NONINVASIVE THERAPEUTIC ULTRASOUND STERILIZATION IN MALE DOGS

THESIS

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by

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1. INTRODUCTION

Canine overpopulation has become a global problem with significant public health impacts. Current population control methods are expensive and/or have questionable efficacy. Various controversies with surgical procedure of sterilization in animal are raised by human and animal activist. The painful invasive procedure are unethical and questionable (Leoci et al., 2015).

Sterilization is a technique that intentionally makes the animals unable to reproduce, thus a method of animal birth control. Sterilization methods include surgical and medicinal means for both the sexes (Slatter, 1993).

The complications associated with invasive procedure are unavoidable thus there is always a need for a contraceptive method which could solve the purpose by keeping the animal intact. Male dog contraceptive approach needs to be long-effecting, with minimal side effect and easy to perform with low cost (Verstegen, 2010).

Therapeutic ultrasound (U/S) is a promising canine contraceptive procedure which consists of inaudible high frequency (greater than 20 kHz) mechanical vibrations (pressure waves) (Millis et al., 2004). The waves are transmitted by propagation through molecular collision and vibration, with a progressive loss of the intensity of the energy during passage through the tissue (Straub et al., 2008). When absorbed by tissue, U/S energy waves are converted to heat and are very effective in deep heating.

The temperature of the scrotal testis of mammals is normally several degrees (3–4°C) below that of the body core. An elevation of testicular temperature through therapeutic ultrasound results in impairment of spermatogenesis (Jung and Schuppe, 2007). Thus, it is possible to affect sperm production by raising testicular temperature. It also alter membrane permeability and suppress spermatogenesis. There is significant reduction in testicular volume and testicular degeneration with widespread tubular atrophy degeneration (Paltiel et al., 2002).
Another effect of therapeutic ultrasound is mechanical. Therapeutic ultrasound markedly alters the permeability of the membrane to ions and other substances: U/S increases the sodium and decreases the potassium concentration in the fluid of the seminiferous tubules and decreases the sodium concentration and increases the potassium concentration in the fluid of the rete testis (Straub et al., 2008).

Despite previous promising studies, a standard therapeutic ultrasound treatment for contraceptive purposes has not yet been identified. Identification of the treatment parameters, including the reduced number of applications, the shortest intervals of applications, the most effective target area of the testis, the speed of the sound head, and the ultrasonic variables (frequency, intensity, duty cycle, etc.), to produce durable contraception has yet to be determined (Leoci et al., 2015).

The sterilization procedures are performed to decrease the aggressive behavior, make the animal docile, easier to handle and also to prevent the unwanted mating and mounting activities or for certain testicular/inguinal pathologies (Edwards, 2008).

There are several medicinal and surgical procedures for sterilization of dogs. However, the procedures are questionable on humanitarian or other grounds. There is no systematic study on noninvasive sterilization procedures in canine. The objective of the current investigation was to determine the most practical and effective application protocol for dog sterilization. Therefore, the present work has been planned with the following objective:

**Objective**

1. To establish a noninvasive therapeutic ultrasound procedure for sterilization in male dogs.

2. Evaluation of its clinical efficacy for sterilization by Hormonal Assay-Testosterone and histological examination.
2. REVIEW OF LITERATURE

In the recent years, many efforts were made to identify reliable methods of sterilization. However, surgical methods have remained the mainstay in various species (Howe, 2006). In the present survey, conventional surgical method of castration was widely adopted to castrate the dogs. Thus, the main objective of this study was to establish a noninvasive therapeutic ultrasound procedure for sterilization in male dogs and to compare it with conventional procedure.

Andersson and Forsberg (2002) in their study noticed increased body weight by 47 percent in 122 castrated dogs. Although it might not only be due to primary regulators of metabolism but also gonadal hormones had influenced bodyweight either directly by acting on centres in the brain that regulate activity, or indirectly by altering metabolism at the cellular level (Salmeri et al., 1991).

2.1 Invasive procedures

Sally (1976) reported vasectomy as a substitution for castration in canine species. Vasectomy is indicated in cases where owners do not want to breed the dog due to hereditary problems such as hip dysplasia. Vasectomy does not alter the male hormones as does castration. Young dogs still develop normal male characteristics and there is no problem of weight gain from decreased metabolic rate due to loss of testosterone. Intact male dogs have more drive to exercise and therefore keep in shape. It is also prefer not to castrate their dogs due to psychological identification problems.

Tsuruta et al. (2012) studied on orchiectomy results in irreversible sterility and may trigger a number of side effects such as underdevelopment of external genital organs, disturbances in the musculoskeletal system, obesity, increased risk of diabetes mellitus or hypothyroidism and behavioral problems. Therefore, alternative methods of contraception have been developed during the last decades. The methods of non-surgical contraception, used adequately, are currently considered to be simple and effective.
2.2 Minimum invasive procedures

Vijay and Vipin (2013) concluded that laparoscopic technique permitted clear visualization and identification of abdominal organs and vas deferens. The advantages of surgical laparoscopy over the conventional open surgical exploratory laparotomy include faster patient recovery because of smaller surgical sites, improved visualization of abdominal organs, lower postoperative morbidity with lower infection rates, and less postoperative pain and stress. It has proved a boon in animal birth control programme where the extensive surgery is required in minimum time in a large population. The post-surgery recovery is quick and uneventful.

Swahney (2016) reported in pinhole technique of castration as effective, less painful, field oriented, required no instrumentation, ethical and observed with less complications. Pinhole technique may also be regarded superior as greater degree of testicular degeneration was observed in both the specie, calves and goat.

2.3 Non invasive procedures

Fahim et al. (1975) proposed that the combined thermal and mechanical (non-thermal) effect of therapeutic ultrasound application could cause an ion exchange between the fluid in the seminiferous tubules and rete testis, creating an environment not suitable for spermatogenesis which might explain the spermatogenesis suppression.

Tsuruta et al. (2012) reported that therapeutic ultrasound treatment depleted developing germ cells from the testis. They also reported that a combination of elevated temperature, high power and high frequency is the key to reducing sperm count.

Vandevoort and Tollner (2012) compares two methods of ultrasound exposure is direct application to the surface of the scrotum with therapeutic ultrasound at 2.5 W/cm² for 30 min in non-human primate species. The testicles that compare in size with those of men that ultrasound treatment results in reduced sperm numbers and quality. This study provides a proof of
principle that testicular ultrasound exposure has the potential to be a viable approach for contraception in humans.

Leoci et al. (2015) used three treatments of therapeutic ultrasound 48 h apart at 1 MHz, 1.5 W/cm lasting 5 min each, treating the entire testicular area, lead to irreversible testis damage and azoospermia in the dog. No notable side effects other than infertility and no influence on testosterone production were reported during this study. Confirming its apparent permanence through longer observational studies is the next required step to establish whether therapeutic U/S can serve as the basis for a new, irreversible male dog contraceptive. The mechanism of action of U/S contraception was first supposed.

### 2.4 Chemical procedures

Dixit et al. (1975), Samanta (1998), Canpolat et al. (2006) and Aiudi et al. (2010) reported intratesticular injection of some agents such danazol, calcium chloride, ethanol and chlorhexidine digluconate. Intratesticular injection of hypertonic saline used for animal castration. Hypertonic saline is a solution that is inexpensive and easy to administrate. 20% hypertonic saline solution was inject bilaterally into the rat testes at different areas with a total amount of about 0.5 to 1 cc in each testis, that the coagulation necrosis was observed in all testes.

Hill et al. (1985) trial the chemical, surgical, and mechanical castration, in bulls for chemical castration in male ruminants intratesticular lactic acid tannic acid and zinc sulphate by Feher et al. (1985) alpha-hydroxypropionic acid by Cohen et al. (1995), by Formalin Ijaz et al. (2000) and Castrate-Quin by Soerensen et al. (2001) have been used.

Fordyce et al. (1989) studied in chemical castration by injection of sclerosing or toxic agents (e.g. 88% lactic acid) into the testicular parenchyma to cause irreparable damage and loss of function.16 Chemical castration requires additional procedural time and technical skill, and almost twice the healing time compared with surgical castration.
Al-Asadi and Al-Kadi (2012) studies on chemo sterilization with injection of sclerosing or toxic agents like three per cent formalin in the testicular parenchyma leading to irreparable damage and loss of function of testis but it required longer procedural time and technical skills and almost twice the healing time compared with surgical castration.

