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Effects of intratesticular vs intraepididymal calcium chloride sterilant on testicular morphology and fertility in dogs

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ABSTRACT

Background: Both stray and free-roaming owned dogs contribute to the serious global dog overpopulation problem. Many dog owners are unwilling to have their pet castrated for various reasons, including a reluctance to have their dog's behavior changed. A non-surgical method of sterilizing both stray and owned dogs would help to prevent unwanted litters. Previous studies have shown that intratesticular injection of calcium chloride dihydrate ($CaCl_2$) in alcohol is a promising and cost-effective alternative to surgery for stray dogs, with testosterone significantly decreased and sexual activity eliminated. The aim of this study was to compare the use of a solution of 20% $CaCl_2$ in 95% ethanol injected into the testicles or into the head of the epididymis.

Methods: A total of 148 dogs divided into 4 groups (2 experimental and 2 control) were respectively injected with $CaCl_2$ or saline solution into the testicle or epididymal head (ultrasound-guided). The animals were examined at 0, 3, 6, and 9 months for sperm quality, concentration of testosterone in serum, and side effects; at 0 and 5 months with contrast-enhanced ultrasound (CEUS) to enhance the morphological aspects/alteration of the testicular parenchyma or epididymis; and at 9 months when all were castrated for histological examination.

Results: All dogs treated with $CaCl_2$ became sterile with azoospermia achieved over the 9-month study. The concentration of testosterone in serum significantly decreased following intratesticular treatment with $CaCl_2$. No adverse effects were noted.

Conclusions: A single, bilateral intratesticular injection of 20% $CaCl_2$ in 95% ethanol was confirmed to be a reliable method for induction of sterilization in male dogs. The approach showed long-term efficacy and may reduce sexual behavior, with the additional benefits of low-cost and ease of use, making this nonsurgical method appropriate for use in stray dogs.

Sterility was also achieved if injected in the head of the epididymis but no significant decrease in serum concentration of testosterone occurred. Moreover, performing the intraepididymal injection into the epididymal head was as time consuming as orchiectomy. This approach may be optimal for use in owned dogs where anatomical integrity and testosterone maintenance is preferred by the owner.

1. Introduction

Dog overpopulation is a serious global problem that is complicated by the infeasibility of neutering all stray dogs and the presence of free-roaming, intact owned dogs. Dogs with an identifiable owner but allowed to roam and freely mate make up almost 46% of all roaming dogs [1]. Many owners of male dogs are reluctant to have their dog castrated due to concerns about surgery or changes

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in behavior or anatomy.

A recent study demonstrated that even by sterilizing 100% of the intact stray dogs annually, it would not be possible to obtain a sterility rate greater than 86% after 20 years due to the high introduction of new intact animals [2]. Necessary steps to reduce the stray dog population include increasing the neutering rate and controlling reproduction of companion animals.

Alternative methods to surgical sterilization that are easy to administer and affordable would offer immense benefits, allowing animal welfare organizations, public health programs, and governments to reduce dog overpopulation even with limited resources [3]. A nonsurgical alternative may also appeal to dog owners who are averse to surgical castration.

Intratesticular and intraepididymal injections of sclerosing agents represent promising methods for non-surgical sterilization. Such methods have been studied for decades and warrant continued investigation and refinement. Sclerosing agents that have been used effectively include calcium chloride dihydrate ($CaCl_2$) in solution [4–11]. Previous research has shown that an intratesticular injection of a 20% solution of $CaCl_2$ in alcohol produced long-term azoospermia with a durable reduction of testosterone and reduced aggressive and sexual behavior [4,5]. While both injection techniques result in azoospermia, intratesticular injection results in decreased spermatogenesis, whereas intraepididymal injection blocks sperm transport to deference tubules but does not alter spermatogenesis.

Both techniques of injecting sclerosing agents can be performed percutaneously. While the method is obvious for intratesticular injection, the epididymal route is possible since the epididymal tubule is coiled at the head of the epididymis and remains coiled also at the caudal portion. The epididymal head is also the site used to perform testicular sperm aspiration (TESA) [12].

While several works on intraepididymal injection refer to *cauda epididymis* as the area to inject [13–16], we chose to inject the head due to the functional characteristics of the head, body and tail sections. The head of the epididymis is near the top of the testis. Sperm entering the caput epididymis are incomplete - they lack the ability to move forward (motility) and to fertilize an egg. It stores the sperm for 2–3 months while they mature. In the lower portion of the epididymis - the tail - the sperm are stored until they are transported to the ejaculatory duct during ejaculation [17].

Our goal was to inject the sclerosing/necrotizing agent directly into the place where sperm are still immature. With the impairment of the epididymal head, sperm do not become motile; in the occurrence of regained ejaculation capacity, sperm would be unable to reach oocytes, leaving the dog infertile. Injecting the tail of the epididymis may result in fertility if sperm flow is regained. No long-term studies have been conducted to assess whether the effect of injection into the tail of the epididymis is permanent.

