


Efficacy of *Azadirachta indica* (Neem) leaf extract and hypertonic saline solution as intratesticular chemical sterilizing agents in dogs

Eficácia do extrato de folha de Azadirachta indica (Neem) e solução salina hipertônica como agente químico esterilizante intratesticular em cães

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ABSTRACT

Castration refers to induced sterility via physical, chemical, or hormonal methods. Chemical castration is an efficient and reliable technique in contrast to other sterilization procedures as it is less painful to physical methods and cost-effective to hormonal methods. *Azadirachta indica* (neem), is a charismatic plant as its leaves possess anti-inflammatory, anti-microbial, and anti-androgenic chattels. To abate the escalating human population in South Asia, neem oil and neem leaf extract have been effectively used as a contraceptive agent. The key determinant of the current study was to evaluate Neem as a chemical sterilizing agent, (either necrotic or apoptotic), in dogs injected intratesticular in comparison to a hypertonic saline solution. Pre- and post-injection testicular width size and blood samples for serum testosterone levels were collected on alternative days. Results disclosed substantial changes in testicular width size, histopathological profile, and serum testosterone level. A non-significant ($P > 0.05$) pre-injection testicular width readings in contrast to a significant increase ($P < 0.05$) three days post-injection was noted in all the competitive groups. The mean values recorded for testicular width size at the end of the trial study via neem leaf extract, 30% HSS and, control groups were 27.7362 ± 2.3315 mm, 30.9594 ± 4.6861 mm, and 24.5023 ± 2.5387 mm, respectively. A declining trend, regarding serum testosterone level being statistically significant ($P < 0.05$) was recorded in treated groups (A, B) in contrast to the control group (C) as the values were 1.5357 ± 0.7819 ng, 1.2669 ± 0.9095 ng, and 2.4517 ± 0.1827 ng in groups A, B, and C, respectively. Histopathological findings advocated the presence of apoptotic bodies in the neem treated group whereas the presence of degenerated interstitial cells, necrosed seminiferous tubules, damaged germinal epithelium, and ceased spermatogenesis was also studied in both competitive groups. Thus, the apoptotic effect and anti-inflammatory property of neem leaf extract resulted in less painful castration and verified *Azadirachta indica* as a better substitute for chemical castration in contrast to hypertonic saline solution.

Keywords: Neem. Castration. Chemical castration. *Azadirachta indica*. Canine. Population control.

RESUMO

A castração consiste na indução da esterilidade por meio físico, químico ou hormonal. A castração química é uma técnica eficiente e confiável, em contraste com outros procedimentos de esterilização, pois é menos dolorosa para os métodos físicos e econômicos para os métodos hormonais. *Azadirachta indica* (neem), é uma planta carismática, pois possui folhas anti-inflamatórias, antimicrobianas e antiandrogênicas. Para diminuir a crescente população humana no sul da Ásia, o óleo de nim e o extrato de folhas de nim têm sido efetivamente usados como agente contraceptivo. O principal determinante deste estudo atual foi avaliar o Neem como um agente esterilizante químico (necrótico ou apoptótico) em cães injetados intratesticularmente em comparação com uma solução salina hipertônica. O tamanho da largura testicular pré e pós-injeção e as amostras de sangue para os níveis séricos de testosterona foram colhidas em dias alternados.

