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Towards an improved pain assessment in castrated horses using facial expressions (HGS) and circulating miRNAs

E. Dalla Costa[1], F. Dai[1], C. Lecchi[1], F. Ambrogi[2], D. Lebelt[3], D. Stucke[4], G. Ravasio[1], F. Ceciliani[1], M. Minero[1]

1 Università degli Studi di Milano, Dipartimento di Medina Veterinaria, Via Dell'Università 6, 26900 Lodi, Italy

2 Università degli Studi di Milano, Dipartimento di Scienze Cliniche e di Comunità, Via Vanzetti 5, 20133 Milano, Italy

3 Equine Research & Consulting, 07140 Sencelles, Spain

4 Landratsamt Ravensburg, Veterinäramt, Friedenstraße 2, 88212 Ravensburg, Germany

Corresponding author: Cristina Lecchi. Email: cristina.lecchi@unimi.it

Abstract

Background. Pain in horses is an emergent welfare concern and its assessment represents a challenge for equine clinicians. This study aimed at improving pain assessment in horses through a convergent validation of existing tools: we investigated whether an effective analgesic treatment influences the Horse Grimace Scale (HGS) and the concentration of specific circulating miRNAs.

Methods. Eleven stallions underwent routine surgical castration under general anaesthesia. They were divided into two analgesic treatment groups: castration with the administration of pre-operative flunixin; castration with pre-operative flunixin plus a local injection of mepivacaine into the spermatic cords. HGS and levels of seven circulating miRNAs were evaluated pre-, 8- and 20h post-procedure.

Results. Compared to pre-castration, HGS, miR-126-5p, miR-145 and miR-let7e increased significantly in horses receiving flunixin at 8h post-castration (Friedman test, $p<0.05$). Both behavioural and molecular changes occurred in horses receiving flunixin only, confirming that the addition of local mepivacaine is an effective analgesic treatment.

Conclusions. Combining the use of HGS and circulating miRNAs, particularly miR-145, could be meaningful to monitor acute pain conditions in horses. Our results further validate the HGS as a method to assess acute pain in horses and point out miR-145 as a promising biomarker to identify pain.

Introduction

Pain assessment in horses is an important clinical issue and a welfare concern (1). Nevertheless, pain assessment is still tricky as no gold standard is available for equine clinicians. During the past decade, several pain scales including both physiological and behavioural parameters were developed (see (2) for a review), among which the Composite Pain Scale (CPS) and the Horse Grimace Scale (HGS) seemed the most promising. The CPS is a multidimensional scale focusing on the presence of pain-related behaviours and the changes in the frequency of standard behavioural patterns and physiological parameters (3). CPS is reported to be reliably applied to assess acute orthopaedic and visceral pain (4,5). However, CPS needs experienced observers to assess pain-related behaviour and the palpation of the painful area. The HGS, a facial-expression-based pain coding system, consists of the sum of six Facial Action Units (FAUs) (6,7). The HGS is reported to be a reliable tool for identifying acute pain in different conditions (6,7), allowing to classify horses in pain with an accuracy greater than 70% (8). Further validation with other indicators is required to evaluate its potential application to identify the efficacy of analgesic treatments. Recently, microRNAs (miRNAs) were investigated in equine medicine as an innovative approach to determine acute pain, identifying the increase of circulating miR-23b-3p, miR-145-5p and miR-200b-3p in horses with acute laminitis (9). Further studies on these molecular indicators are required to understand better whether their modulation is linked with acute pain perception, inflammation or both; covering this gap is pivotal to start an effective pain treatment. Even though the existing pain assessment measures allow equine clinicians to make considerable progress in identifying acute pain in horses, further studies are needed to understand whether these measures can be used as valid tools to support clinical decisions. As miRNA modulate pain neuronal pathways, their knowledge may be useful to link the molecular background behind pain to already known and established measurement tools, such as HGS and CPS.

Castration provides a good pain model for studying convergent validity of pain indicators. Firstly, the clinical status of the horse before castration is healthy and pain-free. Secondly, castration is a standard procedure, meaning that is equal for all the subjects. Moreover, it is well known that animals experience both acute and chronic pain following surgical castration (10–12). Finally, horse castration is a common husbandry painful procedure, with more than 240,000 horses castrated in Europe annually (13). Post-castration pain has an important impact on equine welfare (2,14); however, in most countries there are no regulations, nor recommendations, requiring pain-relieving treatment following castration. Pain management following this procedure is still frequently suboptimal (15–17). When analgesic treatments are used, phenylbutazone and flunixin are the NSAID drugs most commonly administered (16,18). As previously reported (6,17,19), the administration of flunixin does not seem to provide sufficient post-castration pain relief, although in equids is generally prescribed for inflammation and pain associated with soft tissue conditions (20). The administration of a local anaesthetic in the spermatic cord has been demonstrated to decrease pain associated with castration (12,17,21). In their

study, Abass and colleagues (17) found that the administration of mepivacaine before surgical castration significantly reduced post-surgical pain scores and cytokine levels.

Based on the above considerations, this study aimed at improving pain assessment in horses undergoing surgical castration through a convergent validation of existing tools: we investigated whether an effective analgesic treatment (the use of a local anaesthetic in the spermatic cords) influences the HGS and the concentration of specific circulating miRNAs.

Methods

Animals and husbandry

Eleven stallions, of different breeds and aged between 1 and 3 years (mean=2.36±0.67), were referred to the equine hospital (Havelland Equine Hospital, Germany) for routine surgical castration under general anaesthesia for husbandry purposes (Table 1). A physical examination and a behavioural evaluation were performed by an equine veterinarian to ensure that all the stallions were healthy and without signs of cryptorchidism. For five days, all subjects were housed in standard single horse boxes (4×3 m with an outside window) on wood shavings (German Horse Span Classic, Wismar, Germany), and in visual contact with other conspecifics. They were fed twice a day with hay (approx. 3 kg/100 kg body weight per day), and water was provided ad libitum by automatic drinkers. Food was withheld from all horses for 8 hours before and 5 hours after anaesthesia (22). Horses were monitored at least 3 times a day by an experienced veterinarian or nurse, who collected data about heart rate, respiratory rate, appetite, defecation, and apparent signs of pain.

