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Chemical castration in cattle with intratesticular injection of sodium chloride: Effects on stress and inflammatory markers

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ABSTRACT

Intratesticular injection (ITI) of sodium chloride (NaCl) is efficient for chemical castration of young calves, but its effects on calves welfare are unknown. Two experiments were conducted to evaluate the effects of ITI of 20% NaCl on stress and inflammatory markers in calves less than 20 days old and to assess the efficiency of ITI of 30% NaCl in 5 months old calves. In Experiment 1, control calves were only restrained and compared to calves submitted to castration through surgery (SC) and ITI with 20% NaCl (n = 9/group). No differences were observed for the eye corner temperature measured by thermography from 60 s before to 60 s after the procedures ($P > 0.05$). In the SC group, acute serum cortisol levels increased at 30 and 60 min after the procedure, but increased levels in the ITI group occurred only at 30 min ($P < 0.05$). Chronic discomfort markers were measured at 0, 24, 48, 72 and 96 h after the procedures (D0, D1, D2, D3 and D4, respectively). The serum levels of the paraoxonase 1 (PON1) enzyme and cortisol did not differ among groups ($P > 0.05$). Scrotal temperature was higher at D1 in the SC group than for the other groups, but lowest at D4 compared to the control (both $P < 0.05$). In Experiment 2, histological sections of testes were compared after ITI with either 30% NaCl or 30% calcium chloride ($CaCl_2$), to intact calves (control). After 60 days, intact seminiferous tubules and mediastinum were observed after ITI with 30% NaCl, whereas coagulative necrosis, inflammatory infiltration and calcification occurred after ITI with 30% $CaCl_2$. Efficient chemical castration through ITI of 20% NaCl in young calves was followed by slight stress and inflammatory responses compared to surgical castration. However, ITI of 30% NaCl was ineffective for chemical castration of 5 months old calves.

1. Introduction

Castration of calves is a widespread practice among beef breeders to prevent aggressive behavior, sexual activity and bull breeding [1], with additional benefits for carcass finishing, since decreased circulating testosterone levels prevent the pH increase in the meat [2–4]. Castration can be done using physical, immunological or chemical methods. Physical castration can be performed through either surgery (orchiectomy) or emasculation, using Burdizzo clamps, stenotic elastic rings or strips to interrupt the blood supply to the testicles [5]. Immunological castration is based on reducing testosterone levels through immunocontraception, inducing the production of antibodies against GnRH [6]. Chemical castration can be accomplished by intratesticular injection (ITI) of compounds that induce destruction of testicular cells through caustic or osmotic processes, such as lactic acid [7], $CaCl_2$ [8] and NaCl [9].

Surgical castration is widely used in cattle, even though it is often performed under inadequate conditions, resulting in a

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significant degree of pain and bacterial contamination [10,11]. Besides the pain, calves show various behavioral changes during and after the surgical procedure such as: agitation, leg movements, tail swing, disoriented walk, prostration, reduced interest in their mothers, and decreased feed intake [12]. Such signs of discomfort can be assessed by markers capable of identifying processes of stress, pain and inflammation, such as the serum levels of cortisol [13], the eye corner temperature [14] and acute phase proteins such as the paraoxonase 1 (PON1) enzyme [15].

Currently, consumers are increasingly questioning management practices that may be associated with pain and discomfort on the animals that provide the meat they consume [16,17]. Thus, the use of alternative castration methods that can improve animal welfare is of interest for the cattle industry. The ITI is a minimally invasive chemical castration technique that is efficient when using NaCl on calves that are 30 days old or younger [9] and $CaCl_2$ in 7–8 months old buffalos [8]. However, the effects of ITI with NaCl on the welfare of castrated young calves are still unknown and its effectiveness for chemical castration of older calves has not yet been evaluated. The objectives of this study were to evaluate the effects of ITI with 20% NaCl on markers of stress and inflammatory responses in calves up to two weeks old, and to test the efficiency of 30% NaCl ITI for chemical castration of 5-months old calves.