Os resultados obtidos revelaram alterações substanciais no tamanho da largura testicular, perfil histopatológico e nível sérico de testosterona. Observou-se uma leitura não significativa ($P > 0,05$) da largura testicular da pré-injeção, em contraste com um aumento significativo ($P < 0,05$) três dias após a injeção em todos os grupos competitivos. Os valores médios registrados para o tamanho da largura testicular no final do estudo via extrato de folhas de nim, HSS a 30% e grupos controle foram $27,7362 \pm 2,3315$ mm, $30,9594 \pm 4,6861$ mm e $24,5023 \pm 2,5387$ mm, respectivamente. Uma tendência decrescente, com relação ao nível sérico de testosterona sendo estatisticamente significativa ($P < 0,05$), foi registrada nos grupos tratados (A, B), em contraste com o grupo controle (C), pois os valores eram $1,5357 \pm 0,7819$ ng, $1,2669 \pm 0,9095$ ng e $2,4517 \pm 0,1827$ ng nos grupos A, B e C, respectivamente. Os achados histopatológicos advogaram a presença de corpos apoptóticos no grupo tratado com nim, enquanto a presença de células intersticiais degeneradas, túbulos seminíferos necrosados, epitélio germinativo danificado e espermatogênese interrompida também foi estudada nos dois grupos competitivos. Assim, o efeito apoptótico e a propriedade anti-inflamatória do extrato de folhas de nim resultaram em uma castração menos dolorosa e confirmaram que a *Azadirachta indica* foi um melhor substituto para a castração química do que a solução salina hipertônica.

Palavras-chave: Neem. Castração. Castração química. *Azadirachta indica*. Canina. Controle de população.

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Received: June 29, 2020
Approved: October 14, 2020

How to cite: Ali M, Ijaz M, Manzoor A, Mohy-Ud-Din MT, Hassan F, Tabassum R, Bhutta ZA, Ali W, Muneeb M, Mehtab U, Zafar MA. Efficacy of *Azadirachta indica* (Neem) leaf extract and hypertonic saline solution as intratesticular chemical sterilizing agents in dogs. *Braz J Vet Res Anim Sci.* 2020;57(4):e171582. <https://doi.org/10.11606/issn.1678-4456.bjvras.2020.171582>

Introduction

The increasing number of stray dogs harms environmental health status and also contributes and has a direct relation with zoonotic diseases. With no control strategies, the number of unwanted dogs is increasing (Jana & Samanta, 2006). During the early '90s in the United States, up to 9.1 million dogs were euthanized in animal sheds due to overpopulation. Castration or sterilization is the most reliable method to overcome this problem and helps to control the overpopulation of stray dogs (Kutzler & Wood, 2006). Castration is an approach that affects the functioning of testicular tissues using different chemicals, resulting in deterioration and atrophy by reducing the blood supply or by surgical removal (Currah et al., 2009).

Overpopulation of dogs and other canines have major consequences worldwide, including public health concerns as

well as overall animal welfare. As a result, the World Health Organization (WHO) rabies control program focuses on dogs as they are in close relation to humans, and castration is the best way to prevent unwanted pregnancies (Blanton et al., 2009). Additional advantages of castration include good behavior and sound health apart from sterility. Furthermore, castration helps in controlling the aggressiveness, urine marking, and excessive libido of dogs (Bloomberg, 1996). Moreover, castration has vast applications in the urology field to treat prostate or testicular cancer issues (Damber, 2005).

Castration can be done by many techniques like physical, mechanical, hormonal, and chemical methods. The use of a Burdizzo (castration device) and a rubber ring at the testicular base is an example of a noninvasive, mechanical way of castration, while physical and surgical method removal involves the excision of testes (Stilwell et al., 2008). Surgical excision of testicles is an efficient technique despite some negative aspects such as it being painful to the dog, time-consuming, requires surgical expertise, and there may be excessive bleeding that could prove to be fatal. Additionally, the dog requires post-operative care to minimize the risk of infection and swelling (Bretschneider, 2005).

Chemical castration is considered a better substitute in comparison to a physical procedure, as it can be applied to a large number of animals at the same time and also requires less trained staff. This technique has been widely used through intratesticular administration of some toxic agents (sclerosing in nature), in several species including feline, caprine, canine, and also rodents. Intra-epididymal injection of these necrotic agents results in azoospermia by producing fibrous occlusions, while intratesticular injection results in reduced testosterone production, which results in a decline in spermatogenesis and testicular necrosis (Kutzler, 2015). Different chemical substances used to control the canine population, particularly in metropolitan

areas where overpopulation of stray dogs is a major concern (Soto et al., 2018). Chemical castration agents includes such as potassium permanganate, formalin, glycerol, zinc gluconate + arginine, sodium fluoride, calcium chloride, and a hypertonic saline solution (Kwak & Lee, 2013).

