

## Calcium Chloride as a Non-surgical Sterilant for Male Dogs and Cats: a History and Summary of Research

### **Background**

In the search for a nonsurgical sterilant, a broad range of agents has been injected directly into the testes of male animals from a range of species including livestock, companion animals, and laboratory rats and mice. Chemicals used in intratesticular injections include hypertonic saline solution (Emir 2008), glycerol (Immegart 2000), chlorhexidine digluconate (Pineda 1980), cadmium chloride (Parizek and Zahor 1965, Cameron 1965, Chatterjee 1968), and zinc salts (Oliviera 2007, Levy 2008). Talsur was a zinc-based chemosterilant used in India in 1990, but was quickly withdrawn due to excessive scrotal swelling (Animal People News, May 2007). Of 400 dogs treated, more than 35% had scrotal swelling, and 140 were eventually surgically castrated (Animal People News, November 2011), resulting in some hesitancy in the field to pursue a similar approach with a different agent. The site of testicular injection is important to the mechanism of action: injection into the ductus deferens or epididymides blocks sperm transport and results in azoospermia, while injection into the testicle results in testicular atrophy, decreased spermatogenesis, and reduction of androgen concentrations (Oliveira 2007).

Neutersol, a solution of zinc gluconate neutralized with L-arginine, received FDA approval in 2003 for use in young male dogs (3-10 months of age) and was marketed in the United States until early 2005. Ark Sciences subsequently acquired rights to the technology and marketed the formula in Latin America under the name EsterilSol™. Ark Sciences launched the product in the U.S. in February 2014 under the name Zeuterin™. Its FDA application for expanded use in male dogs 3 months old and above (rather than just 3-10 months) is under review. This product is injected into the dorsal cranial portion of the testis beside the caput epididymis (Figure 1), and its efficacy is 99.6%. Original data available via the FDA submission is included here as reference:

- The pilot study involved 27 animals. The treated dogs were azoospermic at study termination and did not produce offspring when mated with females in heat. Five dogs developed adverse reactions; in subsequent studies a smaller needle was used to minimize these reactions. Fifteen months after injection, 1 group had serum testosterone levels 39% higher, and the other group 19% lower, than that of the control group.
- In the dose-determination study, all dogs in the highest two of three dose groups were azoospermic for the duration of the study. Three of ten dogs in the lowest dose group became oligospermic, but sperm values in these animals were too low to be considered fertile according to definition used by the study (sperm concentrations  $< 2 \times 10^6$ , average ejaculate volume 0.79 mL,  $< 70\%$  sperm motility). No progeny resulted from mating tests. The average serum testosterone levels were 41-52% lower than the average testosterone levels of the control dogs. Twenty-one of 30 treated dogs had testosterone levels in the same range as the control group 24 months after injection. (Note that testosterone concentrations can range widely among intact dogs (Lowseth 1990)).
- The large-scale clinical evaluation study involved 270 dogs. Effectiveness was 99.6%, with treatment success defined as an animal that displayed aspermia, azoospermia, necrospermia, or oligospermia (less than  $20 \times 10^6$  spermatozoa per mL) 6 months post-injection. Seventeen dogs displayed scrotal pain during the first 7 days post-injection, and 2 dogs were reported licking and biting the scrotum 3 and 5 days post-injection. Eleven dogs were surgically castrated: 1 due to chewing damage to the scrotum, 1 due to severe scrotal infection, and 9 based on owner request. Nine animals (it is not clear

whether these were the same 9 that were castrated) displayed sexual behaviors. Testosterone levels were not measured.

- The safety study involved 24 dogs. At week 8, the testosterone levels of all treated dogs were less than those of dogs in the control group. One dog that moved during injection developed a lesion and had to have a testis surgically removed. Five of 6 dogs that received twice the recommended dose experienced signs of pain when examined 24 hours after injection. All treated dogs displayed discomfort when sitting down, and swelling at 24-48 hours after injection.

A small number of animals treated with Neutersol (1.1% in the 270-dog clinical trial) may experience adverse reactions that can require intervention including scrotal ablation. This is believed to be primarily due to leakage of the solution from or near the injection site that can lead to skin irritation and ulceration and excessive licking or chewing of the site by the dog.

### **Calcium Chloride**

Calcium chloride dihydrate ( $\text{CaCl}_2$ ) has been the subject of renewed interest as a potential injectable sterilizing agent for male dogs and cats that may reduce testosterone levels more significantly, and might carry less risk of severe injection-site reactions, than other injected sterilizing agents. Additionally,  $\text{CaCl}_2$  has spurred discussion because it can be made from readily available ingredients, raising questions about the legality, ethics, appropriateness, and advisability of its use in the many countries in which Zeuterin/EsterilSol is not available and is not projected to come to market in the immediate future.

### **Mechanism of Action**

Chemosterilization is hypothesized to result from edema that follows intratesticular injection of  $\text{CaCl}_2$ , leading to necrosis and fibrosis and degeneration of seminiferous tubules (and germ cells) and the interstitial (Leydig) cells (Jana 2005). As testosterone concentrations fall, the integrity of the seminiferous tubule is further compromised. Jana *et al.* have also proposed a role for free radicals in the mechanism of action of  $\text{CaCl}_2$  injection. According to this hypothesis,  $\text{CaCl}_2$  causes production of free radicals in testicular tissue, leading to lipid peroxidation and destruction of cellular structures, and also directly impairing spermatogenesis and androgenesis. Studies from this group have demonstrated decreased activities of catalase, glutathione peroxidase, glutathione S-transferase, and superoxide dismutase, decreased levels of reduced glutathione, and increased levels of TBARS and conjugated dienes, with increased doses of  $\text{CaCl}_2$  (Jana 2001, 2005, 2007, 2011).