In an attempt to find an acceptable non-surgical method for sterilizing both owned and stray dogs, the objective of the current study was to compare a percutaneous injection of $CaCl_2$ in alcohol into the head of the epididymis to an injection into the testicle. The project evaluated feasibility, efficacy, sperm production, concentrations of testosterone in serum and tissue response. The overall goal of the current project was to determine if chemical sterilization of the head of epididymis would be an effective method for sterilizing free-roaming dogs.

2. Materials and methods

2.1. Animals

For the study, 148 healthy, crossbred male owned dogs (n = 148) were selected. The dogs were 18–26 months of age (mean = 21.71 month, SD = 2.73 month) and weighed 6–26 kg (mean = 18.71 kg, SD = 6.29 kg).

Good health status was confirmed by routine blood test (complete blood count and metabolic panel) and clinical exam (including abdominal ultrasound). To assess the fertility of the dogs, a breeding soundness examination (including physical and ultrasonographic exams and evaluation of semen quality) was performed before the start of the study [18]. Every dog chosen for the study showed sexual interest when exposed to a bitch in estrus and 20 of them were aggressive toward personnel conducting the study.

Dogs were routinely de-wormed and vaccinated. The dogs were housed with owners, fed standard commercial dog food twice a day, and given water *ad libitum*. Procedures were conducted at the University of Bari, DETO, Section of Veterinary Clinic and Animal Production during scheduled study time points.

Investigations were conducted in accordance with the Principles for the Care and Use of Research Animals, promulgated by the European Community (EU Directive 2010/63/EU for animal experiments).

The Ethical Committee of the Department of Emergency and Organs Transplantation, University of Bari Aldo Moro, Italy (DETO 11/04/2016) approved this study.

2.2. Experimental protocol

At baseline (T_0), the dogs, divided into 4 equal groups of 37 each were lightly sedated. Based on the scrotal width, a dose of $CaCl_2$ was administered via intratesticular injection (ITca) or epididymal injection (IEca). The experimentally treated animals were compared to control groups receiving saline injection only (NaCl 0.9%), via intratesticular injection (ITsa) or epididymal injection (IEsa). Dogs that exhibited aggression when approached by the study personnel were equally divided (n = 5) among the groups.

The animals were examined at 0, 3, 6, and 9 months (T_0, T_3, T_6, T_9) for semen analysis, to determine the concentration of testosterone in serum and to detect any adverse side effects associated with treatment. During the first two weeks, the dogs were under clinical observation (see Sec. 2.6). Contrast-enhanced ultrasound (CEUS) was performed before injection (T_0) and after 5 months (T_5) to evaluate any morphological alteration in the testicular parenchyma (see Sec. 2.7).

At month 9 (T_9) injected dogs were castrated and the testicles were stored for histological evaluation.

At T_0 and T_9, dogs were checked for sexual behavior in the presence of a bitch in heat and behavior when approached by members of the research team unfamiliar to the dogs.

The experimental protocol is outlined in Supplemental Table 1.

2.3. Treatment

To prepare the solution, 20 g of calcium chloride dihydrate powder (Sigma Aldrich Corporation) was brought to a final volume of 100 ml of 95% ethanol (Baker Analyzed ACS, JT Baker), mixed, and sterilized in Falcon tubes by autoclaving.

The dogs were lightly sedated with tiletamin chlorhydrate (250 mg/5 ml) and zolazepam chlorhydrate (250 mg/5 ml) at a dosage of 0.02 ml/kg (Zoletil 100. Virbac®, Italy) with an intramuscular (IM) injection. The testicular widths were measured with a caliper. According to the scrotal width, the correct dosage of an experimental solution was injected into each testicle. For ITca, dogs with diameters less than 10 mm were injected with 0.1 ml, diameters of 10–14 mm were injected with 0.25 ml, diameters of 15–18 were injected with 0.5 ml, diameters of 19–22 mm were injected with 0.8 ml, diameters of 23 mm or more received an injection of 1.0 mL [5]. The same dosage of saline solution was injected into animals in

the control group (ITsa). Injections were completed as previously described [5].

Groups IEca and IEsa were injected with 25% of the volume used for groups ITca and ITsa considering that the epididymal volume is approximately 25% of the testicle.

No further intratesticular injections were performed on any of the animals throughout the rest of the study.

The injections were performed using a 23 G needle avoiding seepage of the solution. Intraepididymal injections were ultrasound-guided. The epididymal head was ultrasonically located and injected percutaneously, avoiding seepage of the $CaCl_2$ solution. For the ultrasonography (MyLab™ ClassC, ESAOTE s.p.a., Genoa, Italy), dogs were positioned in lateral recumbency, transmission gel was spread and two-dimensional, grey scale, real-time ultrasound images were produced using a linear 13-4 MHz probe (L4-15 appleprobe VET, ESAOTE s.p.a., Genoa, Italy).

2.4. Sperm analysis

Semen was collected by digital manipulation [5,19,20] and examined through the use of a computerized device (Computer Assisted Semen Analyzer System; CASA) [21,22] within 15 min. Results were compared to those indicated as guide values [23].