Zara Hedge (2013) stated that several non-surgical methods exist, some of which have received regulatory approval or are undergoing field trials. Chemical castration targets the destruction of gonadal cells in males causing infertility by azoospermia, or lack of sperm production. This approach has been studied for nearly 50 years, and FDA approval has been given to Zeuteri, developed by Ark Sciences, for use in male dogs. Zeuterin (known internationally as EsteriSo) is a zinc gluconate compound buffered with arginine which causes testicular atrophy, seminiferous tubule collapse, and intra-testicular scar tissue formation. Only one inexpensive intratesticular injection is necessary for irreversible sterility and 40-50% testosterone reduction. The current formulation requires training and certification to administer, which optimizes efficacy while minimizing adverse effects.

2.5 Anamnesis, clinical observation and examination

Hopkins et al. (1976) concluded that castration in male dogs results in a rapid or gradual decline of indoor urine marking, inter male aggression, and mounting of other dogs in approximately 50 to 70 percent of the dogs. However, roaming to find a potential mate is reduced in 90 percent of the dogs. The adrenal gland normally does not increase its testosterone production in amounts sufficient to have any influence on maintenance of the undesirable behavior.

Johnston (1991) reported castration also provides certain health benefits for the male dog, such as prevention of prostate gland enlargement, perineal hernias, and testicular tumors. Benign prostatic hypertrophy or hyperplasia is testosterone dependent and occurs frequently in older intact male dogs. Prostatic enlargement generally results in straining related to defecation and/or urination. Benign prostatic hypertrophy is both prevented and treated by castration.
Salmeri *et al.* (1991) concluded that castration before puberty to prevent unwanted behaviour is as effective as castration of the adult animal in eliminating the behaviour once it occurs.

Shikone *et al.* (1994) claimed that cell death following heating was not apoptosis but necrosis, although this was not accompanied by inflammation. However, Ohta (1996) in subsequent studies have shown that apoptosis involved.

Hart (2001) observed that a small percentage of castrated male dogs are predisposed to the progression of cognitive impairment. Cognitive impairment refers to the behavioural changes that occur in senile dogs as a result of disturbances of memory, learning, and the circadian rhythm.

Adin (2011) reported that complications of both prescrotal and scrotal techniques include dehiscence, scrotal swelling, haemorrhage, subcutaneous bruising, scrotal hematoma and self-trauma to the surgical site. Dogs with minor complications may need no intervention, while others may require veterinary care. Dogs with severe scrotal hematoma may experience necrosis of the scrotal skin, necessitating a scrotal ablation.

### 2.6 Haematological examination

Murata (1997) reported significant increase in circulating white blood cells and neutrophils while significant reduction of lymphocytes following after castration. These values returned to baseline by seven days after castration. These changes were likely due to reactive leukocytosis and stress.

Earley and Crowe (2002) concluded that, surgical castration results in increased haptoglobin and decreased gamma-interferon production. Haptoglobin exerted a suppressive effect on lymphocyte function, and reduction of gamma-interferon production result in suppression of the immune system’s cell-mediated immunity and response to antigens. So, their study indicated that castration-associated leukocyte depression may be limited or eliminated by pre-surgical administration of a local anesthetic and a systemic analgesic.
Mohammad et al. (2008) observed significant elevation in mean total leucocyte count one day after surgery in eight surgically castrate, significantly decreased. Both values returned to normal on 7th day post castration. The percentages of monocytes, eosinophils and basophils in the blood did not change significantly during the observation period.

2.7 Hormonal assay

Yang et al. (2005) showed the mechanism of the decrease libido is controlled by many factors, such as the nervous system, hormone, sexual pheromone, among which, the hypothalamus–pituitary–testis axis is very important.

Tao et al. (2006) stated that the luteinizing hormone concentration has been shown to have decreased significantly on day 3 and subsequently recovered to the normal level by in unilateral cryptorchid monkey.

Yan et al. (2007) also reported heat-induced germ cell degeneration is usually accompanied by alteration in sertoli cell morphology and function, a decrease in testes fluid, and increase in serum FSH with no changes in serum testosterone and LH levels.

Gesquiere et al. (2011) found that testosterone level was lower of high average daily maximum temperatures. This decrease of testosterone in high temperature is consistent with reduced libido under heat stress, which indicated that testosterone is possibly involved the regulation of libido.

2.8 Histological examination

Austin et al. (1961) showed that the local heating of the scrotum of bulls for few days causes a reduction in the number of sperm ejaculated. Semen quality was reduced within one week after heat stress, continued to decrease for 4 wk, and returned to pretreatment values by 8 weeks after cessation of thermal stress. Total sperm output was not influenced by the treatments. When the bulls were exposed to 40°C, spermatids and spermatozoa in the final stages of development were influenced the greatest.
Wettemann et al. (1976) reported that the spermatogenesis as a continuous process and boars are constantly sensitive to exposure to elevated ambient temperature, and may be more sensitive to lesser increases in ambient temperature than gilts.

Hochereau (1979) observe the size and organization of the seminiferous tubules and the seminiferous epithelium and stated that degeneration of germ cells was frequently observed in high temperature.

Miragia and Hayashi (1993) reported a decrease in the percentage of tubules with spermatogonia from 100 to 89% at day 15 after heating the testes of rats to 43°C for 30 min. It showed that spermatocytes and spermatids are major cells which were affected also spermatogonial number increased after 2 week postoperation and this increase is noticeable in bilateral group compared with unilateral group.

Yin et al. (1997) studied and reported that the spermatocyte and spermatid are most susceptible to heat. Exposure of the testis to the relatively elevated temperature of the abdomen is known to cause germ-cell loss. However, the mechanisms underlying this process are un-known. The study reported that, using experimentally induced unilateral cryptorchidism, demonstrates a clear temporal relationship between the onset of DNA fragmentation by day 6 and germ-cell loss on day 7 (demonstrated histologically and by reduction in testicular weight). This association, together with the coincident appearance of MGCs and vacuoles with apoptosis, strongly suggests that abdominal heat stress causes germ-cell loss by apoptosis in the adult testis.

Paul et al. (2008) discussed the effects of hyperthermia on spermatogenesis, the measurement methods of scrotal temperatures, the various modifiable and non modifiable factors that could cause increased testicular temperatures, the molecular mechanism of apoptosis, DNA damage and autophagy, changes in gene expression and the pathways of germ cell apoptosis in response to testicular heat stress.
Aktas and Kanter (2009) observed severe damage to testicular microstructure in response to chronic heat stress. Previous reports have also described damage to testicular tissue in heat stress conditions.

Tsuruta et al. (2012) studied on non-invasive therapeutic ultrasound and its efficacy in reducing sperm count and reported that therapeutic ultrasound as a promising method for a male contraceptive. Depleting spermatocytes and spermatids from testes noninvasively with therapeutic ultrasound has multiple applications.

Leoci et al. (2015) revealed general testicular degeneration with a widespread tubular atrophy and a significant decrease of the testicular parenchyma. Seminiferous tubules were covered by one or two rows of cells with epithelial vacuolization. The basal membrane appeared with slightly reduced diameter and irregular profile. Sertoli cells appeared normal, while irreversible damage was due to the complete lack of spermatogonia and results in azoospermia.

2.9 Effect of heat

Casady et al. (1953) studied on testicular temperature of bulls and reported that temperature must be 2 to 6° C cooler than body temperature to allow normal spermatogenesis and maturation of sperm. Exposure of bulls to increased ambient temperatures results in reduced semen quality.

Waites (1970) concluded that increased testicular temperature, whether caused by exposure to elevated ambient temperature or fever, reduces semen quality. Acute exposure of bulls to heat stress for as few as 12 h can result in reduced semen quality. It is necessary to reduce the heat that bulls receive from the environment during the summer and increase the rate of loss of body heat. Cooling methods become especially important when ambient temperatures are greater than 32°C.

