pain, owners often overlooked signs indicative of benign conditions, allowing them to deteriorate while demonstrating prompt responses to scenarios associated with lower survival probabilities. Despite these limitations, the overall case fatality rate for adult horses in our study was comparable to that reported in wealthier nations like Canada and the United States, known for their high standard of veterinary medicine, advanced infrastructure, and well-established institutions (15, 16).

4.1 Limitations

The study encountered several limitations. Firstly, the challenge of sourcing cases with complete data significantly hindered the research process. Despite a large pool of potential cases, less than 10% of records yielded usable information, highlighting the need for improved medical record keeping. Additionally, accurately gauging the level of owner experience proved challenging due to the heterogeneous nature of the participant group. Another limitation stemmed from the absence of a reliable system to measure the time owners spent with their horses daily, which has potential implications for horse welfare and health. Moreover, the alert chain for abdominal pain symptoms posed a logistical challenge, often involving multiple individuals before reaching the owner, potentially delaying timely intervention. Lastly, the financial implications of veterinary interventions presented a barrier to prompt alertness from owners, contrasting with the swift actions of groomers who prioritized horse well-being over economic concerns.

However, despite these limitations, the study provides an overview of factors that can be leveraged to improve the welfare of horses in Senegal. With its population of 17 million inhabitants and a herd of 1 million equines, including 462,000 donkeys (1), Senegal has fewer than 200 private veterinarians spread across its territory spanning 196,712 km^2. This notably low rate of veterinary coverage poses challenges in managing equine colic, particularly in rural areas and within the study area. Raising awareness and educating owners is crucial, as abdominal pain management in horses is directly linked to its early detection, a responsibility typically shouldered by owners. This imperative for primary horse education extends to encompassing correct horse handling, feeding, grooming, and pain assessment. Collaboration with local community figures, private veterinarians, and state structures such as the Kébémer Stud Farm is imperative. Various mediums such as local community radio, social media platforms, and direct interaction between professionals and horse owners offer avenues for effective communication and awareness-raising. Recognizing that awareness and communication are as pivotal as increasing the number of private veterinarians and their responsiveness is essential for enhancing equine welfare in Senegal.

5 Conclusion

The findings underscore the insufficient and disparate level of knowledge among owners regarding detecting abdominal pain signs in both foals and adult horses, directly impacting mortality rates for foals and financial costs for adult abdominal pain cases. Unlike professionals, owners frequently failed to identify the early, subtle signs of colic with a potentially favorable prognosis in foals and adults, often referring the horse only when the animal's condition had deteriorated. Enhancing equine well-being in Senegal requires sensitizing and training owners to recognize early signs for prompt intervention. Implementing mechanisms tailored to the country's realities, with active engagement from veterinarians and stakeholders in the equine sector, would be crucial steps toward achieving this goal.

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.

Author contributions

BL: Writing – original draft, Writing – review & editing. MS: Writing – original draft, Writing – review & editing. LC: 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.

Acknowledgments

The authors would like to thank Commandant Mamadou BARRO, Director of the National Stud of Kébemer, and his team: Commandant Mamadou DIARRA and Doctor Malal BA for their help and availability.

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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TYPE Original Research
PUBLISHED 24 October 2024
DOI 10.3389/fpain.2024.1463688

OPEN ACCESS

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RECEIVED 12 July 2024
ACCEPTED 08 October 2024
PUBLISHED 24 October 2024

CITATION
Blum S, Gisler J, Dalla Costa E, Montavon S and Spadavecchia C (2024) Investigating conditioned pain modulation in horses: can the lip-twitch be used as a conditioning stimulus?
Front. Pain Res. 5:1463688.
doi: 10.3389/fpain.2024.1463688

Investigating conditioned pain modulation in horses: can the lip-twitch be used as a conditioning stimulus?

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Study objective was to evaluate whether the application of a lip twitch could be proposed as conditioning stimulus in the context of a novel Conditioned Pain Modulation (CPM) assessment paradigm for use in horses. The study was a prospective, experimental, randomized trial. Twelve healthy horses were evaluated in two experimental sessions. The lip twitch was used as the conditioning stimulus in both sessions; electrical stimulation was used as the test stimulus in one session, while mechanical and thermal stimulations were used in the other. Differences between thresholds recorded before and during twitching (Δ) as well as their percent (%) change were computed for each stimulation modality as a measure of CPM. Heart rate and respiratory rate were recorded throughout the experiments to monitor physiological reactions, while the general level of stress and aversiveness toward twitching were scored using *ad hoc* behavioural scales. Based on these scores, interruption criteria were defined. Ten and seven horses completed the electrical and mechanical/thermal experimental sessions respectively. For electrical stimulation, median (IQR) Δ was −2.8 (−3.9, −1.1) mA and% change 87.9 (65.7–118.2)%; for mechanical stimulation, Δ was −18.2 (−6.4, −21.4) N and% change 343.5 (140, 365.3)%; for thermal stimulation, Δ was −3.1 (−9.2, −2.1)°C, while% change was not calculated. Heart rate and respiratory rates varied significantly over time, with higher values recorded during twitching. Median stress and aversion scores did not differ between the two sessions. As lip twitching consistently affected thresholds to all stimulation modalities, it can be proposed as effective conditioning method for CPM assessment in horses. The exclusion of subjects due to severe aversion shows that this paradigm cannot be indistinctively applied to all horses and that stringent interruption criteria are necessary to guarantee adequate welfare during testing.

