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Reproductive Functions and Toxicology Following Scrotal Ultrasound Therapy in Rats

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***Abstract*—** Therapeutic ultrasound involves direct application to the scrotum that might affect normal reproductive functions. We explore possibilities for development of a localized, non-invasive and reversible contraception method with ultrasound therapy in rat model. Animals were divided equally into five groups. Results observed with a gradual decrease in reproductive organs weight, testis volume, testosterone concentration and cauda epididymal sperm count, motility and viability following therapy, whereas sperm abnormalities were continuously enhanced throughout the study in groups II - V. Testis histopathology marked with duration and frequency dependent changes in groups II - V including conspicuous alterations in the seminiferous tubules, erupted germ cells, impaired spermatogenesis with pycnotic nuclei, vacuolization in the Sertoli cells, small Leydig cell nuclei, total necrosis and reduction in seminiferous tubules and wide interstitial space with fibroblast like cells. The fertility was totally lost at 180 days (groups II and III), 15 - 180 days (group IV) and 7 - 30 days of therapy (group V), respectively. A complete recovery was observed in all parameters in group V rats. Ultrasound application affects reproductive organs and fertility in rats. Hence, it may serve as a viable male contraceptive with further confirmation of dose and duration dependent efficacy, side effects and reversibility.

Keywords— *Fertility; Scrotum; Ultrasound; Toxicology; Wistar albino rat*

I. INTRODUCTION

Today, contraception has taken a pivotal part in life. The availability of a greater number of effective contraceptive choices has the potential to reduce unintended pregnancies and abortions by decreasing morbidity and mortality. For men, condom and vasectomy are most opted with low compliance rate. It's still a need for a method with noninvasive mode, effective for longer duration without toxic effects and most important, retrieve normal fertility after withdrawal. Ultrasound, on the hypothesis of suppression of spermatogenesis in a reversible way provides a new direction in the field of noninvasive approach of contraception in comparison to vasectomy which may eliminate the complications related to surgery and failure in reversibility; hence increase the acceptance rate of the technique. As suggested by early reports in various animal models, ultrasound treatment reduced sperm count upto azoospermia and it may serve to be viable approach for birth control method in future. Accordingly, it has been experimentally proven that exposing intra-scrotal testes to elevated temperature results in rapid degeneration of the seminiferous epithelium [1,2]. The research of Dr. Fahim and colleagues showed brief applications of testicular ultrasound waves to be effective at reducing or eliminating sperm, further studies showed similar effect in rats, cats, dogs, rabbits, monkeys and man [2-6].

As direct application of therapeutic ultrasound to the scrotum may affect normal reproductive functions due to thermal effect, so, there is a requirement to assess possible hazards, to assure safety of end-users with risk-benefit analysis. The summarized use of ultrasound waves includes its simple and convenient use, localized and non-systemic with minimum risk of complications, nonsurgical, noninvasive, as well as endurable. In addition, it provides long acting effects as a contraceptive with reversal aspects as opposed to vasectomy. Although, no side effects are reported in the previous studies, its apparent permanence needs to be established through long-term studies. Thereby, in this article, for the first time, we followed the protocol with long term study in rat model with an ultrasound instrument used for physiotherapy with specific intensity, frequency, duty cycle and time. We investigated effects on reproductive functions following scrotal ultrasound therapy through sperm characteristics, fertility status and regulatory toxicology in albino rats.

The paper is organized as follows, Section I contains the introduction of male contraceptive methods. It also discusses role of ultrasound therapy in male contraception, Section II contains the related work explaining different studies performed on ultrasound and its effect on male reproductive functions on various animal models, Section III contains the details about the experimental procedures used in the present study with detailed experimental design and parameters, Section IV describes all results obtained in

the present work done, section V discusses and compares our results with already published data and Section VI concludes research work with future directions.

II. RELATED WORK

As of 2012, a study conducted on rats found that two 15 min. treatments of ultrasound delivered 2 days apart in a warm salt bath effectively lowered their sperm count to below fertile levels [7]. A few studies involved dogs and found that after ultrasound application the dog's ejaculate contained no sperm [8, 9]. An attempt using cup method and direct application was also made previously that sustained reduction in sperm count, percent motility, normal morphology, and sperm vigor with the cup exposure method; and provides proof of principle that testicular exposure with ultrasound can be an effective contraceptive approach in rhesus monkey [10]. These properties make it a promising approach in terms of contraception; so the study is proposed to translate the technique to be opted by men.

III. METHODOLOGY

Ultrasound Therapy Instrument

The mobile ultrasound therapy machine (Model: Intelect®, Chattanooga, USA) provided with 1, 2, 5 and 10 cm^2 ultrasound head applicators along with conductor ultrasound gel was used in the present study. It has fully functional 1 and 3 MHz frequencies with the option of specific intensity/power, frequency, duty cycle and time to be set as required. All ultrasound head applicators are interchangeable due to electronic signature residing on all transducers. Pulsed and continuous therapy option is also available.