Hypertonic saline solution induces chemical castration by creating osmotic shock and necrosis locally, leading to the degeneration of testicular tissues. Necrosis occurs as a result of surpassing physio-chemical stress in cells subsequently leading to cell death (Kaczmarek et al., 2013). Apoptosis referred as programmed cell death and is considered preferable over necrosis, which has uncontrolled cell death. Apoptosis eliminates unwanted effects of necrosis by provoking the inflammation of neighboring cells (Kroemer et al., 1998). Therefore, chemo sterilization of animals must be achieved by using some apoptotic agents instead of necrotizing agents to avoid some adverse effects like inflammation, swelling, and pain (Yostawonkul et al., 2017).

Historically, *Azadirachta indica* (neem) is well accredited for its astounding healing properties. It has been considered as a primeval cure for a modern world and is labeled as a tree for solving global problems. This tree is native to Pakistan, Sri Lanka, and India. Neem is utilized worldwide due to its diversified properties in many fields like agriculture, environmental protection, and in the medical industry. Some remarkable therapeutic potentials of neem are anti-viral, anti-microbial, anti-inflammatory, anti-bacterial, anti-fungal, and anti-hyperglycemic.

Neem oil or neem leaf extract has been used as a contraceptive in humans because of its anti-androgenic property. Dietary intake of neem for 4-6 weeks reportedly caused a marked drop in sperm count, motility, and spermatogenesis (Khillare & Shrivastav, 2003). Neem extract is associated with fertility effects in both males and females by stopping spermatogenesis, preventing the development of follicles, fetus implantation defects, and abortions besides apoptosis of oocytes in rats (Chaube et al., 2014). However, there is little or no literature available on the use of neem leaf extract as a chemical sterilizing agent in dogs. As such, the proposed study on neem leaf extract was designed to evaluate the efficiency of a hypertonic saline solution and neem leaf extract as a chemo-sterilizing agent in male dogs, specifically, to advance the research in this academic field of study.

Materials and Methods

Management of experimental animals

A total of 18 adult and clinically proven healthy male dogs of the same age and breed were obtained from locally available

kennels of Faisalabad, Pakistan, and kept in a dog shed of the Department of Clinical Medicine and Surgery, Faculty of Veterinary Science, University of Agriculture, Faisalabad. All dogs were settled in separate kennels inside the dog shed and provided with a uniform feeding pattern throughout the trial study. During an adaptation period of seven days, the general health status of all dogs was confirmed through clinical and laboratory examinations. The commercially available anti-parasitic drug Albandazole® was given orally and Ivermectin® administered subcutaneously to the dogs affected with parasites. During the accommodation period, any individual exhibiting signs of illness was substituted with a healthy animal. The investigation project was approved by Synopsis Scrutiny Committee, which was then submitted to the Faculty Scrutiny Committee. After approval from both committees, the proposal was submitted to the Graduate Studies Research Board of the university. All of these bodies take into consideration animal ethics while reviewing and approving the research proposal.

Neem leaf extract

An ample amount of neem leaves were collected from University Botanical Garden and sampling leaves were identified and tagged by the Department of Botany, University of Agriculture, Faisalabad. Neem leaves were shade dried and then crushed to make powder and boiled under light for 4 h. This technique that was adopted to make the extract was a modified one (Parshad et al., 1997). Neem extract was then filtered and collected in a flask. The extract was further autoclaved in a 50 ml glass flask and stored at room temperature until use.

Hypertonic saline solution

Commercially available 0.9% normal saline was injected in the control group while the hypertonic saline solution (HSS) was prepared by dissolving sodium chloride (extra pure DAB, Ph Eur, BP, USP Merck™) in distilled water. To prepare 30% HSS, 30 gm NaCl was dissolved in 100 ml of distilled water. After dilution, the prepared HSS was autoclaved and preserved at room temperature.