The sperm rich fraction was evaluated for concentration, total and progressive motility at T_0, T_3, T_6, and T_9.

2.5. Assay for serum testosterone

At approximately 8:30 a.m. at T_0, T_3, T_6, and T_9, dogs received subcutaneous injections of 1000 international units of human chorionic gonadotrophin (hCG) (Creative Biomart, CD Inc.) to stimulate gonadal synthesis of testosterone [24,25]. Blood was collected from the saphenous vein of each dog 120 min after the hCG injections. A portion of blood was allowed to stand for 10–15 min at 4 °C, then centrifuged at 1500 g for 10 min at 4 °C prior to aspiration of serum. Serum samples were stored at −20 °C until thawed and assayed for testosterone concentrations by a chemiluminescence technique (Immulite Immunoassay System, Siemens). Physiological range was considered 100–1000 ng/dl [26].

2.6. Routine clinical observations and behavior

All the animals were kept under routine clinical observations from T_0 to T_9. After the chemical sterilization procedure, continuous observations were conducted for the first 72 h as the dogs were hospitalized, followed by daily observations for up to 15 days at the owner's house, followed by observations as indicated by the study protocol. The parameters evaluated during clinical observation included body weight, appetite, rectal temperature, scrotal and inguinal integument, palpation of testis, heart and respiratory rates, and general attitude. Testicular firmness was evaluated by palpation. The normal testicle is ovoid, smooth, firm, and mildly tender to palpation. The testicle is easily separated from the epididymis, which lies posterior and slightly lateral to the testicle. The epididymis varies in its adherence to the posterolateral surface of the testicle [27].

Sexual mounting and aggressive behavior were evaluated at T_0 and at T_9. An observer recorded whether the dog exhibited interest (mounting) in the estrus female left with each dog for at least half an hour. Aggressive behavior to unknown study personnel when approaching the dog was also recorded. Aggressive behavior was defined as the dog displaying at least one of these behaviors: growling, **t**ooth displays, muzzle punch or snapping without contact.

2.7. CEUS

CEUS is ultrasound with contrast medium injected into the bloodstream, consisting of microbubbles with a diameter of less than 6–8 μm, equipped with a capsule based on phospholipids, secure and capable of easily crossing the pulmonary filter. Its use allows the echo-amplification of the structures under examination, in specific times for each parenchymous organ [28].

CEUS starts enhancing morphological aspects of the testicular parenchyma in about 15 s after being injected into the bloodstream. It has been used to characterize prostatic and testicular diseases, evaluate perfusion kinetics, evaluate prostatic and testicular abnormalities, and distinguish benign or malignant prostatic and testicular disease [29].

All CEUS examinations were performed using a MyLab™ ClassC (ESAOTE s.p.a., Genoa, Italy) with a 9-2 MHz linear probe (L4-15 appleprobe VET, ESAOTE s.p.a., Genoa, Italy) a second-generation contrast agent, sulphur hexafluoride microbubbles (SonoVue, Bracco Imaging, Italy), and dedicated contrast-enhanced ultrasound analytical software (Contrast Tuned Imaging Technology, Esaote, Italy).

In this study, CEUS was used before injection (T_0) and at T_5 to evaluate any alterations in testicular tissue.

2.8. Histology

Left and right testicular tissue sections were collected from each dog after testicular surgical removal. After fixation in 10% buffered formalin solution, tissues were rinsed in tap water and subsequently dehydrated in alcohol, diaphanized in xylene and embedded in paraffin wax. Subsequently, 5 μm thick sections were stained with haematoxylin and eosin, Masson's trichrome modified by Goldner and then observed by Optical Microscope (Leica DM6 B).

2.9. Statistical analyses

All data were summarized for each individual canine subject by measurement (weight, age, concentration of testosterone, sperm concentration, total and progressive motility), group (ITca, IEca, ITsa, IEsa), and time point (T_0, T_3, T_6, T_9) using the Microsoft Excel 2011 program (Microsoft Corporation, Redmond, Washington, USA). These data were described in terms of the average and standard deviation (SD) and presented as mean ± SD in the results, for brevity.

Statistical analyses were conducted using *Statistica* (StatSoft, Inc. Tulsa, OK, USA).

Repeated measures of analysis of variance (ANOVA), with Time as the within factor and Groups the between factor, were used to evaluate the measurements in the four groups across four time points (T_0, T_3, T_6, T_9) for testosterone, sperm concentration, sperm motility and progression. If the result of the overall test showed significance, then planned comparisons were conducted to determine if the measures changed after treatment (T_0 vs T_3-T_9). If assumptions of the F-statistic were violated, the nonparametric Friedman ANOVA was applied. A two-tailed significance level of $p < 0.05$ was identified.

3. Results

Before the injection of sterilant or control saline, the mean weight of the dogs was 18.7 ± 6.2 kg. No changes in body weight during the trial were observed.

