Veterinary and Animal Science 27 (2025) 100423

Contents lists available at ScienceDirect

Veterinary and Animal Science

journal homepage: www.elsevier.com/locate/vas

Stress, lipid profile and inflammatory responses to flunixin meglumine administration in surgical and non-surgical castration in donkeys

Hamidreza Alipour-Khairkhah [a], Saeed Azizi [a,*], Siamak Asri-Rezaei [b]

[a] Department of Surgery and Diagnostic Imaging, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran
[b] Department of Clinical Pathology and Internal Medicine, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran

ARTICLE INFO

Keywords:
Castration
Flunixin meglumine
Inflammatory markers
Lipid profile
Donkey
Equidae
NSAID

ABSTRACT

Donkeys are in the Equidae family but have several differences from horses. There are many studies on the pathophysiology of pain and its clinical signs in horses, but data are limited for donkeys. Therefore, the present study aimed to investigate biochemical effects of flunixin meglumine in donkeys subjected to pain induced by bloodless and surgical castration. Twenty healthy male donkeys were randomly divided into four groups: 1- Surgical castration with flunixin injection, 2- Surgical castration without flunixin injection, 3- Non-surgical castration with flunixin injection, and 4- Non-surgical castration without flunixin injection. Blood samples were collected a day before surgical procedures; four hours, one day, and two days after castration. Serum levels of IL-6, TNF-α, CRP, fibrinogen, cortisol, triglyceride, and cholesterol significantly increased in non-surgical castrated group compared to the other groups. Moreover, the levels of the measured parameters were significantly higher in the non-surgically castrated group compared to the surgically castrated group. Furtheremore, flunixin meglumine administration reduced the levels of the mentioned parameters, and it was significant for TNF-α and fibrinogen in the surgically castrated donkeys. However, in the nonsurgically castrated animals, there was a significant reduction in the levels of all mentioned parameters except for fibrinogen. It was concluded that non-surgical method, compared to the surgical method, was accompanied by more inflammation, stress, and pain; therefore, the surgical method could be suggested as a preferred technique for the castration of donkeys. Furthermore, the injection of flunixin meglumine could be suggested in the castration of donkeys, particularly in the bloodless technique.

1. Introduction

In developing countries, donkeys are used to carry loads of people. They are often owned by poor individuals in society and usually maintained in poor condition. Donkeys frequently suffer from overwork and neglect and do not receive adequate welfare resources (do Nascimento et al., 2023; Regan et al., 2014). There is a misconception among people that donkeys do not feel pain as much as horses or that they have higher pain tolerance than horses (Orth et al., 2020). The stoic behavior of donkeys, which do not express pain clearly, has made their painful conditions not well understood by their owners or veterinarians, resulting in inadequate analgesic treatment (Ashley et al., 2005). Furthermore, providing conditions free from disease and pain, along with adequate nutrition and suitable housing, is considered an essential aspect of good welfare for this species (van Loon et al., 2021).

The most common surgery performed on donkeys is castration (Azizi & Masoudi, 2022). The prevalence of this surgery is due to several reasons, including the control of unwanted behaviors, the prevention of sexually transmitted diseases, and population control (do Nascimento et al., 2023). Moreover, in poor societies, castration is performed to diminish the donkey's aggression and lessen the impulse to wander, thus making them more effective as draft animals and assistants to shepherds. The recommended age for castrating a donkey is 6 to 18 months (Nascimento et al., 2023); however, they are often left intact for 2.5 to 3 years to allow for the development of desirable physical characteristics. There are two methods for castration in donkeys: the surgical method and the non-surgical bloodless method using Burdizzo forceps (Azizi & Masoudi, 2022; do Nascimento et al., 2023). The surgical method can be performed in three ways: open, closed, and semi-closed. Castration in a horse causes persistent pain for several days, requiring analgesic

Abbreviations: NSAIDs, non-steroidal anti-inflammatory drugs; TNF-α, tumor necrosis factor α; IL-6, interleukin-6; CRP, C-reactive protein; SAA, serum amyloid A.

* Corresponding author at: Department of Surgery and Diagnostic Imaging, Faculty of Veterinary Medicine, Urmia University, PO Box 57155-1177, Urmia, Iran.
E-mail address: s.azizi@urmia.ac.ir (S. Azizi).

https://doi.org/10.1016/j.vas.2024.100423

Available online 22 December 2024

treatment. Therefore, similar pain could be anticipated in the castration of a donkey (de Oliveira et al., 2021a,b).

Pain is a clinical symptom often observed during surgery. When an animal experiences pain, its physiological functions are disturbed, and healing is delayed. Pain stimulates the sympathetic system, resulting in increased heart rate, tachypnea, stress, and elevated cortisol secretion (Flecknell, 2008; Price et al., 2003; Taffarel et al., 2015). A valid indicator of stress is changes in the profile of serum lipids. One of the clear symptoms of stress is an alteration in cholesterol levels, along with an increased release of stress hormones and elevated triglyceride levels (Abou-Khalil et al., 2020). Interleukin-6 (IL-6) and tumor necrosis factor α (TNF-α) are the two main proinflammatory cytokines that increase during surgical interventions. These proinflammatory cytokines trigger the liver to synthesize acute-phase proteins such as C-reactive protein (CRP) and fibrinogen. The serum level of interleukin-6 is known as a sensitive indicator of the degree of surgical stress (Esme et al., 2011).

Non-steroidal anti-inflammatory drugs (NSAIDs) are a good choice for managing pain and reducing inflammation in horses. The administration of these drugs in donkeys is similar to that in horses, with the same dosage and time intervals (Grint et al., 2015). However, donkeys have differences in drug metabolism compared to horses (Grint et al., 2015; Mealey et al., 2004). Despite these differences, the recommended dosage for flunixin meglumine injection in donkeys is the same as in horses (Grosenbaugh et al., 2011).

The donkey, a member of the equine family, has many differences from horses in terms of behavior, anatomy, and physiology. Compared to horses, donkeys are stoic species and show more subtle clinical signs of pain (Thiemann & Sullivan, 2019). de Oliveira et al. (2021a,b) developed a validated scale for assessing postoperative acute pain in donkeys, although it is subject to certain limitations (de Oliveira et al., 2021a,b).

Further investigations are necessary to improve our knowledge concerning clinical and biochemical parameters in this species. Donkeys are typically castrated either through a surgical procedure, the most commonly used technique in routine practice, or a non-surgical method, which is the least expensive option for owners. In poor societies, castration in the draft donkeys almost always are performed in a nonsurgical method using Burdizzo forceps with no more postoperative cares like using analgesic or antibiotic therapy in field condition. The present study aimed to investigate the biochemical effects of flunixin meglumine in donkeys subjected to pain induced by bloodless non-surgical and surgical castration.

2. Materials and methods

2.1. Animals and groups

In this research, 20 Iranian native male donkeys with an average age of 3–8 years and a weight range of 150–200 kg were used. The criteria for animal selection included normal health status, normally descended testicles, and the owner's written consent to participate in the study. All donkeys were clinically healthy, confirmed through routine clinical examinations and laboratory tests, including a complete blood count (CBC) and total protein levels. The animals were either purchased for DVM student training or admitted for castration to be used as draft animals in sheep flocks. To acclimate to the environmental conditions, all animals were kept in the animal house of the Veterinary Hospital of Urmia University for two weeks under standard conditions. The Animal Ethics Committee of Urmia University approved the experiments, and their guidelines were followed (Ethic code: IR-UU-AEC-3/7). They were housed in groups of two in a 5 × 7 m outdoor paddock, equipped with shade. The donkeys were fed a mixure of grass, alfalfa hay and concentrate twice a day and provided with water ad libitum. Vaccination against rabies and tetanus, as well as post-castration anti-tetanus serum administration, are not considered standard practices in the management of donkeys. The same surgeon performed castrations on the animals in the afternoon. The donkeys were randomly divided into four equal groups as follows:

1- Group 1 (Surg+Flu): In this group, surgical castration was performed, and flunixin meglumine was administered intravenously immediately after surgery and for two consecutive days following the surgical procedure.
2- Group 2 (Surg-Flu): Donkeys in this group underwent surgical castration and did not receive flunixin meglumine.
3- Group 3 (Burd+Flu): Animals in this group underwent non-surgical bloodless castration using Burdizzo forceps and received flunixin meglumine immediately after the procedure and for two consecutive days afterward.
4- Group 4 (Burd-Flu): In this group, non-surgical castration using Burdizzo forceps was performed without the administration of flunixin meglumine.