Procedure and analgesic treatment groups

A closed technique through a scrotal approach without primary closure of the wound (23) was applied as recommended by the National Equine Welfare Council (24) and the Canadian Veterinary Medical Association (14). One of two equally experienced veterinary surgeons carried out all the surgeries. To evaluate the correct drug doses, horse weight was estimated with a weight tape. The anaesthesia protocol was the same for all the subjects: pre-medication with Romifidine (80 micrograms/Kg i.v., Romifidinehydrochloride, Sedivet, Boehringer Ingelheim Vetmedica), induction with Diazepam (0.1 mg/Kg i.v., Diazepam-Ratiopharm, Ratiopharm) and Ketamine (2.2 mg/Kg i.v., Ketamin 10%, Medistar) intravenously via a jugular catheter. The interval between pre-medication and induction was about 10 minutes. None of these subjects needed an additional injection of ketamine to maintain general anaesthesia. The surgery lasted 10–15 min, all the horses recovered from anaesthesia without assistance and under the visual supervision of a veterinary nurse. No intra-operative complications were reported. All procedures were carried out in the morning, between 9 and 11. Stallions received antibiotic treatment (2–4 mg Trimethoprim and 12 mg Sulfadiazine/Kg p.o. every 12 h, Synutrim 72% Pulver, Vétoquinol) for three days starting from the morning before surgery. Stallions were divided into two breed-matched

treatment groups using a blocked randomization process: castration with pre-operative anti-inflammatory medication (1.1 mg/Kg i.v., Flunixin 5%, Medistar) (N=6); castration with pre-operative anti-inflammatory medication (1.1 mg/Kg i.v., Flunixin 5%, Medistar) and intra-surgical injection (after aseptic preparation of scrotal area and before scrotal incision) of 10 ml mepivacaine 2% (Scandicain 2%, AstraZeneca) into each spermatic cord (N=5). The veterinarian responsible for anaesthesia, aware of group allocation, administered all the medications.

Horse Grimace Scale (HGS) and Composite Pain Scale (CPS) assessment

Thirty minutes video-recordings were collected using two digital video cameras (Panasonic, HDC-SD99) at three-time points: pre-castration (after two days of acclimatisation to the clinical environment); 8-hours and 20-hours post-castration. HGS was measured as previously described (6,7,25): the image set, consisting of 99 still images, was scored by four trained treatment- and time point-blind assessors. The CPS used in this study was the same used in Dalla Costa and others (6) and it is reported in Table S1. CPS was scored by direct observation by a trained treatment-blind veterinarian pre-, 8h and 20h post-procedure.

MiRNAs measurement

Blood was collected at three-time points (pre- and 8h and 20h post-procedure) by jugular venipuncture into Monovette® tubes (Sarstedt Company, Nümbrecht, Germany) and serum was stored at −80°C until RNA extraction. Serum was thawed on ice and centrifuged at 3000×g for 5 min at 4°C. An aliquot of 150 µl per sample was transferred to a new tube, and RNA was extracted using miRNeasy Serum/Plasma Kits (Qiagen) following the manufacturer's instructions. After incubation at room temperature for 5 min, 25 fmol (final concentration) of the exogenous synthetic spike-in control *Caenorhabditis elegans* miRNA cel-miR-39 (Qiagen) was spiked into samples at the beginning of the extraction procedure. Reverse transcription was performed in 15 µl volume reactions using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems) using miRNA-specific stem-loop RT primers (9). The RT-qPCR experiments were designed following MIQE guidelines (26). Small RNA TaqMan assays were performed following the manufacturer's instructions using the selected primer/probe assays (ThermoFisher Scientific) listed in Table 2 (9). Data were normalized relative to the cel-miR-39 expression. MiRNA expression levels are presented as fold changes normalized to cel-miR-39 expression using the $2^{-\Delta\Delta Cq}$ formula.

miRNA target prioritization

The target genes of DE-miRNAs were predicted using MiRWalk 3.0 (27), which includes 3 miRNA-target prediction programs (miRDB (28), miRTarBase (29) and Targetscan (30)). The analysis was performed targeting the entire gene sequence (5'UTR, CDS, and 3'UTR). The list of target genes predicted by at least two out of three tools was included in

further analysis. The functional mRNA enrichment was performed using DAVID bioinformatics resource (31,32) and biological pathways in the KEGG (33) were examined for enrichment. Since inflammatory response and alterations in neuronal excitability are believed to contribute to the neuropathic pain, an enrichment of mRNA targets that encode for immunologically relevant genes and synapse genes was performed comparing the target genes obtained from miRWalk with the Gene List of ImmPort (https://www.immport.org/home) (34) and Synaptic Genes Database (https://syngoportal.org/) (35).