Below follows a summary of both published and unpublished research into the use of  $\text{CaCl}_2$  as an injectable sterilizing agent.

### **Published studies**

In 1976, it was reported that a single injection of  $\text{CaCl}_2$  under the horn buds of young calves prevented horn development (Koger 1976). The effectiveness of this treatment led to investigation of the ability of  $\text{CaCl}_2$  to selectively destroy components of the male reproductive system (Bowman 1978). By 1978,  $\text{CaCl}_2$  had been injected intratesticularly in over 300 calves and a smaller number of colts, pigs, lambs, goats, dogs, and cats (Koger 1978). Koger reported that the treatment resulted in necrosis and atrophy of the testicles, and that the advantages of non-surgical castration included “an apparent reduction in pain and stress, and elimination of hemorrhage, hernia, infection, myiasis and other surgical sequelae” (Koger 1978).

At that time, the optimal  $\text{CaCl}_2$  formulation had not been determined; various concentrations of  $\text{CaCl}_2$  had been used, ranging from 10% to 75% weight/volume (w/v)  $\text{CaCl}_2$ , dissolved in sterile water, 99% ethanol, or 70% isopropanol. The final volume administered depended on the testicular mass. Injection was in the draining head of the epididymis, with 1 of 2 injection techniques: delivery via a single injection of 30% of the solution to the draining head followed by moving the needle to distribute the remaining solution throughout the testes, or multiple separate lateral injections (Koger 1978). After treatment with  $\text{CaCl}_2$ , most animals walked with a stilted gait for 1 to 2 days, experienced swelling and progressive induration for 4 to 5 days, and exhibited testicular atrophy at 2 to 3 weeks after injection (Koger 1978). After 3 months only a small remnant of the testicle remained. Other than the stilted walking in the first 24 to 48 hours following injection, no adverse effects or signs of pain were described. In case studies, behavior became asexual in 1 treated bull and comparable to after surgical castration in a second; 1 treated dog exhibited reduced scent marking after injection and a second dog demonstrated no interest in a female in estrus. Though precise data were not available, it was estimated that in 35% of dogs, 1 testis failed to atrophy and a second injection was required (Koger 1978). If too much solution was injected or the solution leaked outside the tunica, necrosis and sloughing of the scrotal skin followed which healed rapidly and without infection (Koger 1978). The author states that irritating skin antiseptics are avoided in dogs, because they may cause excessive licking of the scrotum that can result in dermatitis (Koger 1978).

In 1978, Bowman, Koger and colleagues published a study of intraepididymal injection of  $\text{CaCl}_2$  in rams. Ten rams were paired based on pre-treatment semen production and randomly assigned to receive  $\text{CaCl}_2$  (50% w/v) in saline, or saline alone. Injection was exclusively into the ventral caudal epididymides, taking care to avoid injection into testicular tissue. Injection volume was 3 ml based on preliminary experiments that found a 5 ml dose resulted in some necrosis of the ventral scrotum while 1 or 2 ml doses did not cause widespread epididymal destruction. Besides slight swelling during the first week, no adverse effects were reported. Ejaculate volume and concentration of spermatozoa were reduced by 12 days after the injection, and fragmented spermatozoa and no sperm motility were observed. The authors concluded the animals were sterile based on the absence of whole motile sperm. The intraepididymal  $\text{CaCl}_2$  treatment did not decrease libido.

After these initial experiments with  $\text{CaCl}_2$ , no further studies were published until 1998, when intratesticular injections of different concentrations of  $\text{CaCl}_2$  were studied in 60 stray dogs (15-16 kg) (Samanta 1998).  $\text{CaCl}_2$  was diluted in distilled water to a final concentration of 0, 5, 7.5, 10, or 12.5%. 1.5 mL of the  $\text{CaCl}_2$  solution plus 0.5 ml of 2% lidocaine (Table 1) were injected into each testicle, and the dogs were followed for 8 weeks. The lowest dose, 5%  $\text{CaCl}_2$ , did not induce fibrosis, but 7.5% and greater percentages of  $\text{CaCl}_2$  did induce necrosis of the testicular parenchyma and subsequent fibrosis. At 10% and 12.5%  $\text{CaCl}_2$ , the testicular tissue was completely replaced by fibrocollagenous bands, and no evidence of spermatogenesis was observed. Testosterone levels were not measured. The author notes that adverse events including fever and painful swelling that had been reported after injection of agents such as cadmium chloride or BCG were not observed in this study, and that  $\text{CaCl}_2$  appeared to be non-irritating to body tissues.

Since this study in dogs, a group of researchers has carried out a series of dose-dependent studies of intratesticular injection of  $\text{CaCl}_2$  in rats (Jana 2002), goats (Jana 2005), dogs (Jana 2007), and cats (Jana 2011). These and other recently published studies are summarized below.

*Rats (Jana et al. 2002)*

Five groups of rats (each group, n = 12) were injected with solutions of 0%, 2.5%, 5%, 10%, or 20% (w/v) CaCl<sub>2</sub> in sterile saline. Injection volume was 0.1 mL per 100 g body weight. The needle was directed from the caudoventral aspect of each testis approximately 5 mm from the epididymal tail, towards the dorsocranial aspect of the testis. The solution was carefully deposited along the entire route from the proximal to the distal end by linear infiltration while withdrawing the needle and the authors state all necessary care was taken to prevent seepage of the solution from the injection site. No injection site reactions or other adverse effects were reported, except for transient testicular swelling in all injected animals, and a transient dose-dependent increase in rectal temperature in animals that received the highest 2 doses of CaCl<sub>2</sub>. No change in eating habits or body weight was observed following injection. In addition, no significant alterations in plasma concentrations of prolactin, corticosterone or fasting blood glucose were observed in any of the treatment groups compared to the controls. Thirty days following injection, plasma testosterone concentrations and epididymal sperm counts were reduced in a dose-dependent manner in all groups except 2.5% (Table 1), but at no treatment dose were these values consistent with castration. At the highest 2 doses, histology revealed a dose-dependent increase in necrosis of the germinal epithelium of the semiferous tubules and Leydig cells. The authors concluded that the 10% and 20% doses were optimal effective doses for induction of chemosterilization. Behavior and mating tests were not reported in this publication but have been carried out by this group and are discussed in the unpublished research section below.