2.2. Castration method

Food restriction was applied to the animals for 12 h before the initiation of the castration procedures. Sedation was performed with a combination of acepromazine 1 % (0.05 mg/kg, Neurotranq, Alfasan, Woerden, The Netherlands) and xylazine 2 % (0.5 mg/kg, Alfasan, Woerden, The Netherlands) administered intravenously. Approximately 10 min after the sedation injection, 2 % lidocaine (1.5 ml, Darou Pakhsh, Tehran, Iran) was injected subcutaneously in the intended area to place the angiocatheter in the jugular vein. Subsequently, a 16-gauge angiocatheter was placed and fixed in the vein. The animals were then positioned in lateral recumbency on a tilt table with the uppermost hind limb tied cranially. A short-duration intravenous anesthesia protocol, combined with local anesthesia, was used for both castration methods. Anesthesia was induced and maintained using a combination of ketamine 10 % (2 mg/kg, Bremer, Hamburg, Germany) and xylazine 2 % (1 mg/kg) administered intravenously via the angiocatheter. The animals' vital signs were regularly monitored during anesthesia by assessing heart rate, respiratory rate, indirect blood pressure, eyeball position, and palpebral and corneal reflexes.

2.2.1. Surgical castration

In the surgical castration groups, 10 ml of 2 % lidocaine was injected into each spermatic cord, and 5 ml was administered subcutaneously at the site of the proposed incisions following preliminary preparation. Surgical castration was performed using an open technique with separate incisions for each testis after preparing the scrotal area. The lower testis was first grasped between the thumb and forefingers, and an initial incision, 8–10 cm in length and approximately 1–1.5 cm from the median raphe, was made at the most dependent part of the scrotal skin. The incision was extended through the subcutaneous tunica dartos and scrotal fascia, and the tunica vaginalis over the cranial pole of the testis was opened and extended proximally. The caudal ligament of the epididymis was severed, and the spermatic cord was fully freed from the parietal tunic and exposed. Reimer's emasculator forceps were used to crush the minimally tensioned spermatic cord, ensuring appropriate haemostasis by applying two ligatures—one simple and one transfixation—on the cord. The emasculator was left in place for approximately 3 min, after which the crushing site was checked for probable haemorrhage following the removal of the testis. The upper testicle was removed using a separate incision, performed in the same manner. Antibiotic therapy was conducted using penicillin (22,000 IU/kg) and streptomycin (10 mg/kg) (Norbrook, Newry, Northern Ireland) for three days to prevent surgery-related infections. Additionally, in the Surg+Flu group, flunixin meglumine 5 % (1.1 mg/kg, Flumax, Rooyan Darou, Tehran, Iran) was administered intravenously immediately after surgery and for two consecutive days thereafter.

2.2.2. Non-surgical castration

In the non-surgical castration groups, the sedation and anesthesia protocols, positioning, and preparation were the same as in the surgical castration groups, with the exception of local anesthesia, which was applied only to each spermatic cord via injection of 10 ml of 2 % lidocaine at the propused crushing site. A Burdizzo forceps was used to crush each spermatic cord for five minutes. The crushing site of the spermatic cord was then inspected to ensure the appropriate application of the forceps. Antibiotic therapy was administered as previously described, to prevent surgery-related infections. Additionally, in the Burd+Flu group, flunixin meglumine 5 % (1.1 mg/kg) was administered intravenously immediately following bloodless castration and continued for two consecutive days every 24 h. Animals were discharged from the hospital 7–10 days after castration.

2.2.3. Clinical and behaviorial pain assessment

Following castration, the donkeys were monitored until complete recovery. The surgical site was evaluated twice daily for potential hemorrhage, infection, and, oedema. The presence of preputial and scrotal oedema was scored using a four-point scale: non = 0, mild = 1, moderate = 2, and severe = 3. Pain behavior in donkeys was assessed using the validated Equine Utrecht University Scale for donkeys composite pain assessment (EQUUS-DONKEY-COMPASS) and the Equine Utrecht University Scale for donkey facial assessment of pain (EQUUS-DONKEY-FAP) (van Dierendonck et al., 2020). These scales were evaluated on a day before the surgical procedures (Day 0), four hours after castration (Day 1), one day after castration (Day 2), and two days after castration (Day 3). A simple descriptive behavior scale was used, with scores based on direct observation and photo-videography for 30 min each day. Video analysis for pain assessment was conducted blindly. Furthermore, each animal served as its own control.

2.3. Biochemical evaluations

2.3.1. Sample collection

Blood samples were collected on days 0, 1, 2, and 3. Samples were collected from the jugular vein using serum separator gel tubes. The samples were transferred immediately to lab and then centrifuged at 3000 rpm for 10 min, and the serum was separated from the tubes and stored at −80 °C.

2.3.2. Parameters evaluation

Plasma cortisol, IL-6, and TNF-α concentrations were measured using commercially available ELISA kits (MyBioSource, USA; cat. No MBS281064, MBS706136, and MBS7606845, respectively) according to the manufacturer's instructions. The minimum detectable levels of cortisol, IL-6, and TNF-α concentrations are 0.4 ng/mL, 3.12 pg/mL, and 9.375 pg/mL, respectively. CRP was determined using Abcam's ELISA kit (ab190527), with a minimum detectable level of 1.198 ng/mL. An ELISA kit from MyBioSource (MBS135523) was used for fibrinogen determination in serum, with a sensitivity of 0.41 ng/mL. Both triglyceride and cholesterol levels were measured using PARS AZMUN commercial kits (cat. No 132500 and 110500, respectively). The basis of measurement for triglyceride and cholesterol is colorimetry for single-point measurement using a photometric method.

2.4. Statistical analysis

Statistical analyses were performed using SPSS (IBM SPSS Statistics for Windows, Version 27.0; IBM Corp). A repeated measures ANOVA was conducted to examine the effect of treatment and time on the measured variables. The normality of the dependent variables was assessed using the Kolmogorov-Smirnov test. Sphericity was tested using Mauchly's test, and when this assumption was violated, the Greenhouse-Geisser correction was applied to adjust the degrees of freedom. Partial eta squared was calculated to assess effect sizes, with thresholds for small, medium, and large effects defined as 0.01, 0.06, and 0.14, respectively. Post hoc pairwise comparisons were conducted using the Bonferroni correction to adjust for multiple comparisons. A significance level of $P < 0.05$ was used for all statistical tests.

3. Results

3.1. Clinical and behavioral assessments

On day 3, the Burd-Flu group exhibited moderate edema (score = 2), while the Burd+Flu group displayed mild edema (score = 1). Interestingly, the Surg+Flu group showed moderate edema (score = 2), whereas the Surg-Flu group developed severe edema (score = 3). No other complications were observed in the donkeys throughout the study.