Animals

Adult male Wistar albino rats (*Rattus norvegicus*), 3 - 4 months old, weighing 200 - 250 g were used in the present investigation. The animals were housed in the experimental animal facility at controlled temperature (23 ± 2 °C), humidity (65 ± 5 %) and 12:12 light/dark schedule. Rats were housed in polypropylene cages in group of five/cage (size 43 x 27 x 15 cm) and fed with rat pellet diet (Ashirwad Industries Ltd., Chandigarh, India) and free access to safe drinking water. Animals were maintained under perfect veterinary supervision and in accordance to the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines on the regulation of scientific experiments on animals [11]. The protocol has the approval of the Institutional Animal Ethics Committee.

EXPERIMENTAL DESIGN

The animals were divided into five groups, containing forty animals in each group:

Group I: Sham control
Group II: Animals were exposed with ultrasound at 1 MHz frequency for five minutes duration on alternate day/three days a week (Monday/Wednesday/Friday) for 180 days
Group III: Animals were exposed with ultrasound at 3.0 MHz frequency with 50 % duty cycle for five minutes duration on alternate day/three days a week (Monday/Wednesday/Friday) for 180 days
Group IV: Animals were exposed with ultrasound at 3.0 MHz frequency for 2.5 minutes duration on alternate day/three days a week (Monday/Wednesday/Friday) for 180 days
Group V: Animals were exposed with a single ultrasound at 3.0 MHz frequency for fifteen minutes duration for 180 days.

Ultrasound Therapy

During ultrasound therapy, the rats were housed in the rat restrainer (BIK Industries, Mumbai, India) by keeping the scrotum, tail and hind legs outside. A thin layer of ultrasound gel (Suja Instruments Corporation, Jaipur, India) was applied on the scrotum and the ultrasound therapy was given by the help of 5 cm^2 head applicator as per experimental schedule. The ultrasound machine was set on the required frequency, time, duty cycle, 2.5 W/cm^2 intensity and switched on head warming feature. To prevent any infection antiseptic/antibiotic ointment was applied after every ultrasound therapy.

Five animals from each group were subjected to fertility test with proven fertile females at 1 : 2 ratio prior to and at 7, 15, 30, 60, 90, 120 and 180 days following ultrasound exposure. Following completion of fertility test at regular intervals, the animals from each group (n = 5) were subjected to terminal sacrification under a intraperitonial overdose of sodium thiopentone (Thiosol Sodium, Neon Laboratories Ltd., Mumbai, India) anesthesia.

PARAMETERS

The following parameters were carried out:

Visible Toxicological Symptoms

Mortality and morbidity, changes in fur, skin, eyes and mucous membrane, tremors, convulsions, salivation, diarrhoea, lethargy, animal behavior, feeding pattern, changes in the level of motor activity, gait and posture, reactivity to handling or sensory stimuli, grip strength and bizarre behavior such as self mutilation, walking backward, etc., were recorded daily in all groups. Libido was recorded by fertility test on the above described durations in all animals.

Body, Reproductive and Vital Organs Weight

The body weights were recorded on 0, 7, 15, 30, 60, 90, 120 and 180 days of ultrasound therapy. The reproductive organs (testes, epididymides, vas deferens, seminal vesicle and ventral prostate) and vital organs (brain, lungs heart, liver, kidneys, spleen, thyroid, pancreas and adrenal) were

excised, freed from adherent tissues and weighed following euthanasia.

Testes Volume

The volume of testes was determined at all sacrification intervals following ultrasound exposure by using water displacement technique (Archimedes principle).

Cauda Epididymal Sperm Characteristics

The cauda epididymis was chipped in 1 mL of normal saline and used for the assessment of sperm motility, viability, concentration and abnormality according to WHO manual [12].

Morphology of Cauda Epididymal Spermatozoa

The spermatozoa obtained from cauda epididymis were fixed in 1:1 ether and ethanol fixative and subjected to Papanicolaou staining [12].

Histopathology of Testis

The excised testis was fixed in 4 % paraformaldehyde fluid, dehydrated in graded ethanol, cleared in xylene and embedded in paraffin wax. Five micron thick sections were stained with haematoxylin and eosin for light microscopic observations.

Electron Microscopy

Cauda epididymal sperm pellets were washed in phosphate buffer (PBS, 0.1 mol/L, pH 7.2) and centrifuged at 3,000 rpm for 10 min. The isolated spermatozoa were immediately fixed in 2.5 % gluteraldehyde in PBS buffer for 30 min and washed thrice in PBS. Thereafter, resuspended in double distilled water and a thin film of spermatozoa was smeared on glass pieces, air dried, mounted on SEM stubs with silver paint, sputter-coated with gold (Model: Q150RS, Quorum Technologies Ltd., West Sussex, U.K.) and observed under scanning electron microscope (Model: Carl Zeiss, EVO 18, Oberkochen, Germany).

Clinical Toxicology

Hematology: Blood samples were collected by cardiac puncture at the time of sacrification of animals. Total red blood corpuscles (RBC), white blood corpuscles (WBC), hemoglobin, hematocrit and standard hematological indices were recorded by automated hematology analyzer (Model: CBC-360Plus, Accurex Biomedica Pvt. Ltd., India).

