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Ultrasonographic alterations in testes of rat following chemical sterilization with bilateral intra testicular injection of calcium chloride

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Abstract
The current study to evaluate the chemo-sterilization effects of single intratesticular injection of calcium chloride ($CaCl_2$) in Wistar rats was undertaken at the Department of Veterinary Gynaecology and Obstetrics. Total of 18 mature male Wistar rats were randomly divided into three groups (n=6) as control (intratesticular injection of normal saline), T_1 (intratesticular injection of 20% $CaCl_2$ solution at the dose rate of 0.1 ml/100 gram body weight) and T_2 (same as T_1 group, but with additional subcutaneous injection of meloxicam at the dose rate of 2 mg/kg body weight for three days). The rats were observed for changes in ultrasonographic measurement of testicle length, width and echogenicity. Ultrasonographic examination of testis for length, width and echogenicity on day 0, 5, 10, 20 and 30, revealed that length remained unchanged in the control group, but a gradual and significant ($p < 0.01$) reduction was recorded in T1 and T2 groups. The mean values of width of testis remained unchanged in control group but there was significant ($p < 0.01$) decrease in the testicular width at day 30 in T1 and T2 groups compared to control group. Echogenicity showed an increase and then significantly reduced trend over a period. On day 30 anechoic testes was observed in T_1 and T_2 groups, while the echogenicity remained unchanged in the testicles of control group rats.

Keywords: Chemical sterilization, calcium chloride ($CaCl_2$), ultrasonography

Introduction
Overpopulation of street dogs and cats is a serious problem in many cities around the world as these animals can be reservoirs or vectors for transmissible diseases which are dangerous to humans as well as economically valuable domestic species. Vaccination against the dangerous zoonotic diseases in all stray animals is practically not possible. Animal population management can be achieved through sterilization.

Population management is ensured mainly by means of surgical castration, namely, orchidectomy. Surgical castration is not just costly and time-consuming, but it also poses the risk of postoperative infection, which needs to be minimized by postoperative care (Jana and Samanta, 2011) [11].

To overcome the problems caused by surgical castration, various agents have been used for the nonsurgical chemical sterilization of male animals, including the injection of steroid hormones. But these treatments failed to achieve the desired result satisfactorily (Jana *et al*., 2002) [12].

Chemical sterilization can be the alternative to both Surgical and hormonal sterilization being less expensive and less labor intensive, which allows mass-scale sterilization quickly and safely (Taylor *et al*., 2017) [21]. Various chemical agents are used for it. However, all these chemical agents, following intratesticular injection had exhibited pyrexia, pain and even severe testicular inflammation (orchitis). Some agents like, glycerol, lactic acid and cadmium chloride had caused selective destruction of testicular tissue (Parizek, 1960; Immegart and Threlfall, 2000) [18, 10] with irreversible damage to testicular tissue (Heath and Arowolo, 1987) [9].

Calcium chloride ($CaCl_2$) is one of the most promising chemical agents, calcium chloride, has been used to chemically castrate a variety of species (Koger, 1978) [13]. Following intratesticular injection, calcium chloride induces necrosis, fibrosis, and degeneration of seminiferous tubules and Leydig cells, which, in a dose-dependent manner, reduces the production of spermatozoa, testosterone, and sperm counts. It results in both local and systemic reactions, including scrotal ulcers, dermatitis, abscesses, and fibrosis in the injected area, as well as peripheral edema, nausea, vomiting, diarrhoea, lethargy, and leukocytosis,

which is followed by intratesticular injection (Canpolat *et al*., 2006b, Leoci, 2012, Kutzler and Wood, 2006) [2, 16, 14].

Materials and Method

Ethical approval

This experimental protocol (VETCOLL/IAEC/2022/19/ PROTOCOL-04) was approved by the Institutional Animal Ethics Committee (IAEC) of the College of Veterinary Science and Animal Husbandry, Kamdhenu University (KU), Sardarkrushinagar, Gujarat, India.

Procurement of Rats

Total 18 Wistar rats were procured from the Laboratory Animal Facility of Torrent Pharmaceuticals, Gandhinagar, and Gujarat, India. All the rats were acclimatized for 7 days before enrolling in the studies. Animal management and treatment procedures compiled with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals, New Delhi, India. Rats were kept in polypropylene cages at the Laboratory Animal Facility in an environmentally controlled room with 22 ± 3 ºC temperature and 30-70% humidity. Light/dark cycles of 12/12 hours were given. Standard pellet food and Water were provided *ad Libidum.*

Experimental design

Rats were divided in three groups, each comprising of 6 rats. The group were named as control group (Normal saline), treatment group T_1 ($CaCl_2$) and treatment group T_2 ($CaCl_2$ + Meloxicam). Meloxicam at the dose rate of 2 mg/kg body weight, was given by subcutaneous route.