Statistical analysis

The HGS consisted of the sum of the weighted scores across four FAUs, as reported by Dalla Costa and colleagues (8). Statistical analysis was performed using SPSS 23 (SPSS Inc.). Statistical significance was accepted at $p \leq 0.05$. Inter-rater reliability was evaluated using Intra-class Correlation Coefficient (ICC), also providing correspondent 95% confidence intervals (36,37). To interpret the ICC measures, the guidelines proposed by Cicchetti (37) were applied to evaluate agreement among observers: poor (ICC<0.40); fair (between 0.40 and 0.59); good (between 0.60 and 0.74); excellent (between 0.75 and 1.00). Data were tested for normality and homogeneity of variance using the Kolmogorov-Smirnov and Levene test, respectively. As data were not normally distributed, non-parametric statistical tests were applied. The Friedman test was used to detect differences in CPS, miRNAs concentrations and HGS in groups across time points. Then, a post-hoc test (using Bonferroni correction) was used to determine whether CPS, miRNAs concentrations and HGS differed in the same treatment group at 8 and 20 hours compared to baseline. Data collected on the two treatment groups pre-castration were analysed with Mann–Whitney U test to investigate possible statistical differences between groups. Spearman's rho test was performed to evaluate whether there was any correlation among miR-126-5p, miR-145 and miR-let7e. To analyse the correlation between significant variables (CPS, HGS, miR-126-5p, miR-145 and miR-let-7e) a Principal Component Analysis (PCA, correlation matrix, Varimax rotation) on standardised data was applied.

Results

Horse Grimace Scale (HGS) and Composite Pain Scale (CPS)

ICC values on average scores for each FAU and correspondent 95%-confidence intervals are reported in Table 3. The inter-observer reliability was "excellent" for "Stiffly backwards ears", "Orbital tightening" and "Prominent strained chewing muscles" with average ICC values ranging from 0.82 to 0.96. "Good reliability" was demonstrated for "Tension above eye area" (ICC=0.71), "Mouth strained" (ICC = 0.694) and "Strained nostrils" (ICC = 0.693). The mean HGS for each treatment group over time is presented in Figure 1A. Pre-castration, no differences between treatment groups were found (Mann–Whitney U test, $p>0.05$). Compared to basal, HGS increased significantly in horses receiving only pre-operative administration of flunixin (Friedman test, $p=0.003$), both at 8 (post-hoc test using Bonferroni correction,

p=0.030) and 20 hours (post-hoc test using Bonferroni correction, p=0.002) post-castration. No differences in HGS were found over time in horses receiving preoperative administration of flunixin followed by local mepivacaine in the spermatic cord.

The mean CPS score for each treatment group over time is presented in Figure 1B. Before castration, no statistical differences were found in CPS between groups (Mann–Whitney U test, p>0.05). Compared to basal, CPS increased significantly in horses receiving only pre-operative administration of flunixin (Friedman test, p=0.013) at 8 hours post-castration (post-hoc test using Bonferroni correction, p=0.014). At 20 hours post-castration, no statistical differences were found in CPS compared to basal values. No differences were found in CPS over time in horses receiving preoperative administration of flunixin followed by local mepivacaine in the spermatic cord.

Castration without mepivacaine alters the abundance of miR-126-5p, miR-145 and miR-let7e

The relative abundance of seven miRNAs was quantified using RT-qPCR to identify differentially expressed (DE) miRNAs. The expression levels of target miRNAs were normalized to the abundance of cel-miR-39. The selected miRNAs were detected in all samples, except for miR-143, the Cq of which were ≥34.6. The results provided the evidence that the abundance of three circulating miRNAs – miR-126-5p, miR-145 and miR-let7e – were significantly modulated in horses receiving only pre-operative administration of flunixin (e.g. without local mepivacaine treatment). In details, compared to basal, miR-145, miR-let7e and miR-126-5p were significantly up-expressed in horses receiving only pre-operative administration of flunixin (Friedman test, p<0.05) at 8 hours (post-hoc using Bonferroni correction, p=0.009, p=0.014 and p=0.006, respectively). An overview of the results is presented in Figure 2 (A-C). No differences in miRNA levels were found over time in horses receiving pre-operative administration of flunixin followed by local mepivacaine in the spermatic cord. A positive correlation was observed between miR-145 and miR-Let7e (R^2=0.724; p=0.000) and miR-126 (R^2=0.580; p=0.000).

miRNA localization, target prediction, and pathway enrichment

Predicted targets of DE-miRNAs were computationally retrieved from miRWalk resources and the mRNA enrichment was performed using DAVID bioinformatics tools. The predicted mRNA targets of over-expressed miRNAs were 753 (407 at 3'UTR, 68 at 5'UTR and 278 at CDS), of which 40 and 70 were immune- and synapse-related, respectively. The list of immune and synapse-related relevant genes (Table 4) was employed in further analysis. To elucidate the associated functions of the DE-miRNAs, Gene Ontology (GO) analysis was performed, including the categories biological process (BP), cellular component (CC) and molecular function (MF). Most BP items mainly included genes involved in ion transmembrane transport (Figure 3A); the enriched CC converged on genes associated with an integral component of the

plasma membrane and channel complex (Figure 3B), while MF on channel activity (Figure 3C). The top 10 significantly enriched KEGG pathways, with the exclusion of cancer and infection-related pathways, are reported in Figure 4. The DE-miRNAs were identified to be predominantly involved in the calcium signalling pathway and adrenergic signalling in cardiomyocytes.

The relationship among CPS, HGS, miR-145, miR-Let7e and miR-126-5p

CPS and HGS were analysed together with the expression levels of miR-145, miR-Let7e and miR-126-5p using a Principal Component Analysis (PCA) on standardised data. The PCA revealed three main components explaining 84,68% of total variation between horses 8h post-castration. The PCA identified two main factors with Eigen value greater than 1. Horses with high positive scores on the first Principal Component accounting for 39.20% of the total variance belong to the group receiving only flunixin (Figure 5). MiR-Let7e and miR-126-5p load on the second Principal Component accounting for 26.65% of the variance, but with opposite sign in respect to miR-145, CPS and HGS.