Wettemann et al. (1976) studied and results indicate that if bulls cannot increase the rate of heat loss from the body when they are exposed to elevated ambient temperatures, semen quality and potential fertility will be reduced. Most important in cattle breeding programs, is that 8 wk are
necessary for resumption of normal semen production after bulls are exposed to elevated ambient temperatures that cause heat stress.

Damber \textit{et al.} (1978) showed that blood flow to the cryptorchid testis is reduced despite the fact that the cryptorchid testis contains a greater percentage and number of blood vessels than the normal testis.

Coleman \textit{et al.} (1984) report that providing shade for bulls during exposure to elevated ambient temperatures will reduce black globe temperature and reduce exposure to radiant energy. The efficiency of shades to provide relief from heat stress in the summer was evaluated.

Barth and Bowman (1994) showed that insulation of the scrotum of bulls to decrease heat loss and increase testicular temperature also influences semen quality.

Akdag \textit{et al.} (1999) reported that steriodogenic functions of the somatic cells appear to be normal when exposed to the core body temperature. The elevated testicular temperature affects the normal functioning of all major cell types to some extent.
3. MATERIAL AND METHODS

3.1 Location and place of work

The research work was carried out in the Department of Veterinary Surgery and Radiology, Teaching Veterinary Clinical Complex (TVCC), College of Veterinary Science and Animal Husbandry, Nanaji Deshmukh Veterinary Science University (N.D.V.S.U.) Jabalpur.

3.2 Meteorological data and features of place

Jabalpur is situated at 23.17° latitude and 79.57° East longitudes at 410.87 mean sea level in the southern part of second agro-climatic zone, including Satpura Plateau and Kymore hills. It has a tropical climate having average rainfall of 1241 mm.

3.3 Study period

The study was performed for a period of approximately six months from December, 2016 to May, 2017.

3.4 Animals

The study was conducted on 18 apparently healthy male dogs irrespective of age and breed presented at Teaching Veterinary Clinical Complex.

3.5 Experimental design

Table 01:

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of animals</th>
<th>Treatment</th>
<th>Treatment regimen</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Six</td>
<td>Castration by Surgical procedure</td>
<td>---------</td>
</tr>
<tr>
<td>II</td>
<td>Six</td>
<td>1 MHz therapeutic ultrasound for 5 min</td>
<td>5 applications on 0, 2, 4, 6 and 8th days intervals</td>
</tr>
<tr>
<td>III</td>
<td>Six</td>
<td>3 MHz therapeutic ultrasound for 5 min</td>
<td>Same as above</td>
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</tbody>
</table>
3.6 Instrumentation

3.6.1 Therapeutic ultrasound machine

Ultrasound is a deep heating modality. At an intramuscular depth of 3 cm, a 10-minute hot pack treatment yielded an increase of 0.8°C, whereas at the same depth, 1 MHz ultrasound has raised muscle temperature nearly 4°C in 10 minutes. At 1 cm below the fat surface, while 5-minute heating raised the temperature 1.1°C; however, at the same depth, 3 MHz ultrasound raised the temperature 4°C in 5 minutes. Non-thermal effects occur when pulsed ultrasound is applied. Non-thermal effects are useful for decreasing oedema and promoting cellular repair (Elektromedizin, AG, Electroson-709, 2.5 cm2 transducer) (Plate 01).

3.6.2 Bard Max core biopsy instrument

Automatic spring loaded Bard Max core biopsy gun having needle size of 16G X 16 cm for dogs or 18G X 16 cm for dogs with sample notch of 1.9 cm was used for collection of tissue core biopsy of treated testicle on 0, 10\textsuperscript{th}, 20\textsuperscript{th} and 40\textsuperscript{th} day intervals of post treatment (Plate 02).

3.6.3 Vernier calipers

The testicular measurements (length and width) were noted by Vernier callipers. Hold the testes in between fingers and Vernier callipers place in between the cranial to caudal border of testes for measurements (Plate 03).

3.6.4 Measuring tape

Scrotal circumference was measured using commercially available measuring tape. Measuring tape were placed in round side of both testes with hold of testes.

3.7 Sterilization

All biopsy instruments were sterilized by placing them in a closed formaline vapourizing chamber for 24 hours. The instruments were removed from formaline chamber before biopsy, soaked in 1.5 per cent chlorhexidine solution for 30 minutes and then rinsed with sterile distilled water and wiped with dry sterile gauge.
3.8 Surgical castration procedure

3.8.1 Animal Preparation

Animal was restrained in dorso ventral recumbency and scrotal area was prepared for aseptic procedure prior to surgery by shaving hairs followed by thorough scrubbing and application of 5% povidone iodine and 1% metronidazole solution at the proposed surgical site.

3.8.2 Anaesthesia

The dogs were fasted for 12 hours before the procedure. Atropine sulphate was administered @ of 0.04-0.06 mg/kg body weight intramuscularly. After a gap of 5 minutes administration of xylazine hydrochloride @ 1-2 mg/kg body weight was done intramuscularly. After sedation Ketamine hydrochloride @ 5-10 mg/kg body weight intramuscularly. Maintenance of anaesthesia was done by ketamine hydrochloride intravenously as and when required given with infusion.

3.8.3 Castration procedure

Group – I (Castration by conventional surgical procedure)

In an open castration, a longitudinal incision on cranial to median raphae on pre-scrotal region was given and separate the covering (tunica vaganalis, tunica albugenia and tunica vasculosa) of testes and then testicle was squeezed out through an incision in the operated site between thumb and figure. The spermatic card was ligation, after ligation the testis was removed. The other testis was removed through the same opening following the similar technique. The incision site was closed in routine manner (Plate04).

3.8.4. Group – II (therapeutic ultrasound 1 MHz)

To facilitate therapeutic ultrasound penetration into underlying tissues, water soluble therapeutic ultrasound gel was applied over the scrotal skin, just before placing the probe of therapeutic ultrasound on the surface of the testical. The therapeutic ultrasound transducer (1MHz) was applied over testicles with circular movements up to time of treatment. Five application applications on 0,2,4,6 and 8th days interval were given for 5 minute (Plate 05).
3.8.5. Group – III (therapeutic ultrasound 3 MHz)

Testicular area was exposed and then therapeutic ultrasound transducer (3MHz) was applied over testicles with circular movements. Five applications of therapeutic ultrasound was made on 0,2,4,6 and 8th days for 5 minute duration each (Plate 06).

3.9 Post operative care

The surgical area was dressed with 5 % povidone iodine and 1% metronidazole soluion daily for three consecutive post operative days. Injection Amoxycillin sodium and Sulbactam sodium combination was administered @ 15 mg/Kg body weight intramuscularly twice daily and injection Meloxicam\textsuperscript{11} was administered @ 0.3mg/Kg body weight intramuscularly once in a day for 3 days after castration in group I.

3.10 Parameter of study

3.10.1 Anamnesis and clinical observation

History of each animal was taken for feeding, watering, defecation, urination, any previous illness or specific injury to external genitalia of the animal. Irrespective of management and breed, animals which were about to attain puberty were selected. Presented for elective sterilization for various reasons control of unwanted matting, to make docile and modify behaviour and temperament of dogs.

On clinical observation, The scrotal area was observed by and palpate thoroughly of the scrotum for to check any wound and any other illness, testes were found free from any pathological lesions.

3.10.2 Clinical examination

Temperature (°F), pulse rate (/min) and respiration rate (/min) were recorded pre-treatment at day 0 to determine the health status of the animals and morphology of testes (length, width) (Scrotal circumference) was measured by measuring tape and testicular length and width thickness was measured by Vernier callipers and recorded on day 0 (pre-treatment), on day 10 and 20th (post-treatment) (Plate 07 and 08).
3.10.3. Haematological estimations

Five ml of blood was collected aseptically from the cephalic or saphenous veins on day 0, 10 and 20 days from the dogs of all three groups. The following haematological parameters were estimated:

- Total leucocyte count (TLC) (x 10^6/µl)
- Differential leucocyte count (DLC) (%)
- Haemoglobin (Hb%) (g/dl)

The haematological parameters were estimated by auto analyzer for estimation of complete blood count (model MEDONIL CA 530).

3.10.4. Hormonal assay (testosterone ng/dl)

Blood was collected from the saphenous vein of each dog. Approximately 3 ml of blood was allowed to stand for 10-15 min at 4°C, and then centrifuged 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 Healthcare) on 0, 20 and 40th days in all the three groups.