KEYWORDS

horse, conditioned pain modulation, thermal threshold, nociceptive withdrawal reflex, pressure pain threshold

1 Introduction

Chronic musculoskeletal pain has a high prevalence in horses as well as in humans. Independently from the originating pathology, it is a frequent cause of poor athletic performance, impaired quality of life and an increasingly perceived welfare concern. The appearance of lameness is often the first recognized clinical sign that a painful

process is ongoing and gait assessment in response to diagnostic analgesia is classically used to anatomically localize the source of pain (1–3). This lameness-centered approach justifies why in recent years a multitude of objective tools have been developed to quantify gait asymmetry and to monitor its changes over time (4–6). Furthermore, a large body of novel research has focused on the description of species-specific behavioural indicators of pain, in horses at rest as well as ridden (1, 2, 7–9). On the other side, quantitative sensory testing methods, allowing to define the pain phenotype on a mechanistic base and largely applied in human medicine, have been rather neglected in horses so far, except for algometry (10). Both peripheral and central sensitization phenomena are known to accompany most chronic painful pathologies, as demonstrated for osteoarthritis and laminitis (11). Thus, developing or further refine methodologies to evaluate sensory function and its modulation could be useful to better characterize individual horses affected by chronic pain and to predict response to therapy. In humans, the Conditioned Pain Modulation (CPM) paradigm has been largely applied in research and clinical settings to assess alterations in central pain processing (12). The classical assumption is that in normal conditions pain inhibits pain while, in presence of chronic pain, temporal summation mechanisms are enhanced and endogenous inhibition is reduced, leading to a generalized pain facilitation (13, 14). Even though this assumption has been repeatedly challenged, exploring the phenomenon of Conditioned Pain Modulation in horses appears as an interesting novel opportunity to understand the role of endogenous pain control in this species, in health first and later in presence of chronic pain conditions.

Finding an adequate, reliable, and easy-to-use conditioning noxious stimulus is certainly the first prerequisite for a successful assessment of endogenous pain modulation. Secondly, quantifiable test stimuli which allow to define pain thresholds are necessary, as CPM is calculated as the difference between thresholds measured before and during or just after the application of the conditioning stimulus. Conditioning and test stimuli are typically applied in distant body regions to evoke CPM.

Aim of the present study was to assess whether the application of a common lip twitch as conditioning stimulus would modify the thresholds to electrical, thermal and mechanical stimuli applied to the forelimbs in a consistent fashion. It was hypothesized that: the application of the lip twitch would be able to increase the nociceptive withdrawal reflex (NWR), the pressure pain (PP) and the thermal (T) thresholds to a clinically meaningful extent in healthy horses. If this would be the case, such a paradigm could be considered further to evaluate CPM in horses.

2 Materials and methods

2.1 Study design

This study was designed as a prospective, experimental, randomized, single cohort trial, which received approval from the Committee for Animal Experimentation of the Canton of Bern, Switzerland (license number BE81/2022). The trial, carried out at the National Equine Center in Bern from November 2022 to January 2023, consisted of two experimental sessions for each subject included. In both sessions, the lip twitch was applied as a conditioning stimulus. As test stimuli, in one session electrical stimulation was used (NWR session) while in the other mechanical and thermal stimulation were used (PP/T session). Nociceptive thresholds were measured before, during and after the application of the lip twitch. For each horse, the sequence of sessions was randomized and at least two weeks elapsed between sessions. The timeline of the experiments is graphically represented in Supplementary Material (Supplementary Figure S1).

2.2 Horses

Twelve healthy horses, mare and geldings, older than three years and belonging to the Swiss Armed Forces were included. Horses were kept in single stalls in large stables under standard housing conditions and were regularly ridden or driven. Prior to inclusion in the study, a complete physical examination was performed by two veterinarians (JG, SB) supervised by an experienced equine specialist (SM).

To be included, horses had to be free of clinically detectable orthopaedic, neurologic or systemic diseases, and have no evidence of pain, lameness or mobility impairment. Horses were excluded if they received anti-inflammatory or analgesic drugs in the two weeks prior to the study. Physical and orthopaedic examinations were repeated before each experimental session to ensure that no changes had occurred between appointments. All horses were tested in the afternoon. At least one hour had to elapse between feeding and/or daily training exercise and testing.

2.3 Instrumentation and monitoring

Two horses per day took part to the experiment. They were collected from their own stall and brought to a large, empty stable to which they were accustomed to. While one horse was tested in the corridor, fixed by two ropes on each side of the halter, the other was kept in a nearby box with visual contact. Testing equipment was placed on the left side of the horse under testing. Horses were generally very calm when fixed in the corridor, as this was the usual place for being groomed and saddled.

The sites foreseen for ECG electrodes placement, on both sides of the withers and on the left chest, were clipped. Sites for electrical stimulation and NWR recordings electrodes were clipped, defatted, and slightly abraded to obtain a proper impedance.

A telemetric ECG monitoring system (Televet 100, Engel Engineering Services GmbH, Germany) was applied on the horse and fixed with an ad-hoc belt following manufacturer recommendation. Recording was started as soon as the test stimulation equipment was in place and continued until 15 min after twitch removal.

Respiratory rate was visually assessed and recorded every two minutes before and after twitching, and every minute during twitching.

10.3389/fpain.2024.1463688

For heart rate and respiratory rate, average values were calculated for the 5 min of baseline recordings preceding test stimulation (baseline), for the 5 min preceding twitch application (baseline stimulation), for the 5 min of twitching (twitch), and for 5 min intervals thereafter up to 15 min after twitch removal. These values were used for statistical analysis.

The twitch, as used for minor procedures in equine veterinary care, consisted in a wooden handle and a double rope to be twisted around the horse's upper lip (Figure 1). During twitching, a round metal sensor (DLM20-BU.500.CP3.M4, Baumer AG, Switzerland) connected to a digital display was placed between the wooden handle and the rope to monitor the force applied (Figure 2). The measured force range was 0–50 N.

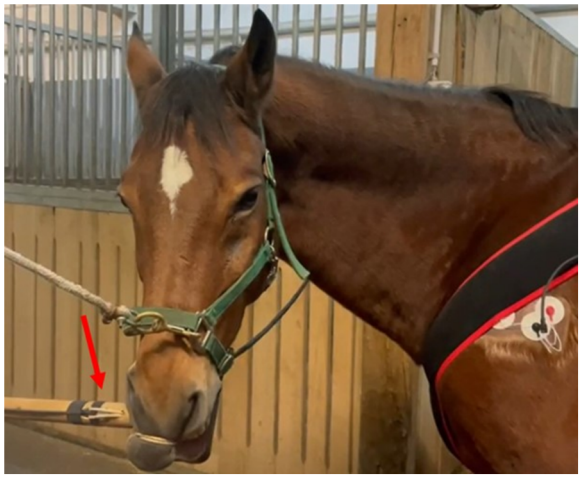

FIGURE 1
The twitch in place around the horse upper lip. The red arrow shows the position of the force sensor.

FIGURE 2
Details of the twitch used in the current trial. On the left, the tip of the wooden handle with the double rope. On the right, the sensor in place under the rope on the back side of the handle.