*Goats (Jana et al. 2005)*

Four groups of 6 goats each (8 kg average body weight, range 6-10 kg) were injected with saline or 10, 20, or 40 mg CaCl<sub>2</sub> per kg body weight in saline in a volume of 2 ml per testicle (Table 1) (correlating to 4%, 8%, or 16% (w/v) CaCl<sub>2</sub> solutions for an 8 kg animal). Animals were also injected with 0.5 ml of 2% lidocaine hydrochloride solution, for a total injected volume of 2.5 ml. Each injection was performed using a 21 gauge needle directed from the caudoventral aspect of each testis approximately 1 cm from the epididymal tail in a similar manner as described for rats. Mild swelling was observed by 24 hours after injection, reached a maximum at 48 to 72 hours after injection, and decreased by 3 weeks. Food consumption did not change and cortisol did not increase significantly. Animals were castrated at 30 days. Testicular weight per kg body weight and plasma concentrations of testosterone decreased significantly in a dose dependent manner, such that mean basal plasma testosterone concentrations were < 0.5 ng/ml in the highest treatment group. Epididymal sperm counts were not performed in this study, but the highest dose of CaCl<sub>2</sub> resulted in similar necrosis of the germinal epithelium of the semiferous tubules and Leydig cells as reported in the rat study.

*Cattle (Canpolat 2006)*

Intratesticular injection of a CaCl<sub>2</sub> solution was compared to absolute ethanol in twelve 12- to 15-month-old bulls (250-400 kg body weight). Each testis was injected with 2 mL of a local anesthetic (2% Citanest), followed by 10 ml of absolute ethanol or 10 ml of a 30% CaCl<sub>2</sub> solution. The authors do not specify in what the CaCl<sub>2</sub> was dissolved, or the testicular size of the animals. Injections were performed from the caudoventral aspect of the testis approximately 1 cm from the epididymal tail. No fever was observed, but a slight increase in firmness of testes was detected on palpation. In both groups, swelling peaked within 48 hours. Ultrasonography was used to measure the testes. Testicles atrophied following ethanol but not CaCl<sub>2</sub> injection; diffuse tubular necrosis and an inflammatory response were seen in microscopic examination of testicles from the ethanol group. Similar lesions were noted in the CaCl<sub>2</sub> group, but the severity and distribution of the lesions were less pronounced. In 2 animals in the CaCl<sub>2</sub> group, swelling

was associated with orchitis and scrotal sloughing. Serum testosterone levels decreased (from about 13 ng/ml to 2.6 ng/ml after 60 days) following ethanol injection but did not change after injection with CaCl<sub>2</sub>. The authors state their results disagree with previous studies since no significant alteration of serum testosterone levels or evidence of severe histopathological damage consistent with sterility were observed in the bulls treated with CaCl<sub>2</sub>.

*Dogs (Jana and Samanta 2007)*

Jana and Samanta conducted a dose-response study in 30 stray dogs (10-15 kg; 2-3 years of age). Dogs were administered intratesticular injections of 1 mL normal saline, or CaCl<sub>2</sub> at doses of 5, 10, 15 or 20 mg/kg bodyweight in a 1 mL solution containing 1% lidocaine hydrochloride (equivalent to 6.25%, 12.5%, 18.75%, or 25% (w/v) CaCl<sub>2</sub> solutions for a 12.5 kg animal). The authors do not specify whether the CaCl<sub>2</sub> and lidocaine were dissolved in water or saline. The authors state that necessary care was taken to avoid the seepage of the solution from the injection site. No fever or marked inflammatory swelling were observed, though all dogs, including controls, showed mild discomfort 1 to 5 min after injection. As in their study with goats (above), mild testicular swelling was observed by 24 hours after injection, reached a maximum at 48 to 72 hours after injection, and decreased by 3 weeks. The authors state the dogs' behavior became calm after 30 days, but no data are presented to quantify this claim. Dogs were castrated, or euthanized and testes collected, 45 days after injection. Testicular weight decreased with increasing dose of CaCl<sub>2</sub>. Epididymal sperm count was reduced in all CaCl<sub>2</sub>-treated animals with the greatest effects seen at the 15 mg and 20 mg per kg of body weight dosage (21 sperm per ml of suspension in the highest dose vs 8350 sperm per ml in controls). Both intratesticular and plasma testosterone levels decreased in a dose-dependent manner, with 90% and 94% reductions in testosterone levels in the 2 highest doses. Cortisol and other measures of stress, including total plasma protein, blood urea nitrogen, fasting blood sugar and PCV, did not change.