All animals in the study tolerated the injections of $CaCl_2$. None of the dogs vocalized, had abdominal muscle contraction or moved excessively at needle puncture of the scrotum during the injection.

During the first two weeks after the $CaCl_2$ injection, the dogs in groups ITca and IEca and the control dogs (groups ITsa and IEsa) did not experience any agitation or marked inflammatory swelling of the testis. Body temperatures were within physiological range (temperatures recorded daily were between 38.1 and 39.2 °C). No adverse side effects were noticed at the 2-week period.

However, beginning after 24 h following injection and continuing for the first 3–4 days, all dogs evidenced a slight increase in firmness of testes on palpation. Changes in testicular firmness were subjectively observed, and slightly more noticeable in dogs in the ITca group. In IEca, the increase in firmness was focused on the epididymis.

At approximately 3 months, the testes became atrophied in dogs in ITca.

No sexual behavior (i.e., loss of libido, mounting behavior) when dogs were exposed to a bitch in estrus and no behaviors associated with aggression directed towards research personnel were observed in any dogs in ITca following treatment at T_9.

In contrast, no testicular changes or alterations of behavior were observed in the IEca group or in the control groups ITsa and IEsa (e.g., all approached and try to mount an estrus female, and dogs previously displaying aggressive behavior to research personnel continued to do so throughout the study).

Group × Time interactions were found for sperm concentration [$F(9,432) = 60.6$, $p < 0.001$], total motility [Friedman $\chi^2 = 96.3$, $p < 0.001$], and progressive motility [Friedman $\chi^2 = 101.9$, $p < 0.001$]. Following treatment, dogs in ITca and IEca groups were azoospermic when semen was collected between T_3 and T_9. Thus, planned comparisons between treatment and control groups were not tested statistically for ITca or IEca. Baseline measures of sperm concentration, total motility and progressive motility were not significantly different after treatment (T_3, T_6, T_9) for control groups ITsa and IEsa (Table 1).

Concentration of testosterone in serum in dogs at baseline (T_0) averaged 550.7 ± 206.0 ng/dL. There was no significant difference between groups at T_0. There was a significant overall Group × Time interaction [$F(9,432) = 28.0$, $p < 0.001$]. Planned comparisons tested differences after treatment for each group. ITca showed a significant and progressive decline in concentration of testosterone after treatment (Fig. 1). No significant differences in testosterone concentration was found over time in the other three groups IEca, ITsa, and IEsa.

Scrotal ultrasonography with the use of microbubbles revealed a reduction in blood perfusion in the areas injected with $CaCl_2$ (T_5, groups ITca and IEca) (Figs. 2B and 3B) compared with healthy testicle imaged at T_0 (Figs. 2A and 3A). The ultrasonography showed anechoic areas in the testicle (Fig. 2B) and the epididymis (Fig. 3B) corresponding to the tissue damage caused by the sclerosing agent injected. No differences in perfusion were seen in control groups ITsa and IEsa.

Histological examination of the epididymal injected dogs (IEca) showed that at T_9, none of the regions in the head, body and tail of the epididymis and ductus deferens contained sperm cells, and necrotic cells were observed, although the identity of these cells could not be determined. Irregularly sparse encapsulations of calcified material in high-density areas of collagen fiber reactions were provoked by the injection.

Necrosis of the seminiferous epithelium, vessel congestion, with the presence of few seminal cells were detected in the area between the testis and epididymis (Fig. 4). In the testicular area adjacent the epididymis, the *ductus deferens* was damaged.

Histological examination of the testicular-injected dogs (ITca) at T_9 showed details of tubular changes, with calcium-containing sequestrations, and depletion of seminal epithelium (Fig. 5).

4. Discussion

The goal of the study was to determine if a percutaneous injection of $CaCl_2$ in alcohol into the head of the epididymis would be an effective method of sterilizing free-roaming dogs. We compared it with intratesticular injection, evaluating feasibility, efficacy, effect on sperm characteristics, and serum concentration of testosterone.

A single, bilateral intratesticular injection of 20% $CaCl_2$ in 95% ethanol was able to sterilize male dogs with azoospermia for at least 9 months. Testicular damage was observed using CEUS and confirmed by histology. Nevertheless, histological findings in the testicular parenchyma were not uniform, which may indicate a partial action of the sterilizing agent on the testicular parenchyma.

At nine months after intratesticular treatment, male dogs exhibited no sexual behavior when in the presence of an estrus bitch nor aggression toward research personnel. The results confirmed previous studies in which azoospermia was reached for at least 12 months [3]. Sterility was also achieved when $CaCl_2$

Table 1
Effects of intratesticular (n = 37) or intraepididymal (n = 37) injection of calcium chloride or saline (controls, n = 37 each) on reproductive parameters. Dosage was in accordance with treatment group and testicular width of each subject. Data expressed in mean values ± standard error within each group. Pre-treatment is baseline T_0 and Post-treatment is nine months following treatment, T_9. *statistically relevant comparison of pre-to post-treatment.

