GMR
The Original

UNDERSTANDING THE CONTRACEPTIVE EFFECT OF COPOLYMER SMA BY MICROSCOPICAL AND ULTRASTRUCTURAL ASSESSMENT OF REPRODUCTIVE TRACT AND SPERMATOZOA IN MALE WISTAR RAT (*RATTUS NORVEGICUS)*

J. Umamageswari[1], S. Balasubramanian[2], K. Krishnakumar[1], S. Rangasamy[1], K. Vijayarani[3], S. P. Preetha[4], N. Pazhanivel[5]

[1] Department of Veterinary Gynaecology and Obstetrics, Faculty of Veterinary College and Research Institute, Udumalpet, Tamil Nadu Veterinary and Animal Sciences University, Tamilnadu, India
[2.] Former Director of Clinics, Tamil Nadu Veterinary and Animal Sciences University, Chennai-07, Tamilnadu, India
[3.] Department of Bioinformatics, Faculty of Madras Veterinary College, Tamil Nadu Veterinary and Animal Sciences University, Chennai-07, India
[4.] Department of Veterinary Pharmacology and Toxicology, Faculty of Madras Veterinary College, Tamil Nadu Veterinary and Animal Sciences University, Chennai-07, India
[5.] Department of Veterinary Pathology, Faculty of Madras Veterinary College, Tamil Nadu Veterinary and Animal Sciences University, Chennai-07, India

ABSTRACT

Background: Of the many biologically active compounds, the use of copolymer styrene-maleic anhydride (SMA), an electrical charge generating and pH lowering polyelectrolyte polymeric compound was one of the most promising approaches to conventional vasectomy and other vas-based male contraceptives. The present work was carried out to study the efficacy of a single intravasal injection of copolymer SMA in inducing antifertility action in male rats and to assess the histopathological changes in the testis and ultrastructural changes in the vas deferens and spermatozoa of SMA-vas-occluded rats

Methods: Thirty adult, male, Wistar albino rats were randomly divided into five groups of six each. Group I (sham control) rats were injected with 10µl of DMSO, and groups II, III, IV and V rats were injected with a therapeutic dose of 5 mg of SMA in 10µl of DMSO (SMA hydrogel) and kept in situ for 15 days, 30 days, 60 days and 90 days, respectively. After ffertility trials with proven females, all the vas-occluded male rats were sacrificed at respective experimental period. Blood samples and reproductive organs were collected for microscopical and ultrastructural assessment of testes, vas, and caudal epididymal spermatozoa.

Result: Intravasal injection of SMA hydrogel in male rat did not affect testicular sperm production but, as spermatozoa pass through the vas deference of copolymer SMA occluded region damages the sperm acrosomal membrane integrity resulting in loss of fertility which was evinced by inhibition of pregnancy in females rats.Further, it was evident from SEM and TEM changes of spermatozoa membrane particularly in the acrosome and mid-piece region of SMA vas occluded groups of rat. The histological examination of testis from SMA vas occluded groups of rats showed mild degenerative changes in the epithelial layer with loss of germ cells in a few seminiferous tubules. While, SEM image of vas occluded rat exhibited formation of a mesh-like structure where sperms were trapped in the copolymer-coated vas lumen, SMA anchored in the mucosal wall by invagination and clinging to the vas lumen to form a plug and damages sperm cell thereby rendering it incapable of penetrating the oocyte and resulting in sterility. Thus, copolymer SMA vas occlusion was found to be a safe procedure for male contraception.

KEYWORDS: Contraception, Copolymer SMA, Ultrastructural spermatozoa, Vas deferens, Male rats

INTRODUCTION

For millennia, the permanent and reliable method of fertility control in animals and humans has been achieved through direct removal of gamete-producing organs. However, the risk involved in surgical procedures makes many people unwilling to undergo what they perceive as a painful and invasive procedure. Also, the cost of surgery and the side effects such as underdevelopment of genital organs, disturbances in the musculoskeletal system, hormonal dysfunctions, urinary incontinence, risk of neoplasms, obesity, etc. make it prohibitive for many owners. Later in the 1970s, chemical agents were tested in various mammalian species by being injected into the testis (Fahim et al., 1982), epididymis (Fahim et al.,1993) and vas deferens (Freeman and Coffey,1973), causing infertility/sterility by inducing azoospermia in male animals; however, due to their adverse side effects, it was not sustainable. Hormonal applications

including progestins, androgens and analogues of gonadotropin-releasing hormone (GnRH) were tried either to directly block reproductive hormone receptor-mediated events or indirectly block conception via negative feedback mechanisms. (England,1997; Lacoste et al.,1989; Garcia et al., 2009; Amory, 2004), but the above approaches resulted in temporary, short-term control of fertility. Correspondingly, an immune-contraceptive method of fertility control via vaccination against GnRH, the luteinizing hormone (LH) receptor, or zona pellucida proteins is also possible but is still at the research stage. Although there are many hormonal and non-hormonal agents available, due to long-term side effects, these contraceptives were not effectively utilized.