Intratesticular injections

After applying local anesthetic intratesticular injection of either normal saline or 20% $CaCl_2$ was given with the use of insulin syringes at the dose rate of 0.1ml/100gram body weight as described by Jana *et al.*, 2002 [12].

Ultrasonography

To evaluate the effect of calcium chloride solution on testis, ultrasonographic examination before, 5, 10, 20 and 30 day after the intratesticular injection was carried out with the use of micro-convex probe with a frequency of 7.5 MHz and Sonosite turbo ultrasonographic device. Echogenicity was graded based on low brightness 1, medium brightness 2, high brightness 3 and very high brightness 4 (Hami *et al*., 2020) [7]. During scanning, the relevant images were frozen on the screen and the measurements were taken with the use of an inbuilt caliper system. Length was measured from anterior to posterior pole and width was measured from medial border to lateral border of testicle.

Statistical analysis

The data pertaining to USG biometrical parameters were compiled as mean ± S.E. and analyzed by analysis of variance method, ANOVA in SPSS 20.0 software and significant differences among the treatments were determined using DMRT. Further, the scoring data such as the USG eco parameter was analyzed by a non-parametric Mann-Whitney U test and presented as median (range).

Results

Length of testes

The mean length of testis between groups showed non-significant difference on day 0 and day 5. On day 10 and 20 there was significant (p<0.05) difference while on day 30 there was highly significant (p<0.01) difference in value of group T1 and T2 with control group but no difference between group T1 and T2 seen in both left and right testis as shown in table 1 and 2.

Table 1: Effect of intratesticular injection of calcium chloride on length (cm) of right testis (Mean ± SE) on day 0, 5, 10, 20 and 30 in male rats

Group Period	Control group (N=6)	Treatment group T_1 (N=6)	Treatment group T_2 (N=6)	P value
0 day	2.23 ± 0.10	$2.29 \pm 0.08_{A}$	$2.43 \pm 0.08_{A}$	0.310
5 day	2.23 ± 0.08	$1.93 \pm 0.08_{AB}$	$2.03 \pm 0.07_{B}$	0.054
10 day	2.21 ± 0.08^{a}	$1.80 \pm 0.12_{B}^{b}$	$1.81 \pm 0.08_{B}^{b}$	0.011*
20 day	2.10 ± 0.07^{a}	$1.70 \pm 0.21_{BC}^{ab}$	$1.46 \pm 0.16_{C}^{b}$	0.034*
30 day	2.13 ± 0.10^{a}	$1.30 \pm 0.18_{C}^{b}$	$1.03 \pm 0.17_{D}^{b}$	0.001**
P value	0.795	0.001**	0.001**	

Note: The mean bearing different superscripts within the rows and subscripts in column differ significantly. p<0.05*Significant, p<0.01**highly significant

Table 2: Effect of intratesticular injection of calcium chloride on length (cm) of left testis (Mean ± SE) on day 0, 5, 10, 20 and 30 in male rats

Group Period	Control group (N=6)	Treatment group T_1 (N=6)	Treatment group T_2 (N=6)	P value
0 day	2.37 ± 0.06	$2.32 \pm 0.06_{A}$	$2.39 \pm 0.06_{A}$	0.672
5 day	2.34 ± 0.10^{a}	$1.93 \pm 0.04_{AB}^{b}$	$2.01 \pm 0.05_{B}^{b}$	0.002**
10 day	2.26 ± 0.07^{a}	$1.89 \pm 0.11_{AB}^{b}$	$1.84 \pm 0.05_{BC}^{b}$	0.005**
20 day	2.28 ± 0.07^{a}	$1.70 \pm 0.21_{B}^{b}$	$1.61 \pm 0.14_{C}^{b}$	0.013*
30 day	2.29 ± 0.07^{a}	$1.22 \pm 0.24_{C}^{b}$	$1.06 \pm 0.17_{D}^{b}$	0.001**
P value	0.850	0.001**	0.001**	

Note: The mean bearing different superscripts within the rows and subscripts in column differ significantly. p<0.05*Significant, p<0.01**highly significant

Width of testes the mean

Table 3: Effect of intratesticular injection of calcium chloride on width (cm) of right testis (Mean ± SE) on day 0, 5, 10, 20 and 30 in male rats

Group Period	Control group (N=6)	Treatment group T_1 (N=6)	Treatment group T_2 (N=6)	P value
0 day	0.80 ± 0.07	0.94 ± 0.06	$0.87 \pm 0.18_{A}$	0.249
5 day	0.82 ± 0.05	0.78 ± 0.05	$0.84 \pm 0.02_{AB}$	0.587
10 day	0.84 ± 0.03	0.79 ± 0.05	$0.80 \pm 0.02_{AB}$	0.466
20 day	0.84 ± 0.05	0.83 ± 0.08	$0.74 \pm 0.04_{B}$	0.502
30 day	0.85 ± 0.04^{a}	0.65 ± 0.09^{ab}	$0.51 \pm 0.06^{b}{}_{C}$	0.011*
P value	0.962	0.081	0.001**	