*Dogs (Chatterjee et al. 2009)*

A second study of CaCl<sub>2</sub> intratesticular injection in stray dogs was performed by Chatterjee *et al.* (2009). Twenty-four dogs (18.5 ± 10 kg; aged 2 to 2.5 years) were anesthetized with ether and divided into 4 groups: a control group that received intratesticular saline injections, and 3 groups that received increasing doses of CaCl<sub>2</sub> (100, 150, or 200 mg/kg bodyweight in 0.5 mL of normal saline (equivalent to 370%, 555%, or 740% (w/v) CaCl<sub>2</sub> solutions for an 18.5 kg animal). After 60 days the testes were surgically removed. Epididymal sperm counts decreased in a dose-dependent manner, with 0.31x10<sup>3</sup> sperm per ml of suspension in the animals receiving the highest dose vs 12.41x10<sup>6</sup> sperm per ml in the control animals. Serum testosterone levels also decreased in a dose-dependent manner, with levels about 2 ng/ml in dogs receiving the highest dose vs 8.5 ng/ml in the control animals (76% reduction). Histologic studies of the testis observed a complete disruption of the tubular architecture, degeneration of interstitial Leydig cells, and absence of germ cells at the highest dose.

*Dogs (Leoci et al. 2014a)*

Leoci *et al.* conducted a 12-month dose-response study in dogs to identify the formulation of CaCl<sub>2</sub> in saline offering the best combination of safety and efficacy. Five groups of 10 dogs each were treated with saline (control group) or 10%, 20%, 30%, or 60% CaCl<sub>2</sub> dihydrate (w/v) in saline. All dogs were 2 to 5 years of age, weighed 18 to 24 kg, and were housed at a private shelter for the duration of the study. Dogs were lightly sedated with acepromazine maleate prior to measurement of scrotal width. Injection volumes were based on scrotal diameter; animals with scrotal diameters of 19 to 22 mm received 0.8-ml injections and those with scrotal diameters 23 mm or greater received 1-ml injections. Blood testosterone concentrations and

semen characteristics were assayed on day 0 (before treatment) and at 2, 6, and 12 months post injection. Semen volume and testicle width were measured at day 0 and month 12. Semen evaluation was conducted by computer assisted sperm analysis (CASA).

All dogs were azoospermic at 2 and 6 months post injection, and all doses significantly lowered semen volume, total sperm count, testosterone levels, and testicular size compared to the control group. At 12 months after injection, serum testosterone concentrations showed a dose-dependent decrease; the serum testosterone concentration in the 60% CaCl<sub>2</sub> group was 83% lower than that of the control group. All dogs treated with 30% or 60% CaCl<sub>2</sub> were azoospermic at 12 months, but 2 dogs that received 20% CaCl<sub>2</sub> and 6 that received 10% CaCl<sub>2</sub> regained some sperm production by 12 months after injection. Though the higher doses resulted in more durable azoospermia, they were also associated with a higher risk of complications.

All dogs experienced slight increases in firmness of testes on palpation from 24 hours to days 3-7 after injection. Dogs receiving 10% or 20% CaCl<sub>2</sub> showed no agitation, fever, or marked inflammatory swelling of the testes. However, some dogs treated with 30% or 60% CaCl<sub>2</sub> showed signs of discomfort and licked the injection site..2 dogs that received 30% CaCl<sub>2</sub> and 6 that received 60% CaCl<sub>2</sub> developed abscesses and underwent surgical castration.

The authors concluded that intratesticular CaCl<sub>2</sub> injection is a promising sterilization agent, and that a 20% solution of CaCl<sub>2</sub> was the most effective dose not associated with a risk of serious complications. However, because some testicular function resumed a year after treatment at this dose, the authors proposed further studies to improve the efficacy of this dose.

#### *Dogs (Leoci 2014b)*

Leoci *et al.* conducted a study to evaluate the long-term efficacy of 2 formulations of 20% CaCl<sub>2</sub>; 20% CaCl<sub>2</sub> in 95% ethanol and 20% CaCl<sub>2</sub> in 1% lidocaine. Each experimental group consisted of 21 dogs and a control group of 10 dogs was injected with saline. Dogs were 2 to 6 years old, weighed 18 to 28 kg, and were housed in a shelter for the duration of the study. Dogs were lightly sedated, their testes measured, and testes injected based on testicular width (1-ml injection if testicular width 23 mm or greater, 0.8-ml injection if testicular width 19 to 22 mm). At 2, 6, and 12 months after injection, semen evaluation and blood testosterone assays were conducted. Blood for serum testosterone assays was collected 2 hours after each dog received a subcutaneous injection of 1000 international units of human chorionic gonadotrophin (hCG). All samples were collected at the same time of day, with hCG injections conducted between 8:00 a.m. and 8:30 a.m.

All dogs injected with CaCl<sub>2</sub> had decreased sperm counts relative to controls at 2, 6, and 12 months after injection. All dogs injected with CaCl<sub>2</sub> in alcohol were azoospermic at 12 months, compared to 81% of those injected with CaCl<sub>2</sub> in lidocaine. At 2 and 6 months after injection, serum testosterone levels decreased in all dogs injected with CaCl<sub>2</sub> relative to baseline and relative to the control group. At 12 months after injection, the mean serum testosterone of dogs treated with CaCl<sub>2</sub> in alcohol remained significantly decreased (63.6% lower than the mean baseline level at time zero), while average levels increased back to baseline levels in dogs treated with CaCl<sub>2</sub> in lidocaine.

The authors conclude that a single, bilateral intratesticular injection of 20% CaCl<sub>2</sub> in alcohol represents an optimal method for sterilizing male dogs, producing azoospermia and significantly reducing serum testosterone for at least 12 months with few inflammatory reactions or other side effects.