3.10.5. Histological examination

Tissue samples were collected by Fine needle aspiration cytology (FNAC) from the testicles for histological examination on 0, 10, 20 and 40th day after treatment in group II and III and on 0 day only in group I.

Immediately after collection, the samples were fixed in 10 per cent buffered formalin for minimum 72hrs. These fixed tissue samples were processed in acetone-benzene sequence, embedded and blocked in paraffin wax 50-60°C melting point (Lillie and Fullmer, 1976). Five to six micron (µm) thick sections were cut with the help of rotatory microtome (spencer), mounted on clean albuminized glass slides and stained with Haematoxylin and Eosin for interpretation (Plate 09).
3.11 Statistical analysis

The quantitative data was analyzed by one-way ANOVA and t-test to compare the mean value within groups and at corresponding intervals among different groups as described by Snedecor and Cochran (1994).
4. RESULTS

The study was conducted on 18 apparently healthy male dogs, divided into three equal groups. Group I of animals (n=6) was subjected to castration by conventional surgical procedure, whereas in group II and group III (n=6 each group), animals were subjected to therapeutic ultrasound application with 1 and 3 MHz transducer respectively with 5 applications for 5 minute on 0, 2, 4, 6 and 8\textsuperscript{th} day intervals. Castration of male dogs provided behavioral and health benefits as well as helped in pet population control. It can help to prevent some undesirable behaviors such as urine or scent marking in the house, mounting other dogs, aggression toward other male dogs, and roaming.

4.1. Anamnesis and clinical observation

The work was conducted on 18 clinical cases of dogs presented for elective sterilization for various reasons like control of unwanted mating, to make docile and modify behavior and temperament of dogs.

History of each animal was taken for normal feeding, watering, defaecation, urination, any previous illness or specific injury to external genitalia of the animal. Irrespective of management and breed, animals which were about to attain puberty and requested for sterilization were selected for the present study.

Slight change in testicular circumference and temperament behaviour of animals was observed on 20\textsuperscript{th} day post-treatment. Gradually animal become more calm and docile.

4.2. Clinical examination

4.2.1 Rectal temperature

In animals of group I, values of rectal temperature (°F) recorded at day 0 ranged from minimum of 99.50 to maximum of 102.50 while on 10\textsuperscript{th} post treatment day it ranged from 100.00 to 102.20 and on 20\textsuperscript{th} day interval values ranged from 100.00 to 102.50. The mean value of rectal temperature
was observed within the normal of 100.87±0.42 at 0 day, 101.38±0.34 at day 10th and 101.00±0.38 at day 20th.

In group II, the rectal temperature ranged from minimum of 99.2 to maximum of 102.40 at 0 day, 100.15 to 102.40 on 10th day, and 98.15 to 102.50 on 20th day with mean value of 100.73 ±0.52 at 0 day, 101.33±0.46 at 10th day and 100.38 ± 0.75 at 20th day respectively.

In animals of group III, the rectal temperature recorded at day 0 ranged from minimum at 100.10 to maximum at 103.45, on 10 day 101.25 to 104.10 and on 20 day from 98.45 to 103.40. The mean values were 101.83±0.45, 102.33±0.46 and 101.40±0.76 on 0, 10th and 20th days respectively.

The mean values of temperature did not show difference in between the intervals in all the groups, however values of group III differed significantly (p>0.05) from group I and II (Table 02, Figure 01).

**Table 02:** Mean values of temperature (ºF) at different time intervals

<table>
<thead>
<tr>
<th>Time interval</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
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<tbody>
<tr>
<td>0 days</td>
<td>100.87&lt;sup&gt;AB&lt;/sup&gt;±0.42</td>
<td>100.73&lt;sup&gt;B&lt;/sup&gt;±0.52</td>
<td>101.83&lt;sup&gt;A&lt;/sup&gt;±0.45</td>
</tr>
<tr>
<td>10 days</td>
<td>101.38&lt;sup&gt;AB&lt;/sup&gt;±0.34</td>
<td>101.33&lt;sup&gt;B&lt;/sup&gt;±0.46</td>
<td>102.33&lt;sup&gt;A&lt;/sup&gt;±0.46</td>
</tr>
<tr>
<td>20 days</td>
<td>101.00&lt;sup&gt;AB&lt;/sup&gt;±0.38</td>
<td>100.38&lt;sup&gt;B&lt;/sup&gt;±0.75</td>
<td>101.40&lt;sup&gt;A&lt;/sup&gt;±0.76</td>
</tr>
</tbody>
</table>

A, B, values in between the groups with different superscript differed significantly (p<0.05)

**4.2.2 Heart rate (beats/min).**

In animals of group I, values of heart rate (beats/min) recorded at day 0 from minimum of 78 to maximum of 84 while on 10th post treatment day it ranged from 75 to 85 and on 20th day values ranged from 75 to 85. The mean values of heart rate (beats/min) observed were within the normal range of 81.33±0.84 at 0 day, 79.67±1.33 on 10th day and 78.17±1.01 at 20th day.
In group II, the heart rate (beats/min) ranged from minimum of 75 to maximum of 78 at 0 day, 75 to 79 on 10\textsuperscript{th} day, and 75 to 80 on 20\textsuperscript{th} day with mean value of 77.33±0.49, 77.66±0.61 and 75.67±1.54 on 0,10\textsuperscript{th} and 20\textsuperscript{th} days respectively.

In animals of group III, heart rate (beats/min) recorded at day 0 ranged from minimum of 77 to maximum of 90, on 10\textsuperscript{th} day 69 to 85 and on 20\textsuperscript{th} day from 65 to 85. The mean values were 76.50±3.15, 76.33±2.43 and 76.50±3.04 on 0,10\textsuperscript{th} and 20\textsuperscript{th} day respectively.

No significant change were observed in between the intervals in all the groups, however heart rate (beats/min) differed in group II and group III from group I (Table 03, Figure 02).

**Table 03: Mean values of heart rate (beats/min) at different time intervals**

<table>
<thead>
<tr>
<th>Time interval</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 days</td>
<td>81.33\textsuperscript{A±0.84}</td>
<td>77.33\textsuperscript{AB±0.49}</td>
<td>76.50\textsuperscript{B±3.15}</td>
</tr>
<tr>
<td>10 days</td>
<td>79.67\textsuperscript{A±1.33}</td>
<td>77.66\textsuperscript{AB±0.61}</td>
<td>76.33\textsuperscript{B±2.43}</td>
</tr>
<tr>
<td>20 days</td>
<td>78.17\textsuperscript{A±1.01}</td>
<td>75.67\textsuperscript{AB±1.54}</td>
<td>76.50\textsuperscript{B±3.04}</td>
</tr>
</tbody>
</table>

A,B, values in between the groups with different superscript differed significantly (p<0.05)

4.2.3. Respiration rate (breaths/min)

In animals of group I, values of respiration rate (breaths/min) recorded at day 0 ranged from minimum of 28 to maximum of 32 while on 10\textsuperscript{th} post treatment day it ranged from 28 to 31 and on 20\textsuperscript{th} day values ranged from 28 to 29. The mean value of respiration rate (breaths/min) observed were within the normal of 29.83±0.75, at 0 day, 29.16±0.54 on 10\textsuperscript{th} day and 28.33±0.21 on 20\textsuperscript{th} day.
In group II, the respiration rate (breaths/min) ranged from minimum of 22 to maximum of 30 at 0 day, 30 to 32 on 10th day, and 30 to 32 on 20th day with mean value of 28.00±1.24, 30.16±0.47 and 30.33±0.33 on 0, 10th and 20th days respectively.

In animals of group III, respiration rate (breaths/min) recorded at day 0, ranged from minimum at 29 to maximum of 30, on 10th day 30 to 28 and on 20th day from 30 to 35. The mean values were 29.33±0.21, 29.16±0.40 and 31.00±0.81 on 0, 10th and 20th day respectively.

The values of respiration rate (breaths/min) at different intervals in animals of all the groups varied non-significant (p>0.05) (Table 04, Figure 03).