Serum Biochemistry: The collected blood was allowed to clot at room temperature. The serum was separated by centrifugation at 3,000 rpm for a period of 10 min. Serum glucose, cholesterol, triglycerides (TGL), urea, bilirubin, lactate dehydrogenase (LDH), alkaline phosphatase (ALP), creatinine, creatinine kinase (CK), serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT), high density lipoproteins (HDL) and low density lipoproteins (LDL) were estimated using reagent kits (Transasia Biomedical Ltd., Mumbai, India), with an autoanalyser (Erba Smartlab, Mumbai, India).

Hormone Analyses: Circulatory levels of serum testosterone (Alkor Bio Company, St. Petersburg, Russia), cortisol and prolactin (DSI, Saronno, Italy) were assayed by ELISA kits. The method of manufacturer reported intra- and inter-assay coefficient of variations from routine assays were 6.82 % and 7.97 % for testosterone, 2.95 % and 4 % for prolactin and 1.9 % and 5 % for cortisol, respectively.

Regulatory Toxicology

Periodical fertility tests were carried out every 15 days in all experimental groups by cohabitating the male with proven fertile females at 1:2 ratio. Success of mating was confirmed by vaginal plug and microscopically for appearance of spermatozoa in the vaginal smear. Thereafter, the females were caged separately and kept under observation for determination of pregnancy. Half of the pregnant animals were allowed to complete the term and the litter size, body weight, body length and body width of litters was recorded. The remaining 50 % of the pregnant animals were sacrificed at 20th day of pregnancy for the implantation record, *viz.*, weights of ovary, uterus and placenta, number of corpora lutea, number of implantation and resorption, if any, litter size, body weight, body length and body width of fetuses.

External and Visceral Examination of Fetuses: Fetuses were examined for external malformation, if any, in a manner starting from head, face, nostrils, eyes, external ear (pinna), trunk to tail and limbs. Fetuses were also fixed in 70 % ethanol for visceral examination and malformation present, if any, were recorded under stereoscopic zoom microscope (Model: SMZ 1000, Nikon, Japan).

Teratology: Selective fetuses/delivered pups from all animals were eviscerated, skinned, fixed in 70 % alcohol, double stained with alcian blue and alizarin red S, macerated, cleared in 0.5 % KOH and processed in graded series of glycerol [13,14]. The skeletal malformations, *viz.*, skull, vertebral column, sternebrae, fore limbs and hind limbs, fore and hind paw, etc., if any, were observed using magnifying glass.

Fertility: On account of above process, the total number delivered females and total number of live and dead litters were recorded to calculate percent fertility of male rats in all ultrasound therapy groups [15].

Statistical Analysis

Data were expressed as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was employed for statistical comparison. The difference between means was analyzed by Holm-Sidak multiple comparison test to detect the inter-group difference by using the statistical software

SPSS version 10.0 (SPSS Inc., Chicago, IL, USA). The $P < 0.05$ was considered as significant.

IV. RESULTS

Visible Toxicological Symptoms

No pre-terminal deaths were recorded. Daily observation of morphological characteristics, *viz.*, skin, fur, eyes and nose showed normal characteristics. No neurological symptoms like tremors, convulsions, autonomic activity, *viz.*, salivation, diarrhoea, lethargic and bizarre behaviour such as self mutilation, walking backward, etc. were observed. Motor activity, gait and posture, reactivity to handling or sensory stimuli and grip strength were recorded normal. All rats were active throughout the ultrasound exposure periods. The scrotum of animals in groups II - IV showed mild to moderate swellings during the initial periods of exposure that normalized within 7 - 10 days. However, significant edema and swelling in the scrotum was observed in group V animals which subsided following 15 days of exposure.

Body Weight

Body weights of sham control (group I) and groups II - IV were notably found close to each other during entire schedule of experiment. There were normal growth rates observed throughout 180 days observation (Data not shown).

Reproductive and Vital Organs Weight

A gradual decrease in the weights of testes, epididymides, vas deferens, seminal vesicle and prostate was noticed in animals of groups II and III and the decrease was found to be significant ($P < 0.05$, 0.01 and 0.001) following 60 days of therapy. Animals of groups IV showed significant reduction in weights of reproductive organs following 7 days of therapy till 180 days. In group V animals, single exposure of ultrasound induced significant reduction in organ weights observed as early as at 7 days of therapy. However, a gradual recovery in the reproductive organs weight was detected after 15 or 30 days of ultrasound therapy in this group and the values became normal when compared to animals of group I (Data not shown).

The vital organs, *viz.*, brain, lungs, heart, liver, kidney, spleen, thyroid, pancreas and adrenal did not show any alterations in their weights in all groups (II - V) when compared with sham control (group I) (Data not shown).