Note: The mean bearing different superscripts within the rows and subscripts in column differ significantly. $p < 0.05$*Significant, $p < 0.01$** highly significant

Table 4: Effect of intratesticular injection of calcium chloride on width (cm) of left testis (Mean ± SE) on day 0, 5, 10, 20 and 30 in male rats

Group Period	Control group (N=6)	Treatment group T_1 (N=6)	Treatment group T_2 (N=6)	P value
0 day	0.94 ± 0.05	$0.87 \pm 0.04_{A}$	$0.89 \pm 0.04_{A}$	0.464
5 day	0.91 ± 0.07	$0.81 \pm 0.06_{A}$	$0.83 \pm 0.06_{AB}$	0.514
10 day	0.85 ± 0.06	$0.76 \pm 0.05_{A}$	$0.74 \pm 0.03_{AB}$	0.296
20 day	0.88 ± 0.09	$0.82 \pm 0.06_{A}$	$0.72 \pm 0.04_{B}$	0.230
30 day	0.83 ± 0.03^{a}	$0.56 \pm 0.08^{b}{}_{B}$	$0.52 \pm 0.08^{b}{}_{C}$	0.017*
P value	0.773	0.008**	0.001**	

Note: The mean bearing different superscripts within the rows and subscripts in column differ significantly. P<0.05*Significant, P<0.01**highly significant

Echogenicity of testes

Echogenicity of the testes showed no changes in control group but in group T1 and T2 showed increase on day 5 and then gradual decrease in the echogenicity was observed was observed. So on day 30 anechoic testes was observed as shown in Table 5.

Table 5: Effect of intratesticular injection of calcium chloride on echogenicity of testis (Median (Range)) on day 0, 5, 10, 20 and 30 in male rats

Group Period	Control group (N=6)	Treatment group T_1 (N=6)	Treatment group T_2 (N=6)	P value
0 day	3	3	3	1.0
5 day	3	4	4	0.001**
10 day	3	3 (3-4)	3	0.368
20 day	3 (3-4)	2 (2-4)	2 (2-3)	0.015*
30 day	3 (3-4)	1 (1-4)	1 (1-3)	0.013*
P value	0.406	0.001**	0.001**	

Note: The mean bearing different superscripts within the rows and subscripts in column differ significantly. $p < 0.05$*Significant, $p < 0.01$** highly significant

Discussion

Ultrasonographic examination showed significant reduction in the length and width over the period were also observed by Hami *et al.* (2020) [7] in rats where they observed on day 7, 14, 21 and 28. Findings of them matched with the present study. Canpolat *et al.* (2006b) [2] in bull, Canpolat *et al.* (2006a) [4]; Canpolat *et al.* (2016) [3] in dogs also found significant decrease in testicular volume after chemical. Rafatmah *et al.* (2019) [19] observed no significant difference in diameter between different days of their study, but noted significant difference between dogs. Though not ultrasonographically but testicular size measured using digital caliper by Silva *et al.* (2018) found significant reduction in testicular size in dogs. Jana and Samanta (2011) [11] found reduction in testicular volume in cats. Leoci *et al.* (2014a) [17] found significant reduction in testicular width after 1 year in dogs. Thakre *et al.* (2021) assessed testicular morphometry using vernier callipers and found significant increase on day 7 as compared to day 0 which then decreased gradually and significantly on day 15 and 30 pos in dogs. Hasan *et al.* (2020) [8] measured scrotal circumference in bucks and found significant decrease. Fesseha and Negash (2020) used cetrimide intratesticularly and observed significant reduction in scrotal width in rats. No significant reduction in testis width was recorded by Ali *et al.* (2020) in dogs. Laku *et al.* (2021) [15] observed increase in testicular diameter on day 7 and reduced on day 60 in bucks.

Alterations in echogenicity over the time were recorded where it first increased and then gradual decrease in echogenity. Similar trends of variation in echogenicity were observed by Canpolat *et al.* (2006b) [2] in bulls, Canpolat *et al.* (2006a) [4]; Canpolat *et al.* (2016) [3], Rafatmah *et al.*, (2019) [19] in dogs; Laku *et al.* (2021) [15] in bucks following the intratesticular injection of various chemical agents. Hami *et al.* (2020) [7] in rats, Cicirelli *et al.* (2022) [5] in dogs, found reduction in the echogenicity but no acute increase in echogenicity was observed by them.

Conclusion

The progressive decrease in the length of the testicle was noticed due to intratesticular injection of $CaCl_2$ for up to 30 days. However, testicle width was significantly decreased on day 30 only. Following administration of intratesticular $CaCl_2$, the marked change in echogenicity was observed on the 30th day with anechoic testicular tissue owing to fluid filling. Meloxicam can be safely used for animal welfare purposes along with intatesticular injection as it does not hinder the sterilization.

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