According to the EQUUS-DONKEY-COMPASS and EQUUS-DONKEY-FAP pain scales, the only observed alterations in the studied animals involved overall appearance, ear position, eyelids, and nostrils. In the Burd-Flu group, obviously tightening of eyelids (score 1), a bit more opened nostrils (score 1), side-down position of ears (score 3), and moderately depressed (score 2) were observed on day 1; however, no notable differences in the mentioned scores was found in the subsequent days. In the Burd+Flu group, the animals were mildly depressed (score 1), with mildly opened nostrils (score 1) were observed on day 1. In the Surg-Flu group, mild depression (score 1), and side-up ear position (score 2) were detected on day 1 and these signs were reduced overtime. In the Surg+Flu group, only a bit more opened nostrils were found without any other alteration (Fig. 1).

3.2. Biochemical evaluations

3.2.1. Interleukin 6

The results for the amount of interleukin-6 (IL-6) are presented in Table 1. On days 1, 2, and 3, the levels of IL-6 in the non-surgically castrated groups significantly increased compared to the surgically treated groups ($P < 0.05$). Moreover, flunixin meglumine administration significantly reduced IL-6 levels in the Burd+Flu group compared to the Burd-Flu group ($P < 0.05$). The serum concentration of IL-6 elevated over time, and a significant difference was found between day 0 (before castration) and days 1, 2, and 3 (after castration) in all experimental groups ($P < 0.05$) (Fig. 2).

3.2.2. TNF-α

Table 2 presents the levels of TNF-α in different experimental groups of the study. On days 1 and 2, the lowest amount of TNF-α was observed in the Surg+Flu group, which was significantly lower than in all other groups ($P < 0.05$). The highest level of TNF-α on days 1 and 2 was observed in the Burd-Flu group, which was significantly higher than in all other experimental groups ($P < 0.05$). The levels of TNF-α significantly decreased in the flunixin meglumine injection groups compared to the groups that did not receive flunixin meglumine in both surgically and non-surgically castrated animals ($P < 0.05$). On day 3 of the study, a significant difference was detected among all studied groups ($P < 0.05$). The highest levels of the marker were detected on day 2 in the surgically castrated groups ($P < 0.05$). However, the highest levels of TNF-α were found on day 3 in the non-surgically castrated groups. Moreover, the levels of TNF-α significantly increased over the duration of the study, which was more remarkable in the Burd-Flu group (Fig. 2).

3.2.3. CRP

Table 3 presents the levels of CRP before and after castration in different groups. On days 1 and 2, CRP levels significantly increased in the non-surgically castrated groups compared to the surgically castrated groups ($P < 0.05$). Moreover, the levels of CRP significantly reduced in the Burd+Flu group compared to the Burd-Flu group ($P < 0.05$). However, the levels of CRP significantly increased on day 3 in the Burd-Flu group compared to the other studied groups ($P < 0.05$). Additionally,

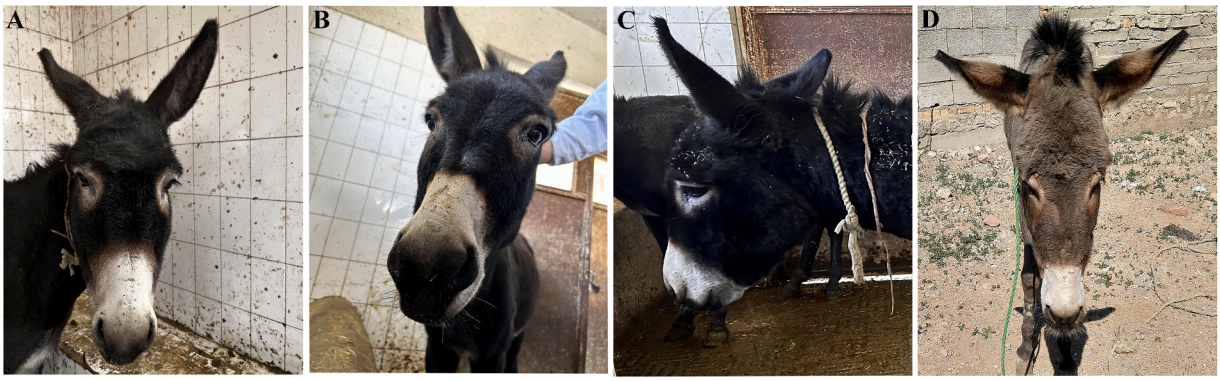

Fig. 1. A: Normal appearance of the donkey, B: a bit more opened nostrils, C: moderate depression, and mildly opened nostrils, D: side-down position of ears, obviously tightening of eyelids, more open nostrils, and severe depression.

Table 1
The means ± standard deviation (95 % confidence interval for mean) of Interleukin 6 (pg/ml) before and after castration in different groups.

	Days				*P* value (Effect size η^2)		
Groups	Day 0	Day 1	Day 2	Day 3	Day	Group	Day × Group
Surg+Flu	12.78 ± 2.05^{A,a}	30.62 ± 3.76$^{A,\ b}$	35.02 ± 2.09$^{A,\ b}$	33.48 ± 0.56$^{A,\ b}$	<0.001	<0.001	<0.001
CI	(9.5–16)	(24.6–36.6)	(31.6–38.3)	(32.5–34.3)	(0.944)	(0.948)	(0.915)
Surg-Flu	16.29 ± 2.38$^{A,\ a}$	54.03 ± 7.29$^{A,\ b}$	47.80 ± 6.22$^{A,\ b}$	42.16 ± 3.25$^{A,\ b}$			
CI	(10.3–22.2)	(35.9–72.1)	(32.3–63.2)	(34–50.2)			
Burd+Flu	13.21 ± 2.07$^{A,\ a}$	104.50 ± 35.16$^{B,\ b}$	113.34 ± 38.33$^{B,\ b}$	69.15 ± 19.81$^{B,\ b}$			
CI	(9.9–16.5)	(48.5–160.4)	(52.3–174.3)	(37.6–100.6)			
Burd-Flu	16.41 ± 2.16$^{A,\ a}$	182.07 ± 9.69$^{C,\ b}$	185.65 ± 3.07$^{C,\ b}$	177.37 ± 9.40$^{C,\ b}$			
CI	(12.9–19.8)	(166.6–197.4)	(180.7–190.5)	(162.4–192.3)			

CI = 95 % Confidence Interval for Mean.
η^2 = Partial Eta Squared.
ABCD: Significant difference between groups.
abcd: Significant difference between days.

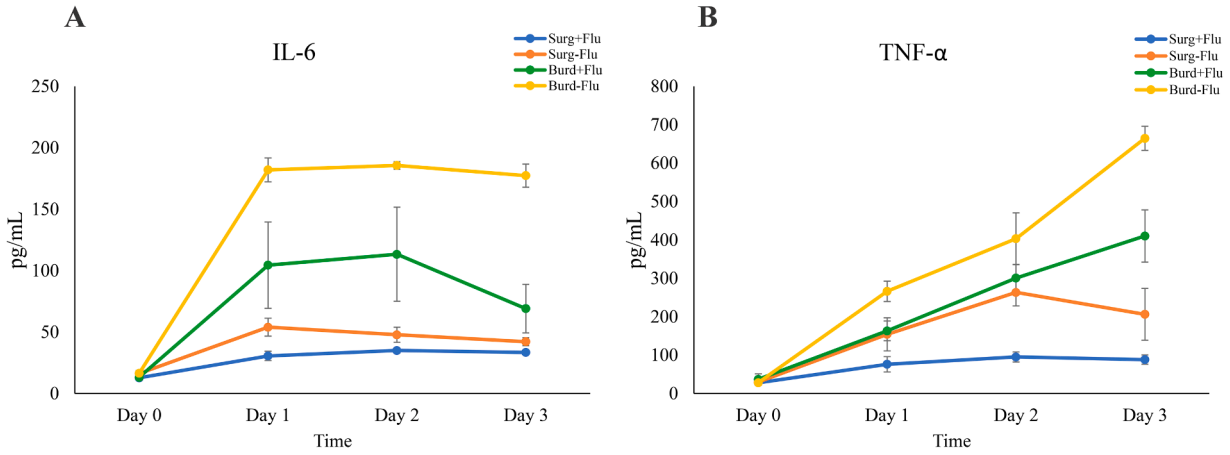

Fig. 2. Levels of IL-6 (A) and levels of TNF-α (B) in different groups of the study on days 0, 1, 2, and 3 after surgery.

the levels of CRP significantly increased over time after castration in the studied groups ($P < 0.05$) (Fig. 3).