2.4 Stress and aversion scores

For each experimental session, the level of stress before and after twitching was evaluated every 2 min using a previously validated stress scale, ranging from 1 (no stress) to 10 (high stress) (15).

Furthermore, the degree of aversion shown during twitch application was scored every minute on a scale developed on purpose and ranging from 1 (no aversion) to 4 (severe aversion) (Table 1).

2.5 NWR session

In horses undergoing the NWR session, the area over the left palmar digital nerve and the left deltoid muscle were shaved and defatted for the application of surface self-adhesive electrodes (Ambu Blue Sensor N, Ambu Sdn, Malaysia). These electrodes, with an active surface of 0.8 cm^2, were used for electrical stimulation and NWR recordings, respectively.

For the determination of the NWR threshold, a continuous threshold tracking device (Paintracker V1, Dolosys GmbH, Germany) was used. This device allows constant current electrical stimulations and electromyographic recording of the evoked muscle responses, with integrated impedance feedback. Stimulation consisted of a train-of-five, 1 ms square pulses applied at a frequency of 200 Hz (standard stimulus used in algology experiments) starting at the intensity of 0.5 mA, with 0.2–0.3 mA step size and three direction changes needed before halving/doubling steps. The interstimulation interval was set at 10 s ± 30%. Recording was started 100 ms prior to stimulus onset and lasted until 400 ms thereafter.

TABLE 1 Scoring system used to evaluate the degree of aversion during twitching. If a stress score >5, or an aversion score >3 was reached, the session was interrupted, and incomplete data sets were discarded. Horses reaching cut-off scores in the first experimental session did not undergo the second experimental session.

Aversion score	Aversion level	Behavioural indicators
1	No	Steady head, eyes open or half closed, ears slowly scanning or still, lips moving freely, tail still or gently swishing. Horse relaxed, calm, accepting, mild signs of sedation possible
2	Mild	Increased head movements, eyes open, increased ears movements or backwards, decreased lips movements. Horse alert, listening, unsettled
3	Moderate	Increased head movements, upward and occasionally against the twitch, pawing/stomping, or freezing with reduced movements but overall increased body tension, eyes open and white showing, tail swishing, defecation. Horse restless, uncomfortable
4	High	Raised head, unsteady and repeatedly moving against the twitch, eyes open and white showing, repeated tail swishing, defecation. Horse agitated, anxious, aggressive behaviour (rearing, barging, pawing against twitch/handler) any time possible or present.

The NWR threshold was automatically tracked based on the last twelve responses to stimulation, analyzing the poststimulation epoch of 80–250 ms after stimulus onset. The Peak z score criterion was selected, with an evaluation cut off value of 10. Noise was evaluated between 130 and 10 ms before stimulus onset and had to be <15 μV for a recording to be considered valid. Baseline NWR threshold was tracked for a minimum of 15 min before application of the lip twitch. Then the lip twitch was applied for 5 min. The NWR threshold was further continuously determined up to 15 min after twitch removal. The mean baseline NWR threshold was determined for the five minutes preceding the application of the lip twitch and the percent change from baseline during conditioning was quantified. Mean NWR thresholds were also calculated for the intervals 0–5- and 10–15-minutes following twitch removal.

2.6 PP/T session

The PP threshold was evaluated using a ProdPro algometer (Top Cat Metrology Ltd, UK). Stimulation was performed through a blunt ended 1 mm diameter pin, pushed against the skin via a pneumatic actuator fixed on the right metacarpus and driven by manually injected air as previously described (16). The actuator was held in place with a boot, and a strap was used to counteract the force generated during stimulation. A dummy actuator, with boot and strap, was applied on the left forelimb at the same height. During stimulation, a constant force rate increase of 2N/s was kept with the guidance of warning LED lights visible on the instrument during operation.

Stimulation was interrupted when a weight shifting to the contralateral limb, a voluntary limb lifting and/or stamping occurred, or when the cut-off force of 25 *N* was reached. At this point, the stimulus was removed, and the peak force (*N*) displayed on the device was recorded as threshold. For stimulations reaching the cut-off, a threshold value of 27.5 *N* was attributed and used for analysis.

Thermal threshold was evaluated on a clipped spot just above the chestnut, on the medial aspect of the antebrachium, using a purpose made hand-held thermode, with a target temperature increase of 0.6°C/sec. The probe (round-shaped, 1 cm diameter) was immediately removed from the skin and the maximal reached temperature recorded as soon as a reaction occurred (see above) or when the cut-off of 52°C was reached. For stimulations reaching the cut-off, a threshold value of 55°C was attributed and used for analysis. Testing sequence (pressure or thermal first) was randomized for each horse and kept constant for consequent measurements. Four threshold measurements for each modality were performed at baseline, with at least 60 s interval between measurements. Thereafter, the lip twitch was applied for five minutes. During the last three minute of application, both thresholds were reevaluated (up to two times each) following the same sequence. Then the twitch was removed, and thresholds were reassessed starting at 5 and 15 min after twitch removal to describe the time course of CPM. At each of these measuring timepoints, thresholds were evaluated 3 times per modality. Whenever more than 2 threshold measurements were obtained for a certain time point, the two closest values were averaged and considered for subsequent statistical analysis.

2.7 Sample size calculation

We considered that a 20% threshold difference due to conditioning would indicate a clinically significant CPM. Therefore, for paired T test, a power of 0.8 and alpha 0.05, assuming a SD of 20%, a minimum of 10 horses were deemed necessary. To compensate for a potential drop-out rate of 20% (if horses would show stress or aversion during one of the sessions), 12 horses were included in this experiment.

2.8 Statistical analysis

Statistical analysis was performed using Sigma Plot for Windows (Sigma Plot Version 14; Systat Software GmbH, Germany). Descriptive statistics was used for demographic data. Continuous data were checked for normality of distribution using Shapiro-Wilk normality test. Given the non-normal distribution of several variables, data were reported as median [interquartile range (IQR)].

Median and maximal individual stress and aversion scores recorded during the two experimental sessions were compared with the Wilcoxon Signed Rank test. To compare thresholds and physiological values recorded at different time points, the Friedman test with Tukey test for posthoc pairwise analysis was implemented. A P value <0.05 was considered statistically significant.