Testes Volume

The ultrasound therapy induced a gradual decrease in the testes volume in all groups which was significant ($P < 0.05$, 0.02, 0.01 and 0.001) following 30 days in groups II, III and IV when compared to group I. The volume of the testes in group V was significantly reduced ($P < 0.05$) at 7 days of therapy and thereafter showed a slow but persistent recovery. However, even after 180 days of therapy testes volume in animals of group V remained non-significantly low in comparison to the sham control (group I) animals (Fig. 1).

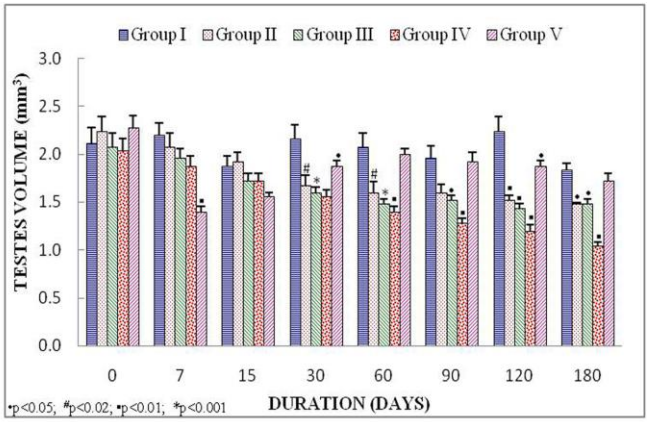

Figure 1. Testes volume (mm^3) in experimental groups following ultrasound therapy for a period of 180 days. Data are represented as Mean ± S.D. Significant differences between the therapy groups and sham control denoted as $•p < 0.05$, $^{#}p < 0.02$, $•p < 0.01$ and $*p < 0.001$.

Cauda Epididymal Sperm Characteristics

Sperm Count

The cauda epididymal sperm count indicated a gradual reduction in groups II and III which was found drastically significant ($P < 0.001$) following 30 days of therapy when compared to sham control (group I). Also, in group IV the sperm count gradually and drastically reduced and depicted at the level of occasional spermatozoa at 180 days of exposure. The cauda epididymal sperm count was found to be drastically reduced ($P < 0.001$) from 7 to 60 days of ultrasound therapy in group V, afterwards this parameter showed a trend of recovery and was found in the normal range during rest of the intervals when compared to sham control (group I) (Fig. 2A).

Sperm Motility

The cauda epididymal sperm motility in groups II and III gradually reduced ($P < 0.001$) following 15 days of therapy. The animals of group IV totally lost sperm motility following 15 days of ultrasound therapy. The sperm motility in group V animals was found to be zero percent at 7 and 15 days of therapy that progressively regained to the level of sham control animal following 120 days of therapy (Fig. 2B).

Sperm Viability

A gradual and significant reduction in the sperm viability was observed in groups II - IV following ultrasound therapy. In group V, the sperm viability drastically reduced ($P < 0.001$) following 7 - 60 days and thereafter, it recovered to the normal level when compared to sham control (group I) (Fig. 2C).

Sperm Abnormality

A gradual enhancement in the sperm abnormality in terms of morphological alterations was noticed throughout the ultrasound therapy in groups II, III and IV. In group V, the sperm abnormality was found to be gradually and significantly ($P < 0.001$) high following 7 - 90 days of therapy. Afterwards, at 180 days of therapy the value was seen in the range of the sham control (group I) (Fig. 2D).