3.2.4. Fibrinogen

Table 4 presents the results for fibrinogen concentration. The fibrinogen concentration was significantly reduced in the Surg+Flu group compared to the other groups over the duration of the study ($P < 0.05$). Additionally, there was an increase in the amount of fibrinogen in almost all groups over the duration of the study (Fig. 3).

3.2.5. Cortisol

Table 5 presents the results for the levels of cortisol. On day 1, the lowest level of cortisol was observed in the Surg+Flu group, while the highest level was detected in the Burd-Flu group. A significant difference was observed among the Surg+Flu, Burd+Flu, and Burd-Flu groups ($P < 0.05$). Following flunixin meglumine injection in the Burdizzo castrated groups, the levels of cortisol significantly reduced over the duration of the study ($P < 0.05$). Additionally, a significant difference was observed between surgically castrated and non-surgically castrated groups on days 2 and 3 ($P < 0.05$). In all experimental groups, the levels of cortisol increased over the duration of the study (Fig. 4).

Table 2
The means ± standard deviation (95 % confidence interval for mean) of TNF-α (pg/ml) before and after castration in different groups.

	Days				*P* value (Effect size η^2)		
Groups	Day 0	Day 1	Day 2	Day 3	Day	Group	Day × Group
Surg+Flu	28.11 ± 6.29 $^{A, a}$	76.21 ± 19.93$^{A, b}$	95.22 ± 12.88$^{A, b}$	88.28 ± 11.93$^{A, b}$	<0.001	<0.001	<0.001
CI	(18–38.1)	(44.4–107.9)	(74.7–115.7)	(69.2–107.2)	(0.956)	(0.969)	(0.917)
Surg-Flu	29.99 ± 6.63 $^{A, a}$	154.26 ± 43.23$^{B, b}$	263.39 ± 35.29$^{B, b}$	206.36 ± 67.33$^{B, b}$			
CI	(13.4–46.5)	(46.8–261.6)	(175.7–351)	(39.1–373.6)			
Burd+Flu	37.02 ± 14.17$^{A, a}$	163.28 ± 25.75$^{B, b}$	300.78 ± 34.80$^{B, c}$	410.44 ± 67.92$^{C, d}$			
CI	(14.4–59.5)	(122.3–204.2)	(245.4–356.1)	(302.3–518.5)			
Burd-Flu	28.23 ± 5.35 $^{A, a}$	266.28 ± 26.53$^{C, b}$	403.34 ± 67.00$^{C, c}$	664.66 ± 31.17$^{D, d}$			
CI	(19.7–36.7)	(224–308.5)	(296.7–509.9)	(615–714.2)			

CI = 95 % Confidence Interval for Mean.
η^2 = Partial Eta Squared.
ABCD: Significant difference between groups.
abcd: Significant difference between days.

Table 3
The means ± standard deviation (95 % confidence interval for mean) of CRP (ng/ml) before and after castration in different groups.

	Days				*P* value (Effect size η^2)		
Groups	Day 0	Day 1	Day 2	Day 3	Day	Group	Day × Group
Surg+Flu	2.36 ± 0. 27 $^{A, a}$	8.98 ± 1.50$^{A, b}$	11.41 ± 1.99$^{A, b}$	10.40 ± 2.91$^{A, b}$	<0.001	<0.001	<0.001
CI	(1.9–2.8)	(6.5–11.3)	(8.2–14.5)	(5.7–15)	(0.939)	(0.931)	(0.839)
Surg-Flu	3.26 ± 1.01 $^{A, a}$	15.10 ± 0.50$^{A, b}$	16.69 ± 1.16$^{A, b}$	9.11 ± 1.11$^{A, c}$			
CI	(0.7–5.7)	(13.8–16.3)	(13.7–19.5)	(14.2–17.1)			
Burd+Flu	3.08 ± 0.45$^{A, a}$	27.82 ± 3.68$^{B, b}$	26.46 ± 5.36$^{B, b}$	18.03 ± 7.85$^{A, b}$			
CI	(2.3–3.8)	(21.9–33.6)	(17.9–34.9)	(5.5–30.5)			
Burd-Flu	2.79 ± 0.16 $^{A, a}$	40.79 ± 3.78$^{C, b}$	37.95 ± 4.42$^{C, b}$	32.96 ± 4.89$^{B, b}$			
CI	(2.5–3)	(34.7–46.8)	(30.9–45)	(25.1–40.7)			

CI = 95 % Confidence Interval for Mean.
η^2 = Partial Eta Squared.
ABCD: Significant difference between groups.
abcd: Significant difference between days.

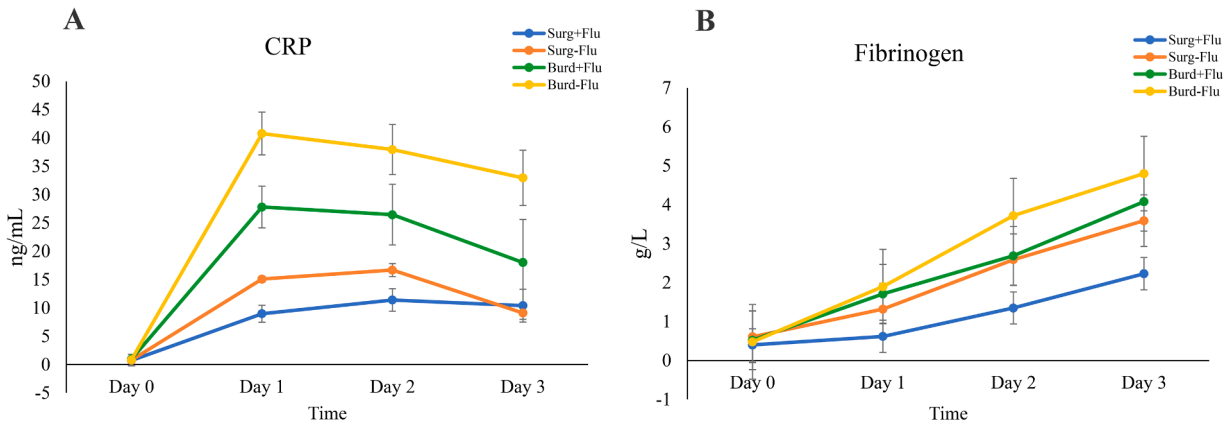

Fig. 3. Levels of CRP (A) and levels of fibrinogen (B) in different groups of the study on days 0, 1, 2, and 3 after surgery.

3.2.6. Triglyceride

Table 6 displays the levels of triglyceride in various groups of the study. The levels of triglyceride increased over time in all groups, and a significant difference was observed between the Burd-Flu group and the Surg+Flu group ($P < 0.05$). Additionally, there was a significant difference between the levels of triglyceride in the non-surgically castrated groups with flunixin injection on days 2 and 3 ($P < 0.05$). The highest levels of triglyceride were observed in the Burd-Flu group on day 3 ($P < 0.05$) (Fig. 5).

3.2.7. Cholesterol

Table 7 displays the levels of cholesterol in various groups of the study. The levels of cholesterol increased over time in all groups, and a significant difference was observed between the Burd-Flu group and the Surg+Flu group ($P < 0.05$). Additionally, there was a significant difference between the levels of cholesterol in the non-surgically castrated groups with flunixin injection on days 2 and 3 ($P < 0.05$). The highest levels of cholesterol were observed in the Burd-Flu group on day 3 ($P < 0.05$) (Fig. 5).