According to recommended standards for reporting of CPM experiments, differences between thresholds recorded before and during conditioning had to be negative to indicate inhibition. Absolute threshold differences ($\Delta = T_{baseline}$-T_{twitch}) as well as percent changes were determined for the different stimulation modalities. For mechanical and electrical test stimuli, the percent (%) change observed during conditioning compared to baseline was calculated as $[(T_{twitch} - T_{baseline})/(T_{baseline})]*100$. A threshold modification of at least 20% was considered clinically relevant. Due to the relative nature of the centigrade temperature scale, the calculation of the percent change from baseline for thermal threshold was not performed, as previously suggested (17).

3 Results

Twelve healthy horses were included in the study, ten Warmblood (all geldings) and two Freiberger (one gelding and one mare). The median (IQR) age of horses was 8 (5.5–16) year-old and they weighed 570 (535–607) kg, with a body condition score of 3 (3.0–3.4). The ambient temperature during the experimental sessions ranged between 5.1°C and 14.8°C.

Complete data were collected from ten horses in the NWR session and from seven in the PP/T session, respectively. In the first session, ten out of twelve horses completed the experiment, while in the second seven out of ten did. The incomplete data collection leading to the interruption of the experiment was due to severe aversion

(score = 4) at the beginning or during application of the twitch in 5 occasions. If this happened during the first experiment, the second did not take place (2 cases, one occurring in the NWR session and one in the PP/T session). Details are presented in Table 2.

Twitch application force was monitored in 9 experimental sessions. The recorded force ranged between 4 and 12 *N*, with a median value of 7.55 *N*. It has to be noticed that force was not constantly applied during twitching, as it was continuously adjusted (slightly released and tensed again) to compensate for the horse's head movements and to avoid slipping, as commonly done in practice.

For the completed sessions, maximal stress scores recorded before and after twitching were always lower than the cut-off. Median stress scores recorded during the 2 stimulation sessions were not statistically different, being 1 (1-1) in both sessions. Aversion scores, attributed every minute during twitch application, varied over time, often reaching a score of 3 (moderate aversion) at one of the observation time points. Median aversion score was 2 (1.75–2.25) during the NWR session and 2 (2-2) during the PP/T session.

3.1 NWR session

Complete data sets were collected for 10 horses. Conditioning significantly affected the NWR threshold (Friedmann test: $P = 0.002$). At baseline, it was 4.4 (2.5–5.8) mA. During twitch application, it raised to a median peak value of 8 (4.7–11) mA (Tukey test: $P = 0.002$) and after removal it decreased to 5.6 (2.0–7.8) mA (interval 0–5 min) and then to 5.1 (2.9–6.9) mA (interval 5–10 min). The peak NWR threshold was reached 4 (2.7–5) minutes after twitch application (Figure 3). The ΔNWR was −2.8 (−3.9 to −1.1) mA. The median percent change during conditioning was 87.9 (65.7–118.2)% (Figure 4).

3.2 Pp/T session

Complete data sets were collected for 7 horses. Conditioning stimulation significantly affected pressure pain and thermal thresholds (Friedman $P = 0.003$ for both stimulation modalities). At baseline, pressure pain threshold was 6.0 (3.2, 6.7) *N*. During twitch application it raised to 25 (11.4, 27.5) *N* and after removal it decreased to 4.2 (3.3, 9.3) N (interval 5–10 min) and then to 3.5 (3.2, 8.300) *N* (interval 15–20 min) (Tukey $P = 0.005$) (Figure 5). The ΔPPT was −18.2 (−6.4, −21.4) *N*. The median percent change during conditioning was 343.5 (140, 365.3)% (Figure 4). At baseline, thermal threshold was 47.6 (45.8, 49.9)°C. During twitch application it raised to 52 (49.1, 55)°C (Tukey $P = 0.04$) and after removal it decreased to 46 (44.1, 48.3)°C (Tukey $P = 0.004$) and then to 48.1 (45.0, 49.1)°C (Tukey $P = 0.03$) (Figure 6). The ΔT was −3.1 (−9.2, −2.1)°C (Figure 4).

3.3 Heart rate and respiratory rate

A statistically significant change in heart rate was observed in both experimental sessions over time (Friedman, $P < 0.001$ for the NWR session and $P = 0.007$ for the PP/T session). Values are reported in Table 2. Overall, median heart rate increased during conditioning compared to baseline and then decreased thereafter. However, only three horses in the NWR session and none in the PP/T session showed >20% increase in heart rate compared to baseline. Two horses in the NWR session (Horse 8 and 9) and three in the PP/T session (Horse 8, 9 and 11) showed a decrease in HR during twitching compared to baseline. Differences between sessions were present only during the baseline stimulation, a higher heart rate being recorded during NWR [39 (34.5–42.1) beats/min] than during PP/T [33.3 (32.6–37.5) beats/min] (Wilcoxon Signed Rank Test $P = 0.03$). A significant change in respiratory rate was observed in both sessions over time (Friedman, $P < 0.001$ for the NWR session and $P = 0.002$ for the PP/T session). Values are reported in Table 2. In both sessions, respiratory rate increased during conditioning and then decreased thereafter. Nine out of 10 horses in the NWR session and all horses in the PP/T session showed >20% increase in respiratory rate. No differences in respiratory rate between sessions were present in any of the experimental phases. Results are reported in Table 3 and figures in Supplementary material (Supplemmentary Figures S2, S3).

TABLE 2 Individual horses included in the study, including their weight, age, sex and breed. For each experimental session it is reported whether the session was completed, interrupted or not performed based on the predefined criteria. Horses for which the first session had to be interrupted, did not participate in the second session (not performed).