2. Material and methods

All procedures were approved by the Ethics in Animal Experimentation Committee (CEEA-UFPel; process # 2258).

2.1. Experiment 1

2.1.1. Experimental design

This experiment was conducted with 27 beef calves from 4 to 20 days of age, with average 36 kg of body weight and kept with their mothers in ryegrass pasture with *ad libitum* access to water. All calves were submitted to the same manual restraining procedures (calves were put down on the ground by gently pulling their legs) and randomly assigned to three groups (n = 9 each). Calves in the control group were only restrained, calves in the second group were castrated through orchiectomy and those in the third group were submitted to ITI of 20% NaCl.

Surgical castration was performed as described elsewhere [9]. Antisepsis of the scrotum was performed with 2% iodine-ethanol solution and 2% chlorhexidine digluconate solution (both from Rioquimica®, São José do Rio Preto-SP, Brazil). Local anesthesia was conducted with 5 ml 2% lidocaine. The orchiectomy was conducted after incision of the scrotum, removal of both testicles and section of the spermatic cords.

The ITI was conducted by dissolving NaCl (Synth®, Diadema, SP, Brazil) [9] and lidocaine [8] in ultrapure water. The final solution with 20% NaCl and 2% lidocaine was sterilized by 0.22 μm filtration and kept in sterile vials at 5 °C. After scrotum antisepsis, each testicle was immobilized and the solution was injected with a 21 G1½ needle, at its distal end. The injected volume (1.5–4.0 ml) varied according to the size of each testicle, as long as the gonad presented firm consistency. For all calves, the ITI was performed by the same technician, using the same criteria to define the injected volume.

2.1.2. Acute discomfort markers

Blood samples were collected from calves of the three groups, at the following time points: during the procedure; 30 min after; and 60 min after. Samples were collected through puncture of the jugular vein with a 21G needle connected to a vacuum collection system (BD Vacutainer®), into 10 ml tubes without anticoagulant. Immediately after collection, samples were centrifuged (1500 × g for 10 min). Serum samples were subsequently stored in liquid nitrogen.

Cortisol levels were quantified by the electrochemiluminescence assay Cortisol II Cobas (Roche Diagnostics, Mannheim, Germany; REF 06687733), in a commercial laboratory, with intra and inter-assay coefficient of variability lower than 10%.

The eye corner temperature was determined by thermography to identify changes in temperature caused by reduced blood flow in the eye corner due to vasoconstriction of the sympathetic nervous in response to pain [18]. Thermography was conducted at the following time points: before (−60 s and −30 s); during (0); and after the procedures (30 s and 60 s). Thermographic images were obtained with the thermograph FLIR® E25 and analyzed by the FLIR® software (FLIR QuickReport™ PC software).

2.1.3. Chronic discomfort markers

Blood samples were collected from the time of the procedures up to four days after, as described above. Chronic serum cortisol levels were determined as described above for acute cortisol. Scrotal thermography was conducted as described above for eye corner thermography.

The quantification of serum PON1 was performed as described elsewhere [15,19]. Briefly, samples were previously diluted in a 1:3 ratio and mixed with a working solution (3.3 μL of the diluted sample in 500 μL of working solution). The working solution consisted of 20 mM Tris/HCI buffer; 1.0 mM $CaCl_2$; and 4.0 mM phenylacetate. The reading was performed in a Cirrus 80ST spectrophotometer, at 270 nm wavelength for 60 s. Enzyme activity was determined by the following formula: Δ Absorbance x 115 × 3. The activity of PON1 was expressed in U/L.

Scrotal temperature and serum levels of PON1 were determined at the time of the procedures (D0); after 24 h (D1); 48 h (D2); 72 h (D3); and 96 h (D4). Chronic serum cortisol levels were determined at D0, D2 and D4.