*Cats (Baran 2010)*

In a study of 4 cats (Baran 2010) 1 cat each was injected with 0 mg, 10 mg, 20 mg, or 40 mg of CaCl<sub>2</sub> in 0.2 ml of saline. The site of injection was not described in the text, but appears to be from the caudal pole of the testis based on the figures presented. Semen could not be collected from the cat that received 40 mg so the authors concluded there was damage to the seminiferous tubules. However, it is not clear from the report if semen was able to be collected from this cat before the testes were injected. In the other 2 cats treated with CaCl<sub>2</sub>, decreased numbers of live and motile sperm were observed in the semen (greater than 20 x 10<sup>6</sup> per ml in controls, less than 20 x 10<sup>6</sup> per ml in treated animals. Exact numbers and the method used for counting sperm were not reported). The cats were followed for 60 days, and no change in blood parameters, attitude, or appetite were observed. The scrotum was swollen or testis reported to be sore in all cats post-injection, but no mention is made of skin irritation or injection site reactions. Testosterone levels were not reported. Based on histological findings, the highest dose was considered suitable for effective chemical sterilization.

*Cats (Jana 2011)*

Jana and Samanta extended their studies to cats (Jana 2011). The authors proposed that CaCl<sub>2</sub> would be a safer option for cats than zinc gluconate, and that it could induce a greater reduction of testosterone. A total of 30 cats were divided into 5 groups: 3 groups received different doses of CaCl<sub>2</sub> in 0.25 ml of saline containing 1% lignocaine hydrochloride, 1 group received saline injections, and the final group was surgically castrated. Injections were directed from the ventral aspect of the testis approximately 0.5 cm from the epididymal tail towards the cranial aspect, and the solution injected while the needle was withdrawn (Figure 2). Injected cats were observed continuously for 72 hours, every hour up to 7 days, every 6 hours up to 30 days and every 12 hours to 60 days. The authors report that their previous experience with CaCl<sub>2</sub> supports that wiping away any excess or spilled solution after injection can avoid tissue necrosis. However, in their earlier published studies, there are no descriptions of tissue necrosis or other injection-related events, so it is not clear how prevalent these adverse events might be in the experience of this group. No fever was detected in treated animals. Mild discomfort was observed in all animals, including controls, 1 to 5 min after injection. Swelling was observed in all animals by 24 hours. Swelling reached a maximum at 48 to 72 hours, then decreased over 4 weeks. Testicular size and epididymal sperm count decreased in a dose dependent manner. At the highest dose, there were 102 sperm per ml of suspension compared to 6540 sperm per ml in the saline-injected cats (98% decrease). A dose-dependent decrease in serum and testicular testosterone levels was observed, but serum cortisol levels were not affected. 60 days after injection, serum testosterone was 2.15 ng/ml in the group that received the highest dose, compared to 7.82 ng/ml in saline-injected animals (73% decrease), and approximately 0.3 ng/ml in surgically castrated animals (96% decrease). Intratesticular testosterone was about 0.05 ng/mg tissue in the group treated with the highest dose, compared to about 0.35 ng/mg tissue in saline-injected animals (86% decrease). As in their previous work, the authors identified signs of increased levels of reactive oxygen species considered responsible for oxidative tissue damage.

**Table 1. Published studies of intratesticular injection of calcium chloride**

Study	Animal	Solution	Volume	Serum testosterone	Testicular testosterone	Sperm count in semen	Sperm in epididymis	Injection site	Notes
Koger 1978	Dog, 3 years old, 40 kg	30 g CaCl <sub>2</sub> in 100 ml 90% ethanol	2 ml per testis	ND	ND	ND	ND	Testis, not specified further	No interest in female in estrus 6 weeks later
Koger 1978	Dog, 3 years old, 6.8 kg	10% CaCl <sub>2</sub> in 70% isopropanol	0.75 ml in one testis and 0.6 ml in other	ND	ND	ND	ND	Testis, not specified further	Reduced scent marking after treatment
Bowman 1978	10 rams	50% CaCl <sub>2</sub> (w/v) in 0.9% NaCl (w/v)	3 ml per testis	ND	ND	0.15x10 <sup>9</sup> vs 3.96x10 <sup>9</sup> in controls	ND	Ventral caudal epididymides	No motile sperm, no change in libido
Samanta 1998	60 dogs (15 to 16 kg)	5%, 7.5%, 10% or 12.5% CaCl <sub>2</sub> Distilled water as control	1.5 ml + 0.5 ml 2% xylocaine per testis	ND	ND	ND	ND	Testis, not specified further	Some regeneration seen at 8 weeks with 5% and 7.5% doses
Jana 2002	60 Wistar rats, 130 g	0, 2.5, 5, 10 or 20 mg CaCl <sub>2</sub> in saline	0.1 ml per 100 g body weight per testis	90% decrease at highest dose	ND	ND	310 per ml in highest dose vs 12410 in controls (98% decrease)	From caudoventral aspect of each testis, 5 mm from epididymal tail, towards dorsocranial aspect, solution dispensed as needle withdrawn. Care to avoid seepage from injection site.	

Study	Animal	Solution	Volume	Serum testosterone	Testicular testosterone	Sperm count in semen	Sperm in epididymis	Injection site	Notes
Jana 2005	24 Black Bengal goats, 1.5 to 2 years old, 6 to 10 kg	10, 20, or 40 mg CaCl <sub>2</sub> per kg body weight, in saline	2 ml + 0.5 ml lignocaine HCl per testis	90% decrease with highest dose	ND	ND	ND	Caudoventral aspect of each testis, 1 cm from epididymal tail, towards dorsocranial aspect, solution dispensed as needle withdrawn. Care to avoid seepage from injection site.	
Canpolat 2006	12 bulls, 12-15 months old, 250-400 kg	30% CaCl <sub>2</sub> (in water?), 100% ethanol in control group	10 ml per testis after 2 ml 2% citanest	No change	ND	ND	ND	From caudoventral aspect approximately 1 cm from epididymal tail towards dorsocranial aspect. Animals restrained.	No effect of CaCl <sub>2</sub>
Jana 2007	30 dogs, 10 to 15 kg, 2 to 3 years old	10, 15, or 20 mg CaCl <sub>2</sub> per kg body weight	1 ml containing 1% lignocaine HCl per testis	90% decrease with highest dose	90% decrease with highest dose	ND	21 per ml in highest dose vs 8350 per ml in controls	From the caudoventral aspect of each testis approximately 1 cm from epididymal tail, towards dorsocranial aspect, solution dispensed as needle withdrawn. Care to avoid seepage from injection site	Dose-dependent decrease in epididymal sperm count

