



ANTIFERTILITY ACTIVITY OF 50% METHANOLIC LEAVES EXTRACT OF MAYTENUS EMARGINATA IN MALE RATS

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Abstract

In order to create a reversible male contraceptive, it is necessary to assess the antifertility activity of *Maytenus emarginata* leaves extract. Male Wistar rats were given oral administration of a 50% methanolic extract of *Maytenus emarginata* leaves at two separate doses: 50, 100 and 200 mg/kg b.wt/day for 60 days. The fertility test was performed and pregnant female with litter number recorded. Immunohistochemistry (IHC) of testis tissue was performed with primary antibody GATA-4. The expression of GATA-4 in testis declined in dose dependent manner of the treatment. For isolation of Leydig and Sertoli cells from adult rats used collagenase enzyme digestion method. The numbers of both Sertoli and Leydig cells reduced significantly after the treatment. Data showed that pregnant female and litter number were declined significantly, followed to decrease fertility. It can be concluded that oral administration of *Maytenus emarginata* leaves extract caused affects spermatogenesis and fertility in treated rats might be due to antiandrogenic nature of treatment.

Key Words- Antifertility, Antiandrogenic, Immunohistochemistry, Spermatogenesis.

1. Introduction

The use of physical barriers or other mildly acidic chemicals by women to block sperm from reaching the uterus dates back to ancient times, when contraception first gained popularity. Newer and more sophisticated forms of female contraception emerged over time. The first descriptions of a barrier approach, which involved covering the cervix, initially surfaced in 1838. The first procedure for female sterilization was reported in 1881. Research on

hormonal birth control techniques continued as science advanced. In 1957, the findings of a clinical research on oral contraceptive were published, and a product appeared on the market in 1959 (potts et al., 2009). Many more female contraceptive methods have been developed since then, with the most popular being female sterilization and intrauterine devices, and the pill and injection agents being slightly less so.

The history of male contraception is much shorter. The oldest method of contraception currently used by men is the condom. There are reports that the first versions were being used in ancient Egypt; (Marfatia et al., 2015) however, the first documented reports about condoms come from 1564 published by Gabriello Fallopio, in which they were described as a method of preventing syphilis infection. Until 1844, condoms were made from the intestines of animals such as sheep or goats. They had some disadvantages, one of which was their high price. However, production costs were reduced by the use of rubber, following the discovery of vulcanisation in 1844, and the condom gained popularity in the 1870 (Youssef et al., 1993). Production switched to latex after 1930; however, this is not an ideal substance, as approximately 4.3% of men and women may be allergic to latex (Wu et al., 2016). However, later discoveries led to the use of plastic and polyurethane in condom production (Marfatia et al., 2015).

Another well-known method of controlling male fertility is vasectomy. There are two types of this procedure: irreversible and reversible vasectomy. All vasectomy procedures aim to close the vas deferens (Dassow et al., 2006). Although the first vasectomy was performed to cause prostate atrophy, it was later acknowledged as a full-fledged contraception method during the Second World War (Leavesley et al., 1980). One of the newest, and most effective, methods is no-scalpel vasectomy (Cook et al., 2014). However, there are continual efforts to identify possible improvements to this procedure.

In 1951, India had a population of around 361 million people, and by 2020, it had grown to more than 1.375 billion. Population explosion is believed to be one of the impediments in the growth of a nation. Poverty, environmental damage, the loss of natural resources, an increase in unemployment, illiteracy, bad health, pollution, and global warming are all caused by overpopulation (Soni et al., 2015; Kent et al., 2020).

Both government and non-government organizations are making all efforts to control the human population, but the outcome has not been very satisfactory. One of the possible reasons could be the limited availability of contraceptive choices (Mishra et al., 2018). The World Health Organisation (WHO) reports that 70% - 80% of the world's population confide in traditional medicines for primary health care (Sethi et al., 2017). The concept of herbal contraceptives which are an effective method for controlling the fertility of animals and humans (Verma and Yadav, 2021).

Approximately fifty percent of human infertility is attributable to male defects, of which 70-90% stems from impaired spermatogenesis (Chen et al., 2015). The Sertoli cells are crucial in controlling the pace of spermatogenesis and spermatozoa production. The actions of Sertoli cells and the quantity of Sertoli cells determine their capacity to control spermatogenesis. The release of spermatids at spermiation, structural support and nutrition for developing

germ cells, phagocytosis of dying germ cells and leftover bodies, and production of a variety of proteins that control pituitary hormone release and mitotic activity of spermatogonia are all functions of the sertoli cell (Johnson et al., 2008).

