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Intratesticular hypertonic sodium chloride solution treatment as a method of chemical castration in cattle

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Castration of male calves is necessary for trading to facilitate handling and prevent reproduction. However, some methods of castration are traumatic and lead to economic losses because of infection and myiasis. The objective of the present study was to evaluate the efficiency of intratesticular injection (ITI) of hypertonic sodium chloride (NaCl; 20%) solution in male calf castration during the first weeks of life. Forty male calves were allocated to one of the following experimental groups: negative control—surgically castrated immediately after birth; positive control —intact males; G1—ITI from 1- to 5-day old; G2—ITI from 15- to 20-day old; and G3—ITI from 25- to 30-day old. Intratesticular injection induced coagulative necrosis of Leydig cells and seminiferous tubules leading to extensive fibrosis. Testosterone secretion and testicular development were severely impaired in 12-month-old animals from G1 and G2 groups ($P < 0.05$), in which no testicular structure and sperm cells were observed during breeding soundness evaluation. Rectal and scrotal temperatures were not affected by different procedures. In conclusion, ITI of hypertonic NaCl solution induces sterility and completely suppresses testosterone secretion when performed during the first 20 days of life.

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1. Introduction

Decreasing production costs and increasing productivity are the main goals of cattle farmers. There is also an increasing concern regarding animal welfare and organic beef production, which has led to reducing the use of vaccines, antibiotics, and anthelmintic administrations. The orchiectomy is the most common technique used to suppress testosterone production by preventing reproduction of genetically inferior animals and reducing their aggressiveness [1]. However, orchiectomy is a traumatic procedure and requires a qualified technician to decrease animal suffering. Furthermore, as in all other surgical procedures, it is necessary to follow the animals individually during postoperative period to prevent common complications such as inflammation, infection, and myiasis, which can predispose the animal to arthritis and septicemic death [1]. To avoid the aforementioned complications, most producers in South America use antiparasite drugs such as fipronil, doramectin, abamectin, and ivermectin to prevent and control *Cochliomyia hominivorax* infestations in the wound of castrated cattle [2–4], which consequently lead to parasite resistance against available drugs.

Castration can be defined as extirpation or suppression of gonadal function and can be classified as hormonal, physical, or chemical. Intratesticular injections (ITIs) to

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promote chemical castration were performed in various species including primates [5], rodents [6], canine [7,8], feline [9], and caprine [10]. Several sclerosing agents have been used to cause testicular parenchyma disorganization such as ethanol [11] and zinc gluconate associated with or without DMSO [12]. Results obtained after hypertonic sodium chloride (NaCl) ITI in rats demonstrated a great potential application for this technique [6,13], because NaCl is atoxic, being marketed without restrictions at a low price. However, the viability of using NaCl to promote chemical castration in livestock animals has not been evaluated.

Several chemical castration methods may cause adverse effects such as pain and inflammation [14] or have restricted use in livestock species. Furthermore, the efficacy of chemical castration is strongly influenced by the age of the animals. Thus, it is necessary to further investigate efficient alternatives to promote sterilization with minimal discomfort and absence of adverse effects when determining the optimal age of castration [15]. On the basis of the previous studies, we hypothesized that the intratesticular administration of NaCl impairs the function of Leydig and seminiferous duct cells, suppressing testosterone and sperm production in a minimally invasive manner. The objectives of the present study were as follows: (1) to evaluate the effect of ITI of hypertonic saline on testicular structure and steroidogenesis; (2) to determine the optimal time at which to perform the procedure in calves during the first weeks of life; and (3) to investigate the effect of treatment on animal fertility after puberty.

2. Materials and methods

2.1. Hypertonic NaCl solution

The hypertonic solution was prepared by dissolving NaCl (200 mg/mL) in ultrapure water. After dilution, the 20% NaCl solution was autoclaved in 50 mL glass flasks and stored at 5 °C until use.

2.2. Animals and experimental groups

All experimental procedures were reviewed and approved by the Federal University of Santa Maria Animal Care and Use Committee. Forty crossbred beef calves (30–35 kg live weight) from a farm in southern Brazil were randomly allocated to five groups (n = 8 per group): negative control (NC)—surgically castrated immediately after birth; positive control (PC)—intact males; G1—ITI from 1- to 5-day old; G2—ITI from 15- to 20-day old; and G3—ITI from 25- to 30-day old. All the animals were identified using numbered ear tags at Day 0 (immediately after birth).