Table 04: Mean values of respiration rate (beats/min) at different time intervals

<table>
<thead>
<tr>
<th>Time interval</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 days</td>
<td>29.83±0.75</td>
<td>28.00±1.24</td>
<td>29.33±0.21</td>
</tr>
<tr>
<td>10 days</td>
<td>29.16±0.54</td>
<td>30.167±0.47</td>
<td>29.16±0.40</td>
</tr>
<tr>
<td>20 days</td>
<td>28.33±0.21</td>
<td>30.33±0.33</td>
<td>31.00±0.81</td>
</tr>
</tbody>
</table>

4.2.3.1 External morphology (scrotal circumference cm²) (length and width cm²)

In animals of group I, testicular measurements (length and width cm²) recorded at day 0 (pre operative) ranged from minimum of 10 to maximum of 28 (cm²) were and therefore animal subjected to Castration by Conventional Surgical procedure.

In group II, testicular measurements (length and width cm²) ranged from minimum of 8.00 to maximum of 18 at 0 day, 8.00 to 18 on 10th day, and 8.00 to 16.50 on 20th day with mean value of 14.25±1.62, 14.25±1.62 and 12.42±1.43 on 0, 10th and 20th days respectively.
In animals of group III, testicular measurements (length and width cm²) recorded at day 0 ranged from minimum of 12 to maximum of 24.55, on 10th day 12 to 24.50 and on 20th day 10 to 21. The mean values were 17.17±2.02, 17.17±2.02 and 15.17±1.62 on 0, 10th and 20th day respectively.

Slight homogenous tenderness and slight change in testicular circumference was observed on day 20th in group III.

The mean values of testicular measurements (scrotal circumference cm²) in group II differed significantly (p<0.05) from group I at day 0. However, no changes were observed in between intervals (Table 05, Figure 04).

<table>
<thead>
<tr>
<th>Time interval</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 days</td>
<td>18.33 ±2.58</td>
<td>14.25 ±1.62</td>
<td>17.17 ±2.02</td>
</tr>
<tr>
<td>10 days</td>
<td>0</td>
<td>14.25 ±1.62</td>
<td>17.17 ±2.02</td>
</tr>
<tr>
<td>20 days</td>
<td>0</td>
<td>12.42 ±1.43</td>
<td>15.17 ±1.62</td>
</tr>
</tbody>
</table>

A, B, values in between the groups with different superscript differed significantly (p<0.05)

4.3. Haematological estimations

Five milliliters of blood was collected aseptically from cephalic or saphenous veins in EDTA coated vaccutainer on day 0, 10th and 20th from dogs of all the three groups. Samples were subjected for estimation of haemoglobin (g/dl), total leukocyte count (×103 /µl) and differential leukocyte count (%) to evaluate the health status of animals prior to treatment and to correlate the values with any inflammatory or degenerative changes.

4.3.1 Haemoglobin (g/dl)

In animals of group I, values of haemoglobin (g/dl) recorded at day 0 ranged from minimum of 11.00 to maximum of 14.20 while on 10th post treatment it ranged from 10.00 to 13.24 and on 20th day values ranged from 10.00 to 14.50. The mean values of haemoglobin (g/dl) observed were within
the normal range with 12.03±0.46, at 0 day, 11.83±0.48 on 10th day and 13.08±0.77 on 20th day.

In group II, the haemoglobin (g/dl) ranged from minimum of 11.20 to maximum of 15.60 on 0 day, 12.40 to 14.95 on 10th day, and 11.55 to 16 on 20th day with mean value of 14.13±0.78, 14.35±0.52 and 13.78±1.06 on 0,10 and 20th days intervals respectively.

In animals of group III, haemoglobin (g/dl) recorded at day zero ranged from minimum of 11.2 to maximum of 15.5, on 10th day 11.1 to 15.1 and on 20th day from 10.1 to 16.2. The mean values of haemoglobin were 13.35±0.85, 13.40±0.82 and 13.61±0.85 on 0,10th and 20th day interval respectively.

The mean values of haemoglobin (g/dl) was differed significantly in between the groups with transient changes within the groups. While no change were observed in between the intervals (Table 06, Figure 05).

**Table 06: Mean values of Haemoglobin (g/dl) at different time intervals**

<table>
<thead>
<tr>
<th>Time interval</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 days</td>
<td>12.03 B±0.46</td>
<td>14.13 A±0.78</td>
<td>13.35 AB±0.85</td>
</tr>
<tr>
<td>10 days</td>
<td>11.83 B±0.48</td>
<td>14.35 A±0.52</td>
<td>13.40 AB±0.82</td>
</tr>
<tr>
<td>20 days</td>
<td>13.08 B±0.77</td>
<td>13.78 A±1.06</td>
<td>13.61 AB±0.85</td>
</tr>
</tbody>
</table>

A,B, values in between the groups with different superscript differed significantly (p<0.05)

**4.3.2. Total leucocytes count (x10³/µl)**

In animals of group I, values of total leukocyte count (x10³/µl) recorded at day 0 ranged from minimum of 9.24 to maximum of 13.00 while on 10th post treatment day, it ranged from 8.25 to 12.00 and on 20th day it ranged from 7.90 to 13.50. The mean value of total leucocytes count (x10³/µl) observed were within the normal range with 10.55±0.55, at 0 day, 10.33±0.65 on 10th day and 10.66±0.92 on 20th day.

In group II, the total leucocytes count (x10³/µl) ranged from minimum of 9.10 to maximum of 10.20 at 0 day, 8.20 to 12.10 on 10th day,
and 9.85 to 11.55 on 20th day with mean value of 10.01±0.27, 10.73±0.54 and 10.58±0.28 on 0,10th and 20th days intervals respectively.

In animals of group III, total leucocyte count (x10^3/µl) recorded at day 0 ranged from minimum of 10.00 to maximum of 10.18, on 10th day 9.10 to 11.15 and on 20th day from 9.00 to 14.28. The mean values were 10.08±0.01, 10.20±0.37 and 11.48±0.73 on 0,10th and 20th day intervals respectively.

The mean total leucocytes count (x10^3/µl) values for the groups were within normal range at each time intervals, and the values did not differ significantly within or between the groups (Table 07, Figure 06).

Table 07: Mean values of total leucocyte count (x10^3/µl) at different time intervals

<table>
<thead>
<tr>
<th>Time interval</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 days</td>
<td>10.55±0.55</td>
<td>10.01±0.27</td>
<td>10.08±0.01</td>
</tr>
<tr>
<td>10 days</td>
<td>10.33±0.65</td>
<td>10.73±0.54</td>
<td>10.20±0.37</td>
</tr>
<tr>
<td>20 days</td>
<td>10.66±0.92</td>
<td>10.58±0.28</td>
<td>11.48±0.73</td>
</tr>
</tbody>
</table>

4.3.3 Differential leukocyte count (%)

4.3.3.1 Neutrophils count

In animals of group I, values of neutrophils (%) recorded at day zero ranged from minimum of 70 to maximum of 82 while on 10th post treatment day it ranged from 75 to 85 and on 20th day interval values ranged from 71 to 80. The mean value of neutrophils (%) observed 75.50±2.33, at 0 day, 77.17±1.28 on 10th day and 74.50±1.88 on 20th day.

In group II, the neutrophils (%) ranged from minimum of 60 to maximum of 90 at 0 day, 72 to 82 on 10th day, and 76 to 80 on 20th day with mean value of 74.33±4.14, 76.50±1.52 and 77.33±0.84 on 0, 10th and 20th days respectively.

In animals of group III, neutrophils (%) recorded at day 0 ranged from minimum of 70 to maximum of 78, on 10th day 75 to 80 and on 20th day
The mean values of 73.33±1.54, 76.00±0.81 and 78.17±1.33 on 0, 10th and 20th day respectively.

The values did not differed significantly (p>0.05) within or between the various groups at different time intervals (Table 08, Figure 07).