4. Discussion

The present study found that administration of flunixin meglumine improved pain scores in castrated donkeys based on the EQUUS-DONKEY-COMPASS and EQUUS-DONKEY-FAP pain scales. Furthermore, surgically castrated animals exhibited lower pain scores

Table 4
The means ± standard deviation (95 % confidence interval for mean) of fibrinogen (g/l) before and after castration in different groups.

	Days				*P* value (Effect size η^2)		
Groups	Day 0	Day 1	Day 2	Day 3	Day	Group	Day × Group
Surg+Flu	0.40 ± 0.10 $^{A, a}$	0.62 ± 0.08$^{A, b}$	1.35 ± 0.09$^{A, c}$	2.23 ± 0.07$^{A, d}$	<0.001	<0.001	<0.001
CI	(0.2–0.5)	(0.4–0.7)	(1.2–1.5)	(2.1–2.3)	(0.931)	(0.864)	(0.573)
Surg-Flu	0.61 ± 0.26 $^{A, a}$	1.32 ± 0.06$^{B, ab}$	2.59 ± 1.18$^{B, bc}$	3.59 ± 0.40$^{B, c}$			
CI	(−0.04–1.2)	(1.1–1.4)	(−0.3–5.5)	(2.5–4.5)			
Burd+Flu	0.52 ± 0.14$^{A, a}$	1.71 ± 0.17$^{B, b}$	2.69 ± 0.43$^{B, c}$	4.08 ± 0.61$^{BC, d}$			
CI	(0.2–0.7)	(1.4–2)	(2–3.3)	(3.1–5)			
Burd-Flu	0.48 ± 0.06 $^{A, a}$	1.90 ± 0.56$^{B, b}$	3.72 ± 0.86$^{B, c}$	4.80 ± 0.60$^{C, c}$			
CI	(0.3–0.5)	(1–2.8)	(2.3–5.1)	(3.8–5.7)			

CI = 95 % Confidence Interval for Mean.
η^2 = Partial Eta Squared.
ABCD: Significant difference between groups.
abcd: Significant difference between days.

Table 5
The means ± standard deviation (95 % confidence interval for mean) of cortisol (ng/ml) before and after castration in different groups.

	Days				*P* value (Effect size η^2)		
Groups	Day 0	Day 1	Day 2	Day 3	Day	Group	Day × Group
Surg+Flu	13.05 ± 2.08 $^{A, a}$	37.51 ± 1.63$^{A, b}$	36.08 ± 2.28$^{A, b}$	44.58 ± 2.65$^{A, c}$	<0.001	<0.001	<0.001
CI	(9.7–16.3)	(34.9–40.1)	(32.4–39.7)	(40.3–48.8)	(0.977)	(0.954)	(0.942)
Surg-Flu	11.70 ± 0.80 $^{A, a}$	47.68 ± 10.32$^{AB, b}$	41.57 ± 6.36 $^{A, b}$	61.52 ± 3.96$^{A, b}$			
CI	(9.7–13.7)	(22–73.3)	(25.7–57.3)	(51.6–71.3)			
Burd+Flu	10.74 ± 1.61 $^{A, a}$	60.87 ± 10.77$^{B, b}$	89.12 ± 12.38$^{B, bc}$	104.52 ± 21.87$^{B, c}$			
CI	(8.1–13.3)	(43.7–78)	(69.4–108.8)	(69.7–139.3)			
Burd-Flu	14.10 ± 4.64 $^{A, a}$	93.33 ± 7.90$^{C, b}$	120.39 ± 11.59$^{C, c}$	175.00 ± 6.27$^{C, d}$			
CI	(6.7–21.4)	(80.7–105.9)	(101.9–138.8)	(165–184.9)			

CI = 95 % Confidence Interval for Mean.
η^2 = Partial Eta Squared.
ABCD: Significant difference between groups.
abcd: Significant difference between days.

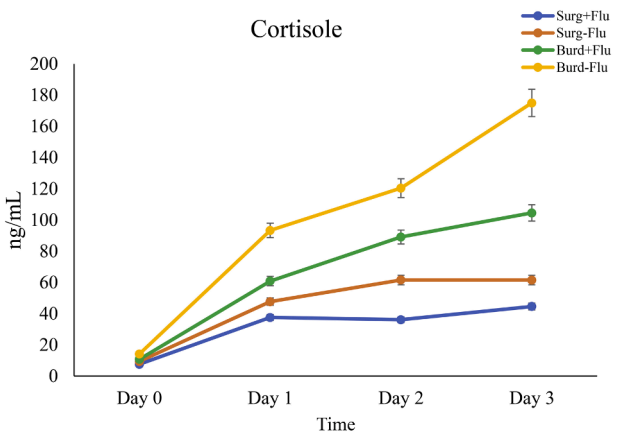

Fig. 4. Levels of cortisol in different groups of the study on days 0, 1, 2, and 3 after surgery.

compared to those castrated non-surgically. van Dierendonck et al. (2020) reported significantly higher COMPASS and FAP scores in donkeys compared to controls at 4 h after surgical castration, with a specificity of 100 % and sensitivity of 57.0 % for EQUUS-DONKEY-COMPASS and a specificity of 94 % and sensitivity of 42.9 % for EQUUS-DONKEY-FAP (van Dierendonck et al., 2020). Another study investigating pain recognition in donkeys post-castration using the Grimace Scale, identified alterations in body posture (Orth et al., 2020). According to the Donkey Pain Scale (DOPS) developed by de Oliveira et al. (2021a,b), lifting the pelvic limbs, characterized by excellent specificity but low sensitivity, was identified as the most relevant specific pain behavior following castration in donkeys (de Oliveira et al., 2021a,b).

In the present study, based on EQUUS-DONKEY-FAP pain scores, changes in ear position, eyelids, and nostrils were predominantly observed in donkeys without flunixin meglumine administration. However, the EQUUS-DONKEY-COMPASS scores did not reveal alterations specific to castration pain in donkeys. Therefore, the EQUUS-DONKEY-FAP pain scores could be a more reliable scale for evaluating pain during donkey castration, especially considering our use of the same animal as its own control.

The findings of the present research underscore the importance of using analgesic agents to ensure the welfare of castrated animals, especially when employing non-surgical bloodless methods, given the stoic nature of donkeys. In a study on the effects of local mepivacaine before castration of horses conducted by Abass et al. (2018), plasma cytokines such as interleukin-6 and TNF-α were evaluated. They observed that both the group of horses that received mepivacaine and the group that did not receive it exhibited a significant increase in pain scores and cytokine levels compared to baseline values. Moreover, the mepivacaine-injected group exhibited significantly lower IL-6 and TNF-α concentrations compared to the group without mepivacaine (Abass et al., 2018). This highlights the potential benefit of local analgesic administration in reducing pain and inflammatory responses during castration procedures (Abass et al., 2018). In another study, serum biomarkers such as IL-1β, IL-6, and TNF-α were assessed for diagnosing chronic back pain in horses, and the findings revealed that IL-1β, IL-6, and TNF-α levels were significantly higher in the horses suffering from chronic back pain compared to the healthy group (Mayaki et al., 2023). In recent research conducted on the effects of intratesticular versus intrafunicular lidocaine in field castration of ponies, pain and immunological response were evaluated. Their findings indicated that levels of TNF-α and IL-6 in the animals with intrafunicular injection were significantly lower than those of the other group (Vullo et al., 2022).

Table 6
The means ± standard deviation (95 % confidence interval for mean) of triglyceride (mg/dl) before and after castration in different groups.