Discussion

Based on the hypothesis that an effective analgesic treatment would affect behavioural parameters and molecular markers, our results show that behavioural parameters such as HGS and CPS and circulating levels of miR-145, miR-Let7e and miR-126-5p were significantly higher at 8 post-castration in horses receiving only a pre-operative administration of flunixin, further validating the HGS as a method to assess acute pain in horses undergoing surgical castration and pointing out miR-145 as a promising biomarker to identify pain. Indeed, miR-145 concentration was meaningfully associated with HGS and CPS. Even though the administration of general anaesthesia and flunixin should be considered for their potential confounding effect, an untreated control group undergoing castration without any analgesic treatment was not included in this study for ethical reasons, as this would have subjected the horses to risks of serious harm. Moreover, it was proven in previous studies (6) that general anaesthesia does not affect HGS and that horses receiving a single preoperative administration of flunixin differences following a routine surgical castration showed a significant increase in HGS scores.

Castration is a painful husbandry procedure, that can be only inconsistently mitigated through NSAIDs (6,15,17,38). The combined use of NSAID with mepivacaine in the spermatic cord is effective in reducing pain associated with castration (12,17,21). The present study demonstrated for the first time that HGS, scored by time- and treatment- blinded assessors, discriminated between groups with different analgesic treatments. Compared to pre-castration, horses treated with a single administration of flunixin showed a significant increase of the HGS up to 20h post-surgery, suggesting that pain persisted much more extended than 8h (39). The additional intra-surgical injection of mepivacaine into each spermatic cord exerted adequate analgesia: post-castration, both HGS and CPS scores were comparable to pre-castration ones. When used in perineural analgesia, the local action of mepivacaine subsides between 1 and 2 hours (40) In the present study, the effect

of local anaesthesia is likely to be responsible for the relevant perioperative pain reduction over 8h post-castration. In humans, a prolonged effect of perioperative local analgesia on postoperative pain up to 24 hours is well known and might be explained by changes of synaptic neuroplasticity due to a reduced post-operative nerve impulse activity (41). The ability of local administration of mepivacaine to reduce the levels of IL-6 and TNF-α, two pro-inflammatory cytokines reflecting the severity of the injury and the inflammatory response, was previously described (17).

MiRNAs are upstream regulators of pain progression and maintenance due to their ability to post-transcriptionally modulate the expression of genes. MiRNA dysregulation is reported in both inflammatory and neuropathic pain within several experimental models and clinical pain disorders. They can, therefore, provide a useful source of pain biomarkers in whole blood, plasma or serum (42). The process of peripheral sensitization or nociceptor hyperexcitability can occur after peripheral nerve injuries, affecting primary afferents and involving modulation of ion channel permeability and inflammatory genes. Additionally, expression signature from blood has the advantage of being minimally invasive, easy to perform and repeatable over time. In the present study, we investigated the alteration of circulating miRNAs in a well-established model of visceral inflammatory pain in veterinary medicine, attempting to quantify the impact of therapy by pre-operative administration of flunixin with or without intra-surgical administration of the local anaesthetic mepivacaine into each spermatic cord on pain manifestation and maintenance. The results showed that castration with pre-operative administration of flunixin alone induced dysregulation of three circulating miRNAs, miR-145, miR-let-7e and miR-126, the levels of which were restored to that of pre-operative amounts with the intra-operative administration of mepivacaine in combination with the pre-operative administration of flunixin. These results suggested that molecular changes related to pain occurred in the absence of local anaesthetic administration. The dysregulation of miR-145 as related to pain in horse laminitis was previously reported (9). Being differently abundant in both pain models, miR-145 may be particularly useful as an unspecific biomarker of pain in horses. Let-7 family, which is highly expressed in horse plasma (43), is involved in stem cell differentiation and cell proliferation and differentiation and the modulation of the immune response, either promoting or inhibiting the inflammatory response (44). Let-7 family is also involved in neuronal processes, including differentiation (45), cell subtype specification (46), regeneration (47), and synapse formation (48), while extracellular let-7 causes neurodegeneration by targeting toll-like receptor 7 (TLR7) (49). Moreover, nociceptor neurons are excitated by extracellular let-7b via TLR7 and its coupling to the TRPA1 ion channel, causing internal currents and action potentials in dorsal root ganglion neurons and, finally, eliciting pain (50). MiR-126 modulates innate immunity (51) by targeting PI3K/AKT and MAPK signalling pathways (52) and its overexpression suppressed inflammation and ROS production by targeting HMGB1 (53). The Gene Ontology and the pathway enrichment analysis showed that DE-miRNAs potentially regulate both pain- and immune-related genes. Calcium signalling, cGMP-PKG and MAPK signalling pathways are significantly more expressed in the group receiving only flunixin. This phenomenon could be

explained by the overproduction of peripheral and central glutamate (Glu), due to the presence of pain perception. Whitin the nervous system, Glu is an excitatory neurotransmitter, involved in a wide range of neural functions (54). The NMDA receptors, a family of Glu receptors (GluRs), are ligand-gated ion channels playing an important role in synaptic plasticity. Cells associated with trauma or disease release Glu, stimulating further Glu release by normal signalling mechanisms, and the resulting excessive Ca^{2+} influx leads to aberrant over-activation of proteases and caspases (55). In the present study, the pathways potentially regulated by DE-miRNAs (calcium signalling pathway, cGMP-PKG pathway and MAPK signalling pathway) could be correlated at an increased intracellular influx of calcium and at the maintenance of painful signal. Thus, in the horses treated only with flunixin, the failure in pain treatment could lead to the genesis of prolonged pain in some individuals. Romifidine (use in this study as premedication drug in all animals) is a α-2 adrenoceptor agonist commonly used to induce sedation and analgesia in horses (56). Moreover, some α-2 adrenoceptor agonists, including romifidine, xylazine and detomidine, modulate the plasma amounts of glucose and some stress-related hormones and metabolites, decreasing the release of insulin from pancreatic β-cells in horses (56). This action should be residual after 8h from the end of castration: however, a higher pain condition in the group treated only with flunixin could increase the circulating cortisol levels and consequently lead to an inhibition of circulating insulin and increasing glycaemia (mimicking a diabetes condition).