**Table 08: Mean values of neutrophil count (percent) at different time intervals**

<table>
<thead>
<tr>
<th>Time interval</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 days</td>
<td>75.50±2.33</td>
<td>74.33±4.14</td>
<td>73.33±1.54</td>
</tr>
<tr>
<td>10 days</td>
<td>77.17±1.28</td>
<td>76.50±1.52</td>
<td>76.00±0.81</td>
</tr>
<tr>
<td>20 days</td>
<td>74.50±1.88</td>
<td>77.33±0.84</td>
<td>78.17±1.33</td>
</tr>
</tbody>
</table>

**4.3.3.2 Eosinophil count**

The values of eosinophil count (%) in group I, recorded at day 0 ranged from minimum of 1 to maximum of 8 while on 10th post treatment day it ranged from 1 to 5 and on 20th day values ranged from 1 to 2. The mean value of eosinophil count (%) observed were 2.83±1.25 at 0 day, 1.50±0.76 on 10th day and 1.16±0.40 on 20th day.

In group II the, eosinophil count (%) ranged from minimum of 2 to maximum of 5 at 0 day, 2 to 5 on 10th day, and 1 to 4 on 20th day with mean value of 1.83±0.74, 2.16±0.79 and 1.83±0.54 on 0, 10th and 20th days respectively.

In animals of group III, eosinophil count (%) recorded at day of 0 ranged from minimum 2 to maximum of 5, on 10th day 2 to 5 and on 20th day from 2 to 8. The mean values of eosinophil count (%) 4.16±0.54, 3.33±0.84 and 2.17±1.28 on 0, 10th and 20th day respectively.

The mean values of eosinophil count (%) was differed significantly (p<0.05) in between the groups with transient changes within the groups. While no change were observed in between the intervals (Table 09, Figure 08).
**Table 09: Mean values of eosinophil count (percent) at different time intervals**

<table>
<thead>
<tr>
<th>Time interval</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 days</td>
<td>2.83&lt;sup&gt;B&lt;/sup&gt;±1.25</td>
<td>1.83&lt;sup&gt;AB&lt;/sup&gt;±0.74</td>
<td>4.16&lt;sup&gt;A&lt;/sup&gt;±0.54</td>
</tr>
<tr>
<td>10 days</td>
<td>1.50&lt;sup&gt;B&lt;/sup&gt;±0.76</td>
<td>2.16&lt;sup&gt;AB&lt;/sup&gt;±0.79</td>
<td>3.33&lt;sup&gt;A&lt;/sup&gt;±0.84</td>
</tr>
<tr>
<td>20 days</td>
<td>1.16&lt;sup&gt;B&lt;/sup&gt;±0.40</td>
<td>1.83&lt;sup&gt;AB&lt;/sup&gt;±0.54</td>
<td>2.17&lt;sup&gt;A&lt;/sup&gt;±1.28</td>
</tr>
</tbody>
</table>

A,B, values in between the groups with different superscript differed significantly (p<0.05)

4.3.3.3 Lymphocyte count

In animals of group I, values of lymphocyte count (%) recorded at day 0 ranged from minimum of 17 to maximum of 22 while on 10<sup>th</sup> post treatment day it ranged from 15 to 19 and on 20<sup>th</sup> day, values ranged from 10 to 25. The mean values of lymphocyte count (%) observed were within the normal range with 18.67±0.95 at 0 day, 18.00±0.73 on 10<sup>th</sup> day and 16.00±2.34 at 20<sup>th</sup> day.

In group II, the lymphocyte count (%) ranged from minimum of 8 to maximum of 25 at 0 day, 9 to 20 on 10<sup>th</sup> day, and 17 to 20 on 20<sup>th</sup> day with mean value of 16.67±2.32, 14.50±1.45 and 16.83±0.74 on 0, 10<sup>th</sup> and 20<sup>th</sup> days respectively.

In animals of group III, lymphocyte count (%) recorded at day 0 ranged from minimum of 10 to maximum of 20, on 10<sup>th</sup> day 10 to 18 and on 20<sup>th</sup> day from 8 to 18. The mean values were 14.17±1.54, 11.33±1.33 and 12.67±1.58 on 0, 10<sup>th</sup> and 20<sup>th</sup> day respectively.

The lymphocyte count (%) showed significant difference (p<0.05) from group III when compare with group I and group II, however no change was observed in between intervals (Table 10, Figure 09).
Table 10: Mean values of lymphocyte count (percent) at different time intervals

<table>
<thead>
<tr>
<th>Time interval</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 days</td>
<td>18.67±0.95</td>
<td>16.67±2.32</td>
<td>14.17±1.54</td>
</tr>
<tr>
<td>10 days</td>
<td>18.00±0.73</td>
<td>14.50±1.45</td>
<td>11.33±1.33</td>
</tr>
<tr>
<td>20 days</td>
<td>16.00±2.34</td>
<td>16.83±0.74</td>
<td>12.67±1.58</td>
</tr>
</tbody>
</table>

A,B, values in between the groups with different superscript differed significantly (p<0.05).

4.3.3.4 Monocyte count

In animals of group I, values of monocyte count (%) recorded at day 0 ranged from minimum of 1 to maximum of 5 while on 10\textsuperscript{th} post treatment day it ranged from 2 to 9 and on 20\textsuperscript{th} day values ranged from 2 to 10. The mean value of monocyte count (%) observed were within the normal range of 2.50±0.56 at 0 day, 5.00±1.21 on 10\textsuperscript{th} day and 5.33±1.26 on 20\textsuperscript{th} day.

In group II, the monocyte count (%) ranged from minimum of 2 to maximum of 15 at 0 day, 2 to 16 on 10\textsuperscript{th} day, and 3 to 8 on 20\textsuperscript{th} day with mean value of 5.50±1.96, 6.83±2.21 and 4.16±0.83 on 0, 10\textsuperscript{th} and 20\textsuperscript{th} days respectively.

In animals of group III, monocyte count (%) recorded at day 0 ranged from minimum at 5 to maximum of 10, on 10\textsuperscript{th} day 4 to 12 and on 20\textsuperscript{th} day from 2 to 12. The mean values were 8.33±1.05, 8.50±1.31 and 5.33±1.93 on 0, 10\textsuperscript{th} and 20\textsuperscript{th} day respectively.

The mean values of monocyte count (%) was differed significantly (p<0.05) in between the groups with transient changes within the groups. While no change were observed in between the intervals (Table 11, Figure 10).
Table 11:  Mean values of monocyte count (percent) at different time intervals

<table>
<thead>
<tr>
<th>Time interval</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 days</td>
<td>2.50±0.56</td>
<td>5.50&lt;sup&gt;AB&lt;/sup&gt;±1.96</td>
<td>8.33&lt;sup&gt;A&lt;/sup&gt;±1.05</td>
</tr>
<tr>
<td>10 days</td>
<td>5.00&lt;sup&gt;B&lt;/sup&gt;±1.21</td>
<td>6.83&lt;sup&gt;AB&lt;/sup&gt;±2.21</td>
<td>8.50&lt;sup&gt;A&lt;/sup&gt;±1.31</td>
</tr>
<tr>
<td>20 days</td>
<td>5.33&lt;sup&gt;B&lt;/sup&gt;±1.26</td>
<td>4.16&lt;sup&gt;AB&lt;/sup&gt;±0.83</td>
<td>5.33&lt;sup&gt;A&lt;/sup&gt;±1.93</td>
</tr>
</tbody>
</table>

A,B, values in between the groups with different superscript differed significantly (p<0.05)

Basophil it was recorded zero data in different groups at various intervals.

4.4. Hormonal assay (testosterone ng / dl)

The values of testosterone (ng/dl) ranged from minimum of 7.10 to maximum of 10.65 at day 0, 3.15 to 4.50 on 20<sup>th</sup> day and 2.90 to 3.90 on 40<sup>th</sup> day in group I with mean values of 9.18±0.55, 4.16±0.21 and 3.61±0.16 at day 0, 20<sup>th</sup> and 40<sup>th</sup> day. In animal, of group II, the level of testosterone varied from minimum 6.10 to maximum 11.20, 5.60 to 10.80 and 4.10 to 9.50 at 0, 20<sup>th</sup> and 40<sup>th</sup> day, with mean values of 8.73±0.74,8.21±0.76 and 6.33±0.80 on 0,20<sup>th</sup> and 40<sup>th</sup> day respectively.

In group III, the values minimum was 8.80 to maximum of 10.20 at day 0, 6.90 to 10.30 on day 20<sup>th</sup> and 5.60 to 8.10 on day 40<sup>th</sup> the mean values 9.05±0.56, 8.56±0.50 and 7.13±0.48 on 0, 20<sup>th</sup> and 40<sup>th</sup> day.