	Days				*P* value (Effect size η^2)		
Groups	Day 0	Day 1	Day 2	Day 3	Day	Group	Day × Group
Surg+Flu	52.19 ± 2.80 $^{A, a}$	57.52 ± 6.17$^{A, ab}$	65.05 ± 5.41$^{A, bc}$	71.55 ± 4.46^{A,c}	<0.001	<0.001	<0.001
CI	(47.7–56.6)	(47.7–67.3)	(56.4–73.6)	(64.4–78.6)	(0.944)	(0.869)	(0.808)
Surg-Flu	46.23 ± 4.38 $^{A, a}$	56.06 ± 3.11$^{AB, a}$	84.64 ± 5.27$^{B, b}$	81.94 ± 7.76$^{A, b}$			
CI	(35.3–57.1)	(48.3–63.8)	(71.5–97.7)	(62.6–101.2)			
Burd+Flu	45.91 ± 2.48$^{A, a}$	74.28 ± 12.76$^{BC, b}$	82.70 ± 8.00$^{B, b}$	81.11 ± 3.94$^{A, b}$			
CI	(41.9–49.8)	(53.9–94.5)	(69.9–95.4)	(74.8–87.3)			
Burd-Flu	45.91 ± 2.48 $^{A, a}$	79.48 ± 5.27$^{C, b}$	103.21 ± 6.00$^{C, c}$	114.10 ± 7.11$^{B, c}$			
CI	(41.9–49.8)	(71–87.8)	(93.6–112.7)	(102.7–125.4)			

CI = 95 % Confidence Interval for Mean.
η^2 = Partial Eta Squared.
ABCD: Significant difference between groups.
abcd: Significant difference between days.

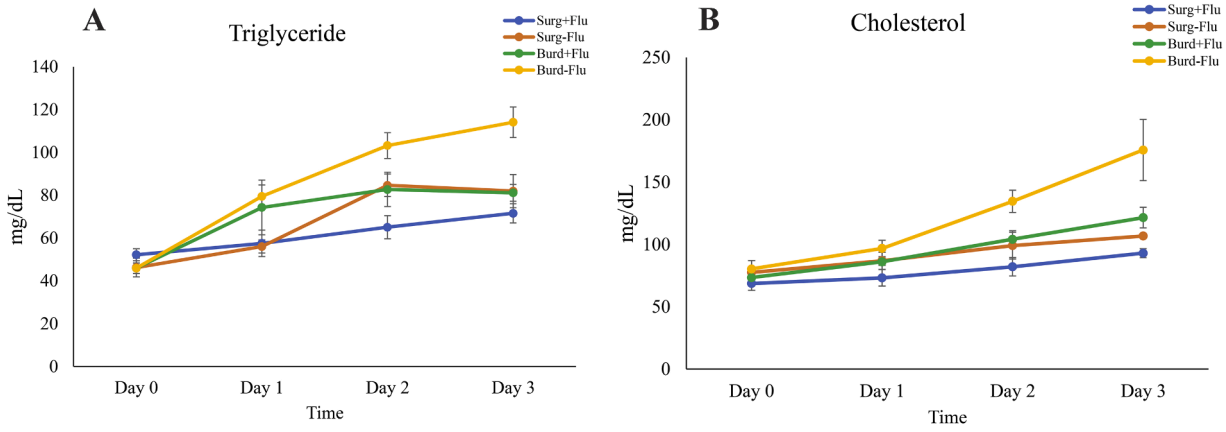

Fig. 5. Levels of triglyceride (A) and cholesterol (B) in different groups of the study on days 0, 1, 2, and 3 after surgery.

Table 7
The means ± standard deviation (95 % confidence interval for mean) of Cholesterol (mg/dl) before and after castration in different groups.

	Days				*P* value (Effect size η^2)		
Groups	Day 0	Day 1	Day 2	Day 3	Day	Group	Day × Group
Surg+Flu	75.25 ± 5.50 $^{A, a}$	73.20 ± 6.61$^{A, ab}$	82.07 ± 7.37 $^{A, bc}$	92.97 ± 3.63$^{A, c}$	<0.001	<0.001	<0.001
CI	(66.4–84)	(62.6–83.7)	(70.3–93.8)	(87.1–98.7)	(0.900)	(0.917)	(0.788)
Surg-Flu	77.53 ± 1.73 $^{A, a}$	86.89 ± 6.88$^{AB, ab}$	99.09 ± 10.75$^{AB, bc}$	106.79 ± 1.96$^{A, c}$			
CI	(73.2–81.8)	(69.8–103.9)	(72.3–125.8)	(101.9–111.6)			
Burd+Flu	73.43 ± 3.24 $^{A, a}$	86.06 ± 2.96 $^{AB, b}$	104.12 ± 6.87$^{B, c}$	121.57 ± 8.32$^{A, d}$			
CI	(68.2–78.5)	(81.3–90.7)	(93.1–115)	(108.3–134.8)			
Burd-Flu	80.37 ± 6.74 $^{A, a}$	96.82 ± 6.57 $^{B, a}$	134.61 ± 8.96$^{C, b}$	175.80 ± 24.51$^{B, c}$			
CI	(69.6–91.1)	(86.3–107.2)	(120.3–148.8)	(136.8–214.8)			

CI = 95 % Confidence Interval for Mean.
η^2 = Partial Eta Squared.
ABCD: Significant difference between groups.
abcd: Significant difference between days.

Consistent with the findings of previous studies, in the present study, the serum levels of both IL-6 and TNF-α significantly increased compared to the baseline values on day 0. These results suggest that levels of TNF-α and IL-6 can be measured to evaluate surgical pain in donkeys. Moreover, the levels of TNF-α and IL-6 significantly reduced in the surgical castration groups compared to the non-surgical castrated groups. Hence, the surgical technique led to lower pain compared to the bloodless technique and could be suggested as a preferred method for castration in donkeys. Furthermore, flunixin meglumine injection significantly reduced the levels of IL-6 and TNF-α. Therefore, administration of NSAIDs could be recommended to alleviate postoperative pain in castration of donkeys, especially in bloodless techniques, for at least three days.

Acute phase proteins serve as valuable biomarkers and are integral components of the innate immune response, increasing in the serum of animals during inflammation. Some widely used indicators of the acute phase response include serum amyloid A (SAA), haptoglobin, fibrinogen, C-reactive protein (CRP), ceruloplasmin, and α1-acid glycoprotein (Fararah et al., 2018; van der Coelen, 2022). In an investigation on arthritis-induced donkeys, serum levels of CRP, total protein, amyloid A, and IL-1β were assessed, revealing these biomarkers as valuable for promptly diagnosing the disease (Fararah et al., 2018). In a study by Jacobsen et al. (2005), serum amyloid A and other acute phase proteins were tested following surgical castration in horses. Serum amyloid A and fibrinogen were measured before (day 0) and on days 3 and 8 after castration in two groups of horses with mild and severe post-operative

inflammation. Fibrinogen concentration significantly increased in both groups compared to the baseline value and remained elevated on the studied days with similar amounts in both groups (Jacobsen et al., 2005). Another study assessed the effects of flunixin meglumine, firocoxib, and meloxicam in horses after surgical castration, evaluating hematological parameters and peritoneal fluid analysis before and 7 days after castration. The results revealed hyperfibrinogenemia compared to the reference values on day 7. Moreover, all administered NSAIDs were reported to be effective in reducing inflammation (Gobbi et al., 2020). Hauck et al. (2017) evaluated the effects of single and triple penicillin injections following castration in horses and found that fibrinogen levels significantly increased post-castration on days 3 and 8. Additionally, three administrations of penicillin significantly reduced fibrinogen levels on day 8 (Haucke et al., 2017). In the present study, consistent with previous research, castration, both surgical and non-surgical methods, significantly increased CRP and fibrinogen levels compared to baseline values. The higher levels of these inflammatory markers following castration indicate that the animals could be experiencing a high level of pain. Both surgical and non-surgical castration induced acute inflammation in the experimented donkeys. However, higher levels of fibrinogen and CRP were observed in the bloodless non-surgically castrated groups compared to the surgically castrated groups. Therefore, donkeys castrated by the bloodless method might experience more severe inflammatory responses to the castration trauma, suggesting that more pain could be anticipated in these animals. Overall, administration of flunixin meglumine reduced the levels of CRP and fibrinogen in castrated animals. Thus, using NSAIDs due to their anti-inflammatory properties could be beneficial in improving post-operative pain in castration of donkeys.