Experimental protocol

All 18 dogs were randomly arranged into three groups A, B, and C as the neem treated group, HSS treated group, and a control group, respectively. The intratesticular injection was administered under the effect of local anesthetic agent 0.2-0.5ml, 2% Lidocaine HCl (Anestex, Fagra S/A, SP, Brazil) subcutaneously. A dose of the test block was injected under the testicular width of each testis (Table 1), which was determined with a Vernier caliper at its widest point (Leoci et al., 2014).

Intra-testicular injection

With the dog lying on its back (dorsal recumbency), the scrotum was shaved and cleaned with an antiseptic, povidone-iodine solution. Each intratesticular injection was carried out using a sterilized 21-gauge needle inserted from the caudoventral point of each testis approximately 1 cm from the epididymal tail towards the dorso-cranial side of each testis so that the solution was deposited over the entire pathway by linear infiltration during removal of the needle from the proximal end to the distal end.

Evaluation criteria

Clinical assessment

During the complete experimental trial, clinical observations like scrotal swelling, dermatitis, licking of the injection site, self-induced trauma, skin lesions, and scrotal ulcers were noticed carefully once daily for three consecutive days after the injection, and thereafter, once weekly until the trial termination. All the testicles were observed for their physical features such as color, size, shape, and constancy. General visible alterations including marked atrophy and necrosis and deterioration of tissue and lesions were also noticed. Biometric features (i.e., the width of each testis) were measured by Vernier's caliper before treatment, daily for three consecutive days post-injection, and then once a week. Data was documented as a mean width of the left and right testicles separately (Hoei-Hansen et al., 2003).

Histological examination

At the end of the three-week experimental trial, the testicles of all the animals were removed by adopting both open and closed surgical methods of castration. Collected samples were washed with 0.9% normal saline by keeping in mind the anatomical structures. Then, the samples of testicles were preserved in NBF (neutral buffer formalin) in 100 ml glass containers for each right and left testicle separately (Abshenas et al., 2013).

Statistical analysis

Data were analyzed with the ANOVA technique under LSD by SPSS 20.0 (Heinisch, 1962).

Results and Discussion

Clinical assessment

No itching and scrotal dermatitis were noticed in all groups until the end of the experimental trial.

Table 1 – Volume of neem leaf extract and hypertonic saline injected in each dog's testis based on testis width

Testis width (mm)	Volume/testis (ml)
10-12	0.2
13-15	0.3
16-18	0.5
19-21	0.7
22-24	0.8
25-27	1.0

Source: Macêdo et al. (2018).

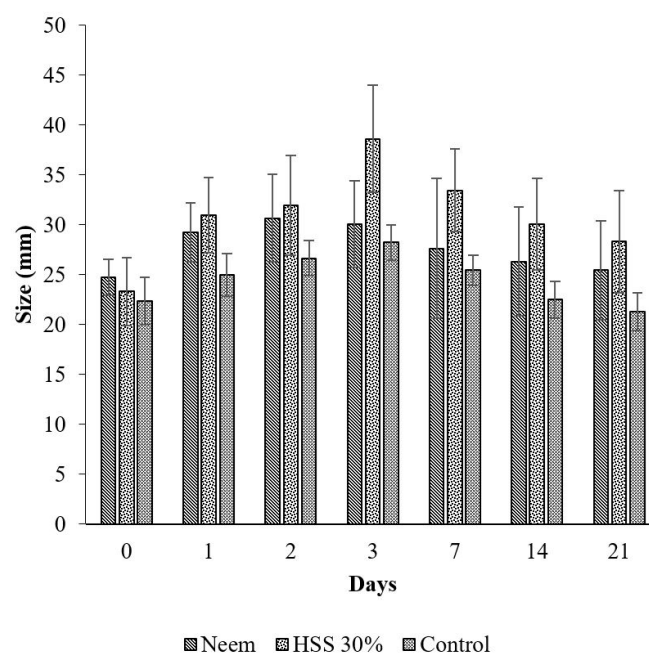


Figure 1 – Comparison testes width (mm; Mean ± SD) at different time intervals of three groups via bilateral intratesticular injection of neem leaf extract (group A), 30% HSS (group B), and normal saline solution (group C).