Taken together, our results further validate the HGS and circulating levels of miR-145 as specific pain indicators, thus improving pain assessment in horses undergoing surgical castration. The combined use of HGS and the miR-145 expression in serum could help the veterinarian in the identification of horses with an acute pain condition. Furthermore, our findings confirmed that the addition of local mepivacaine is useful for reducing pain in horses undergoing surgical castration. As our results were collected in a research setting, further studies should address the feasibility and applicability of these indicators in equine practice.

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Author Contributions

The contributions of each author were as follows: C. L., F. D., M. M., E. D. C., D. L., D. S., conceived and designed the experiments; C. L., D. L., D. S., E. D. C. performed the experiments; E.D.C., F.A., analysed the data; C. L., F. C. carried out the laboratory analysis; C. L., F. D., M. M., E. D. C., D. L., F. A., G. R., E. C., F. C., D. S. wrote the paper.

Competing interests

None declared.

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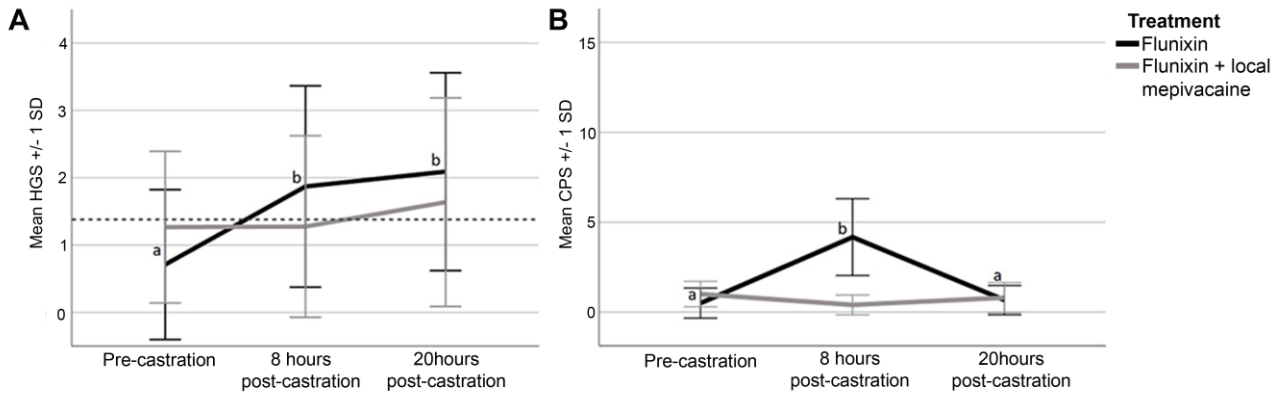
Ethics statement

Surgical castration was carried out in compliance with the European Communities Council Directive (No. 86/609/EEC). This study was registered as an animal experiment at the Brandenburg State Veterinary Authority (V3-2347-A-42-1-2012) in compliance with German legislation on animal experiments. Horses underwent routine surgical castration for husbandry purposes at the request of their owner voluntarily. No animals underwent surgery or were directly used to record data for this study. Verbal informed consent was gained from each participant before taking part in this research. Written consent was deemed unnecessary as no personal details of the participants were recorded. No animals received less than the standard analgesic regimen for the study. This study employed a strict “rescue” analgesia policy: if any horse was deemed to be in greater than mild pain (assessed live by an independent veterinarian), then additional, pain-relieving medication would immediately be administered and the animal removed from the study. The choice of medication and dosage would be based on the severity of pain identified through the clinical examination of the individual horse.



399

400 **Figure 1** – The charts represent mean (A) HGS+/-1 SD and (B) CPS+/-1 SD over time in the two treatment groups.
401 Differences within the treatment groups are indicated as follows: a,b $p<0.05$ (post-hoc test using Bonferroni correction).
402 Pain threshold as described by Dalla Costa and colleagues (8) is represented by the dashed grey line.