2.2. Experiment 2

Nine calves aging between 120 and 150 days and average live weight of 125 kg (from 119 to 137 kg) were randomly assigned to three groups (n = 3 each): in two groups, calves were submitted to ITI with either 30% NaCl or 30% $CaCl_2$; whereas calves in the control group were only restrained. The restraining procedures for calves in all three groups were the same described for Experiment 1. Both hypertonic solutions were prepared using 2% lidocaine (20 mg/ml) in saline, filtered in 0.22 μm filter and stored in sterile vials at 5 °C until use. After scrotum antisepsis, each testicle was immobilized and the solution was injected with a 21 G1½ needle, at its distal end. The injected volume varied from a minimum of 4.0 ml to a maximum of 8.0 ml, which was defined considering the size of each testicle, as long as the gonad presented firm consistency. As occurred in Experiment 1, that procedure was always conducted by the same technician. The group submitted to ITI with $CaCl_2$ was considered as a positive control, since such chemical is known to be effective on promoting sterility in older animals [8].

Sixty days after the procedures, calves from all three groups were surgically castrated, as described for Experiment 1. Immediately after castration, samples of testicular parenchyma were placed in 10% formalin buffered solution. Thereafter, fragments were removed from that solution, dehydrated in alcohol, cleared in xylene and embedded in paraffin. Sections of 5 μm were obtained using an automatic microtome (RM2245, Leica Biosystems, San Diego, CA, USA), stained with hematoxylin and eosin (HE) and blindly evaluated by an experienced veterinary pathologist. Testicle samples from calves of the control group were considered as

physiological parameters.

2.3. Statistical analysis

The effects of the castration methods on serum levels of cortisol (both acute and chronic) and PON1, and on eye corner and scrotal temperatures were evaluated by analysis of variance with repeated measures, using the SAS® Mixed Procedure. Data without normal distribution were transformed, but the results were presented in the original scales.

3. Results

In Experiment 1, the average eye corner temperature varied between 38 and 39 °C. There were no differences in eye corner temperature among castration methods ($P > 0.05$), at any period (Fig. 1A).

Scrotal temperature declined in all treatments ($P < 0.001$) after the procedures (Fig. 1B). A treatment per moment interaction indicated that scrotal temperature was higher at D1 for surgically castrated calves than for calves at other groups, but lowest at D4 only for calves in the control group ($P = 0.05$).

An interaction between treatment and moment influenced the acute cortisol concentration, indicating that surgically castrated calves had higher serum cortisol levels 30 min and 60 min after castration ($P < 0.05$), compared to the levels observed during surgery (Fig. 2A). However, serum cortisol levels of calves submitted to 20% NaCl ITI were higher after 30 min ($P < 0.05$), but declined after 60 min to levels similar to those observed at 0 min, during the ITI ($P > 0.05$). There was no effect ($P > 0.05$), of the castration method on the serum levels of chronic cortisol (Fig. 2B) and PON1 (Fig. 2C).

The histological sections of testes samples from calves of Experiment 2 are shown in Fig. 3. Out of the six testicles submitted to ITI of 30% NaCl, all presented intact seminiferous tubules and five (83.3%) presented intact mediastinum, whereas only one out of six (16.7%) samples showed both such features after ITI of 30% $CaCl_2$ (Fig. 4). Coagulative necrosis and infiltration of inflammatory cells were observed in testicles submitted to both chemical castration methods, but no testicle in the control group presented such lesions. Calcification was observed only in testicles of calves submitted to $CaCl_2$ ITI (Fig. 4).