Testicular width

Testicular width, (separately for each testicle, left and right), was measured at different time intervals during the experimental trial (Figure 1). Pre-injection readings of testicular width in all groups were non-significant ($P > 0.05$). A marked rise ($P < 0.05$) was observed in the testicular width of all three groups up to three days post-injection. On the seventh day, there was a slight decrease in testicular width that was noticed in the neem leaf extract-treated group in comparison to the hypertonic saline solution treated group. However, the control group showed a significant size reduction ($P < 0.05$) as opposed to both treated groups on the seventh day. A similar trend was again noticed on day 14 where the neem leaf extract group observed a significant reduction ($P < 0.05$) in contrast to the HSS group and the control group attained almost normal testicular size. On day 21, the testicular width of the neem leaf extract group and HSS group were non-significant ($P > 0.05$), in comparison to the control group. The mean values recorded

for testicles width via neem leaf extract, 30% HSS, and control groups were 27.7362 ± 2.3315 , 30.9594 ± 4.6861 , and 24.5023 ± 2.5387 mm, respectively.

Serum testosterone concentration

Taking into account the serum testosterone concentration levels in all three groups, the neem leaf extract group, 30% HSS, and control were statistically non-significant ($P > 0.05$) at the baseline. A decreasing trend in both the competitive groups (neem and 30% HSS) was noticed throughout the trial, being statistically significant ($P < 0.05$) as compared to the control group at the end of the 21st day. The results of neem leaf extract and 30% hypertonic saline solution (or HSS) groups were non-significant to each other ($P > 0.05$) at the end of the trial, as the testosterone level dropped to a level below the normal range (2-5 ng/ml) within the three weeks (Figure 2). Mean values recorded for serum testosterone levels were 1.5357 ± 0.7819 , 1.2669 ± 0.9095 , and 2.4517 ± 0.1827 ng/ml for neem leaf extract, 30% hypertonic saline solution, and the control group, respectively.

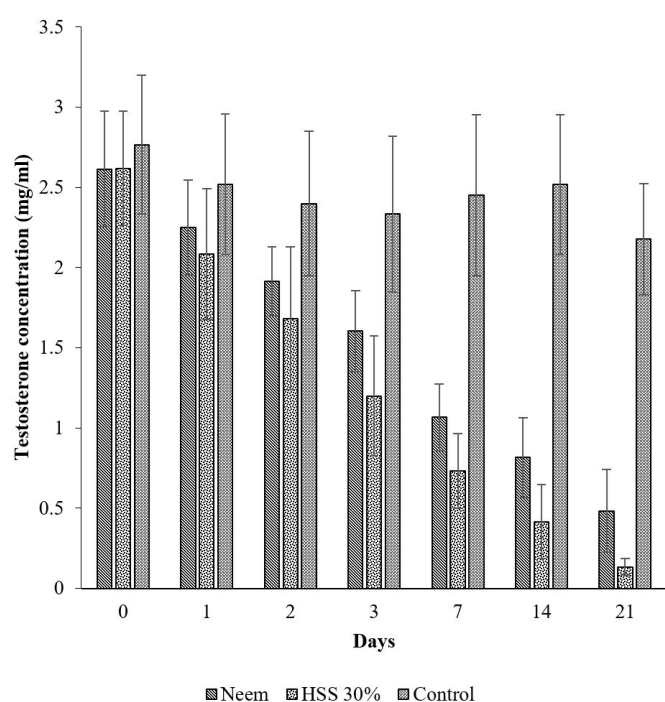


Figure 2 – Comparison of the testosterone concentration of three groups at a different time interval (days).