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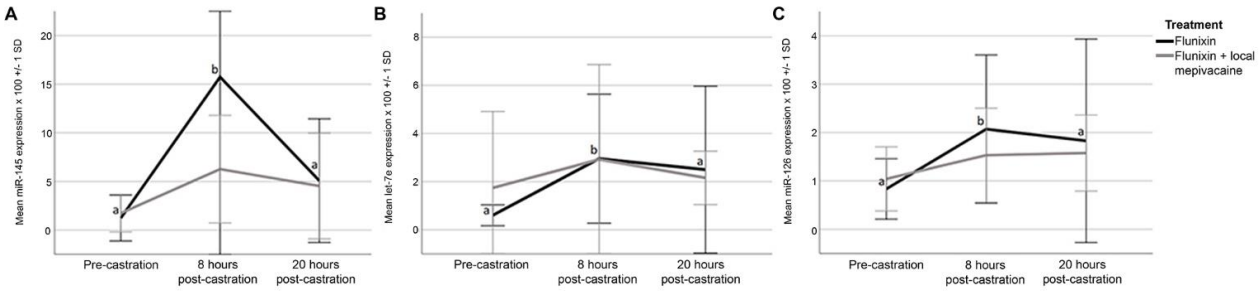
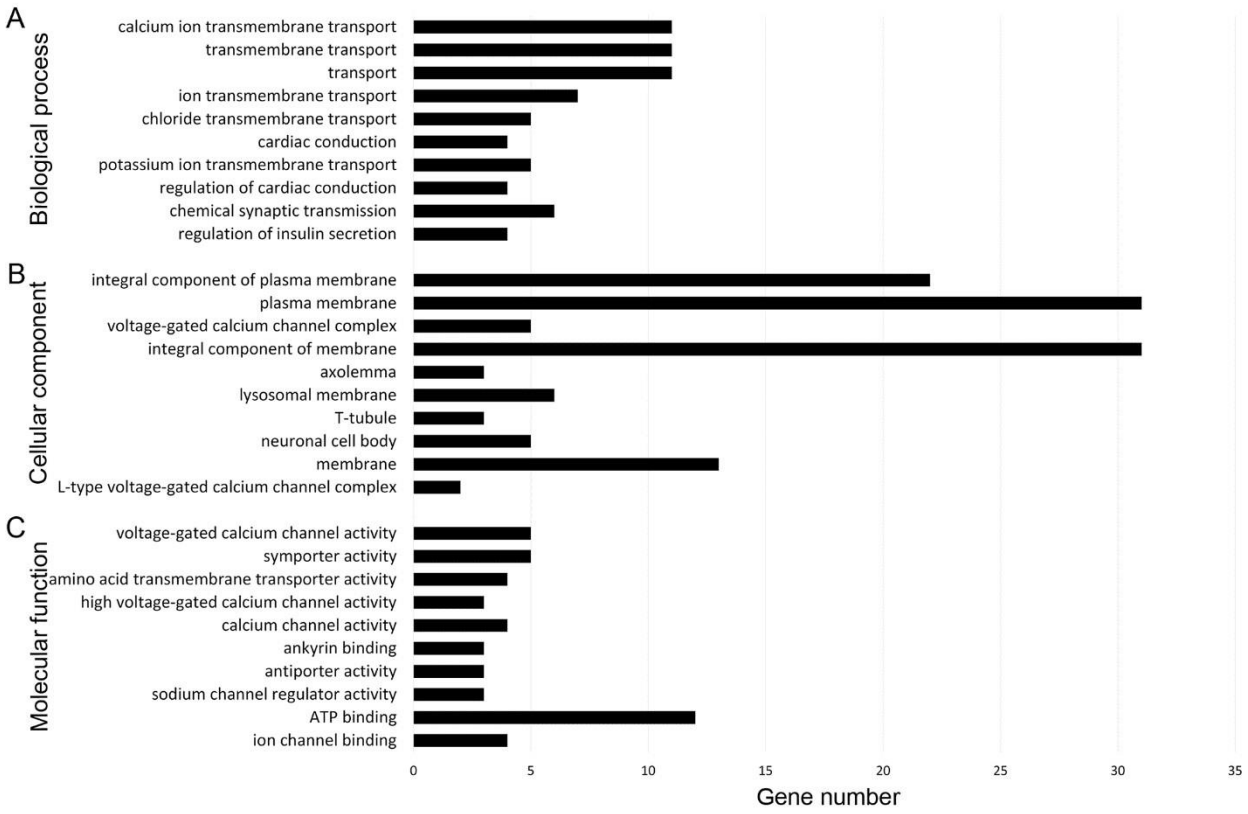


404

405 **Figure 2 -** The charts of circulating DE-miRNAs over time in the two treatment groups of miR-145 (A), miR-let7e (B)

406 and miR-126 (C). Significance was declared for ^{a,b} $p<0.05$ (post-hoc test using Bonferroni correction).

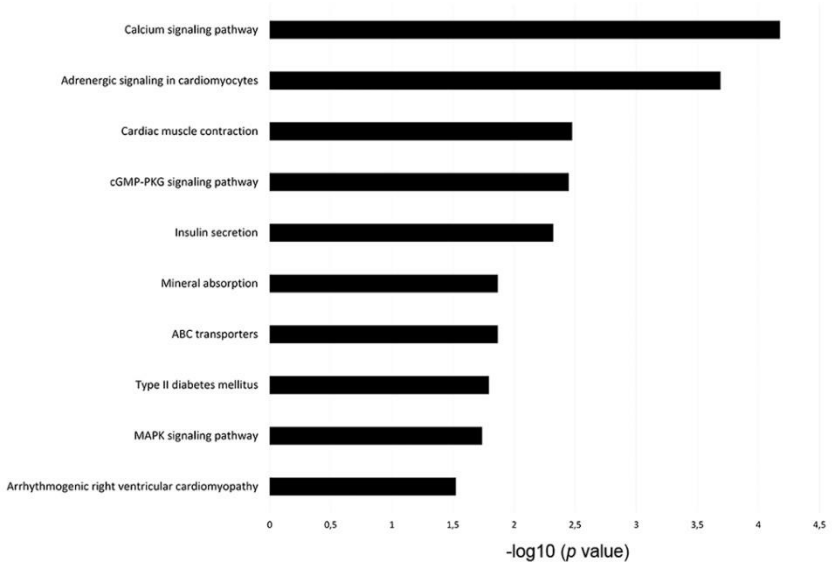
407



408

409 **Figure 3** - Enriched gene ontology (GO) of terms potentially regulated by DE-miRNAs. The target genes were annotated
410 by DAVID at three levels: (A) biological process, (B) cellular component and (C) molecular function. The top 10
411 significantly enriched items are shown.