To perform ITI or surgical orchiectomy, animals were adequately contained and the site of procedure (scrotal skin) was disinfected using iodine-ethanol solution and 2% chlorhexidine (Riohex 2%; Rioquímica, SP, Brazil). Both procedures were performed under local anesthesia (5 mL 2% lidocaine; Anestex, Fagra S/A, SP, Brazil). To conduct ITI, calves were treated with up to 4 mL (according to testicular size, avoiding rupture) of hypertonic solution in each testicle using a 40 × 0.8 mm needle. The incision for surgical castration was executed using a scalpel followed by testicular removal by manually twisting the testicle. Animals showing signs of discomfort, pain, or distress received an im injection of flunixin meglumine (2.2 mg/kg).

2.3. Scrotal thermography and rectal temperature

Three animals from groups G1, NC, and PC were submitted to scrotal thermography using an infrared camera (FLIR ThermaCAM E25) at five time points: 0, 3, 6, 12, and 24 hours after procedure to evaluate inflammatory response after ITI or surgical orchiectomy. Simultaneously, rectal temperature was measured.

2.4. Testicular histopathology

Testicles fixed in 10% formalin and embedded in paraffin from three animals from each group (G1, G2, G3, and PC) were analyzed 60 days after procedures. Blocks were sectioned using a microtome, mounted on slides and stained with hematoxylin-eosine before being blindly evaluated by an experienced veterinary pathologist.

2.5. Serum testosterone

Five 12-month-old animals from each group (G1, G2, G3, NC, and PC) received an im injection of 0.012 mg GnRH analog buserelin acetate (Sincroforte; Ourofino, SP, Brazil) to induce a testosterone peak. Before blood collection, the site of the jugular vein was trichotomized and disinfected (iodine-alcohol). Blood samples were collected using 40 × 0.8 mm needles at 0 and 3 hours after GnRH injection. Samples were then centrifuged at 1800 × g for 20 minutes to obtain serum samples, which were labeled and stored (1 mL per animal) at −80 °C. Serum testosterone was measured using chemiluminescent assay (Siemens ADVIA Centaur Testosterone Test Kit; REF05476206).

2.6. Breeding soundness evaluation

Five animals from each group (G1, G2, G3, NC, and PC) aged 24 months were subjected to breeding soundness evaluation. Scrotal circumference and semen characteristics after electroejaculation were recorded. Sperm motility and vigor were subjectively evaluated using direct light microscopy. Mass activity (wave motion) was determined by placing a drop of semen on a microscope slide (prewarmed) and examining the edge of the drop (magnification, ×40 or ×100). Mass activity was classified from 1 (absent) to 5 (intense).

2.7. Statistical analysis

All continuous data were tested for normal distribution and normalized when necessary. Testosterone levels in different groups and at different time points were submitted to ANOVA. Multicomparison between groups was performed by the Tukey test. The assessment of treatment effects on scrotal and rectal temperatures was calculated as repeated measures data and analyzed using the mixed procedure of SAS software with a repeated measure statement. Main effects of treatment group, day, and their interaction were

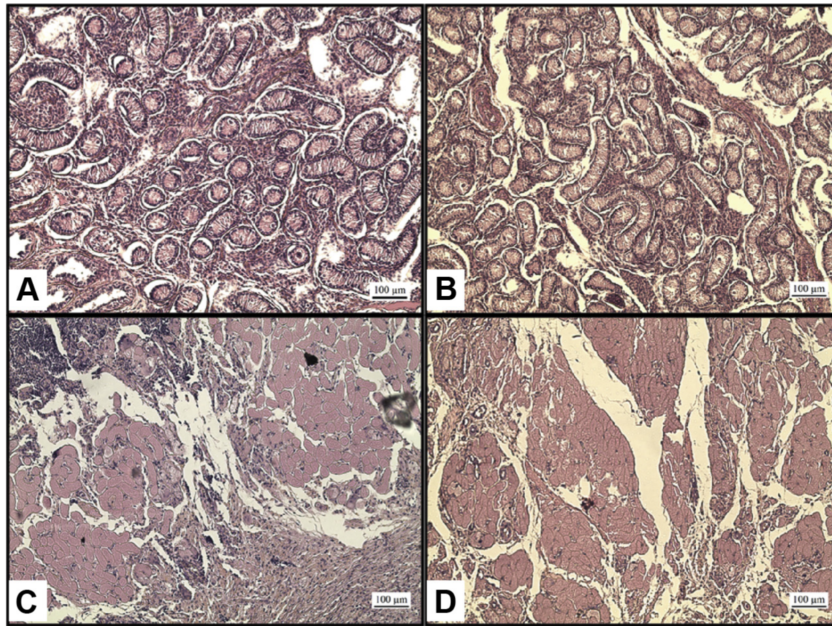

Fig. 1. Hematoxylin-eosine–stained histologic section of testis from control and treated groups. Intact seminiferous tubule (A) and normal testicular parenchyma (B) from animals submitted to surgical orchiectomy. Coagulative necrosis of Leydig cells and seminiferous tubule (C) and severe disorganization of parenchyma cells (D) from animals submitted to 20% NaCl intratesticular injection.

determined. A value of $P < 0.05$ was considered statistically significant.