Leydig cells (LC) are present in the interstitial compartment of the testes and their main function is to produce testosterone, which is essential for spermatogenesis and development of secondary sexual characteristics.

GATA4 is predominantly expressed in somatic cells within the testis (Sertoli cells, Leydig cells and other interstitial cells), and its expression remains consistently abundant in both embryonic gonads and adult testes (Chen et al., 2015).

2. Materials and Methods

2.1 Identification of Plant and preparation of extract

The plant *Maytenus emarginata* was identified by taxonomist at Department of Botany, University of Rajasthan, Jaipur (Rajasthan, India). The leaves of *Maytenus emarginata* were collected, shade dried, crushed and powdered. This powder was subjected to soxhlet extraction with 50% methanol for 24 hrs (8 hrs. X 3 days) according to the WHO protocol 1983.

2.2 Animal model

Colony-bred, healthy adult fertile male albino rats (*Rattus norvegicus*) weighing between 150-200 gm were used and kept under controlled environment for the present study.

2.3 Treatment protocol

The experiment was designed to examine antifertility effects, possible mode of action / effects nature of the extract and reversibility. The experiment was divided into five groups for analysis of leaves extract activity. Each group consisting of 10 animals rats of similar body weight, size and age.

Experiment

Group-I: Control vehicle treated rats (sterile distilled water) alone orally for 60 days.

Group-II: *Maytenus emarginata* leaves extract at a dose of 50 mg/kg.b.wt./day for 60 days

Group-III: *Maytenus emarginata* leaves extract at a dose of 100 mg/kg.b.wt./day for 60 days.

Group-IV: *Maytenus emarginata* leaves extract at a dose of 200 mg/kg.b.wt./day for 60 days.

Group-V: *Maytenus emarginata* leaves a dose of 100 mg/kg.b.wt./day for 60 days were kept for a recovery period of 30 days.

2.4 Fertility test and scarification schedule

Fertility test of individual rat was done prior to the experiment and after 55 days of treatment. To assess fertility of each male rat was cohabitated with proestrous females in 1: 2 ratio, vaginal smear was examined every morning for positive mating and number of litters delivered was noted.

2.5 Immunohistochemistry

On paraffin-embedded testis slices that were 5 m thick, deparaffinized in xylene, and rehydrated in ethanol, immunohistochemistry (IHC) was carried out. After blocking endogenous peroxidase activity with 0.3% H₂O₂ in methanol, antigen retrieval was heated up in tris buffer (10mM Tris Buffer with 1mM EDTA, pH 8.0 - 9.0). Sections were first rinsed in phosphate buffer saline (PBS), followed by an overnight incubation at 4°C with the primary antibody, rabbit polyclonal GATA4 antibody (Origene TA325501). After 30 minutes at 37°C, the slides were exposed to the secondary antibody (biotinylated anti rabbit). Finally sections were treated with diaminobenzidine (DAB) H₂O₂ mixture and counterstained with hematoxylin and got observation at 100X magnification (Lasheen et al.2015).

2.6 Isolation of Sertoli and Leydig cells

For isolation of Leydig and Sertoli cells from adult rats, we used established procedure (Gautam et al., 2016) modified from that described for the culture of Sc from adult hamster testes (Majumdar et al., 1995). To extract the testes, animals had their cervical vertebrae dislocated. Testes were removed from their capsules, placed in a flask with an enzyme solution (1 mg collagenase in 10 mL PBS), and digested in a water bath for three minutes at a temperature of 37 °C (Digestion I). The seminiferous tubules were able to disperse because to this quick digestion. Following digestion, Leydig cells were isolated using the supernatant solution. This solution is then mixed with DMEM medium, centrifuged at 2000 rpm for five minutes, the supernatant is discarded, the pellet is resuspended in DMEM media, and the filtrate is used for microscopic viewing (Salva et al., 2001).

The pelleted tissues were chopped and suspended in 30 mL of enzymatic solution (1 mg collagenase/10 mL PBS), and kept for digestion in shaking water bath at 140–150 oscillation per min at 37 °C for 10 min (Digestion II). Supernatant was discarded and pellet resuspend in DMEM and centrifuge again at 500 rpm/ for 5 min. Discard supernatant and resuspend pellet in DMEM media for visualization under the microscope at 400X magnification.