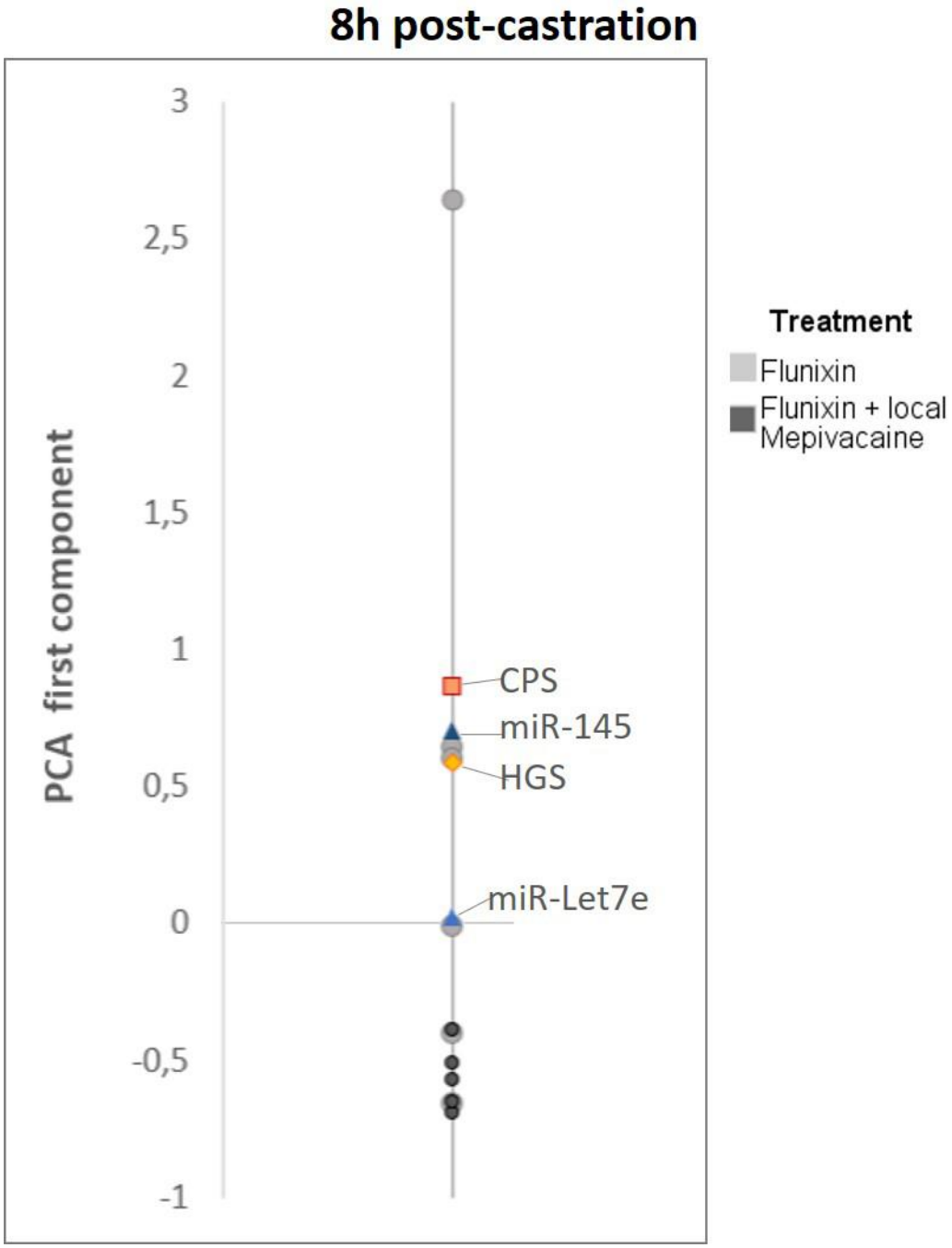
412



413

414 **Figure 4** - Pathway enrichment analysis for pain- and immune-related genes potentially regulated by DE-miRNAs. Genes
415 regulated by DE-miRNAs were retrieved and enriched in KEGG using DAVID. The p-value was negative 10-base log-
416 transformed. The top 10 enriched KEGG pathways are reported.

417



418

419 **Figure 5** – Loadings of CPS, HGS, miR-145, miR-126-5p and miR-Let7e 8 hours post-castration along the first two PCA
420 Components. Horses receiving only pre-operative administration of flunixin are presented in black dots, while horses
421 receiving preoperative administration of flunixin followed by local mepivacaine in the spermatic cord are presented in
422 grey dots. Proximity in space between indicators indicates categories which are related.

423

Table 1 - Breed and age distribution within the two treatment groups.

Treatment group	**Breed**	**Age**
Flunixine	Islandic Horse	3 years
	German Warmblood	1 year
	Frisian	2 years
	Quarter Horse	2 years
	German Warmblood	3 years
	Tennesse Walker	2 years
Flunixine + local Mepivacaine	Islandic Horse	3 years
	German Warmblood	3 years
	Black Forest Coldblood	3 years
	Black Forest Coldblood	2 years
	Quarter Horse	2 years

427 **Table 2** - List of TaqMan™ probes (ThermoFisher Scientific, Monza, Italy) and assay IDs.

miRNA	**Assay ID**	**Related to**	
cel-miR-39-3p	000200	Exogenous spike-in	Exogenous spike-in
hsa-miR-200b	002251	Pain	Lecchi et al., 2018 doi: 10.1017/S1751731117001525.
hsa-miR-23b	000400	Pain	Lecchi et al., 2018 doi: 10.1017/S1751731117001525
hsa-let-145-5p	002278	Pain	Lecchi et al., 2018 doi: 10.1017/S1751731117001525
hsa-miR-143	002249	Pain	Kreth et al., 2018 doi: 10.1213/ANE.0000000000002444
hsa-let-7e-5p	002406	Inflammation/immunity	Hildebrand et al., 2018. https://doi.org/10.3389/fimmu.2018.01224
hsa-miR-126-5p	000451	Inflammation	Marques-Rocha et al., 2015 doi: 10.1096/fj.14-260323
mmu-miR-221	000524	Inflammation	Marques-Rocha et al., 2015 doi: 10.1096/fj.14-260323

428

429

Table 3 - Results regarding the inter-observer reliability analysis on HGS. Average scores of ICC, with the 95%-confidence interval reported in brackets, are presented for each FAU of HGS.