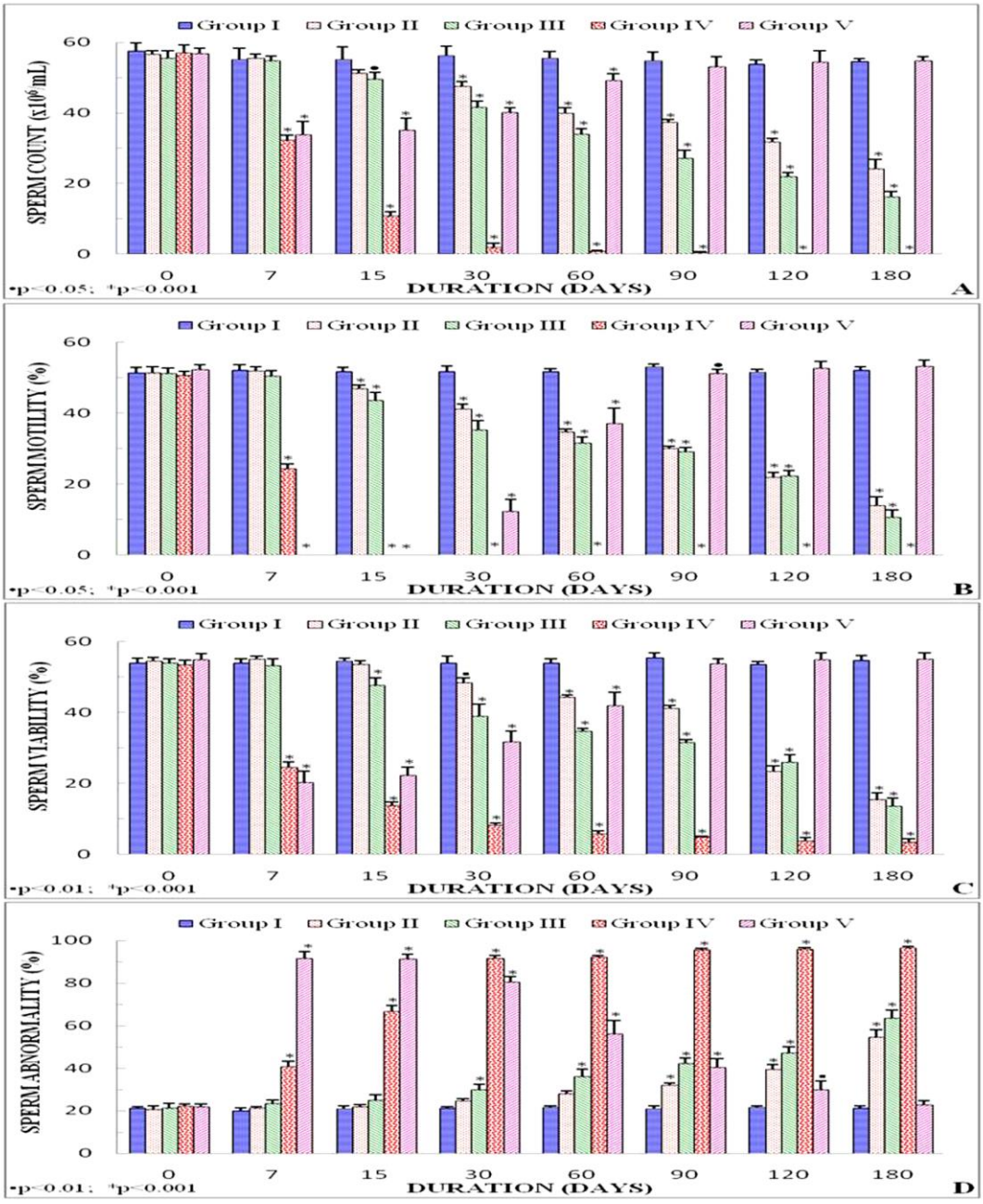

Figure 2. Sperm parametric analysis in sham control and following ultrasound therapy in different groups for a period of 180 days. **(A)** Cauda epididymal sperm count showing gradual reduction in groups II and III, drastic reduction of sperm count to occasional sperms in group IV and reversible decrement in group V, **(B)** A gradual and drastic reduction of sperm motility in groups II and III, total loss of sperm motility in group IV following 15 days and zero percent sperm motility at 7 and 15 days which progressively regained in group V, **(C)** Similar trend was noticed in sperm viability in all groups as sperm motility, and **(D)** A gradual enhancement in the sperm abnormality in group II, III and IV, and a reversible enhancement in group V. Data are represented as Mean ± S.D. Significant differences between the therapy treated groups and sham control denoted as •$p < 0.05$, ▪$p < 0.01$ and *$p < 0.001$.

Morphology of Cauda Epididymal Spermatozoa

The normal morphology of spermatozoa was observed in sham control group and pre-therapy intervals in all groups. The sperm abnormality was found to be mainly head-tail separation, acrosomal damage, bent mid-piece and coiled tail following ultrasound therapy in all groups. The morphology of spermatozoa resumed to normal range at

180 days of ultrasound exposure in group V (Data not shown).

Histopathology of Testis

The histology of testis of sham control showed round or oval seminiferous tubules with the epithelium containing Sertoli cells and germ cells of various stages covering the complete spermatogenesis. The basal lamina was thick showing closer association with spermatogonia and Sertoli cells. Germ cell differentiation appeared normal and the spermatocytes and spermatids were prominent with well defined nuclei and granular cytoplasm. The interstitium occupied with distinct Leydig cells and intertubular elements (Fig. 3A).

The testis of groups II - IV showed exposure and time dependent changes in the histoarchitecture. During initial periods of exposure, the testis exhibited conspicuous alterations in the seminiferous tubules with active spermatogenesis in most of the tubules. However, with the increase of the duration, lumen contained numerous erupted germ cells with unaltered diameter of the seminiferous tubules and normal Leydig cells (groups II and III, 90 - 120 days) (Fig. 3B). The impaired spermatogenesis at stage of primary spermatocyte with pycnotic nuclei, vacuolization in the Sertoli cells and small Leydig cell nuclei were also noticed (groups II, III and IV, 150 - 180 days) (Fig. 3C). The ultrasound therapy at 7 - 15 days induced total necrosis of seminiferous tubules filled with eosinophilic material, reduction in the size of seminiferous tubules and widening of interstitial space with proliferation of fibroblast like cells (groups IV and V) (Fig. 3D). After 15 days of therapy in group V, the regeneration in the spermatogenesis was seen (Fig. 3E). Active spermatogenesis was noticed following 180 days of therapy with normal feature of successive stages of transformation of the seminiferous epithelium into spermatozoa, normal and prominent lamina propria and the interstitium with normal Leydig cells (Fig. 3F).