Horse	Weight (kg)	Age (years)	Sex	Breed	NWR session	PP/T session
H1	550	16	Gelding	Warmblood	Completed	Interrupted
H2	660	19	Gelding	Warmblood	Interrupted	Not performed
H3	560	7	Gelding	Warmblood	Completed	Completed
H4	600	5	Gelding	Warmblood	Completed	Completed
H5	650	16	Gelding	Warmblood	Completed	Completed
H6	455	3	Gelding	Freiberger	Completed	Completed
H7	570	16	Gelding	Warmblood	Completed	Interrupted
H8	530	7	Gelding	Warmblood	Completed	Completed
H9	455	3	Mare	Freiberger	Completed	Completed
H10	610	9	Gelding	Warmblood	Completed	Interrupted
H11	570	16	Gelding	Warmblood	Completed	Completed
H12	570	7	Gelding	Warmblood	Not performed	Interrupted

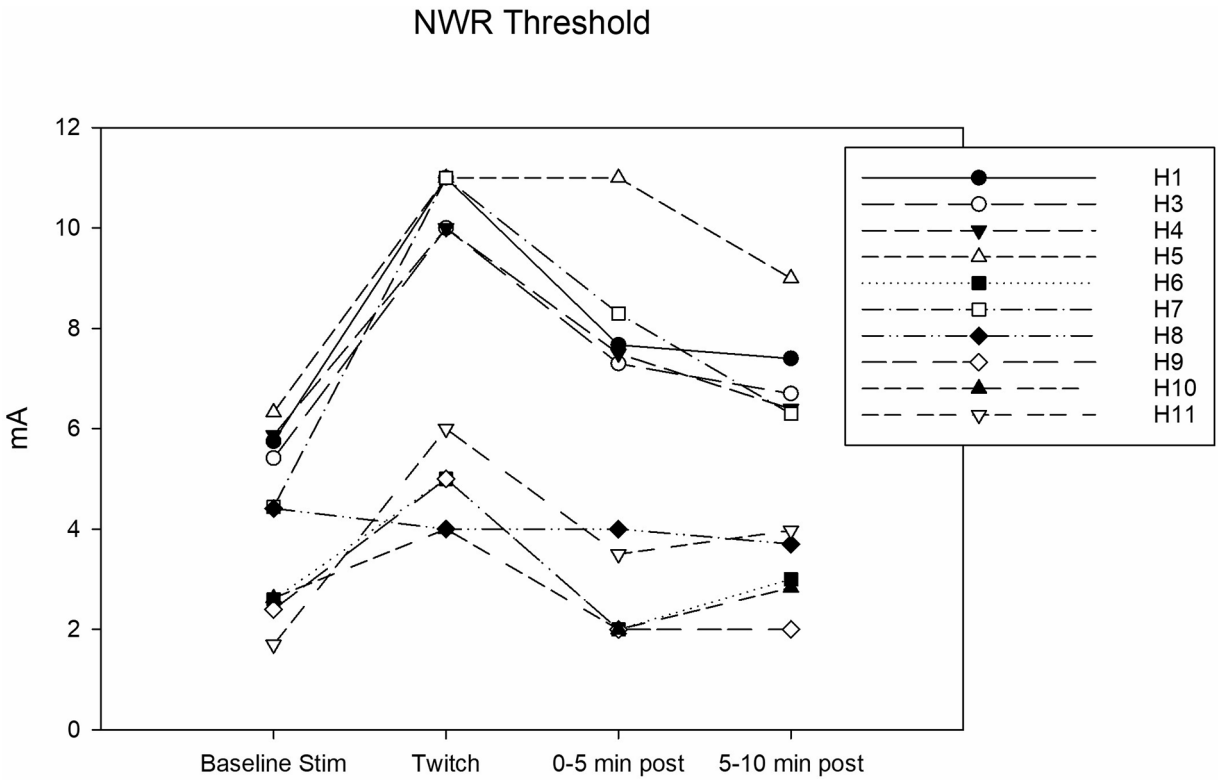

FIGURE 3
Individual nociceptive withdrawal reflex (NWR) thresholds in mA determined during the following experimental phases: baseline stimulation, twitch, and post twitch (intervals 0–5 min and 5-10 min after twitch removal). All the horses that completed the session are represented.

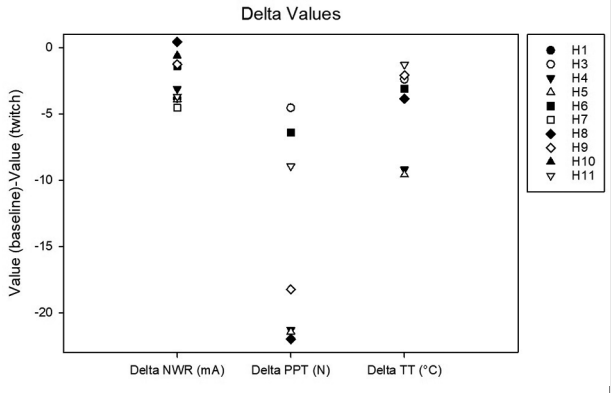

FIGURE 4
Delta values calculated as the difference between baseline and twitch threshold values for the nociceptive withdrawal reflex (NWR), for pain pressure (PP) and for thermal (T).

4 Discussion

In the current investigation, the utilization of a lip twitch acted as a reliable conditioning stimulus, effectively dampening responses to concurrent nociceptive inputs. This was evidenced by a remarkable elevation in thresholds to electrical, mechanical, and thermal stimulations in the healthy equine subjects under study. Notably, for both electrical and mechanical stimulations, the threshold increase surpassed the hypothesized minimum of 20%, and for thermal stimulations, the observed increase paralleled findings reported following the administration of opioid analgesics in previous studies (18, 19).

The common practice of employing a lip twitch as a method of restraint traces its roots back to ancient Greek and Roman times. Initially, its usage was associated with the observation that horses subjected to twitching tended to exhibit greater tolerance to painful procedures performed on distant body regions, thereby rendering them less hazardous to handle. Already more than two centuries ago, it was hypothesized that pain produced by pressure on the upper lip could diminish the perception and consciousness of pain in other areas (20). In accordance with the results of the present study, other authors reported reduced responses to noxious stimulation during twitching. Lagerveij (21) described a weaker behavioural response to repeated needle pin prick stimulations along the back in horses in presence of the lip twitch and similar observations were described later for donkeys (22). Furthermore, using a semi-quantitative approach, it could be shown that twitching was able to considerably increase thresholds to electrical (23) and thermal stimulations (20) in horses. While weaknesses in reporting and differences in stimulation paradigms do not allow direct data comparison, current evidence and clinical experience unequivocally indicate that twitching evokes a certain degree of