2.7 Ethical aspects

The study was carried out under the supervision of ethical committee of the Department of Zoology, University of Rajasthan, Jaipur and CPCSEA guideline was followed (CPCSEA, 2006).

3. Results

After administration of plant leaves (Sharma and Mali, 2016), the levels of serological parameters (acid phosphatase, alkaline phosphatase, serum glutamic oxaloacetic transaminase, serum glutamic pyruvic transaminase) were not altered, indicative that the clinical non-toxicity of the extracts. When *Maytenus emarginata* leaves extract treated males were caged with females after withdrawal of treatment, mating, sperm motility and density was affected. Fertility of plant leaves extract (Table 1) treated males was decreased and number of litters decline as comparative control group; however, Group V animals showed return of fertility after 30 days recovery period. Reduced fertility rate and pregnancy outcomes in females mated with treated males were also observed.

Results of Immunohistochemistry

Antiandrogenic natures of leaves extract of plant observed by Sharma and Mali, 2016. Poor standards or fewer Leydig and Sertoli cells being generated, or perhaps both, may be to blame for decreases in the production of testosterone, LH, and FSH. We stained with GATA 4 following extract administration to further investigate if extracts have an impact on cell growth and number. The highest levels of GATA 4 expression were seen in Sertoli cells and Leydig cells in immunohistochemically stained sections from the testis of control rats. While Sertoli cells and Leydig cells in the treated groups had little GATA 4 expression, this suggests that the numbers of both cells were decreased and compromises the regeneration of Sertoli cells and Leydig cells after administration of leaf extracts. Degenerative changes were also visible in seminiferous tubules and lumen was disrupted (Photomicrograph-I).

Results of isolation of Sertoli and Leydig cells

Sertoli cells were present in polygonal shaped and round nucleus in control group animals. Leydig cells were present in round shaped with specific nucleus and granular cytoplasm in control group animals. After treatment aggregation behaviour were altered and cells are presented in unit cell. It may be possible that extracts affects specific biomolecules which are present on plasma membrane of Sertoli and Leydig cells and regulate aggregation nature. Similarly results of Immunohistochemistry decreased density of both cells were also observed in culture of Sertoli and Leydig cells (Photomicrograph IIA & IIB).

Discussion

The results presented in this study indicated that the treatment with leaves extracts by adult male rats reduces the number of female's impregnation. In addition, the number of implantations and the number of viable foetuses were also decreased; this could be reflect and may be due to the decrease in sperm motility and sperm density observed (Soni and Mali,2017; Mohan et al.2012). Complete spermatogenic arrest is not necessary for male contraception; fertility can be eliminated by altering structure or function of spermatozoa (Ain et al., 2018). The approach to the development of a male contraceptive can be either to inhibit the production of sperm, interfere with sperm function and structure, interrupt sperm transport, interrupt sperm deposition or prevent sperm-egg interaction (Lampiao, 2013).

The spermatozoa require being mobile and alive in order to be have potential to engaged in the process of fertilization. The evaluation of potential drug effects on these individuals' fertility is an essential part of preclinical studies since this could affect the viability and development of their offspring (Krepkova et al.2021).

Transcription factor GATA4 has been implicated in the development and function of the mammalian testis (Viger et al., 2008). Postnatally, GATA4 is found mainly in Sertoli cells and adult Leydig cells (Kyronlahti et al., 2011). In Sertoli cells, GATA-4 is expressed throughout fetal and postnatal development. Expression of steroidogenic genes is modulated by many transcription factors. The nerve growth factor IB (nur77), the GATA binding protein-4 (Gata 4), and the steroidogenic transcription factor 1 (SF-1) are essential transcription factors associated with the expression of genes at steroidogenic enzyme system (Yildizbayrak and Erkan, 2018). Schrade and colleague reported that silencing of Gata 4 in Leydig cells caused the down regulation of genes in the androgen biosynthesis (Schrade et al.2015). Moreover, in the control group the numbers of Leydig cells and Sertoli cells obtained greater than treated groups show antispermatogenic and antiandrogenic nature of extracts.