The results for cortisol serum levels in the present study demonstrated that cortisol concentration increased over time. Additionally, no significant difference was observed between the surgically castrated groups on various days with or without flunixin meglumine administration. However, the levels of cortisol in non-surgically castrated groups were significantly higher than those in surgically castrated groups, and the levels of cortisol significantly reduced in bloodless castrated donkeys when they received flunixin meglumine post-operatively. These results align with those observed in earlier studies. In a study comparing stress in surgically and chemically castrated donkeys, findings indicated that the chemically castrated group showed a significantly increased cortisol level in the plasma compared to the surgically castrated group (Abou-Khalil et al., 2020). Similarly, in a recent study by Ibrahim et al. (2021), the subcapsular technique was compared with the open technique for castration in donkeys, and they reported an increase in serum cortisol levels in the open method at the end of surgery time and 12 h postoperatively (Ibrahim et al., 2021). In a research conducted by Ayala et al. (2012) on horses with various clinical diseases, including castration surgery and stress conditions, the levels of cortisol in serum were evaluated. They reported statistically significant differences between the healthy control group and all other groups (Ayala et al., 2012). However, Taravat et al. (2017) investigated the effects of surgical closed castration on cortisol levels in healthy horses and reported no significant difference in cortisol levels before surgery (day 0), 10, and 30 days after castration (Taravat et al., 2017) which is in contrast with the results of our study. This discrepancy could be attributed to differences in surgical techniques (open vs. closed) and, mostly, inappropriate sampling time in their methodology.

Hyperlipidemia is a prevalent condition in donkeys, often occurring secondary to other primary diseases. When hyperlipidemia arises in the absence of underlying clinical disease, it is considered a primary disease (van der Coelen, 2022). In the present study, serum levels of triglycerides (TG) and cholesterol increased over the course of the study compared to reference values, with significant increments observed on days 2 and 3. Additionally, in bloodlessly castrated donkeys that did not receive flunixin meglumine, the levels of both TG and cholesterol were significantly higher compared to other castrated donkeys. Overall, the results of the study showed that administration of flunixin meglumine reduced the levels of TG and cholesterol in the castrated animals. Lipid profile evaluation was conducted in a study by Abou-Khalil et al. (2020), comparing surgical and chemical castration in donkeys. They reported a significant increase in serum total cholesterol levels in the chemically castrated group on day 45 compared to baseline values, with a significant difference observed between chemically castrated and surgically castrated animals. Additionally, they reported an increase in serum triglyceride levels in both surgical and chemical castrated groups, with significant differences noted in the chemical castrated group on days 45 and 60 compared to day 0 (Abou-Khalil et al., 2020). In a study conducted by Dunkel and McKenzie (2003) on clinically ill horses, severe hypertriglyceridemia was identified in all horses with systemic inflammatory response syndrome (Dunkel & McKenzie, 2003). Similarly, in a recent study by Ibrahim et al. (2021), comparing the subcapsular technique with the open technique for castration in donkeys, an increase in serum levels of cholesterol and triglycerides was reported. Significant differences were observed in the levels of triglycerides on days 2 and 7 in the open method (Ibrahim et al., 2021). Overall, consistent with the aforementioned studies, measuring serum levels of cortisol, triglycerides, and cholesterol could be suggested for assessing stress and the related pain in the castration of donkeys. These biomarkers may provide valuable insights into the physiological response to castration and aid in evaluating the welfare and well-being of the animals.

Castration induces acute pain, leading to stress and an elevation in cortisol secretion. A valid indicator of stress is the alteration in the serum lipid profile in donkeys. IL-6 and TNF-α levels increase following surgical interventions. These proinflammatory cytokines trigger the liver to synthesize acute-phase proteins such as CRP and fibrinogen. Additionally, serum levels of interleukin-6 are a well-established, sensitive indicator of surgical stress (Esme et al., 2011). In the present study, serum levels of IL-6, TNF-α, CRP, fibrinogen, cortisol, and lipids were assessed to explore the relationship between these primarily inflammatory and stress biomarkers and post-castration behavioral alterations in donkeys. Detecting these biomarkers could help in understanding the pain experienced by these stoic animals, whose clinical expression of pain may be difficult to detect.

The study design of this experimental study appears sound, with each animal serving as its own control for the evaluation of behavior. However, it was conducted under controlled conditions, which had some limitations. The relatively small sample size, consisting of 20 donkeys due to the cost of the experiment, may limit the generalizability of the findings. The pain assessment tools used in this study were limited to camera videomotion and direct observation for 30 min. Therefore, further comprehensive research using more appropriate tools may be needed to assess animal behavior, particularly when comparing surgically castrated to non-surgically castrated donkeys. Moreover, additional studies measuring other relevant proinflammatory and inflammatory biomarkers in larger populations are needed to draw more reliable conclusions and extrapolate the results to field conditions.

The results of this study cannot be directly extrapolated to horses or other equids, such as mules, without further research, as donkeys differ significantly from horses in their physiology, behavior, and pain response. Additionally, the findings may not be applicable to other breeds of donkeys due to potential differences in metabolism among breeds. This study was conducted in a controlled animal house, which may not accurately reflect the conditions under which castrations are performed in low-resource field settings without proper postoperative care. Furthermore, in poor communities, the cost of surgery, the availability of trained veterinarians, and the use of appropriate anesthesia methods are practical limitations. Therefore, regardless of the technique used, proper postoperative care and analgesic therapy should be encouraged from an animal welfare perspective.

5. Conclusion

This study evaluated the effects of flunixin meglumine injection following surgical and non-surgical castration in donkeys. Castration significantly affected proinflammatory markers (IL-6, TNF-α), acute phase proteins (CRP, fibrinogen), cortisol, and lipid profile components (TG, cholesterol). Non-surgical castration induced higher levels of inflammation, stress, and pain compared to the surgical method, which is therefore recommended as the preferred technique. Flunixin meglumine, particularly with bloodless castration using burdizzo forceps, effectively reduced pain and supported donkey welfare. Nonetheless, the cost of castration and analgesic therapy remains a challenge for animal welfare in low-income settings.

Funding

This work was supported by Urmia University, Urmia, Iran.

Ethical statement

The Animal Ethics Committee of Urmia University approved the experiments, and their guidelines were followed (Ethic code: IR-UU-AEC-3/7).

Declaration of generative AI in scientific writing

During the preparation of this work the author(s) used chatgpt in order to grammar check and make the text native. After using this tool/service, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the published article.

CRediT authorship contribution statement

Hamidreza Alipour-Khairkhah: Writing – original draft, Methodology, Investigation, Conceptualization. **Saeed Azizi:** Writing – review & editing, Supervision, Project administration, Methodology, Conceptualization. **Siamak Asri-Rezaei:** Writing – review & editing, Visualization, Methodology, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

The present study was extracted from the DVSc dissertation of Hamidreza Alipour-Khairkhah conducted at Urmia University. The authors appreciate the vice chancellor of research of Urmia University for financial support.

References

Abass, M., Picek, S., Garzón, J., Kühnle, C., Zaghlou, A., & Bettschart-Wolfensberger, R. (2018). Local mepivacaine before castration of horses under medetomidine isoflurane balanced anaesthesia is effective to reduce perioperative nociception and cytokine release. *Equine Veterinary Journal, 50*(6), 733–738.

Abou-Khalil, N. S., Ali, M. F., Ali, M. M., & Ibrahim, A. (2020). Surgical castration versus chemical castration in donkeys: Response of stress, lipid profile and redox potential biomarkers. *BMC Veterinary Research, 16*, 1–10.