In 1979, Prof. Guha proposed a radically new technique of male contraception in order to develop non-invasive, and non- hormonal contraceptives, styrene-maleic anhydride in humans.. The polymer, once injected into the lumen of the vas deferens, partially blocks the lumen and impairs the fertilizing ability of spermatozoa that pass in the vicinity of the lumen because of its electrical charge and pH-lowering action and it also has long term effectiveness. Following this, Misro et al. (1982) developed a revolutionary occlusive polymer named RISUG (Reversible Inhibition of Sperm Under Guidance), on the impression that contraception with SMA could be reversed at any time following vas occlusion. After 18 years of study on the human, the Indian Council of Medical Research has now approved RISUG for mass use, and it is now undergoing phase III clinical trials in India (Guha, 1996 and 2007; Sharma et al., 2008). Thus, a safe, non-hormonal, male contraceptive technique with one-time intervention, which gives long-term contraceptive effect with minimal side effects, is required in mass fertility control programs in animals especially in dogs and cats. To meet these demands, the present study was carried out with the following objectives: to study the efficacy of a single intravasal injection of copolymer SMA in inducing sterility in male rats and to assess the histopathological changes in the testis and ultrastructural changes in the vas deferens and spermatozoa of SMA-vas-occluded rats.

MATERIALS AND METHODS

Adult male and female albino rats of the Wistar strain, weighing 120 ± 10 g (10-12 weeks old), were purchased from the Laboratory Animal Medicine Unit, Madhavaram Milk Colony, Chennai. were maintained in the Centralized Laboratory Animal House under standard condition of humidity (50-60 per cent), temperature (25°C- 27°C) and light (12 h light and 12 h dark schedule) in polypropylene cages. They were fed with rat pellet diet (Nutrivet Life Sciences, Pune, India) and water ad libitum. The experiments were conducted in accordance to the guidelines regulated by the Committee for Control and Supervision of Experiments on Animals (CPCSEA). The protocol has the approval of the Institutional Animal Ethical Committee (IAEC No. 36/SA/IAEC/2018). Styrene Maleic Anhydride (SMA), a copolymer prepared by Radical addition-fragmentation chain transfer (RAFT) polymerisation technique at the Translational Research Platform for Veterinary Biologicals, TANUVAS, Tamil Nadu (India), was used to induce vas occlusion in male rats.

Experimental Design and *In vivo* Vas occlusion

Thirty adult, male, Wistar albino rats were randomly divided into five groups of six each. Group I (sham control) rats were injected with 10µl of DMSO, and groups II, III, IV and V rats were injected with a therapeutic dose of 5 mg of SMA in 10µl of DMSO (SMA hydrogel) and kept in situ for 15 days, 30 days, 60 days and 90 days, respectively. For vas occlusion, rats of groups I to V were sedated with injectable anaesthesia. After aseptic preparation of the midventral abdomen, the animal was positioned in dorsal recumbency and a single median incision was made just above the scrotum to expose the vas deferens of the inguinal region. Group I rats were injected with DMSO, while groups II to V rats were injected with a therapeutic dose of SMA hydrogel into the vas deferens bilaterally. The vas deferens of both control and treated rats were replaced in their original position, and the surgical incision was closed as per standard procedure. Postoperatively, rats were treated with antibiotics and anti-inflammatories for three consecutive days.

Fertility trial in vas-occluded rats

Fertility was assessed by cohabitating the control and SMA-treated male rats from each group with proven female rats at the ratio of 1:2 for three oestrous cycles (12 to 15 days), and the presence of vaginal plug/appearance of spermatozoa in the vaginal smear assessed the success of mating. After 15-19 days of mating, pregnancy was confirmed by ultrasonography and pregnant female rats were maintained in separate cages until delivery of pups.