4. Discussion

Although it is known that 20% NaCl ITI impairs testosterone secretion and testicular development in beef calves younger than 20 days of age [9], the present study is the first one to report that such chemical castration method is also beneficial for animal

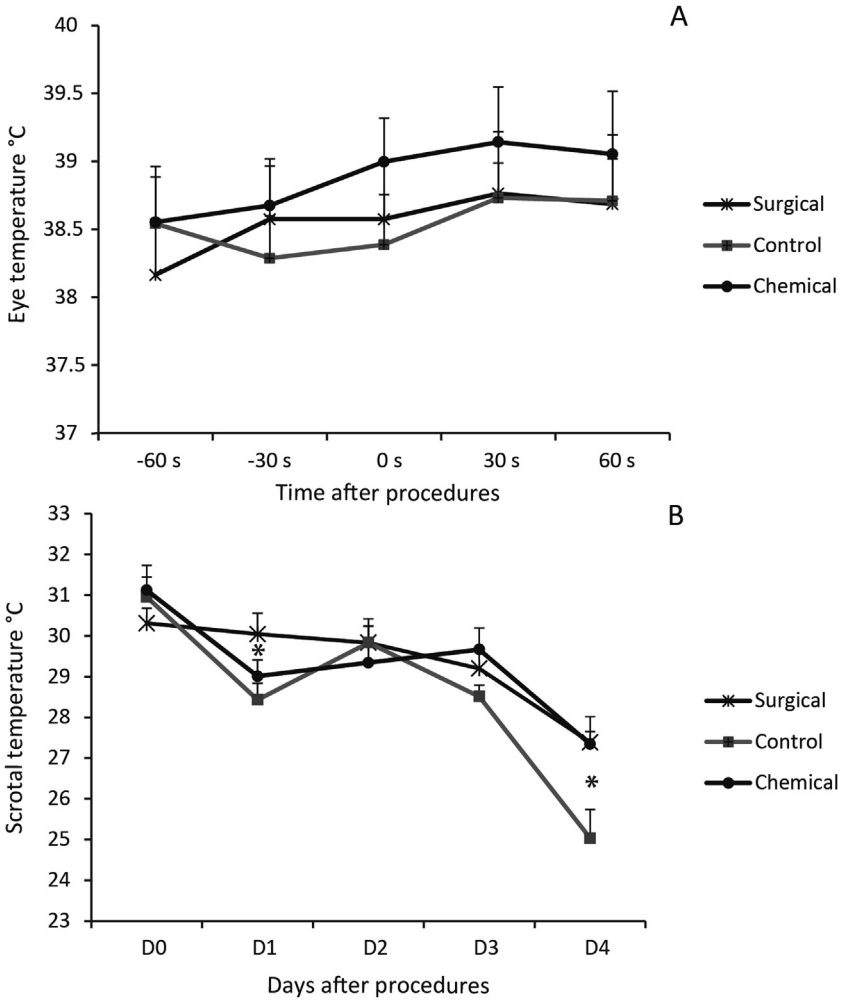

Fig. 1. Means ± SEM for eye corner (A) and scrotal (B) temperature (°C) in 4–20 days old male calves submitted to surgical castration, chemical castration through intratesticular injection of 20% NaCl or only restrained (control), at distinct time points relative to the procedures - Experiment 1: ($n = 9$ per group). *At D1, surgical castration differs from control and chemical castration; at D4 surgical and chemical castration differ from control ($P < 0.0001$).