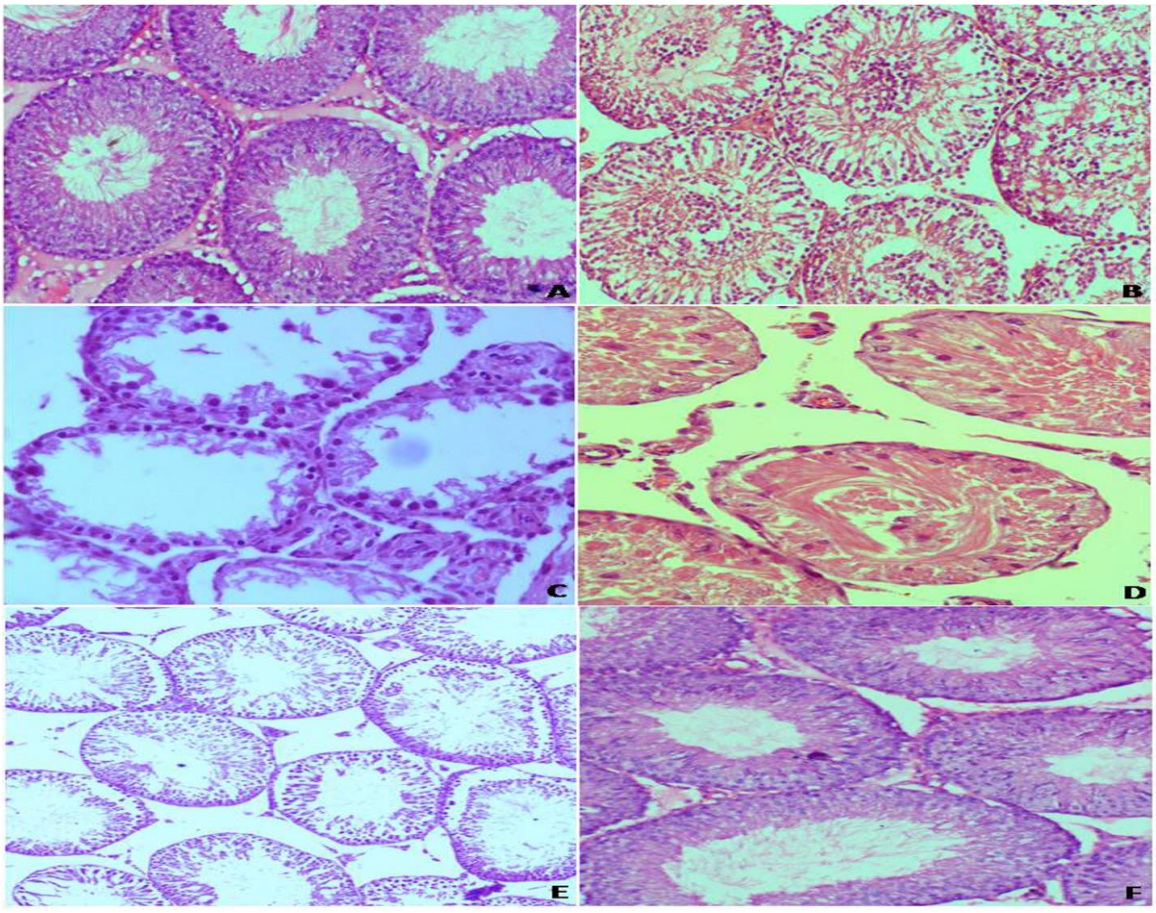

Figure 3. Histoarchitecture of testis in experimental groups during the entire course of the study. (A) Sham control (group I) rat testis showing normal features with successive stages of transformation of the seminiferous epithelium into spermatozoa. (B) The rat testis (groups II and III) following 90 - 120 days of ultrasound therapy showing erupted germ cells with unaltered diameter of the seminiferous tubules and normal Leydig cells. (C) The rat testis (groups II, III and IV) following 150 - 180 days of ultrasound therapy showing impaired spermatogenesis at stage of primary spermatocyte with pycnotic nuclei, vacuolization in the Sertoli cells and small Leydig cell nuclei. (D) The rat testis (groups IV and V) at 7 - 15 days of ultrasound therapy induced total necrosis of seminiferous tubules filled with eosinophilic material, reduction of seminiferous tubules and widening of interstitial space with proliferation of fibroblast like cells. (E) In group V (after 15 days of therapy), the regeneration in the spermatogenesis was seen. (F) In group V (following 180 days), active spermatogenesis was noticed with normal feature of successive stages of spermatozoa. HE x 200.

Electron Microscopy

The spermatozoa of sham control (group I) and pre-therapy in all groups exhibited normal morphology. During ultrasound therapy, ultrastructure of spermatozoa as observed by scanning electron microscopy (SEM) showed head-tail separation, acrosomal damage, membrane damage at certain regions, bent mid-piece and coiled tail in all groups. Normal morphology of spermatozoa was noticed at 180 days of ultrasound therapy in group V (Fig. 4).