Study	Animal	Solution	Volume	Serum testosterone	Testicular testosterone	Sperm count in semen	Sperm in epididymis	Injection site	Notes
Chatterjee 2009	24 dogs, 2 to 2.5 years old, 18.5 ± 10 kg	0.1 ml saline per 100 g, or 100, 150, or 200 mg CaCl <sub>2</sub> in 0.5 ml saline per kg	18.5 ml or 9.25 ml per 18.5 kg body weight <sup>a</sup>	76% decrease with highest dose	ND	ND	310 per ml in highest dose vs 1.241 x 10 <sup>7</sup> per ml in controls	Needle directed from caudoventral aspect of testis approximately 5 mm from the epididymal tail towards the dorsocranial aspect. Solution deposited while withdrawing needle.	
Baran 2010	4 cats, 1 to 3 years old, 3.5 to 4.0 kg	0, 10, 20, 40 mg CaCl <sub>2</sub> in saline	0.2 ml per testis	ND	ND	Greater than 20x10 <sup>6</sup> in controls, less than 20x10 <sup>6</sup> in treated	ND	Testis, not specified further	Cat that received highest-dose unable to ejaculate. Cats that received lower doses became oligospermic.
Jana 2011	30 cats, 2 to 3 kg, 9 to 12 months old	5%, 10%, 20% sterile CaCl <sub>2</sub> in saline	0.25 ml in saline containing 1% lignocaine HCl per testis	75% decrease with highest dose	86% decrease with highest dose	ND	102 per ml in highest dose vs 6540 per ml in controls	Needle directed from ventral aspect of testis approximately 0.5 cm from epididymal tail towards cranial aspect, dispensed as needle withdrawn.	Dose-dependent decreases in epididymal sperm count and testosterone levels
Leoci 2014a	50 dogs, 18 to 24 kg, 2 to 5 years old	0, 10%, 20%, 30%, 60% CaCl <sub>2</sub> in saline	1 ml for testicular width > 23 mm; 0.8 ml for 19 to 22 mm	70% decrease with highest dose at 12 months after injection	ND	(x10 <sup>6</sup> ) 0: 311.4 10%: 29.8 20%: 7.5 30%: 0 60%: 0 12 months after injection	ND	Needle directed from caudal pole of each testis approximately 5 mm from epididymal tail, directed towards opposite pole.	Dose-dependent decrease in sperm count, increased risk of adverse events at two highest doses.

Leoci 2014b	52 dogs, 18 to 28 kg, 2 to 6 years old	0, 20% CaCl <sub>2</sub> in 95% ethanol, 20% CaCl <sub>2</sub> in 1% lidocaine	1 ml for testicular width > 23 mm; 0.8 ml for 19 to 22 mm	63.6% decrease with ethanol formulation at 12 months after injection	ND	(10x <sup>b</sup> ) 0: 311.4 lidocaine; 7.1 ethanol: 0	ND	From ventral aspect of each testis approximately 0.5 cm from epididymal tail towards cranial aspect of testis.	
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<sup>a</sup> Appears to be an error in the description of dose in this publication.  
 Abbreviation: ND, not determined.

Unpublished studies

1. Goats

A pilot study in the U.S. attempted to replicate calcium chloride use in goats (Schouten *et al.* 2012, personal communication, Elaine Lissner). Four juvenile pygmy goat kids 4 to 6 weeks of age were injected (Figure 4) with 0.5 to 1 ml per testis, depending on testicular size, of 20% CaCl<sub>2</sub> by weight dissolved in either 75.5% ethanol (food grade) or 2% lidocaine solution. The injections were well tolerated by all goats, with brief vocalizations at the moment the needle punctured the skin but no signs of further distress. The scrotum became swollen, hard and slightly tender to the touch in the days following injection, but behavior, including appetite and activity level, remained within normal limits and the animals showed no apparent signs of distress and no adverse clinical signs. Body temperatures remained normal and the goats' weight continued to increase. One animal experienced testicular shrinkage; the testicular tissue felt hard and somewhat atrophied after several months. The CaCl<sub>2</sub> injection appeared to reduce testicular growth in the other 3 adolescent goats, since their testicles were only slightly larger 2-3 months after injection than when injected, as contrasted with a volume increase of approximately 10 fold expected with normal maturation. By feel, those goats' scrotal contents were harder than normal and thought to include a mixture of scarring and pathologic swelling.

Two of 4 goats developed skin reactions, 1 at 4 days and 1 at 11 days after the procedure, which continued to develop over several weeks. Though the lesions in both animals were reported to come close to requiring veterinary care, both eventually resolved without intervention. In these animals, the reactions appeared more extensive on the ethanol side than the lidocaine side. Conversely, the animal that had injections in an ethanol base in both testes experienced no skin reaction. Injections on this goat were performed using a 30-gauge, ½-inch needle (insulin syringe) rather than a 25-gauge, 1-inch needle with syringe filter.

Two to 3 months after injection, testosterone levels of the goat with testicular shrinkage were about 30-50% of normal (2.53 ng/ml) and were substantially reduced in 2 of the other 3 goats (less than 1 ng/ml) compared to expected levels of 4-9 ng/ml at this age (Georgie *et al.* 1984). Based on testicular shrinkage in 1 goat and the dramatically reduced testosterone levels in 2 others, the researchers concluded that 3 of the 4 animals were likely to have been successfully sterilized by the procedure. Serum testosterone results are shown in Table 2.