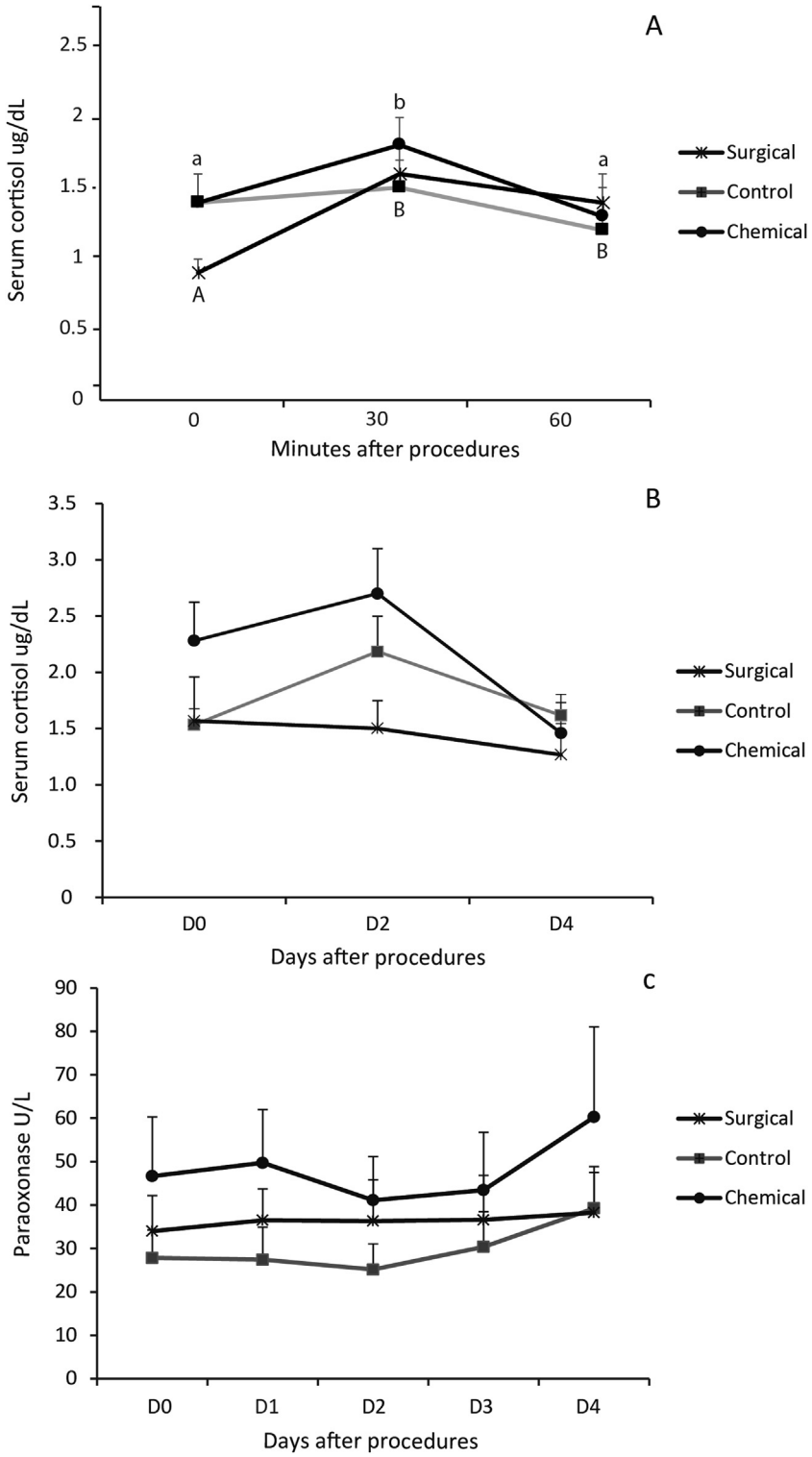

Fig. 2. Means ± SEM for serum levels of acute (A) and chronic (B) cortisol and paraoxonase activity (C) in 4–20 days old male calves submitted to surgical castration, chemical castration through intratesticular injection of 20% NaCl or only restrained (control), at distinct time points relative to the procedures - Experiment 1: paraoxonase and acute cortisol (n = 9 per group); chronic cortisol (n = 6 per group) [a,b]Differences among time points for chemical castration ($P < 0.05$). [A,B]Differences among time points for surgical castration ($P < 0.05$).

welfare. Pain and stress manifestations are expected to occur after castration through physical methods [10,20], after either surgery [21] or emasculation using Burdizzo clamps or rubber rings [22]. Increased cortisol is an indicator of the occurrence of pain and stress in such situations [2], although cortisol levels may eventually be similar after castration through different methods [13]. In Experiment 1, no relevant pain manifestation was observed in chemically castrated calves, which showed only a mild reaction at the time of needle insertion in the scrotal skin, similarly to that described elsewhere for water buffalos [8]. Calves submitted to 20%