In addition to antiandrogenic function of extracts, the extracts also affect both cells population and may capable of disturbing with pubertal development of Leydig cells. The decline protein level of testes also indicates less numbers of Sertoli cells (Mohamed and Saddek, 2019). Sertoli cells also synthesize and secrete various growth factors which are necessary for germ cells maturation and normal spermatogenesis. The number of both cells reduced due anti androgenic nature of extract. The formation of cells cluster property in culture condition was also affected after extracts treatment. The aggregation behaviour of both cells in culture condition altered due to some changed in plasma membrane or protein present on membrane of cells. The changes in concentration of Lutenising Hormone (LH), Follicle Stimulating Hormone (FSH) and testosterone that could disrupt spermatogenesis, which further affects sperm quality and male fertility (Seriana et al.,2021) .

From the results of current investigation, it can be concluded that *Maytenus emarginata* is effective in the terms of male contraceptives due to their fertility regulative. The results of Immunohistochemistry and culture of Leydig and Sertoli cells reflected that leaves extract effective for contraception

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Table 1 : Fertility, Motility, Density of Male Rats Administered with Methanolic Extract of *Maytenus emarginata* leaves for 60 Days

Treatment	No. of Mated Males	No. of Mated Females	No. of Pregnant Females	Sperm Motility % (Cauda)	Sperm density (mil/ml)	Fertility Test (%)
					Testes	
Group-I Control or Vehicle treated	10	20	18	69.54 ± 0.49	60.43 ± 2.84	90.00%
Group-II 50mg/kg b.wt / day for 60 days	10	20	15	61.57 ± 2.63*	53.05 ± 2.71*	75% (-15%)
Group-III 100mg/kg b.wt / day for 60 days	10	20	9	56.44 ± 2.75*	47.17 ± 1.93***	45% (-50%)
Group-IV 200mg/kg b.wt / day for 60 days	10	20	4	52.16 ± 1.80***	39.85 ± 2.05***	20% (-70%)
Group-V Recovery (withdrawal of 90 days)	10	20	19	69.34 ± 0.49 ^{ns}	59.76 ± 2.84 ^{ns}	95%

(Mean±SEM of 10 Animals) Animals of treated Groups II, III, IV and V Compared with treated vehicle Control Group I.

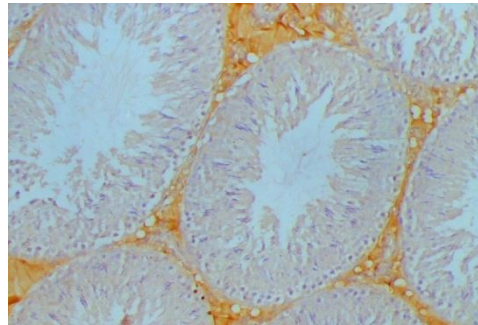
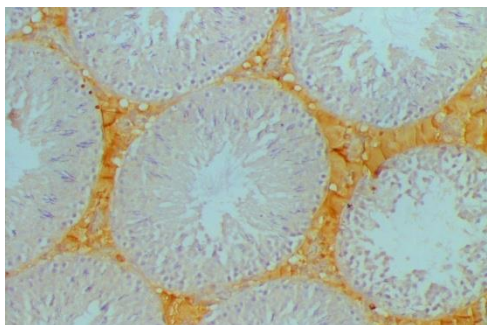
*** = highly significant ($p \leq 0.001$)

** = significant ($p \leq 0.01$),

* = significant ($p \leq 0.05$),

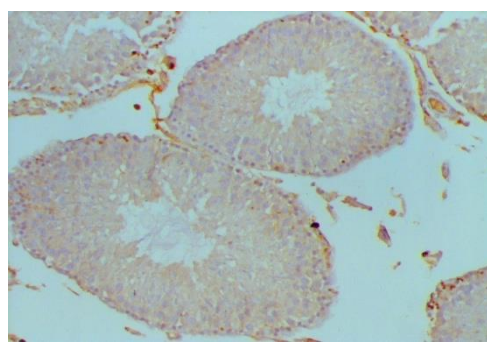
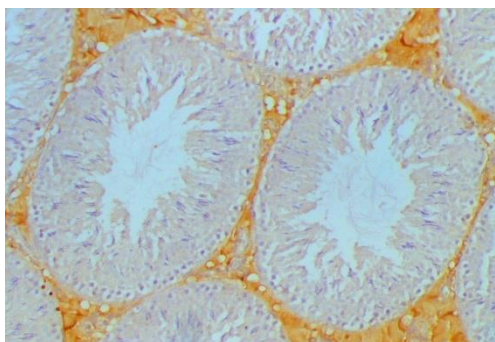
ns = non-significant