**Table 2: Serum Testosterone Levels in Goats Treated with CaCl<sub>2</sub>**

Goat (birth date)	Treatment	Testosterone (ng/ml) <sup>a</sup>				
		4/11	4/20	5/9	6/27	7/09
1 (2/26/2012)	Ethanol, both sides	1.35	ND	ND	ND	2.53
2 (3/8/2012)	Ethanol side 1 Lidocaine side 2	0.63	ND	3.7 7	8.85	ND
3 (3/16/2012)	Ethanol side 1 Lidocaine side 2	2.49	0.92	2.6 6	0.22	ND
4 (3/16/2012)	Ethanol side 1 Lidocaine side 2	ND	1.55	0.5 7	0.93	ND

<sup>a</sup> Injection date for goat 1 was 3/23/2012, for goats 2, 3 and 4 was 4/20/2012. Abbreviations: ND, not determined.

Surgical castration was performed on July 10, 2012. Independent histological evaluation (Necropsy Services Group, Davis, CA) reported that in all specimens, the tubules of the epididymis were empty (negative spermatids) and concluded that all of the testicles were effectively sterile. Re-examination of testicular tissues to evaluate the distribution of interstitial testosterone-producing cells (Leydig cells) was hampered by incomplete fixation of the interior of the testicular samples; however, it appeared to show an apparent normal number and distribution of Leydig cells in all the specimens, with the exception of goat 4, in which there was an apparent absence of interstitial cells in both right and left testicles.

The researchers propose several possible contributing factors to the skin reactions and poorer testosterone results than in other studies. They did not change the needle between drawing the fluid and injection (a practice recommended for Esterilol injections, but not described in published studies of CaCl<sub>2</sub> injections so should not be a factor contributing to differences in frequency of injection site reactions). Second, use of a fine 30-gauge insulin syringe and needle (as was done in animal #1, but not subsequent animals) may reduce or prevent skin reactions. Third, the two-sided study design in goats #2-4, using an ethanol base in 1 testis and lidocaine base in the other, may have affected results. This theory is supported by later studies (Leoci, above) showing reduced swelling from CaCl<sub>2</sub> in ethanol than lidocaine. Lastly, the growing goat may be a difficult model, as its testes normally expand from egg size to grapefruit size as it grows. Published goat studies (Jana *et al.* 2005) used adult goats (age 1.5 to 2 years) and a larger volume of fluid for the large adult testes (2.5 ml instead of 0.5 to 1.0 ml).

All 4 goat kids are healthy as of 7/28/2012 and have been adopted out as pets.

## 2. Rats

The Masters thesis of Kuladip Jana (Jana 2004) includes studies of the fertility and sexual behaviors of male rats injected with CaCl<sub>2</sub>. Two groups of rats were studied; 12 injected with saline (0.1 ml of normal saline per testis per 100 g body weight), and 12 injected with CaCl<sub>2</sub> (20 mg CaCl<sub>2</sub> in 0.1 ml normal saline per testis per 100 g body weight). On the 28<sup>th</sup> day after injection, vehicle and CaCl<sub>2</sub>-treated rats were allowed to mate with virgin female rats that were in day 1 of the 4-day estrous cycle; 2 females were housed with 1 male rat per cage and were observed for 4 hours. The male rats were removed from the cages and vaginal smears taken from the females. Appearance of sperm in the smears was considered evidence of successful mating. On the 10<sup>th</sup> post coital day, implantation sites in the uterine horns of the female rats were counted. Behavior of most of the CaCl<sub>2</sub>-treated males was asexual. A summary of the behavior and mating results is presented in Table 3.

**Table 3. Behavior and fertility of male rats injection with CaCl<sub>2</sub>**

Treatment (no. of rats)	No. of matings	No. of sperm per microscopic field in vaginal smears (mean)	No. of implantation sites in uterine horns (mean)
Males introduced to females 28 days post-injection			
Vehicle (12)	11	24	4.5
CaCl <sub>2</sub> (12)	4	2.5	0.0
Males introduced to females 90 days post-injection			
Vehicle (12)	10	20	5.0
CaCl <sub>2</sub> (12)	0	0.0	0.0

A second experiment was carried out with a longer timeframe to determine if the observed effects were permanent. Fertility tests, plasma testosterone levels, and testicular and epididymal histomorphology were studied 90 days after injection. As in the previous study, 12 male rats were treated with vehicle and 12 with CaCl<sub>2</sub>. On the 91<sup>st</sup> day after injection, the male rats were introduced to female rats in estrus as in the experiment described above. No sexual behavior was observed, no successful mating occurred, and no implantation sites were observed with any of the CaCl<sub>2</sub>-treated rats. Testosterone levels remained low in the CaCl<sub>2</sub>-treated rats (0.32 ng/ml vs 10.80 ng/ml in vehicle-treated animals), and no mature or immature germ cells were identified upon microscopic examination of their testes. The epididymal section of the CaCl<sub>2</sub> treated rats exhibited only fibrous and hyaline tissue with no spermatozoa.