Ashley, F., Waterman-Pearson, A., & Whay, H. (2005). Behavioural assessment of pain in horses and donkeys: Application to clinical practice and future studies.

Ayala, I., Martos, N. F., Silvan, G., Gutierrez-Panizo, C., Clavel, J. G., & Illera, J. C. (2012). Cortisol, adrenocorticotropic hormone, serotonin, adrenaline and noradrenaline serum concentrations in relation to disease and stress in the horse. *Research in Veterinary Science, 93*(1), 103–107.

Azizi, S., & Masoudi, H. (2022). Strangulating left colon volvulus following nonsurgical castration in a 6-year-old donkey. *Veterinary Research Forum, 13(1), 145-147*.

de Oliveira, M. G. C., de Paula, V. V., Mouta, A. N., Lima, I. O., de Macêdo, L. B., Nunes, T. L., Trindade, P. H. E., & Luna, S. P. L. (2021b). Validation of the donkey pain scale (DOPS) for assessing postoperative pain in donkeys. *Frontiers in Veterinary Science, 8*, Article 671330. https://doi.org/10.3389/fvets.2021.671330

de Oliveira, M. G. C., Luna, S. P., Nunes, T. L., Firmino, P. R., de Lima, A. G. A., Ferreira, J., Trindade, P. H., Júnior, R. A., & de Paula, V. V. (2021a). Post-operative pain behaviour associated with surgical castration in donkeys (Equus asinus). *Equine Veterinary Journal, 53*(2), 261–266.

do Nascimento, R. C. M., Graboschii, A. C. G., da Fonseca, L. S., Silva, A. R., Souto, P. C., da Fonseca, L. A., Goulart, M. O. F., & Escodro, P. B. (2023). Pain assessment and acute phase response in donkeys submitted to inguinal orchiectomy. *Journal of Equine Veterinary Science, 123*, Article 104223.

Dunkel, B., & McKenzie, H. C., III (2003). Severe hypertriglyceridaemia in clinically ill horses: Diagnosis, treatment and outcome. *Equine Veterinary Journal, 35*(6), 590–595.

Esme, H., Kesli, R., Apiliogullari, B., Duran, F. M., & Yoldas, B. (2011). Effects of flurbiprofen on CRP, TNF-α, IL-6, and postoperative pain of thoracotomy. *International Journal of Medical Sciences, 8*(3), 216.

Fararah, K. M., Elshemy, M. A., Abdalgalil, A. S. A., Khalil, A. H., & Elsaber, A. E. (2018). Clinicopathological and ultrasonographic changes during experimentally induced arthritis in donkey. *Benha Veterinary Medical Journal, 35*(1), 250–262.

Flecknell, P. (2008). Analgesia from a veterinary perspective. *British Journal of Anaesthesia, 101*(1), 121–124.

Gobbi, F. P., Di Filippo, P. A., de Macêdo Mello, L., Lemos, G. B., Martins, C. B., Albernaz, A. P., & Quirino, C. R. (2020). Effects of flunixin meglumine, firocoxib, and meloxicam in equines after castration. *Journal of Equine Veterinary Science, 94*, Article 103229.

Grint, N., Murrell, J., & Whay, H. (2015). Investigating the opinions of donkey owners and veterinary surgeons towards pain and analgesia in donkeys. *Equine Veterinary Education, 27*(7), 365–371.

Grosenbaugh, D., Reinemeyer, C., & Figueiredo, M. (2011). Pharmacology and therapeutics in donkeys. *Equine Veterinary Education, 23*(10), 523–530.

Haucke, K., Kuhn, M., Lübke-Becker, A., Mählmann, K., & Lischer, C. (2017). Two regimes of perioperative antimicrobial prophylaxis for equine castration: Clinical findings, acute-phase proteins, and bacterial cultures. *Journal of Equine Veterinary Science, 57*, 86–94.

Ibrahim, A., Mahmoud, U. T., Ali, M. M., & Ragab, S. M. (2021). Evaluation of the subcapsular technique for primary closure castration in donkeys (Equus asinus). *Scientific Reports, 11*(1), 14080.

Jacobsen, S., Jensen, J., Frei, S., Jensen, A. L., & Thoefner, M. (2005). Use of serum amyloid A and other acute phase reactants to monitor the inflammatory response after castration in horses: A field study. *Equine Veterinary Journal, 37*(6), 552–556.

Mayaki, A. M., Razak, I.-S. A., Adzahan, N. M., Mazlan, M., & Abdullah, R. (2023). Investigation of potential serum biomarkers for the diagnosis of chronic back pain in horses. *Macedonian Veterinary Review, 46*(1), 79–87.

Mealey, K. L., Matthews, N. S., Peck, K. E., Burchfield, M. L., Bennett, B. S., & Taylor, T. S. (2004). Pharmacokinetics of R (−) and S (+) carprofen after administration of racemic carprofen in donkeys and horses. *American Journal of Veterinary Research, 65*(11), 1479–1482.

Orth, E. K., Navas González, F. J., Iglesias Pastrana, C., Berger, J. M., le Jeune, S. S., Davis, E. W., & McLean, A. K. (2020). Development of a donkey grimace scale to recognize pain in donkeys (Equus asinus) post castration. *Animals, 10*(8), 1411.

Price, J., Catriona, S., Welsh, E. M., & Waran, N. K. (2003). Preliminary evaluation of a behaviour–based system for assessment of post–operative pain in horses following arthroscopic surgery. *Veterinary Anaesthesia and Analgesia, 30*(3), 124–137.

Regan, F. H., Hockenhull, J., Pritchard, J. C., Waterman-Pearson, A. E., & Whay, H. R. (2014). Behavioural repertoire of working donkeys and consistency of behaviour over time, as a preliminary step towards identifying pain-related behaviours. *PLoS One, 9*(7), Article e101877.

Taffarel, M. O., Luna, S. P. L., De Oliveira, F. A., Cardoso, G. S., de Moura Alonso, J., Pantoja, J. C., Brondani, J. T., Love, E., Taylor, P., & White, K. (2015). Refinement and partial validation of the UNESP-Botucatu multidimensional composite pain scale for assessing postoperative pain in horses. *BMC Veterinary Research, 11*, 1–12.

Taravat, S., Tabatabei-Naeini, A., Nazifi, S., & Rahnama, S. P. (2017). The effect of castration on cortisol changes and blood glucose in horses. *Comparative Clinical Pathology, 26*, 617–620.

Thiemann, A. K., & Sullivan, R. J. (2019). Gastrointestinal disorders of donkeys and mules. *The Veterinary Clinics of North America. Equine Practice, 35*(3), 419.

van der Coelen, I. (2022). *ACUTE PHASE PROTEINS IN DONKEYS AND MULES* Ghent University.

van Dierendonck, M. C., Burden, F. A., Rickards, K., & van Loon, J. P. (2020). Monitoring acute pain in donkeys with the equine utrecht university scale for donkeys composite pain assessment (equus-donkey-compass) and the equine utrecht university scale for donkey facial assessment of pain (equus-donkey-fap). *Animals, 10*(2), 354.

van Loon, J. P., de Grauw, J. C., Burden, F., Vos, K. J., Bardelmeijer, L. H., & Rickards, K. (2021). Objective assessment of chronic pain in donkeys using the donkey chronic pain scale (DCPS): A scale-construction study. *The Veterinary Journal, 267*, Article 105580.

Vullo, C., Crupi, R., Di Paola, R., Cuzzocrea, S., Gugliandolo, E., Biondi, V., & Catone, G. (2022). Intratesticular versus intrafunicular lidocaine to reduce perioperative nociception and immunological response in ponies undergoing field castration. *Veterinary Sciences, 9*(12), 664.