Photomicrograph-I. The testes histoarchitecture of after administration of *Maytenus emarginata* leaves extract according to different dose level groups rats. (Magnification: X100)



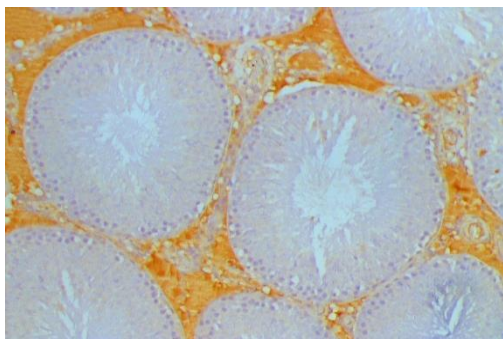
Group-I Testes of control group animals shows normal expression of GATA4 antibody with Leydig cells and Sertoli cells

Group-II The photomicrograph of testes consists to reduced expression of GATA4 antibody.



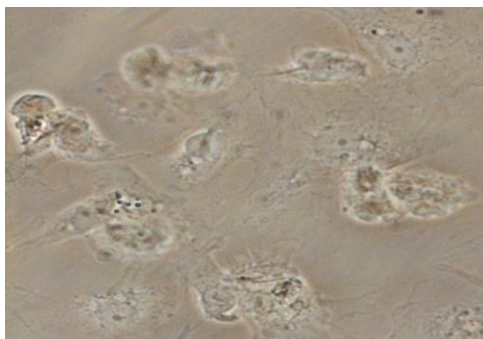
Group-III The photomicrograph of testes consists to reduced expression of GATA4 antibody and increased lumen size of seminiferous tubules

Group-IV The photomicrograph of testes consists to reduced expression of GATA4 antibody and increased lumen size of seminiferous tubules

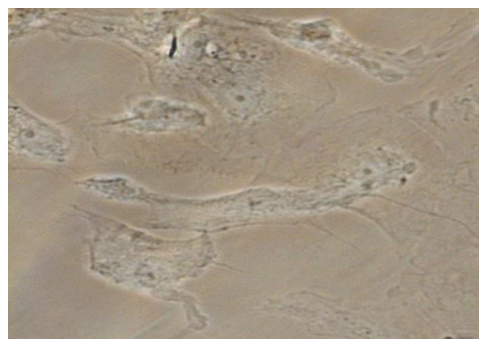


Group-V The photomicrograph of testes of recovery group rats shows normal expression of GATA4 antibody

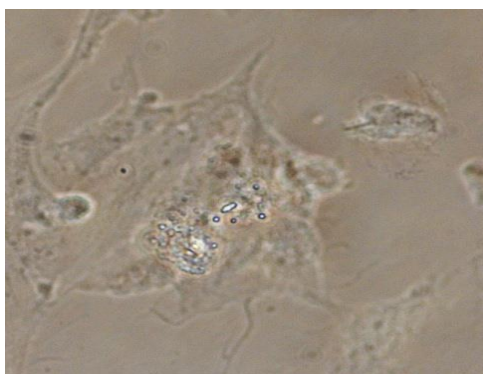
Photomicrograph-IIA The Sertoli cells after administration of *Maytenus emarginata* leaves extract at different dose level group rats. (Magnification: X400)



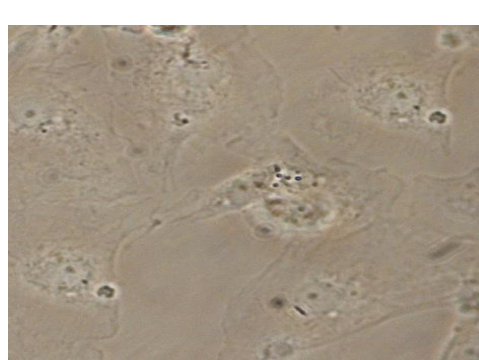
Group-I The photomicrograph of Sertoli cells were occupied the round shaped nuclei and irregular polygonal shape with well-formed. The cells were presented in large number and got aggregated and form clusters



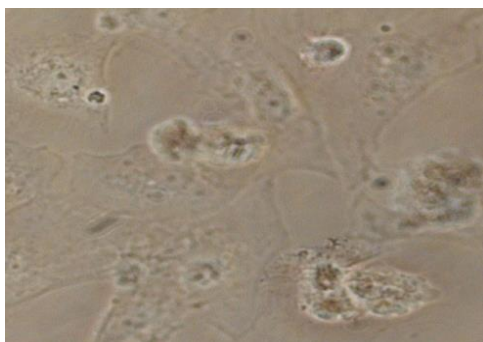
Group-II The cells population were decreased.