There was highly significant (p>0.05) difference between mean values of hormonal assay (testosterone ng/dl) on day 0, when compared with values at day 20 and 40 in group I.

The testosterone values significantly differed in animal of group I whereas compared to animals of group II and III.

The values of testosterone (ng/dl) showed declining trends in all the three groups at intervals of day 0, 20<sup>th</sup> and 40<sup>th</sup>. However, this decreased was significantly in group II and III as compare to group I, however, no significant difference among hormonal levels in groups II and III was observed.
Similar to day 20\textsuperscript{th}, the hormonal level in group I, was significantly (p>0.05) lower as compared to those in Groups II and III on day 40\textsuperscript{th}. However, the hormonal levels in groups II and III were at par and do not differ from each other on day 40 (Table 12, Figure 11).

**Table 12:** Mean values of hormonal assay (testosterone ng/dl) at different time intervals

<table>
<thead>
<tr>
<th>Time interval</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 days</td>
<td>9.18\textsuperscript{A}±0.55</td>
<td>8.73\textsuperscript{A}±0.74</td>
<td>9.05\textsuperscript{A}±0.50</td>
</tr>
<tr>
<td>20 days</td>
<td>4.16\textsuperscript{B}±0.21</td>
<td>8.21\textsuperscript{A}±0.76</td>
<td>8.56\textsuperscript{A}±0.50</td>
</tr>
<tr>
<td>40 days</td>
<td>3.61\textsuperscript{B}±0.16</td>
<td>6.33\textsuperscript{A}±0.80</td>
<td>7.13\textsuperscript{A}±0.48</td>
</tr>
</tbody>
</table>

A,B, values in between the groups with different superscript differed significantly (p<0.05)

### 4.5 Histology

Histological observation revealed that in group I, the seminiferous tubules were lined by the stratified germinal epithelium comprised of various spermatogenic cells. The tubules were placed close to each other with little amount of intertubular tissue (plate10 a). Under high magnification, the larger spermatogonial cells with round nucleus were seen in the basal part of the seminiferous tubules and in center portion of the lumen spermatocytes were seen. Peritubular cells were also visible just out side the lamina propria (plate10 b).

In group II, on 10\textsuperscript{th} day of treatment increased connective tissue with fibrocytes was seen which was slightly more in group III in comparison to group II. The seminiferous tubules showed spermatogenic cells without any significant alteration in group II and III in comparison to the group I. However, some of the seminiferous tubules were irregular in out line.

On 20\textsuperscript{th} day, of treatment connective tissue was increased in comparison to 10\textsuperscript{th} day, which was more pronounced in group III in comparison to group II. The seminiferous tubules became irregular in both the
groups and vacuolization was seen in the epithelial cells of it seminiferous tubules.

On 40th day of observation in group II, there was increased intertubular space with presence of connective tissue. In group III, the connective tissue was abundant and densely packed in comparison to group II. However, the seminiferous tubules were more irregular in shape in group II comparison to group III (plate11 a). Under high magnification, it was observed that in group II and group III seminiferous tubules were covered by one or two rows of cells with epithelial vacuolization. In group III, most of the seminiferous tubules were filled with clumps of cells and basement membrane was discontinuous (plate 11 b). The spermatogenic series was seen in group II, However, some of seminiferous tubules were empty in group III (plate 12 a and 12 b).

In both the groups (II and III) depletion of testicular elements and spermatogonial cells with presence of dead spermatozoa in the center portion of the seminiferous tubules. However, abundant increased connective tissue was more seen in group III in comparison to group II, Indicating the proliferation of the connective tissue because of generation of more heat in the superficial part of the testes in group III.
5. DISCUSSION

Sterilization in dogs is indicated for various reasons to check the population (Patronek et al. 1996), the unwanted behavior (Hart and Eckstein 1997), minimize hormonal disorder and neoplasia (Johnston, 1991, Slatter, 1993).

There are various procedures for sterilization in dogs, like vasectomy, castration (Johnston, 1991), hormonal and chemical procedure, (Aiudi et al. 2010) used from years in veterinary practice. However, each procedure has its merit and demerit.

Veterinary surgeons are continuously making their effort for a better, minimum invasive and non invasive procedure for sterilization with least complication, Sterilization by therapeutic ultrasound has been tried successfully in human, dogs and rhesus monkey (VandeVoort and Tollner 2012). Therapeutic ultrasound procedure has been reported as a better substitute to conventional surgical castration without any adverse effect (Leoci et al. 2015).

5.1. Anamnesis and clinical observation

The work was conducted in 18 clinical cases of dogs presented for elective sterilization for various reasons in dogs.

In present study, all the dogs were maintained in proper care and feeding by the pet owners. No systemic or local effect like inflammation or any injury were recorded during post treatment intervals. On clinical observation and palpation of the scrotum, testes were found free from any apparent pathological lesions, light homogenous tenderness was observed on day 20 in animals of group III.

A slight decrease in testicular circumference area were observed in animals of group II and III on 30th day post treatment. The temperament and behavior of animal also gradually decreased to mild calmness.

Similar, observations were also reported by (Millis et al. 2004) in dogs (Leoci et al. 2015) who has also recorded no local and systemic side effect after therapeutic ultrasound treatment in dogs and animal became calm.
A slight decrease in scrotal circumference and change in temperament and behavior of animals were observed in present study. No change was observed over the scrotum. Therapeutic ultrasound procedure was found easy to apply over the scrotum and performed in minimum time.

5.2. Clinical examination

5.2.1 Temperature, heart rate and respiration rate

The non significant variation in temperature, heart rate and respiration rate between the groups or intervals, may be because of individual variation, breeds and physical and surgical stress (Taelman et al., 2009) and inflammation. Millis et al., (2004) stated that during physiotherapy treatment of the Therapeutic ultrasound intensity for 5 to 10 minute dogs start whine or act uncomfortable. No such observation was recorded, in the present study it indirectly support the present findings of no variation in temperature, heart rate and respiration rate between the groups or intervals.

Protocols used in this study did not cause pain or discomfort (Leoci et al. 2015), as therapeutic ultrasound was used in continuous mode only for 5 minute duration (Millis et al. 2004).

The variation was non significant in temperature, heart rate and respiration rate in present study (VandeVoort and Tollner 2012), who recorded that low intensity therapeutic ultrasound exhibit both thermal and non-thermal effects on tissue.

In present study, temperature, heart rate, and respiration rate did not showed significant change after surgery as well as after therapeutic ultrasound treatment. Slight change was found in between the groups.

External morphology (scrotal circumference cm²) (length and width cm²)

Heat near the testes can greatly reduced fertility. Heat may cause shrinkage of testes about a 3rd by weight in study on ram, Hoffman et al. (2010) reported marked shrinkage of testes after heat application continuously for two week. In a study on rats he reported less responsiveness of testicles.
The shrinkage in scrotum circumference in the present study was more marked in group III, and may be due to aggregation of therapeutic ultrasound by 3MHz transducer which produces heat on superficial skin area where as 1MHz transducer produced less heat with more penetration power therefore reduction in scrotum circumference was not noticeable.

VandeVoort and Tollner (2012) reported that higher intensity result is more serious damage and likely sterilization. They further noticed that male with smaller testes showes greatly reduction in sperm number following therapeutic ultrasound treatment.

In the present study scrotum circumference reduced non significantly in group II and group III as compare to group I. The scrotum circumference decreased was more observe in animals of group III. The finding correlated with observation of (Paltiel et al. 2002).

The non significant decrease in group III may be the effect of more intensity of therapeutic ultrasound application of 1MHz on scrotum.

5.3 Heamatological estimations

Measurement of blood parameter is the important means to determine the health status of experimental animals.

The mean values of haemoglobin concentration (g/dl) in group II differed significantly from group I and III in between the intervals. It may be the individual variation in the animals. The animals of group II may possibly, be more healthy with better haemoglobin concentration (g/dl) than animals of group II.

It is similar to the result of Sawhney (2016) who also reported no significant difference in heamatological parameters following castration in buck and calves.

In present study, the values of heamatological examination observed to be within normal range all the intervals, significant change was found in between the groups. Earley and Crowe (2002) concluded normal
haemoglobin levels as the indicate animal healthy during pre and post treatment.