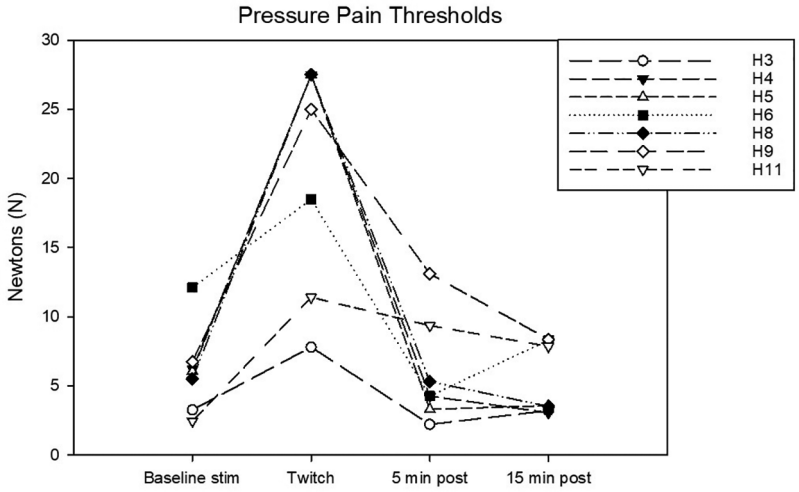

FIGURE 5
Individual pressure pain (PP) thresholds in N determined during the following experimental phases: baseline stimulation, twitch, and post twitch (intervals 5–10 min and 15–20 min after twitch removal). All the horses that completed the session are represented.

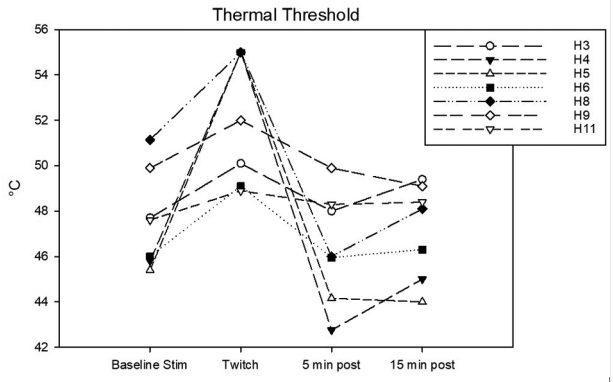

FIGURE 6
Individual thermal thresholds (T) in °C determined during the following experimental phases: baseline stimulation, twitch, and post twitch (intervals 5–10 min and 15–20 min after twitch removal). All the horses that completed the session are represented.

antinociception. Interestingly, the physiological mechanism responsible for such effect has been and continues to be the subject of significant scientific debate in equine veterinary research. Among the proposed theories, the "pain inhibits pain" phenomenon, an acupuncture-like effect and stress-induced analgesia are the most mentioned (20). In common, they all assume a central inhibitory effect, potentially also responsible for the sedation and immobilization concomitantly observed. The supposed implication of the endogenous opioid system in its mechanism of action inspired the current study, aiming at testing whether the lip twitch could be used as a conditioning stimulus in a CPM experimental paradigm.

In human studies evaluating CPM, the primary conditioning methods employed include cold pressor pain, which involves immersing the forearm into cold water; noxious heat stimulation, typically administered through a water bath or contact thermode; and the ischemic arm technique, utilizing a tourniquet inflated at a predetermined pressure to induce pain. Among these techniques, the cold pressor pain test consistently demonstrates the most robust and reliable CPM effect (24–26), with the ischemic arm technique following closely behind in terms of efficacy.

In domestic animals, few CPM paradigms have been described. While in calves the ischemic arm technique was deemed adequate to evaluate CPM (27), only continuous mechanical stimulation gave reliable results in dogs (28). Most of the evidence obtained so far suggests that the conditioning stimulus must be noxious to evoke CPM, and that the intensity of pain evoked is associated to the degree of CPM efficacy (12, 29). Furthermore, there seems to be an additive effect of distraction on CPM efficacy, but distraction itself cannot explain the entire CPM effect (30).

TABLE 3 Medians and interquartile ranges for heart rate and respiratory rate for the two sessions (NWR and PP/T) for the following phases: baseline, baseline stimulation, twitch and post-twitch (intervals 0–5 min, 5–10 min after twitch removal).

	Session	Baseline	Baseline Stim	Twitch	0–5 min post	5–10 min post	*P* value
Heart rate (beats/min)	NWR	35.4 (34.4,38.0)	39.1 (34.8,40.9)	41.9 (37.1,43.2)	36.7 (34.8,40.3)	34.2 (32.1,35.8)	<0.001
	PP/T	33.4 (33.3,39.6)	33.4 (32.6,37.5)	36.4 (34.5,37.0)	37.4 (34.6,43.1)	33.1 (30.2,39.7)	0.006
Respiratory rate (breaths/min)	NWR	12 (9.6,14.8)	11.8 (10.0,12.4)	27.3 (15.3,31.7)	14.2 (12,16)	11.8 (10.3,12.8)	<0.001
	PP/T	12 (12,14)	12 (12,18)	28 (16,36)	12 (12,18)	12 (8,20)	0.002

Interestingly, distraction was also thought to be partly implicated in the lip twitch efficacy in early reports, under the so-called hypothesis of "divertive pain" (21). In human subjects, various stimulation modalities are employed as "test stimuli" to gauge the extent of CPM. These include thermal, mechanical, chemical, and electrical stimuli, each utilized with phasic, tonic, or summation approaches. Noxious stimuli can be administered either to a remote location or to the same body part as the one undergoing conditioning stimulation. Additionally, the tissues targeted for stimulation may be superficial (e.g., skin) or deep (e.g., muscles, viscera), providing a comprehensive assessment of pain modulation mechanisms (12). In the context of the present study, three distinct stimulation modalities were applied across two experimental sessions. The adoption of multiple test stimuli within the same experimental setup has been recommended to enhance mechanistic comprehension and confirm test validity (31).

During one session, electrical stimulation was employed to elicit the NWR using a continuous automated threshold tracking device. This device relies on a quantifiable neurophysiological outcome, specifically electromyographic activity recorded within a defined post-stimulation time epoch, to establish and modify the input, namely the stimulation intensity.