Group-III The photomicrograph of Sertoli cells showed the cells population were reduced and aggregation nature also altered

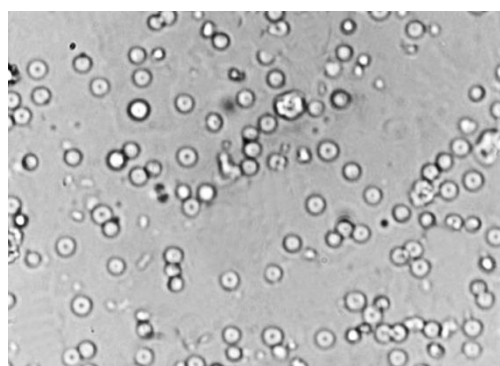
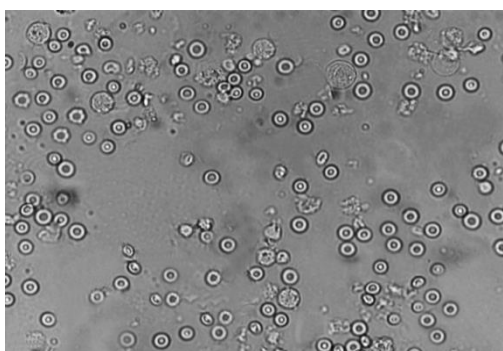


Group-IV In this group, the cells numbers remarkable decreased and aggregation nature altered.



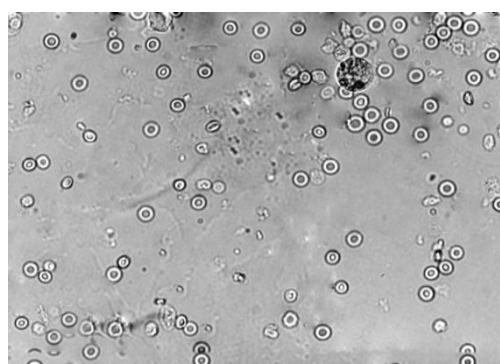
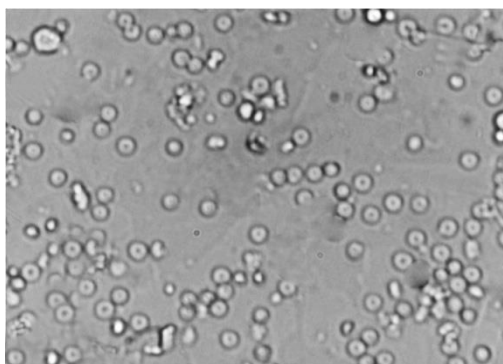
Group-V In this group, cells numbers increased and recover up to control group

Photomicrograph-IIB The Leydig cells after administration of *Maytenus emarginata* leaves extract at different dose level group rats. (Magnification: X400)



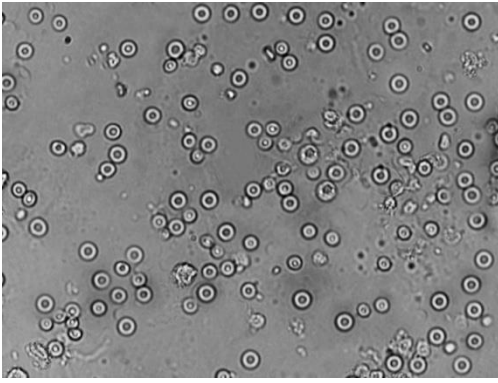
Group-I The photomicrograph of Leydig cells was occupied in round shaped and present in proper numbers

Group-II The cells population were reduced



Group-III The photomicrograph of Leydig cells showed the cells population were reduced and aggregation nature also altered

Group-IV In this group, the cells numbers remarkable decreased and aggregation behaviour altered



Group-V In this group, cells numbers increased and recover up to control group