Facial Action Units	Intraclass Correlation Coefficient (ICC)
Stiffly backwards ears	0,962 (0,952 – 0,971)
Orbital tightening	0,834 (0,789 - 0,872)
Tension above the eye area	0,712 (0,633 – 0,778)
Prominent strained chewing muscles	0,821 (0,772 – 0,862)
Mouth strained	0,694 (0,609 – 0,764)
Strained nostrils	0,693 (0,607 – 0,763)

Table 4 - Immune- and synapse-related target genes of differentially expressed miRNAs. Immune-related genes were retrieved from ImmPort (https://www.innatedb.com/redirect.do?go=resourcesGeneLists) and synapse-related genes from Synaptic Genes Database (https://syngoportal.org/).

Immune-related genes	Synaptic-related genes
ACVR1B, ACVR1C, ACVR2A, ADRB2, AEN, AP3B1, ARG2, BACH2, BMP3, CASP3, CCL7, CCR7, DDX17, FASLG, FGF11, FLT1, GDF6, GHR, IGF1, IGF1R, NGF, NR4A2, OLR1, OSMR, PLXNA4, PLXND1, PPP3CA, PRLR, PTAFR, PTGFR, SEMA4F, SEMA4G, SOCS1, TGFBR1, TGFBR2, TGFBR3, THBS1, TNFSF9, TPT1, VAV3	*ABHD17C, ABL2, ABR, ACTB, ADGRL3, ADRB2, AKAP9, AP1G1, ARF6, ARHGEF15, ATP2B4, ATP6V1C1, BCR, BEGAIN, BSN, BTBD9, CACNA1D, CACNA1E, CACNB2, CACNB4, CAPRIN1, CAPZB, CPEB1, CRK, CRKL, CTNND1, DAGLA, DAPK1, DICER1, DLGAP4, DMD, DVL3, EEF2K, EIF4EBP2, EPHA3, ERBB4, EXOC2, FARP1, FBXO45, FZD4, GABRA6, GIT1, HIP1, IGF1, IGF1R, ITGB3, KCNC2, KCNJ11, KPNA1, LRFN4, NECTIN1, P2RX1, PLXNA4, PLXND1, PPP1R9A, PPP3CA, PRKAR2A, PTPRD, RAB5C, RAPH1, SEMA4F, SKP1, SLC1A2, SLC8A2, SLITRK4, SNAP23, STX3, SYT1, SYT11, SYT2*

439

440 # Supplementary data

Behaviour	**Criteria**	**Score/30**
Posture	Normal movements, stands quietly with equal weight distribution among all four legs or stand-resting with weight distribution among only three legs	0
	Occasional weight shift, temporarily showing discharge positions, slight muscle tremors	1
	Non-weight bearing, abnormal weight distribution	2
	Analgesic posture (attempts to urinate), prostration, muscle tremors	3
Head movement / notable gesture	Natural head movements, head straight ahead for the most part	0
	Intermittent head movements laterally or vertically, looking at flanks (1–2/5 min), lip curling/teeth grinding with or without chewing movements (1–2/5 min)	1
	Intermittent and rapid head movements laterally or vertically, frequent looking at flank (3–4/5 min), lip curling/teeth grinding with or without chewing movements (3–4/5 min)	2
	Continuous head movements, excessively looking at flank (>5 times/5 min), lip curling/teeth grinding with or without chewing movements (>5 times/5 min)	3
Movement	Stands relaxed or quiet movement	0
	Reduced movement or mild agitation	1
	Reluctance to move or moderate agitation	2
	Refusal of movement or uncontrollable forwards movement	3
Pawing on the floor	Quietly standing, no pawing	0
	Occasional pawing (1–2 times/5 min)	1
	Frequent pawing (3–4 times/5 min)	2
	Excessive pawing (>5 times/5 min)	3
Kicking at abdomen	Quietly standing, no kicking	0
	Occasional kicking at abdomen (1–2 times/5 min)	1
	Frequent kicking at abdomen (3–4 times/5 min)	2
	Excessive kicking at abdomen (>5 times/5 min), intermittent attempts to lie down	3
Auditory stimulus (click one's tongue)	Pays attention to people and noises	0
	Exaggerated response to auditory stimulus	1
	Excessively aggressive response to auditory stimulus	2
	Stupor, prostration, no response to auditory stimulus	3
Appetite (hay fed by hand)	Eats hay readily or is not allowed to eat hay	0
	Hesitates to eat hay	1
	Shows little interest in hay, eats very little or takes hay in mouth but does not chew or swallow	2
	Neither shows interest in nor eats hay	3
Sweating	No obvious signs of sweat	0
	Damp to the touch	1
	Wet to the touch, beads of sweat are apparent over the horse's body	2
	Excessive sweating, beads of water running off the animal	3
Touch response (approaching the horse slowly (left side), allowing it to sniff your hand, slowly touching the neck and withers, stroking along the back down to the flank and in direction of the painful area/genitals)	Contacting, no defence reaction to touch	0
	Contacting, trying to avoid touch through shifting of weight or taking a step to the side	1
	Contacting, trying to escape from touch by walking or trotting and / or aggressive defence by head swinging with ears pinned back	2
	aggressive defence reaction to touch with kick or bite (open mouth with exposed teeth) threatens or cannot be touched et all	3
Physiology	**Criteria**	**Score/12**
Heart rate	24-44 bpm	0
	45-52 bpm	1
	53-60 bpm	2
	> 60 bpm	3
Ventilation rate	8-13 breaths pm	0
	14-16 breaths pm	1
	17-18 breaths pm	2
	> 18 breaths pm	3
Digestive sounds	Normal	0
	Decreased motility	1
	No motility	2
	Hypermotility	3
Rectal temperature	36,9 –38,5 °C	0
	36,4–36,9 °C or 38,5–39,0 °C	1
	35,9–36,4 °C or 39,0 –39,5 °C	2
	35,4–35,9 °C or 39,5–40,0 °C	3
CPS Overall Score		**/42**

441 **Table S1 -** Composite Pain Scale (CPS) according to Bussières and others (2008) modified considering Bohnet (2007)

442 and (Søndergaard and Halekoh 2003)