The methodology employed in the current study builds upon several previous reports that have delineated the NWR and its pharmacological modulation in horses. Furthermore, the NWR model has been used in humans to assess CPM (32, 33). The utilization of continuous tracking, as opposed to a singular threshold definition, permits the ongoing assessment of treatment efficacy or procedural effects in a continuous manner, thereby facilitating a more precise determination of onset and duration of action. Given that the threshold determination process was automated and continuous, with randomized yet brief interstimulation intervals, it would have posed a risk of interference to combine this stimulation modality with another. Consequently, the other two modalities, thermal and mechanical, were administered in a distinct session to ensure data integrity and prevent potential confounding factors. In the PP/T session, an interstimulation interval of at least 60 s was adhered to between two consecutive stimuli. Additionally, stimulation was avoided during the initial 2 min following twitch application. This measure was implemented based on findings from previous reports, which suggested that a certain time is necessary for the onset of twitch action to manifest effectively. While thermal testing was performed with a hand-held device, the mechanical was based on a fixed-mounted design. Advantage of this last one was that no additional contact with the horse was necessary during the experiment. Conversely, in the case of thermal stimulation, prompt removal of the thermode upon reaching the threshold was practiced. This approach served to mitigate the potential risk of sensitization, as cooling of the sensor may occur with a slight delay even after the stimulus is discontinued. Given that various stimulation modalities exhibit differential responses to analgesic agents, the concurrent use of multiple tests aimed to investigate whether twitching exerts a specific inhibitory effect on particular afferent inputs, thus offering deeper insights into its mechanisms of action. For instance, electrically induced NWR has demonstrated heightened sensitivity to alpha-2 agonists and local anesthetics, whereas its responsiveness to opioids is comparatively reduced. Conversely, the sensitivity of thermal stimuli applied at slow increasing rates is known to be diminished by opioids. Considering the observed effects across all three modalities, it is reasonable to infer that both opioid and non-opioid mechanisms of endogenous pain inhibition were activated by the lip twitch in the horses of the present study.

The contribution of the endogenous opioid system in the mechanism of action of the lip twitch has been substantiated by several studies. Most of the reports indicate an early (21–23) or even immediate (20) rise in β-endorphin after application, followed by a continuous rise with a peak occurring at around 5 min and a tendence to decline thereafter. Such a decline was hypothesized to explain the biphasic effect observed in case of prolonged twitch application, with sedation observed in the first 5 min followed by restlessness, aversive behaviour and high sympathetic tone thereafter (34). In Lagerweji (21), the observed increased in β-endorphin levels were interpreted as a demonstration that twitching acts as acupuncture in inducing sedation and analgesia. However, a large body of evidence has shown that in equines β-endorphines are released during early stages of stress (35) and in acute painful conditions such as during colic (36). Its precursor, proopiomelanocortin (POMC) is produced in the anterior pituitary in response to increasing levels of hypothalamic corticotropin releasing hormone (CRH). In presence of a stressor, the activation of the autonomic nervous system and hypothalamic pituitary adrenal (HPA) axis promotes the secretion of circulating catecholamines, β-endorphines, adrenocorticotropic hormone (ACTH), and cortisol. All these substances have been abundantly used to monitor animal welfare and emotional responses to stressors in the literature (35). In equines, β-endorphin release appears to occur early during stressful events. Through a negative feedback mechanism, it inhibits the secretion of CRH, suggesting a role in modulating the stress cascade. Furthermore, this release pattern may facilitate active coping strategies and mitigate pain, as previously shown (35, 36). In the present study, stress hormones were not measured, thus their potential correlation with the observed antinociceptive effects cannot be directly investigated.

On the contrary, heart rate and respiratory rate were measured before, during and after twitching. While heart rate and heart rate variability have been monitored in several other twitch studies (20–22, 34, 37, 38), previous data about respiratory rates could not be found in the literature. Our heart rate findings overlap with those previously reported by some authors (20, 38). We observed individual variations in the heart rate response to twitch, with some horses increasing and other decreasing frequency. Overall, the extent of variation was rather low (always lower than 20%), and the same horses which responded with bradycardia to the first challenge did the same at the second occasion, indicating that there is a rather individual predisposition for the direction of response. As most of the horses displayed bradycardia in response to twitch in other studies (21, 34, 37), a trigemino-vagal reflex with a shift toward parasympathetic dominance was hypothesized and associated to the analgesic and sedative effect observed. On the contrary, in a study in donkeys, heart rate and heart rate variability

data indicated increased sympathetic tone in these animals, which also showed aversive behaviour (22). Thus, the present and past evidence regarding twitch effects on heart rate is rather inconclusive and points toward individual differences and external factors that might induce a shift of the autonomic balance toward either the sympathetic or parasympathetic dominance. Further work on the collected heart rate variability data, not included in the current report, might contribute to a better understanding of this phenomenon.

Differently from what observed for heart rate, an impressive, clinically relevant rise in respiratory rate was consistently observed during the twitch application in all the horses of the present study. Interestingly, as soon as the twitch was removed, this parameter immediately normalized. Release of plasma catecholamines, adrenaline, noradrenaline and dopamine, classically accompanies the activation of the sympathetic adrenomedullary system, which reflects the most immediate, but also generally short lasting, response to stress. Catecholamines relax bronchioles and increase ventilation, thus preparing the organism for a flight and fight reaction (39). In a study in horses, adrenalin and noradrenalin increased significantly after twitch application, with adrenalin levels remaining higher than noradrenalin longer after removal. Hematocrit increased quickly after application too, indicating that twitching acts as a potent stressor, while cortisol increase was delayed, as expected due to its physiological function and release pattern (20). These observations would substantiate the hypothesis that the effects of the twitch, commonly targeted in clinical practice, are at least partially mediated by a stress-related sympathetic activation, one of the mechanisms known to be implicated in endogenous pain inhibition (40). Whether stress is rather related to pain, thus to a physiologically mediated event or to forceful restraint, thus rather an emotional challenge, or both cannot be distinguished at present. Further investigations, including both physical challenges and emotional stressors, such as social isolation, confinement in unfamiliar environments, and novel object tests (41), are necessary to explore how these factors differently affect endogenous pain modulation in horses.Concerning behaviour, the literature reveals a wide spectrum of possible reactions to the lip twitch, spanning from deep sedation to freezing, clear aversion to handling, escape behaviour, or even dangerous fight attitudes. Most reports described sedative effects or even lethargy in a high proportion of subjects. Interestingly, the administration of naloxone reversed sedation, leading to an increase occurrence of aversive behaviour (20, 21). These findings suggest a potential association between sedation and the levels of circulating b-endorphins or other endogenous opioids. In the study by Schelp (20) and in a study involving donkeys (22), it was highlighted that not all subjects displayed sedation following twitching. However, antinociception was still evident, suggesting that the lip twitch might activate both opioidergic and non-opioidergic mechanisms of endogenous pain inhibition.