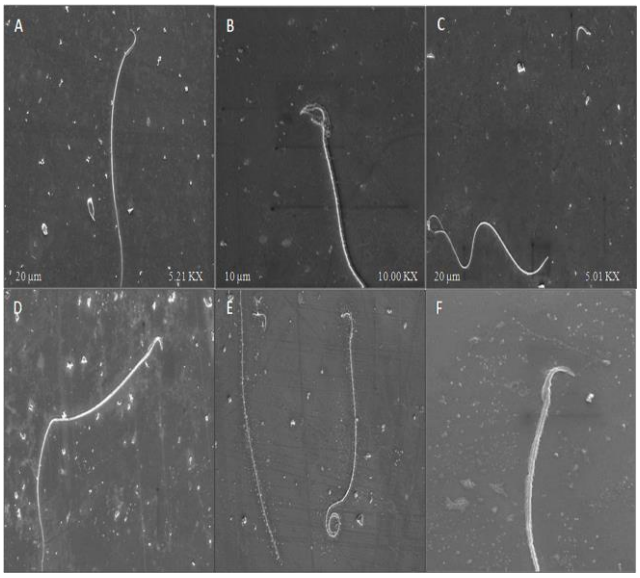

Figure 4. Scanning electron micrographs of spermatozoa following ultrasound therapy in experimental groups. (A) Group I animals showing distinct normal morphology comprising typical oval shaped head, slender mid-piece and long tail. The whole spermatozoa look intact membranes and organelles. (B-F) Group II-V animals during therapy showing the spermatozoa with acrosome damage, nuclear degeneration, head-tail separation, bulging and bent mid-piece, coiling in tail and disrupted plasma membrane. Scale (10 - 30 µm) and magnification (5 - 10 KX) of the micrographs showing on the figures.

Clinical Toxicology

Hematology

Hematologic parameters, RBC and WBC counts, hemoglobin, hematocrit (PCV) and standard hematological indices varied within the sham control/pre-therapy range. No ultrasound therapy related effects on any of these parameters were observed in all groups II - V when compared with sham control (Data not shown).

Serum Biochemistry

Serum glucose, cholesterol, triglycerides (TGL), urea, bilirubin, lactate dehydrogenase (LDH), alkaline phosphatase (ALP), creatinine, creatinine kinase (CK), serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT), high density lipoproteins (HDL) and low density lipoproteins (LDL) did not change significantly all through the ultrasound therapy in animals of all groups (Data not shown).

Hormone Analyses

The circulatory levels of testosterone decreased progressively and significantly ($P < 0.01$, 0.001) in animals of groups II & III. The level of testosterone was markedly low ($P < 0.001$) throughout the ultrasound therapy in group IV animals. In group V, the testosterone level decreased markedly following 7 - 60 days of therapy and afterwards was recorded normal (Fig. 5). The serum cortisol and prolactin did not alter due to ultrasound therapy in animals of any group (Data not shown).

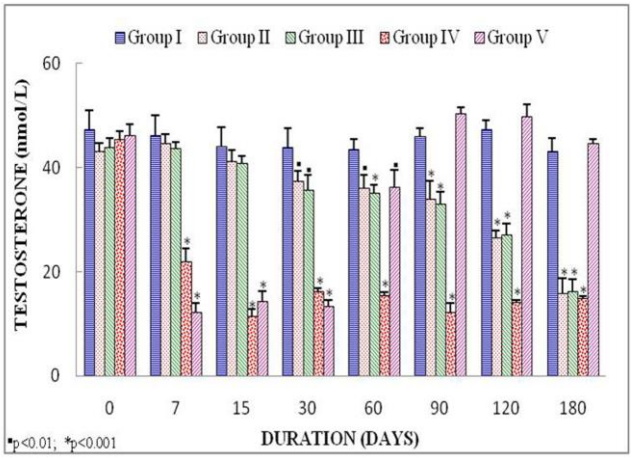

Figure 5. Testosterone level in serum prior to and following ultrasound therapy in different groups. The concentration found to be progressively and significantly decreased in groups II & III, markedly low levels in group IV and reversible decrement in group V. Data are represented as Mean ± S.D. Significant differences between the therapy groups and sham control denoted as ▪$p < 0.01$ and *$p < 0.001$.

Regulatory Toxicology

Pregnancy/Implantation and Teratology

The pregnancy/implantation record of caesarian females and full term delivery of groups II - V were comparable to that of group I (sham control) animals throughout the study period (Data not shown). External examination, visceral malformation and teratogenic malformations of all fetuses/pups revealed no abnormality in groups II - V rats when compared with sham control (Data not shown).

Fertility

A gradual inhibition of fertility in groups II & III animals was recorded; thereafter total sterility was recorded at 180 days of therapy. The fertility reduced to 40 % following 7 days of ultrasound therapy in animals of group IV, afterwards cent percent sterility was noticed throughout the exposure periods of 180 days. The total fertility loss in group V was found at 7 to 30 days of ultrasound exposure. Resumption of fertility to 60 % was noticed at 60 days of therapy in group V, thereafter all animals were found to be

100 % fertile during rest of the experimental duration of 180 days (Table 1).