Collection of blood, reproductive organs and epididymal spermatozoa

At the end of the experimental schedule, all the rats were euthanised by the carbon dioxide inhalation method as described in the CPCSEA guidelines. After sacrifice, blood samples were collected for hormonal analysis. The testis along with the epididymis was removed and transported in 0.9% normal saline at 37˚C to the andrology laboratory to ascertain the testicular function.

All the procedures from excision of the epididymis to sperm collection were carried out in room temperature as described earlier (Varisli et al., 2013). Spermatozoa recovered from rat cauda epididymis using the mincing method were examined for motility, concentration, live and dead and abnormal spermatozoa percentage of cauda epididymal spermatozoa as per the standard protocols (Dostal et al., 1996; Campos-Pereira et al., 2015). The ultrastructural changes of epididymal spermatozoa in rats were examined by Atomic Force Microscopy (AFM), and Scanning Electron Microscopy (SEM) as per the protocols (Lohiya et al., 1998; Kumar et al., 2005).

Histopathological analysis of reproductive organ

The effect of copolymer SMA on histological changes in the reproductive organs like testis and vas deferens collected from control and vas-occluded rats. Tissues were paraffin sectioned, processed by alcohol-xylene method and determined by haematoxylin and eosin staining using a phase contrast microscope of required magnification.

SEM Examination of vas deferens

The infiltration of copolymer SMA into the vas deferens of SMA-treated rats was studied by Scanning Electron Microscopy. The vas deferens harvested from control and SMA treated rats were fixed immediately in 2.5 per cent glutaraldehyde in 0.1 M phosphate buffer and dehydrated in series of graded alcohols and dried to critical point drying with CPD (EMS-850) unit by using liquid CO_2. The processed samples were mounted over the stub's samples with double-sided carbon conductivity tape, and a thin layer of gold coat over the samples were done by using an automated sputter coater for 3 min and scanned under SEM (Model : JOEL- JSM 5600) at required magnifications as per the procedures of Bozzola and Lonnie (1999).

Assessment of Serum testosterone levels

After separating serum, samples were stored at 4°C until assay was carried out as described earlier (Manivannan et al., 2005). The estimation of testosterone concentration in control and copolymer SMA treated rats were performed using specific commercial enzyme-linked immunosorbent assay (ELISA) kits (Cusabio Biotech Co., Ltd. Inc., China) according to the manufacturer's instructions.

Statistical analysis

The data collected in the study were analysed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test for cauda epididymal rat sperm characterisation, and hormonal estimation between groups. The fertility analysis was performed by Chi-square followed by the McNemar test with the aid of SPSS (23.0 version) statistical software. The values presented as means $\pm$ SE; $p < 0.05$ and $p < 0.01$ were considered statistically significant and highly significant, respectively.

RESULTS

Vas occlusion with copolymer SMA in rats

All experimental rats belonging to control and treatment groups were found to be active, and healthy and did not show any sign of distress following surgical interventions. No mortality was noticed throughout the study period in either control or copolymer SMA treated group of rats. The vas deferens harvested from sham-operated control and copolymer SMA vas occluded rats were subjected to SEM. The SEM images of vas deferens of sham control rats showed that the patency of the lumen was clearly visualized without any changes in the mucosal wall and muscular layer of vas deferens **(Fig. 1)**. On the other hand, the SEM images of vas deferens occluded with copolymer SMA showed that the vas deferens lumen filled with the copolymer SMA appeared as a whitish amorphous substance **(Fig. 1)**. The lumen was occluded with copolymer SMA hydrogel which formed a mesh-like structure where sperms were trapped in the copolymer-coated vas lumen. The copolymer SMA is anchored in the mucosal wall by invagination and clinging to the vas lumen to form a plug. The muscular layer appeared normal with no ultrastructural changes **(Fig. 1)**.

Effect of copolymer SMA on fertility in female rats

Vaginal smears of all mated female rats showed the presence of detached sperm heads and tails along with vaginal exfoliative cells indicating successful mating (**Fig 2**). After 15-19 days post-mating, 75% of female rats (9/12) mated with sham-operated males were found to be pregnant on ultrasound examination. However, 17 (2/12), 8 (1/12), 0 (0/12), and 0 (0/12) % of the female rats mated with group II, III, IV, and V male rats, respectively, were found to be pregnant. A highly significant difference ($P < 0.01$) in the pregnancy rate was observed with group II, III, IV, and V rats when compared with the sham-operated control.