### 3. Dogs

A field study in Kolkata, India involved 52 dogs that were treated with Chemisterol®, a patented agent containing 20% CaCl<sub>2</sub> and 1% lignocaine HCl in saline. The dose injected was based on testicular width: 0.25 ml for widths 10-14 mm, 0.5 ml for 15-18 mm, 0.8 ml for 19-22 mm, and 1 ml for 23 mm or above. A 21/22-gauge needle was used, and injections were from the ventral aspect of the testes approximately 0.5 cm from epididymal tail toward the cranial aspect (Figure 3), with solution deposited along the entire route while withdrawing the needle from the proximal to distal end. Swelling reached a maximum at 48 to 72 hours after injection and decreased over 4 weeks. By 60 days after injection, testicle size was reduced 60% to 70%. In this field study, the researchers quantified adverse events not observed in their previous study with this or similar formulations (see Jana 2007 in published studies section above). Adverse events included 13 dogs that experienced elevated rectal temperatures, 11 dogs that experienced moderate pain, 7 dogs (13.5%) with ulcers at the injection site, and 3 dogs (5.8%) with severe ulceration that were castrated surgically. Ulcers were thought to be due to poor injection technique, reinforcing the need to avoid seepage of solution from the injection site. In some cases a light sedative was used to help restrain the dogs so seepage would be less likely. Separate needles were used for drawing up and injecting solution to further avoid getting solution on the skin near the injection site (personal communication, Kuladip Jana).

### 4. Cats

Farms in rural Northern California are using calcium chloride, 20% in saline in about a 10:1 ratio with a 2% lidocaine solution, to sterilize goats and barn cats. These farms consider it more humane than the usual farm method for neutering goats (banding), and more humane and less prone to infection than the usual farm method of neutering barn cats (cutting). A handful of juvenile goats (bucklings) have been neutered with calcium chloride. Though no fertility or testosterone testing has been done, the owner reports that they are "not acting bucky" (mounting/ flapping lips/ urinating on self) and is very satisfied. The farms report the injections have been successful, and the injection is being requested by neighboring farms (personal communication, Elaine Lissner).

### **Summary**

Intratesticular CaCl<sub>2</sub> injection has been conducted in multiple species, including dogs and cats. In general, the treatment results in necrosis and fibrosis and a decrease in testicular size associated with a decrease in testosterone levels. Animals have been assumed to be sterile based on structural disruption detected through microscopic observation and histopathology, and on reduced sperm counts, but the only specific test of post-treatment fertility has been an unpublished study conducted in rats (Jana 2004). There is not a consensus on the criteria that must to be met to establish sterility (for example, whether sperm count must be zero, or whether

mating tests must be conducted), and therefore whether any publication summarized above has definitively demonstrated sterility of treated animals remains an open question.

The formulation of the  $\text{CaCl}_2$  injection has not been standardized (Table 1); published work has included  $\text{CaCl}_2$  dissolved in water, saline, or alcohol, and several studies have incorporated a local anesthetic in the injection.  $\text{CaCl}_2$  concentrations have not been standard, with some reports injecting a specific mass of  $\text{CaCl}_2$  based on animal weight, and others injecting different volumes of a standard solution of  $\text{CaCl}_2$ . Injection volume has been adjusted based on testicular mass or, in some cases, the mass of the animal, with the goal being the injection of enough material to cause necrosis in the bulk of the testicular tissue without injecting excess material that can leak out and result in skin necrosis or adversely affect adjacent tissues. The recent studies of Leoci *et al.* provide a promising formulation (20%  $\text{CaCl}_2$  dihydrate in 95% ethanol) that appears to induce sterility in dogs for at least 12 months with minimal side effects.

The injections appear to be well tolerated, with animal responses being similar to expected response to any injection in the area, including anesthetic. Swelling is reported to peak within 2 to 4 days and to decrease over a period of 3 to 4 weeks. Scrotal skin reactions are reported when solution seeps from the injection site, though little detail has been provided about these reactions and studies have been too small to catch rare severe reactions. Studies have also been too small to allow a direct comparison of the incidence of injection site reactions associated with  $\text{CaCl}_2$  injection to that reported for zinc gluconate.

Effectiveness appears to vary from no change to complete destruction of sperm production depending on volume, vehicle, and  $\text{CaCl}_2$  concentration. Alcohol appears superior to saline, lidocaine, or water as vehicle. Testosterone reduction appears dose-dependent, with 75%-95% reduction reported at the highest doses. Multiple studies using intratesticular (as opposed to intra-epididymal) injection have reported changes in animal behavior, but further studies will be needed to determine and quantify the effect of partial testosterone reduction on sex-linked behavior.

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Figure 1. Injection of a dog with Esterilsol (Source: Esterilsol Veterinary Instruction Manual)



Figure 2. Injection of an adult cat with  $\text{CaCl}_2$ , demonstrated in Kolkata, India, 2010. From “Nonsurgical sterilization for dogs and cats: Calcium chloride,” copyright Parsemus Foundation, 2010.



Figure 3. Injection of a juvenile dog with  $\text{CaCl}_2$ , demonstrated in Kolkata, India, 2010. From “Nonsurgical sterilization for dogs and cats: Calcium chloride,” copyright Parseus Foundation, 2010.

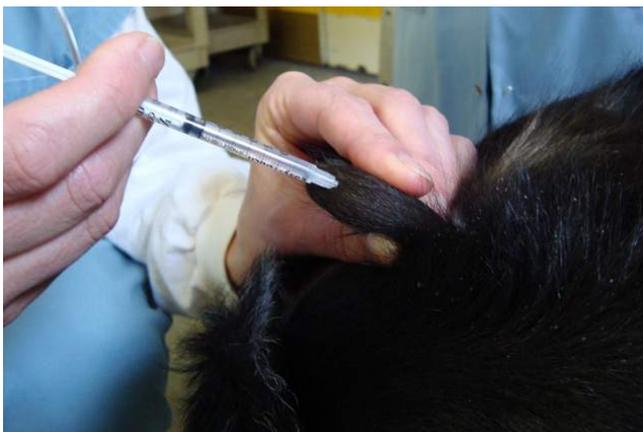


Figure 4. Injection of a pigmy goat with  $\text{CaCl}_2$ ,