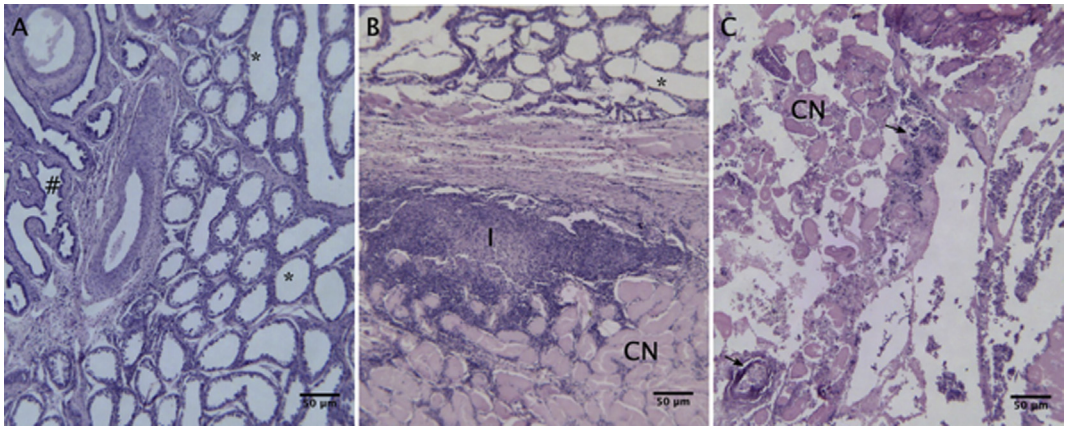

Fig. 3. Histological sections of the testicular parenchyma (hematoxylin-eosin staining) for 5-months old male calves submitted to chemical castration through intratesticular injection of 30% NaCl (B) or 30% $CaCl_2$ (C) and only restrained (control) (A) - Experiment 2. *Typical prepubertal seminiferous tubes; #Typical rete testis; I: inflammatory cells; CN: extensive coagulative necrosis; Arrows: calcification areas.

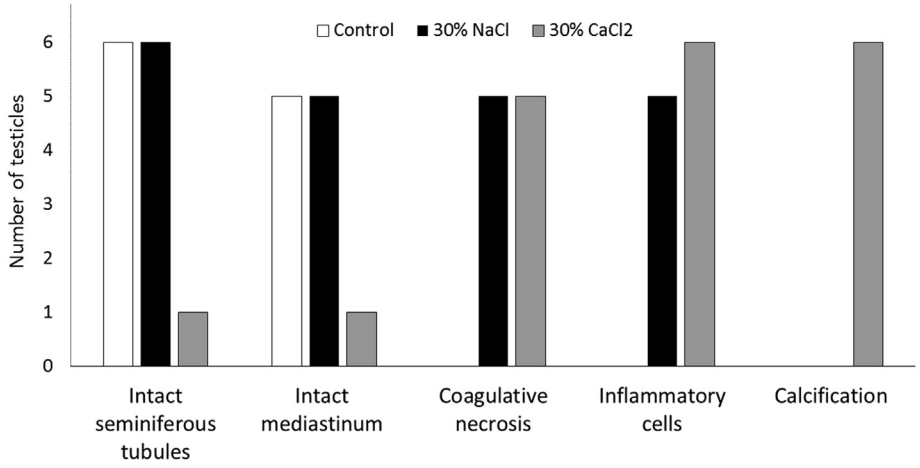

Fig. 4. Histological features in testicular tissues of 5-months old male calves submitted to chemical castration through intratesticular injection of 30% NaCl or 30% $CaCl_2$ and only restrained (control) - Experiment 2 (n = 6 testicles per group).