Variable	Time	Treatment			
		$CaCl_2$ intratesticular (ITca)	$CaCl_2$ intraepididymal (IEca)	Saline control intratesticular (ITsa)	Saline control intraepididymal (IEsa)
Sperm concentration (n. × 10^6/mL)	Pre-treatment	167.4 ± 19.1	384.4 ± 29.4	398.8 ± 34.2	452.2 ± 35.5
	Post-treatment	0*	0*	403.8 ± 31.5	470.0 ± 34.7
Total sperm motility (%)	Pre-treatment	95.1 ± 0.9	94.0 ± 1.1	93.5 ± 1.1	93.0 ± 1.2
	Post-treatment	0*	0*	90.7 ± 1.3	91.0 ± 1.3
Progressive sperm motility (%)	Pre-treatment	80.3 ± 1.8	90.9 ± 0.9	89.4 ± 1.3	88.7 ± 1.2
	Post-treatment	0*	0*	86.9 ± 1.5	87.2 ± 1.7
Serum testosterone concentration (ng/dL)	Pre-treatment	530.0 ± 35.1	545.9 ± 34.4	551.2 ± 31.6	575.6 ± 35.3
	Post-treatment	174.4 ± 8.6*	557.8 ± 32.7	555.5 ± 28.7	561.1 ± 28.4

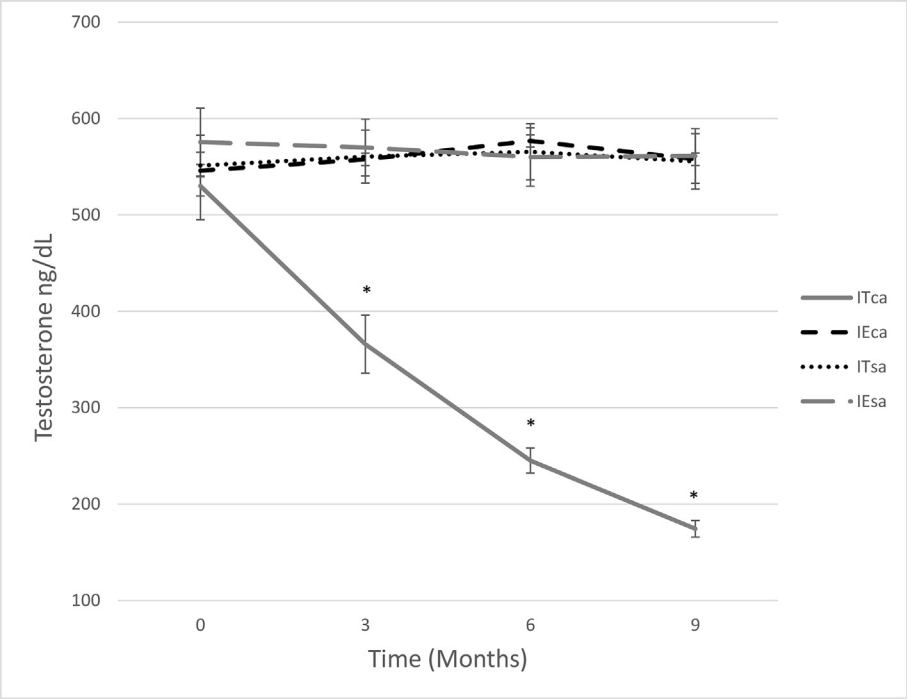

Fig. 1. Effects of injection on serum testosterone levels in each group over time. Following the intratesticular injection of $CaCl_2$ in group ITca (n = 37), testosterone decreased significantly over 9 months, although testosterone levels at 9 months were still within physiological range. No significant differences in testosterone levels were found over time in the other three groups: intraepididymal injection CaCl2 (IEca), intraepididymal injection saline (IEsa), and intratesticular injection saline (ITsa) (each n = 37). Data are displayed as mean ± SE. * indicates statistically significant difference between pre-treatment baseline and post-treatment measurements for ITca.