Table 1. Fertility profile of the experimental animals during ultrasound therapy.

Mating Schedule	Group I	Group II	Group III	Group IV	Group V
0 day	100	100	100	100	100
7 days	100	100	100	40	0
15 days	100	80	80	0	0
30 days	100	60	60	0	0
60 days	100	60	60	0	60
90 days	100	40	40	0	100
120 days	100	20	20	0	100
180 days	100	0	0	0	100

V. DISCUSSION

Male contraception is still in need of an ideal method that is reliable, reversible and presents least number of side effects. A few preliminary studies verify the concept that elevation in testicular temperature results in impairment of spermatogenesis, using different modes to increase temperature like hot water, infrared, microwave, etc. Ultrasound presents a thermogenic effect that can be used clinically as a physical medicine for healing conditions involving muscles, bones and joints and ultrasound, when compared with the other techniques was found to be more effective at lower temperatures because of its combined effects [3]. A recent, but still explorative hypothesis is utilizing ultrasound as male contraceptive wherein exposing intrascrotal testes to ultrasound could result in rapid degeneration of the seminiferous epithelium and this approach can be utilized for designing an effective male contraceptive

Towards optimizing a contraception protocol, ultrasound intensity and time of application are required to be standardised. Studies on different animal models have suggested ultrasound to be utilized as a male contraceptive; however studies on long term effects of the exposure with probability of reversibility of the effect are still missing.

The main objective of the present study was to determine if modern therapeutic ultrasound machine could form the basis for a male contraceptive, and to elucidate most effective frequency that impacts reproductive functions through sperm characteristics, fertility status and regulatory toxicology, leading to infertility in rats. We explored how the reproductive organs and their functions are affected by scrotal application of therapeutic ultrasound therapy (1 to 3 MHz @1.5 W/cm^2) on male rats.

As previously reported, we utilize the frequency and intensity of ultrasound commonly used during therapy of ultrasound. However, for the first time we report a long term study and reversibility effects.

The treatment procedure in the present study was easy to administer as the rats remained calm in the restraint during the therapy. It was observed that direct application of ultrasound at 1 MHz (group II) and 3.0 MHz with 50% duty cycle for 5 min (group III) to scrotum induced a duration dependent changes. A gradual and slow decrease in testis volume, spermatogenesis, sperm parameters followed by fertility and testosterone level were observed with increase in sperm abnormalities. At these exposure levels, the animals did not exhibit azoospermia upto the end of the schedule; could be due to requirement of continuous therapy to get the desired results. However, at higher frequency, *i.e.*, 3 MHz for 2.5 min, azoospermia and loss of fertility was observed as early as at 7 days of therapy and continued up to 180 days. In animals with single exposure at 3 MHz for 15 minutes, total fertility loss was found at 7 days of therapy, but thereafter gradual resumption of fertility was observed. The effect in single exposure was instant and drastic with later on, regaining normal reproductive organs and their functions.

The mechanism of ultrasound action for contraception was first proposed by Fahim et al. [2] suggesting that 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. In 2013 an automated multi-step algorithm for segmenting the prostate boundary from ultrasound images was suggested [16]. Alternatively, Tsuruta et al. [7] 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. On the other hand, it has been also reported that ultrasound treatment at 1 MHz for 5 - l0 min. with intensities 1 - 4 W/cm^2 had no measurable effect on sperm counts, reproductive organ weights, or on testicular histology [7,17]. Dumontier et al. reported that ultrasound exposure temporarily interrupted the spermatogenic process in rats and inducing sterility for 150 days [5]. However, we speculated an important finding that therapeutic ultrasound provide spermicidal properties, as indicated by loss of sperm motility that may result in safe and effective mode of contraception. The effects appear to be reversible at a synchronized frequency, time, voltage per area in rats. Future considerations may involve study carried out in higher animal model with the single effective frequency that includes the time of contraceptive effect that lasts and recovery period after withdrawal of exposure to reflect possible maximum effect. Confirming its apparent permanence through DNA damage in spermatozoa following recovery of sperm concentration and morphology is also required step to elaborate the studies as a matter of concern.

VI. CONCLUSION, RECOMMENDATIONS AND FUTURE SCOPE

The long-term periodic application of therapeutic ultrasound waves in a specified frequency to rat scrotum resulted in deleterious effects on sperm characteristics and

reproductive organs. It causes gradual decrease in fertility; however, the impact is not permanent and fertility resumes eventually. The frequency with 3 MHz provided promising results without any toxicity, to be carried forward in higher animals. This type of effects if observed in human can be detrimental and limit the use of ultrasound as a contraceptive. Thus, before commercial usage there is a requirement to confirm efficacy in providing contraceptive effect in humans, any side effects of the repeated usage, reversibility of the effect and also long-term effect must be verified in higher animal models or humans. Therefore, the promising, but unproven technology require further exploration to achieve an adequate totality of evidence and will represent a major breakthrough to combat world population growth.

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CONFLICT OF INTEREST

No potential conflict of interest was reported by the authors.

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