NaCl ITI had an acute response after the procedure, restoring their serum cortisol levels up to physiological levels earlier than surgically castrated calves. Chemically castrated calves also presented a more favorable response to chronic stress compared to surgically castrated calves, since their scrotal temperature was lower 24 h after 20% NaCl ITI and similar throughout the remaining evaluated periods. However, calves castrated through both methods presented higher scrotal temperatures 96 h after the procedure than calves in the control group. Such temperature increase could be attributed to an increase in the local blood supply due to the release of inflammatory mediators. Furthermore, surgically castrated calves do not have normal scrotal thermoregulation due to anatomical changes after orchiectomy. Similarly, after intratesticular injections, testicles move closer to abdominal wall (personal observation), potentially affecting thermoregulation. Agents other than NaCl have also been used for chemical castration in cattle, such as $CaCl_2$ [23], ethanol [24] and lactic acid [25]. Both $CaCl_2$ [23] and lactic acid [25] were associated with lower cortisol release than castration by emasculation, but were also followed by pain manifestation. Nonetheless, to our knowledge, there are few studies investigating the impact of chemicals used for ITI on stress and inflammation markers in calves.

The lack of effect of the tested castration methods in the eye corner temperature in our study also suggests that the acute pain manifestation after 20% NaCl ITI was mild. Decreased eye corner temperature related to painful and stressful procedures was detected by thermography in dogs by Travain et al. [14], supposedly initiated by vasoconstriction induced by the sympathetic system activated by acute pain [26]. Rapid reduction in the eye corner temperature was also reported in bulls 2 min after surgical castration, but such temperature was raised after 15 min concomitantly with an increase on circulating cortisol levels, indicating an activation of the parasympathetic nervous system, leading to an increase on peripheral blood flow [26].

None of the tested castration methods induced inflammatory reaction in young calves capable to alter systemic markers, as indicated by the similar serum level of PON1 across groups. Although both PON1 [15] and cortisol [26] are related to situations of inflammation and discomfort, there are currently no markers considered specific and reliable for stress and pain [27]. Also, the secretion of both markers may be affected by the management practices conducted prior to castration, which may by themselves constitute sources of anxiety for the animals [28]. For this reason, the experimental design applied in our study tried to control such factor, by submitting the non-castrated control calves to the same restraining procedures applied to the calves in the other groups.

When applied to calves up to 20 d-old, ITI of 20% NaCl resulted in extensive testicular fibrosis, replacement of parenchymal

components by dense connective tissue and coagulative necrosis of Leydig cells and seminiferous tubules. Nevertheless, in Experiment 2, most testicles submitted to 30% NaCl ITI presented intact areas of seminiferous tubules and mediastinum 60 days after the procedure, indicating that such method is not effective for chemical castration of older calves, even with a higher concentration of NaCl (increased by 10% points compared to Experiment 1). Testicles from calves in the control group did not present the histological characteristics observed in testicles submitted to ITI of $CaCl_2$, such as coagulative necrosis, inflammatory cell infiltration and calcification, as also observed in testicles of chemically castrated buffaloes [8]. Chemical castration with $CaCl_2$ ITI was also effective in domestic cats, although with lower concentration of $CaCl_2$ and with distinct histological features observed in the testicles [29]. Our results agree with the findings of Andrade Neto et al. [9], which reported that testosterone secretion at 12 months of age was fully impaired after GnRH injection when ITI with 20% NaCl was conducted in calves at most 20 days-old, but were similar to basal levels from intact males when ITI was performed in calves older than 20 days of age. Thus, compared to ITI with 30% $CaCl_2$, ITI with NaCl is less effective for chemical castration of 5-months old calves, even when using concentrations greater than 20%, since it appears unable to induce damages in the testicular parenchyma extensive enough to warrant permanent sterilization after 20 days of age.

In conclusion, efficient chemical castration through ITI of 20% NaCl in young calves was followed by slight stress and inflammatory responses compared to surgical castration. Chemical castration using ITI of NaCl with concentration increased to 30% was inefficient in older calves (with nearly 5 months of age).

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