Histopathological findings

Histopathological findings discovered that both neem leaf extract and hypertonic saline solution severely damaged the parenchymal cells and induced necrosis of testicular tissues. However, mild to negligible necrotic changes were observed in the normal saline-treated group (Table 2).

In the control group, there was no disintegration of seminiferous tubules, no vacuolization, and there was a presence of normal Leydig and Sertoli cells. The germinal epithelium was lined with spermatogonia, (primary and secondary spermatids), thus indicating the normal ongoing process of spermatogenesis (Figure 3i).

In the neem leaf extract-treated group, there were several findings of apoptosis of seminiferous tubules along with necrosis of testicular parenchyma, interstitial Leydig, and Sertoli cells. Blebs of cellular debris were visible inside seminiferous tubules that are the indicator of the apoptosis of germ cells. Partial vacuolization was also observed in the neem leaf extract-treated group. Disruption of spermatogonia, primary spermatocytes, secondary spermatocytes, and spermatids revealed that the spermatogenesis completely stopped and there was no further production of germ cells thereafter (Figure 3ii, 3iii).

In the 30% hypertonic saline solution group, there was severe disintegration of seminiferous tubules, along with coagulated necrosis and complete vacuolization of germ cells. The germinal layer was destroyed and the disorganization of parenchyma cells was observed (Figure 3iv).

Discussion

The final results showed that the intratesticular use of neem leaf extract causes necrosis and apoptosis of seminiferous tubules accompanied by reduction of serum testosterone levels in comparison to severe coagulative necrosis caused by 30% HSS. These changes are relative to the findings observed by 20% HSS (Emir et al., 2008).

Concerning serum testosterone levels, both the neem leaf extract and 30% hypertonic saline solution markedly reduced the levels in contrast to the control group. The findings are following the results of Emir et al. (2008), who noted that

Table 2 – Histopathological results comparison between the normal saline, neem leaf extract-treated group, and the 30% HSS group

Histopathologic characteristics	Normal saline solution (NS)	Neem leaves extract group	30% hypertonic saline group
Coagulative necrosis of testicular parenchyma tissue	+	++	+++
Changes in germinal epithelium	+	++	+++
Infiltration of inflammatory cells	+	++	+++
Coagulated necrosis and vacuolization of Leydig and sertoli cells	+	++	+++
Pyknosis of nuclei	+	++	+++
Parenchymal cells alterations	+	++	+++

Mild +, Moderate ++, Severe +++.