In the current study, a notably high occurrence of aversive behaviour was observed, surpassing what is typically encountered in a mixed population of equine patients treated in field or clinical conditions. This heightened prevalence could potentially be attributed to the specific population of horses included in the study, all of which were selected for demanding public tasks within the armed forces. It is plausible that their personality traits are distinct and predispose them to more proactive reactions during forced restraint. However, this hypothesis would require further investigation to be confirmed. Furthermore, the test stimuli applied before twitching to obtain baseline thresholds might have caused a subliminal arousal state, that might have modified subsequent behavioural reactions to the conditioning. This hypothesis might be at least partly confirmed by the observation that in the NWR session, the heart rate during baseline stimulation was significantly higher than during baseline in absence of stimulation. Future specific HRV investigations on the presented HR data will possibly contribute to define the role of the autonomic balance in the twitch action and in endogenous pain modulation mechanisms in horses.

In summary, while several factors such as individual predisposition, past experiences, environment, and coexisting stressors could contribute to determine the behavioural response, predicting a specific pattern appears unrealistic at present. The complexity and variability of equine behaviour underscores the need for further research to better understand the dynamics at play in response to the lip twitch.

In the current study, it was observed that some horses exhibited sensitization to the lip twitch. It is highly probable that many of these horses had previous experiences with this restrain method. This likelihood could explain the exclusion of certain horses during the first experimental session and, most likely, their subsequent exclusion during the second session as well. Research has indicated that sensitization to aversive events can occur in horses after relatively few exposures and such sensitization can persist for extended periods of time (42). While it would certainly be interesting to address this issue specifically in future investigations, it can be deduced that for certain subjects, lip twitching constituted a stressful, potentially fear-inducing, and aversive event.

The current study has several limitations. First, horses needed to be retrained to perform the testing, and this might have induced a basic level of stress influencing the whole procedure and potentially the CPM results. Future studies will need to compare antinociceptive extent of conditioning with and without restrain to evaluate whether this factor has a relevant influence. To this end, different conditioning and test stimulations modalities will need to be used, as the ones adopted in the present study cannot be applied in freely moving animals. Thus, this limitation is inherent to the lip twitching, which must be performed by a handler holding it continuously as conditioning stimulus. Second, the limited number of subjects included, and in particular of females, precludes thoroughly investigating the role of influencing factors on the different behavioural and physiological response patterns observed. This would need a bigger sample size to provide credible results. Third, as mentioned above, plasmatic concentrations of stress hormones, catecholamines and endogenous opioids were not measured. Blood sampling implies an additional handling, and an increased severity level, even if performed through an indwelling catheter. As the focus of the current investigation was on antinociception, it was decided to minimize sources of distraction and the number of interventions needed around the experiment.

In conclusion, based on the current findings, the question of whether lip twitching should be utilized as a conditioning method in CPM studies appears debatable. On one hand, if conditioning is to effectively induce pain, aversive behavioural signs must be inherently tolerated to some extent; on the other, it is imperative to establish stringent cut-off criteria to prevent exposing sensitive individuals to elevated stress and excluding them from testing. Similar considerations should apply to the general use of the lip twitch as a restrain technique in horses.

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 authors.

Ethics statement

The animal study was approved by Cantonal Committee for Animal Experiment, Canton Bern, Switzerland. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

SB: Writing – original draft, Writing – review & editing, Data curation. JG: Writing – review & editing. EC: Writing – review & editing. SM: Writing – review & editing. CS: Writing – review & editing, Writing – original draft.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. The horses were provided free of charge by the Swiss Armed Forces. Two of the authors (S. Blum and J. Gisler) received temporary funding from the Swiss Armed Forces - Veterinary Department.

Acknowledgments

We would like to thank all those involved in the completion of this thesis. Especially the people who helped us with the data collection. Namely: Dr. med. vet. Alessandro Mirra Dipl. ECVAA PhD, Dr med. vet. Larissa Mattei, Dr. med. vet. Ekaterina Gamez and med. vet. Nadja Karol. We thank Manuela Hofer and Fritz Schmid from the NPZ for their support during the experimental sessions. A special thanks goes to med. vet. Dany Elzahaby for his help during the submission process.

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.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpain.2024.1463688/full#supplementary-material

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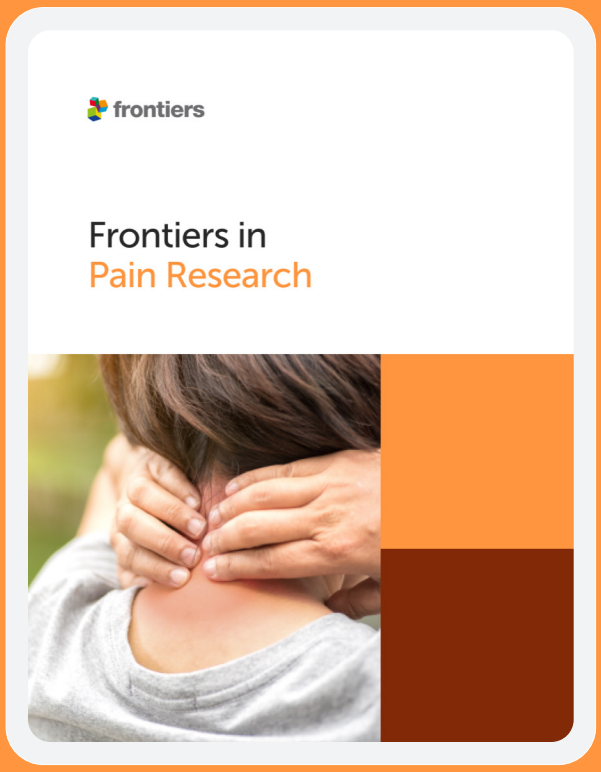

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