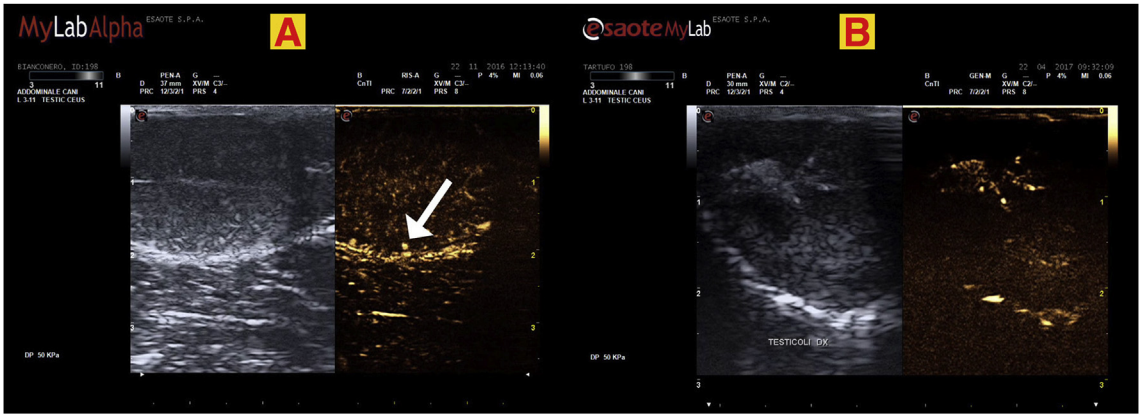

Fig. 2. Ultrasonography on the left, CEUS on the right. **A**: The normal testis at T_0. Normal testicle with the hyperechoic tunica albuginea (arrow). The grey-scale ultrasonography shows a homogeneous parenchyma. Normal macrovascular and microvascular flow on contrast-enhanced ultrasonography. **B**: Intratesticular $CaCl_2$ injection (group ITca) at T_5. Reduced intratesticular perfusion. The grey-scale ultrasonography shows a non-homogeneous parenchyma and an area without testicular perfusion corresponding to the injection site.

sterilant was injected into the head of the epididymis, with azoospermia for at least 9 months, but no significant decrease in serum concentrations of testosterone were recorded. Sexual and aggressive behaviors remained unchanged in this group.

When a drug is injected into the epididymis or the vas deferens, the resultant inflammatory reaction can result in occlusion of the epididymal lumen or the vas deferens and prevent sperm movement [15,30].

In this study, microscopic evaluation of tissues following epididymal injection revealed the lack of sperm cells in the head, body and tail of the epididymis confirming the blockage and absence of immature spermatozoa. Moreover, the testicular area adjacent the epididymis, corresponding to *rete testis* and *efferent ductules*, was damaged. This finding demonstrated that an intraepididymal injection may have a more extensive impact on adjacent tissues. More precisely, an injection into the head of the epididymis may damage both testicular and epididymal areas that transport and mature spermatozoa.

In terms of efficiency, injecting the head of the epididymis was not simple and required an experienced technician and echography guidance. The procedure took around 15 min - as long as orchiectomy [31] - making it less desirable for field settings and spay/neuter programs due to the time and resources required.

For contraception of stray male dogs, desirable methods require a suppression of sexual behavior. Results from the current study for intratesticular injection and previous research on $CaCl_2$

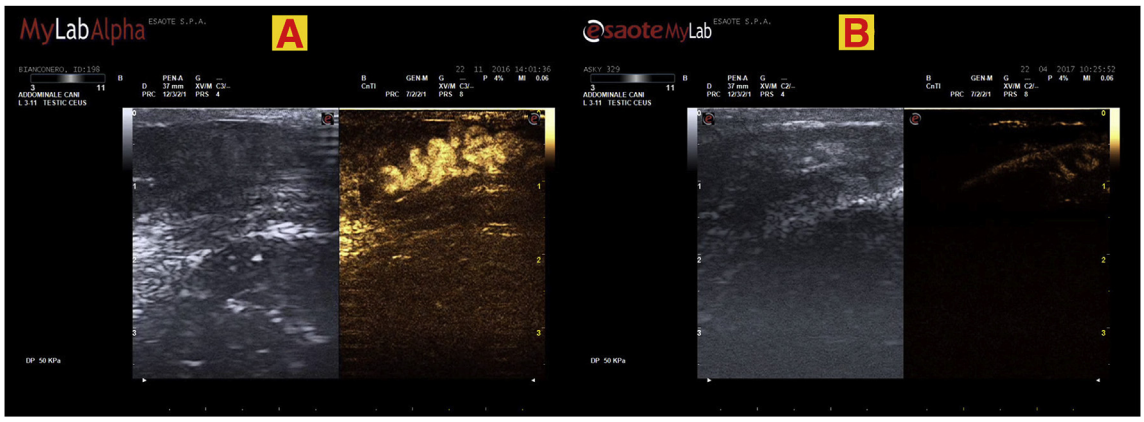

Fig. 3. Ultrasonography on the left, CEUS on the right. **A**: The normal epididymis at T_0. The grey-scale ultrasonography shows a normal aspect of epididymal tubule enhanced by the passage of microbubbles. **B**: Intraepididymal $CaCl_2$ injection (group IEca) at T_5. Reduced epididymal perfusion. The grey-scale ultrasonography shows the epididymis without perfusion and completely anechoic.