3. Results

Intratesticular injection of 20% NaCl was efficient in causing testicular lesion. In the histologic examination, total dehydration of testicular parenchymal cells and extensive testicular fibrosis (Fig. 1C, D) was observed when compared with the control group (Fig. 1A, B). Furthermore, it was observed that intact parenchyma (Fig. 1A, B) was substituted by dense and loose connective tissue with vascular components (Fig. 1C) associated with coagulative necrosis of Leydig cells and seminiferous tubules (Fig. 1D).

Calves from G1 and G2 groups treated with hypertonic NaCl solution had basal concentration of testosterone even after GnRH injection. This concentration was similar to that observed in surgically castrated calves from the NC group (Fig. 2). However, animals from G3, which were treated from 25- to 30-day old, responded to GnRH injection and had a significant increase in serum concentration of testosterone (20.5 ± 8.5 and 89.2 ± 32.2 ng/dL at 0 and 3 hours, respectively), similar to basal concentration in intact males (111 ± 26.6 ng/dL). As expected, intact animals experienced a peak in testosterone at 3 hours after GnRH administration (815.5 ± 42 ng/dL; Fig. 2).

Thermograms of the scrotum revealed only a significant effect at the moment of evaluation ($P < 0.05$), but no effects of group and time versus group interaction were observed (Fig. 3A), with means between 26.3 ± 1.6 °C and 34.3 ± 1.7 °C. Rectal temperature was not affected by different treatments but varied according to the time of measurement ($P < 0.05$; Fig. 3B), with means between 38.5 ± 0.3 °C and 39.3 ± 0.2 °C.

During breeding soundness evaluation, it was observed that intact males had scrotal circumference from 28 to 31 cm (29.6 ± 0.5 cm), whereas only one animal from G3 had a testis-like structure of approximately 8 cm. Semen analysis in samples obtained from intact males had sperm motility from 50% to 80% (68 ± 5.8%), vigor ranging from 3 to 4, and mass activity from 2 to 4 (0 = absent and 5 = intense). All castrated animals (NC) and all animals submitted to ITI were aspermic (Supplementary Table 1). Because only animals from the positive control group had sperm cells, we did not conduct any further semen analysis.

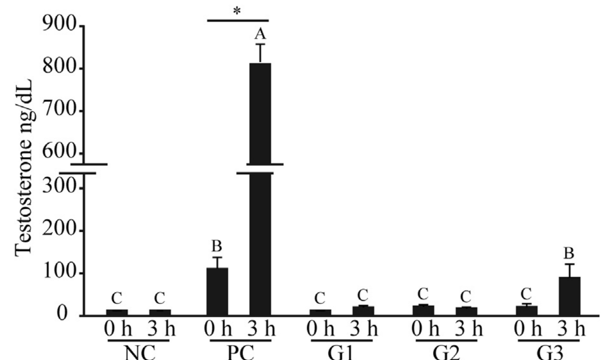

Fig. 2. Testosterone levels in 12-month-old control and treated animals (n = 5 per group). Blood samples were collected 0 and 3 hours after im administration of GnRH analog in surgically castrated (NC) and intact animals (PC) and animals submitted to 20% NaCl intratesticular injection from 1 to 5 (G1), 15 to 20 (G2), and 25 to 30 (G3) days old. Asterisk indicates the testosterone peak observed in intact animals, validating the model. Different letters indicate significant differences ($P<0.05$).