The mean values of total leucocytes and neutrophils were observed to be within normal range in different groups at different intervals. Similar findings were also reported by Mohammad et al. (2008) in lambs and Mahalingam et al. (2009) in dogs.

In the present investigation significant difference of lymphocytes between the groups may be the effect of surgery in group I, and other temperature induced by therapeutic ultrasound in group II and III. The temperature stimulates the hematopoietic system to release more lymphocytes causing an increase in their number in the blood stream. Aziz et al. (2010) also reported an increase in lymphocytes under the influence of exposure to radiation, increased temperature. Findings of the present study corroborates the results of (Abdolmaleki et al. 2013), and Sisodia et al., (2013).

Rotkovska et al. (1993) reported no change in complete blood count between irradiated and non irradiated mice, however significant decrease in total leukocytes was observed following irradiation in mice.

Significant decrease in monocytes in group II and III may be attributed to damage of cells due to ultrasound temperature. Similar finding has also been reported by (Sisodia et al, 2013) in albino mice with 10GHz MW exposure. It also correlated with the study of Rifat et al. (2014).

Monocytes count (%) of group III differed significantly from group I and group II, however no change was observed between the intervals. Mean values of eosinophils count (%) at group III differed significantly from group I and group II, while no change observed in between the intervals.

In the present study Mean values of eosinophils count (%) at group III differed significantly from group I and group II, while no change observed in between the intervals.
5.4 Hormonal assay (Testosterone ng/dl)

The level of Testosterone showed significant difference from control values (group I) of day 0 to 40\textsuperscript{th} day post treatment. However, in group II and III non significant declining were noticed at day 20 and 40. The severe significant decline in the values of testosterone from day 0 to day 20 and 40 was due to castration, where testicles were removed in group I. A slight non significant decline in values of testosterone in group II and III was similar to report of Fahim \textit{et al.} (1977) who reported that therapeutic ultrasound method does not affect testosterone production by Leydig cells. Leoci \textit{et al.} (2015) also noticed that testosterone levels remained within physiological range with all application protocols. However he further reported that several treatment with therapeutic ultrasound for longer period may cause permanent sterilization.

Raphael \textit{et al.} (2014) reported that 30\% of surgical castrated dogs showed reduced testosterone concentration (1 ng/dL) after 6 month postoperatively. The results simulates the present study where in group I the testosterone level reduced almost half the values than preoperative values, subsequently testosterone level showed gradual decline on subsequent intervals.

Sanni \textit{et al.} (2012) have also reported significantly decreased plasma testosterone level following castration in rabbits. The result indicates the cession of testosterone production following castration.

Hoffman (2010) observe that testosterone produced by leyding cells from heated testes may not secret as effectively as a normal testes. He further reported that testes become less responsive like partial secondary hypogonadism and heat was found effective to reduced sperm count drastically in monkey. The study further suggested a time lag form the application of therapeutic ultrasound and decreased testosterone level.

In animals of group II and III the decline values of testosterone might be due to heat stress which down regulated two enzymes required for testosterone biosynthesis namely cytochrome and steroidogenic. Acute
regulatory protein in Leydig cells which leads to reduction in testosterone biosynthesis by following therapeutic ultrasound treatment. The similar findings were also reported by Tian et al. (2015) in rats.

5.5 Histology

The finding of the present study of group II and III corroborates with the report of Leoci et al. (2015) who reported that elevation of testicular temperature result to impairment of spermatogenesis. Jung and Schuppe (2007) concluded from his study in male dogs that sperm production directly affected by raising the testicular temperature. The sign of hypoplasia were more marked in animals of group II at 40 day intervals as revealed histologically by testicular degeneration with a widespread tubular atrophy and a significant decrease of the testicular parenchyma. These findings are similar to the report of Leoci et al. (2015), who noticed irreversible testicular damage and azoospermia in the dogs by applying therapeutic ultrasound treatment on testicle. He further added that effect seems to be irreversible histologically.

Sailer et al. (1997) observed minor sperm chromatin abnormality with heat stress on mouse testicular cells. They concluded that spermatogenesis is disrupted by scrotal exposure to higher environmental temperature.

Soto et al. (2005) reported depletion of spermatocytes and spermatogenesis from non invasive with multiple application of therapeutic ultrasound, they further reported that histologically effect seems to be irreversible.

Findings of Tsuruta et al. (2012) also supported the present observation and reported that therapeutic ultrasound treatment depleted developing germ cells from the testis and subsequently decreased the size of sperm reserve in the epididymis in rats. However, Fahim et al. (1977) observed contraceptive effect following single treatment of 1 MHz.
6. SUMMARY, CONCLUSIONS AND SUGGESTIONS FOR FURTHER WORK

6.1 Summary

The present study was conducted with the objectives to establish a noninvasive therapeutic ultrasound procedure for sterilization in male dogs and evaluation of its clinical efficacy for sterilization by clinical, hematological, hormonal assay (Testosterone ng/dl) and histological examination.

The study was conducted on 18 apparently healthy male dogs irrespective breed, divided into three equal groups. Animals of group I (n=6) subjected to castration by conventional surgical procedure, Where as animals of group II and III (n=6 each) were subjected to therapeutic ultrasound treatment over testicles with 1 and 3 MHz transducer respectively for 5 minutes and five applications on zero, 2nd, 4th, 6th and 8th days intervals.

All the animals were adult and presented for elective sterilization for various reasons like control of unwanted matting, to make docile and modify behavior and temperament of dogs.

Anamnesis was taken for feeding and hygiene, any previous illness or specific injury to external genitalia of the animals.

In all the animals clinical observation and examination, rectal temperature, pulse rate, respiration rate were evaluated and morphology of testes were measured at 0, 10 and 20th day post-treatment. Hormonal assay (testosterone ng/dl) at day 0, 20, 40th day and histological examination was carried out on day 0, 10, 20 and 40th day post-treatment was also observed.

On clinical observation and palpation of the scrotum, testicles were found free of any pathological lesion. On 20th day post treatment interval mild change in temperament was observed and behaviors of animal gradually changed to calmness.

The mean values of temperature and heart rate did not show difference in between the intervals in all the groups, however values of group
III differed significantly (p>0.05) from group I and II. The values of respiration rate (breaths/min) at different intervals in animals of all the groups remain transient and were non significant.

Slight homogenous tenderness was observed on day 20 in group III only. The mean values of testicular measurements (scrotal circumference cm²) in group II differed significantly from group I at day 0. However, no changes were observed in between intervals.

In hematological estimation, transient changes were observed in the mean values of haemoglobin, lymphocyte and monocytes count within the groups, while no change were observed in between the intervals. The mean value of total leucocyte and nutriophils were within the normal range in all the group at different intervals.

The values of testosterone showed highly significant difference in between mean values of day 0, when compared with values at day 20 and 40 in group I (p>0.05). The testosterone value differed significantly in animals of group I when compared to animals of group II and III at day 20 and 40.

The values of testosterone (ng/dl) showed declining trends in all the three groups at intervals of day 20 and 40. However, this decrease was significantly lower in group I as compare to group II and III. However, no significant difference among hormonal level in groups II and III was observed. On day 20 and 40 the hormonal level in Group I was significantly lower as compared to those in Groups II and III. However, the hormonal levels in Groups II and III were at par and do not differ from each other on day 40.

In both the groups (II and III) depletion of testicular elements and spermatogonial cells with presence of dead spermatozoa in the center portion of the seminiferous tubules were abundant. However increased connective tissue was more seen in group III in comparison to group II, indicating the proliferation of the connective tissue because of generation of more heat in the superficial part of the testes in group III.
6.2 Conclusion

On the basis of observation of the present study like scrotal circumference, hormonal assay (testosterone ng/dl) and histological examination it may be conducted that:

1. Three treatments with 1MHz lasting 5 min each seems to cause progressive sterilization in dogs.

2. Sterilization by therapeutic ultrasound may be feasible alternative to castration by conventional surgical procedure.
6.3 Suggestion

1. The therapeutic ultrasound may be studies in a larger population of dogs for a longer duration of observation.

2. Therapeutic ultrasound may also be trial in other species like goat and calves.
7. REFERENCES


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