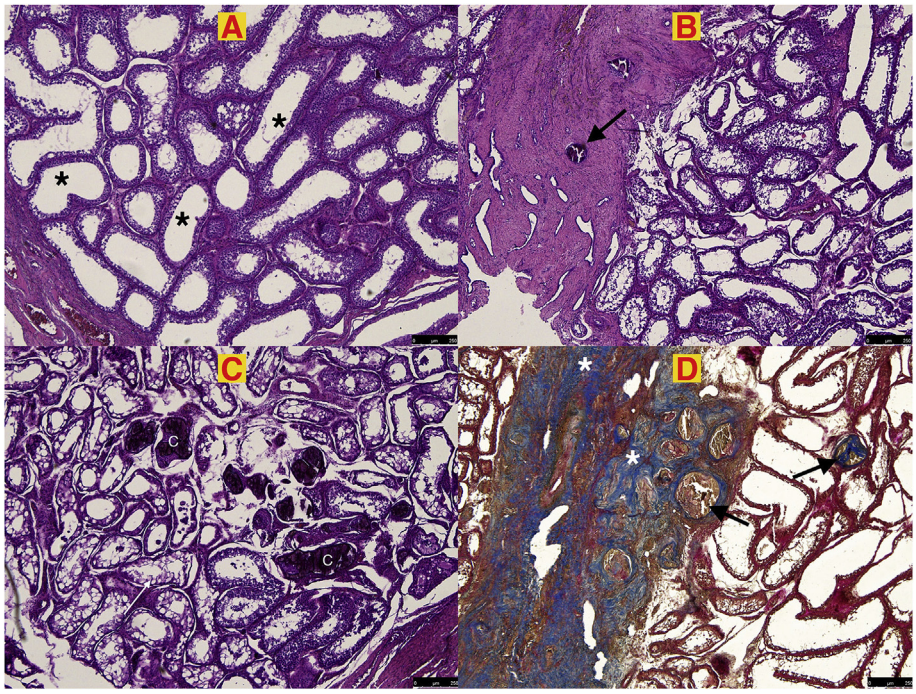

Fig. 4. **A**: Epididymal injection group IEca at T_9. Section of epididymal tubules in the head. No evidence of mature sperm cells (asterisks). (H&E 40×). **B**: Group IEca. Testicular parenchyma close to the epididymis. Small mineral aggregates were seen (arrow). The seminiferous epithelium was partially degenerated with a small number of seminal cells. (H&E 20×). **C**: $CaCl_2$ epididymal treatment IEca. Histological section showing calcium deposits (C) between parenchyma and epididymis. The seminiferous tubules (arrow) appeared empty and showed epithelial degeneration and lack of sperm cells. (H&E 10×). **D**: Epididymal injection. Histochemical section showing exuberant connective tissue (asterisks) surrounding necrotic tubules (arrows) in the testicular parenchyma close to epididymis. (Masson's Trichrome 20×).

demonstrated a significant decrease in concentrations of testosterone in serum, but measurements remained at the lower end of normal physiological concentration [3]. While the goal of this study was not to evaluate behavior changes following chemical sterilization, exposure of the dogs in the ITca group to a female in estrus indicated a lack of sexual behavior. Additionally, aggression toward the research personnel disappeared in the ITca injected dogs. This result supports the concept that a reduction in testosterone concentrations to the low end of the physiological range may affect behavior [3] but further detailed study examining the effects on behavior in response to treatment are needed.

On the contrary, no significant change in serum concentrations of testosterone was observed in dogs that received intraepididymal injections of $CaCl_2$ or in control animals. The lack of change in the intraepididymal group may be explained by the fact that testosterone is produced by Leydig cells residing in the testicular parenchyma which was not damaged during the epididymal injection. Sexual and aggressive behavior remained unchanged for the intraepididymal and control groups, supporting the relationship between testosterone and specific behavior patterns. It may be possible that during the course of the study, dogs initially displaying aggression toward research personnel became habituated to being examined and manipulated by them by the end of the study. However, since aggressive behaviors disappeared only in the aggressive dogs of the ITca group, a correlation between aggression and testosterone is likely.

CEUS and histology showed a normal testicular parenchyma, except for the testicular area just below the epididymal head,