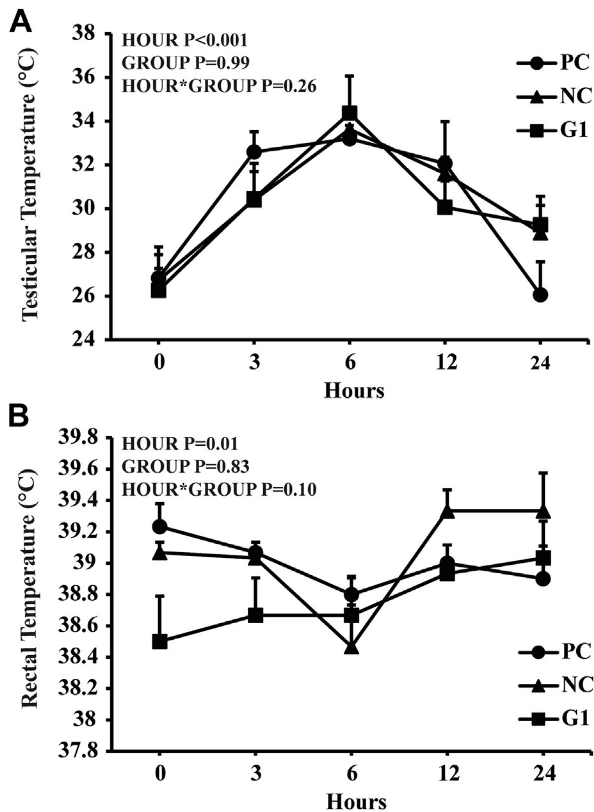

Fig. 3. Testicular temperature (A) and rectal temperature (B) from control and treated groups (n = 3 per group). Temperature was measured in intact animals (PC) and surgically castrated (NC) and animals submitted to 20% NaCl intratesticular injection from 1 to 5 (G1) days old.

4. Discussion

In the present study, a minimally invasive technique was investigated using an ITI of nontoxic solution as an alternative to surgical orchiectomy in calves during early life. The ITI injection of 20% NaCl was efficient in causing testicular lesion, because it resulted in total dehydration and fibrosis of testicular parenchyma leading to testicular atrophy. The substitution of intact parenchyma by dense and loose connective tissue also compromised Leydig cell function. In rats, coagulative necrosis was observed after 20% NaCl ITI leading to testicular atrophy [13]. Therefore, we conclude that hypertonic NaCl solution compromises the two major functions of testis tissue: testosterone synthesis by Leydig cells and germ cell production in seminiferous epithelium.

Other sclerosing agents, ethanol [11] and zinc gluconate [8] associated with or without DMSO [12], are also efficient in causing parenchyma disorganization leading to sterility. However, some of these compounds are toxic, causing pain, and pyrexia or even inflammation [14] after ITI. The main advantage of the approach used in the present study was the use of a nontoxic compound, decreasing the chance of having undesirable adverse effects. However, further studies are being planned to investigate stress and inflammation responses after NaCl ITI injection.

Few studies have investigated subsequent reproductive function after chemical sterilization. Aiming to confirm if the lesions observed histologically were sufficient to suppress libido, we evaluated testosterone levels after puberty. Therefore, we used a well-established model of inducing testosterone release by GnRH im administration, which result in a peak of testosterone 3 hours after injection in animals with functional testicular tissue [16,17]. Intratesticular injection completely suppressed testosterone secretion in animals from G1 and G2 groups, which experienced basal levels of testosterone after GnRH injection, similarly to what was observed in surgically castrated calves from the NC group. However, animals from G3, which were treated between 25 and 30 days after birth, responded to GnRH injection. A significant increase in serum testosterone was observed, reaching levels similar to basal concentration witnessed in intact males. Intact animals experienced a testosterone peak 3 hours after GnRH administration, reaching levels (815.5 ± 42.1 pg/dL) similar to those reported in previous studies [16]. The absence of response to GnRH in animals submitted to ITI until 20 days after birth confirms that these animals did not have responsive Leydig cells in contrast to intact males from the same age validating the model. Moreover, a decrease in testosterone synthesis was observed after calcium chloride ITI in dogs and bucks [10,18].

To compare the inflammatory response induced by ITI with that of the surgical orchiectomy, scrotal and rectal temperatures in animals submitted to different procedures were evaluated. Thermograms of the scrotum showed only a significant effect at the time of evaluation ($P < 0.05$), whereas no effects of group and time versus group interaction were later observed. These results confirm that scrotal temperature fluctuations occurred with changes in environmental temperature and not as a consequence of local inflammation. Despite scrotal temperature being approximately 5° lower than the temperature measured inside the testicles, there is a high correlation between the two, validating the model of scrotal thermography to indirectly evaluate temperature oscillation inside testicles [19]. Rectal temperature was not affected by different treatments and varied according to the time of measurement, but the averages remained within the normal range of 36.7 °C to 39.1 °C [20]. In contrast, Cohen et al. [21] reported an increase in rectal temperature (40.3 ± 0.1 °C) in surgically castrated calves but not in chemically castrated and intact calves, suggesting that a surgical approach causes enhanced inflammation when compared with a chemical approach. However, in that study the animals were between 7- and 9-month old at the time of treatment.