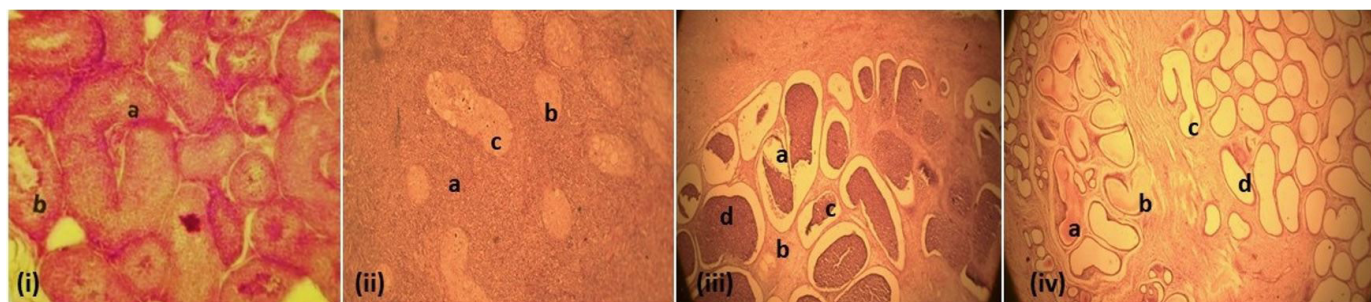


Figure 3 – (i) Effect of normal saline on testicular parenchyma (a) seminiferous tubules (b) in an adult male dog; intact seminiferous tubules and normal testicular parenchyma can be noted; (ii) Effect of neem leaf extract on (a) Leydig cells, (b) seminiferous tubules, and (c) germinal layer. Necrosis of Leydig cells, seminiferous tubules, and disrupted germinal epithelium layer; (iii) Effect of neem leaf extracts causing (a) partial vacuolation of seminiferous tubules, (b) damage to interstitial cells (c) damage to parenchymal cells, and (d) stoppage of spermatogenesis; (iv) Effect of 30 % hypertonic saline solution on (a) testicular parenchyma and seminiferous tubules in an adult male dog, (b) severe necrosis of seminiferous tubules and Sertoli cells, (c) diminishing of germ cells (d) complete vacuolation.

20% hypertonic saline causes a reduction in the testosterone levels of rats. The normal physiological functioning of the testis was disturbed because of testicular degeneration, ultimately leading to depressed spermatogenesis and apoptosis of seminiferous tubules along with necrosis of Leydig cells that caused a severe drop in testosterone concentrations. The concentration of testosterone is dependent on the number of Leydig cells inside the testicles; the more normal Leydig cells were, the greater the chance there will be more testosterone concentration, and vice-versa. Similar findings except for the apoptotic changes were explained by Jana & Samanta (2006), who stated that secularizing properties of calcium chloride also causes a drop in serum testosterone levels in dogs. These observations are also co-related to the experimental study output by Leoci et al. (2014), who explained that upon administering an intratesticular injection of calcium chloride in dogs, a marked decline in serum testosterone concentration was noticed. The same results were also concluded by Abshenas et al. (2013), who injected the essential oil of *Eugenia carophyllata* in dogs to achieve chemical castration. The atrophied testicles indicated a decreased level of testosterone.

In terms of testicular width size, a marked decrease in width of testicles in comparison to the control group was observed on completion of the trial study, with a significant increasing trend in width size during the first three days post-injection. Our research observations are concerning Leoci et al. (2014), who concluded that the administration of calcium chloride intratesticular causes the reduction of testicular size after the induction of inflammation. The same results were also explained by Oliveira et al. (2012), who revealed that the zinc gluconate sterilizing agent causes the degeneration of testicular tissues ultimately leading to a reduction in testicular size and cessation of spermatogenesis after few days of the intratesticular injection with infiltration of inflammatory

cells recorded in histopathological findings. Experimental findings of Vanderstichel et al. (2015) presented a direct relation between the testicular width and serum testosterone concentration. Apart from testicular changes that might be caused by the necrotizing agent, the researchers clarified the alterations in testicular size related to testosterone levels. This study further explained that over time testosterone level and testicular width were also decreased.

Conclusion

Intratesticular injection of *Azadirachta indica* (neem) leaf extract causes the apoptosis of seminiferous tubules and necrosis of testicular parenchyma and is a better alternative in comparison to 30% HSS. This method of sterilization is safe and effective not only for animal welfare but also as a way to prevent zoonotic diseases and increasing disease-free dog populations in the South Asian sub-continent.

Conflict of Interest

The authors declare they have no conflicts of interest.

Ethics Statement

It is stated that the research work carried out in the Department is approved dually by the Department Synopsis Scrutiny Committee, which is then submitted to Faculty Scrutiny Committee. After approval from both these committees, the proposal is submitted to Graduate Studies Research Board of the university. All these bodies, while reviewing and approving the research proposal take into consideration, animal ethics seriously. Hence, the article under reference, being a part of proposal “Comparative evaluation of single bilateral intratesticular injection of *Azadirachta indica* (Neem) leaf extract and hypertonic saline solution as chemical sterilizing agent in dogs” has been approved by these bodies, which take serious concerns of animal ethics and welfare.

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Financial Support: No funding received.