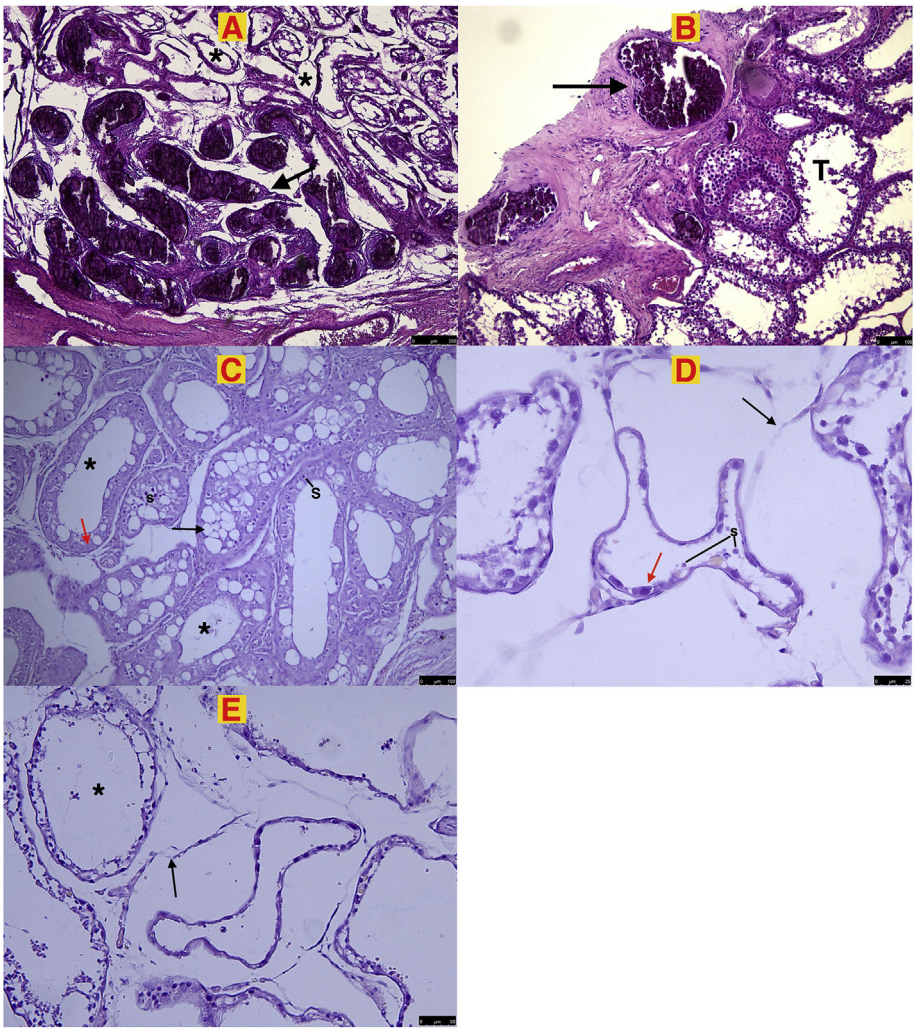

Fig. 5. A: $CaCl_2$ intratesticular treatment, ITca. Histological section showing some calcium deposits (arrows) due to peripheral injection with calcium chloride. Note the severe necrosis of the germinal epithelium of seminiferous tubules (asterisks). (H&E 20×). **B:** $CaCl_2$ intratesticular injection, ITca. Histological section showing several calcium deposits (arrow). The inner portion of the testes showed a severe depletion of seminal epithelium reduced to one cell layer (asterisks). (H&E 20×). **C:** ITca: Histological section showing severe changes in the germinal epithelium of different empty seminiferous tubules (asterisks) with large vacuolations and almost total disappearance of sperm cells. S: spermatogonia; s: spermatocytes; (red arrow): Sertoli cell; (black arrow): vacuoles. (H&E 20×). **D:** ITca: The germinal epithelium reduced to one layer of spermatogonia (red arrow), as well as very few primary and secondary spermatocytes (s), placed on an irregularly displaced basal membrane (black arrow). (H&E 40×). **E:** ITca: Histological section showing some empty seminiferous tubules (asterisk) lined by a unique sheet of cells (sometimes none) on an irregularly distorted basal membrane, showing occasional splitting (arrow). (H&E 40×). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

confirming the integrity of the endocrine structures of the testis. This is the first study that highlights testicular aspects of CEUS after a chemical agent injection either in the testis or epididymis in the dog. It shows that the damage included a reduction of blood perfusion, thus explaining the necrotizing action of $CaCl_2$ on the germinal epithelium.

5. Conclusions

A single, bilateral intratesticular injection of 20% $CaCl_2$ in 95% ethanol is an easily performed and reliable method for induction of sterilization in male dogs. The approach showed long-term efficacy and probably reduced sexual behavior, therefore fulfilling the principal requirements for application to a population of stray canines.

Sterility was also achieved when $CaCl_2$ was injected into the epididymis, but no drop in serum testosterone level occurred. Moreover, performing the intraepididymal injection was as time consuming as orchiectomy and is not easily performed without ultrasound guidance. This approach may be optimal for use in owned dogs where anatomical integrity and testosteronemia maintenance can induce reluctant owners to sterilize their animals. The use of both methods together in stray and owned dogs may help fight the global problem of dog overpopulation.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

RL was involved in the concept and design of the study, analysis and interpretation of results, semen sampling and evaluation, and preparation of this manuscript. RL, GA, were involved in the revision of study design. GA and VC performed the intratesticular injection of the dogs. GA performed the ultrasonography. VC was

involved in the clinical care of the dogs, blood sampling, and acquisition of data. LB performed statistical analysis. CI performed histology. GML, RL and LB were involved in revision of the manuscript. All authors have read and approved the manuscript.

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List of abbreviations used

ANOVA	Analysis of variance
$CaCl_2$	Calcium chloride
X g	Centrifugal force X gravity
°C	Centigrade
dL	Deciliter
F	F-statistic (Fisher)
G	Gauge
g	Gram
hCG	Human chorionic gonadotropin
I.U	International unit
IM	Intramuscular
Kg	Kilogram
x	Magnification
µL	Microliter
mg	Milligram
mm	Millimeter
p	p-value
SD	Standard deviation
SEM	Standard error of the mean
SC	Subcutaneous
Vs	Versus

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.theriogenology.2019.01.006.

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