When evaluating reproductive characteristics in animals after puberty (24-month old), it was observed that intact males had scrotal circumference similar to those reported by Brito et al. [22] in Angus and crossbred bulls. Intact males had sperm parameters in the normal range, whereas all castrated animals (NC) and all animals submitted to ITI were aspermic. These results are in agreement with those observed using zinc gluconate ITI in dogs, in which the procedure decreased testosterone synthesis leading to azoospermy [8]. Similarly, the method used in the present study was efficient with only one administration.

In the United States of America, it has been reported that most orchiectomies are performed without local analgesic

drugs on calves weighing less than 90 kg [15]. Comparing different physical methods of castration, it was noted that they all induct stress and pain, even with anesthesia [23,24]. However, procedures performed in younger animals are associated with less discomfort and stress, independent of the method used, and body weight loss increased with the age [1].

In contrast to previous studies, we evaluated the effect of ITI at different time points in the first month of life. One of five animals from the G3 group had a concentration of testosterone greater when analyzed at 12 months, indicating the presence of functional Leydig cells. Furthermore, the same calf had a testis-like structure with a consistency resembling a normal testis, but adhered to scrotal wall and a discontinuous epididymis with a gap between head and tail. On the basis of these observations and the aforementioned results, we can conclude that NaCl ITI is a safe technique when used during the first 20 days of life. The procedure proposed in the present study can provide an alternative approach to surgical orchiectomy in cattle, being less traumatic and invasive, decreasing production losses, linked to infection, inflammation, and myiasis.

4.1. Conclusions

Intratesticular injection of 20% NaCl hypertonic solution induces coagulative necrosis of Leydig cells and seminiferous tubules and extensive testicular fibrosis. These observed lesions compromise testicular development and testosterone synthesis leading to sterility when performed during the first 20 days of life. The NaCl ITI represents a viable alternative to surgical orchiectomy in calves.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.theriogenology.2014.07.020.

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Supplementary Table 1
Breeding soundness evaluation.

Animal ID	Group	Scrotal circumference (cm)	Aspect	Mass motility	Motility (%)	Vigor	Volume (mL)	Conclusion
1	G1	N/A	Aspermic	N/A	N/A	N/A	N/A	Rejected
2	G1	N/A	Aspermic	N/A	N/A	N/A	N/A	Rejected
3	G1	N/A	Aspermic	N/A	N/A	N/A	N/A	Rejected
4	G1	N/A	Aspermic	N/A	N/A	N/A	N/A	Rejected
5	G1	N/A	Aspermic	N/A	N/A	N/A	N/A	Rejected
6	G2	N/A	Aspermic	N/A	N/A	N/A	N/A	Rejected
7	G2	N/A	Aspermic	N/A	N/A	N/A	N/A	Rejected
8	G2	N/A	Aspermic	N/A	N/A	N/A	N/A	Rejected
9	G2	N/A	Aspermic	N/A	N/A	N/A	N/A	Rejected
10	G2	N/A	Aspermic	N/A	N/A	N/A	N/A	Rejected
11	G3	N/A	Aspermic	N/A	N/A	N/A	N/A	Rejected
12	G3	N/A	Aspermic	N/A	N/A	N/A	N/A	Rejected
13	G3	N/A	Aspermic	N/A	N/A	N/A	N/A	Rejected
14	G3	8 cm	Aspermic	N/A	N/A	N/A	N/A	Rejected
15	G3	N/A	Aspermic	N/A	N/A	N/A	N/A	Rejected
16	PC	29	Creamy	++++	80%	4	2	Approved
17	PC	31	Milky	+++	80%	4	3	Approved
18	PC	30	Milky	+++	60%	3	2	Approved
19	PC	28	Milky	++	50%	3	5	Approved
20	PC	30	Milky	+++	70%	3	3	Approved
21	NC	N/A	Aspermic	N/A	N/A	N/A	N/A	Rejected
22	NC	N/A	Aspermic	N/A	N/A	N/A	N/A	Rejected
23	NC	N/A	Aspermic	N/A	N/A	N/A	N/A	Rejected
24	NC	N/A	Aspermic	N/A	N/A	N/A	N/A	Rejected
25	NC	N/A	Aspermic	N/A	N/A	N/A	N/A	Rejected

Abbreviations: N/A, not applicable; NC, negative control; PC, positive control.