Effect of copolymer SMA vas occlusion on testicular function

The present study indicated that no significant differences were observed in sperm count, motility percentage, live and abnormal epididymal spermatozoa between the sham-operated group and copolymer SMA treated groups of rat after 15, 30, 60, and 90 days of vas occlusion period. Similarly, no significant differences were observed in the serum testosterone level of different groups of vas occluded rats.

Ultra-structural changes of epididymal spermatozoa

AFM images of rat epididymal spermatozoa showed normal head and mid-piece region of sperm cells recovered from copolymer SMA vas occluded rats **(Fig. 3)**. Similarly, the SEM images of epididymal spermatozoa recovered from copolymer SMA vas occluded rats revealed normal sperm morphology which included normal sperm head with distinct acrosomal envelope, mid-piece, and principle piece of tail regions with no morphological changes at different magnifications **(Fig. 3)**. In addition, the TEM images of epididymal spermatozoa showed no ultrastructural changes in the head, mid-piece, and tail regions (**Fig. 3**).

Histopathology of reproductive organs in rats

Testicular cross-sections from a sham control group of rats showed normal histological structure of seminiferous tubules containing Sertoli cells and germ cells at different stages of spermatogenesis in the tubular lumen. Whereas in the copolymer, SMA treated group of rats, most of the seminiferous tubules showed normal configuration except

in a few seminiferous tubules, wherein, mild degenerative changes with loss of germ cells in the lumen were observed (**Fig. 4**). The epididymal cross-section showed an intact basement membrane with well-organized epithelial cell lining and lumen filled with spermatozoa in the sham control group of rats (**Fig.4**). While in the copolymer SMA treated group of rats, a few of the epididymal ductular lumen showed low sperm cell density (**Fig.4**). The histopathological section of the vas deferens in the control group of rats showed no histological changes in the vas lumen, mucosal folds, and musculature tissues. Whereas, in the copolymer, SMA treated group of rats showed distended vas lumen and occupied with eosinated polymeric material, which appeared as glassy pink with mild degenerative changes noticed in the mucosal epithelium and the muscular layers appeared normal **(Fig. 4)**.

4. Discussion

Intra vasal injection of SMA hydrogel in male rats did not affect testicular sperm production as well as the morphology of epididymal spermatozoa. But, as spermatozoa pass through the SMA vas occluded region, damage to sperm acrosomal membrane integrity occurred, resulting in loss of fertility which was evidenced by inhibition of pregnancy in females that were mated with copolymer SMA vas occluded male rats. Thus, from the present study and from previous reports copolymer SMA vas occlusion was found to be a safe procedure, as it did not bring about significant changes in testicular function evidenced by normal testosterone concentration and sperm production.

Fertility trials of copolymer SMA treated groups of rat showed 0% fertility when mated with normal adult female rats at 60 to 90 days of post vas occlusion period. Similar to the results of the present study, 100 per cent inhibition of fertility was achieved in female rats which were mated with copolymer SMA injected male at vas occlusion period of 7 to 90 days (Lohiya et al., 2010), and 180 days (Misro et al., 1979a). However, in the present study few female rats mated with male rats after 15 and 30 days of vas occlusion, were found pregnant. The low fertility recorded in group II and III vas occluded rats may be due to partial removal of injected copolymer SMA by spontaneous dissolution during natural body process and/or by improper injection of copolymer into the vas lumen, leading to escape of spermatozoa from polyelectrolytic action of SMA resulting in pregnancy (Lohiya et al., 1998).

CONCLUSION

The loss of pregnancy achieved by intravasal injection of copolymer SMA was clearly explained in studies that the intrinsic property of copolymer SMA was pH lowering action, which is spermicidal and its polyelectrolytic nature induced a surface charge imbalance on the sperm membrane system resulting in acrosomal membrane damage and causes impairment in the gamete interaction (Guha et al., 1985b; Guha, 2007). Further, it was evident from SEM and TEM changes of spermatozoa of SMA vas occluded region, which revealed severe damage to the spermatozoa membrane, particularly in the acrosome and mid-piece region thereby rendering the sperm cell incapable of penetrating the oocyte and resulting in loss of fertility after vas occlusion.

Informed consent

All animal study procedures complied with the appropriate local animal welfare regulations and were approved by applicable legal bodies.

Declaration of Competing Interest

The authors declare no conflict of interest in connection with this study.

Acknowledgements

The authors thank the Dean, Madras Veterinary College and Directorate of Clinics, TANUVAS for supporting and GOI for providing necessary financial assistance under the DBT-TANUVAS Canine Research Centre and Networks project on “Canine Contraception”

Figures

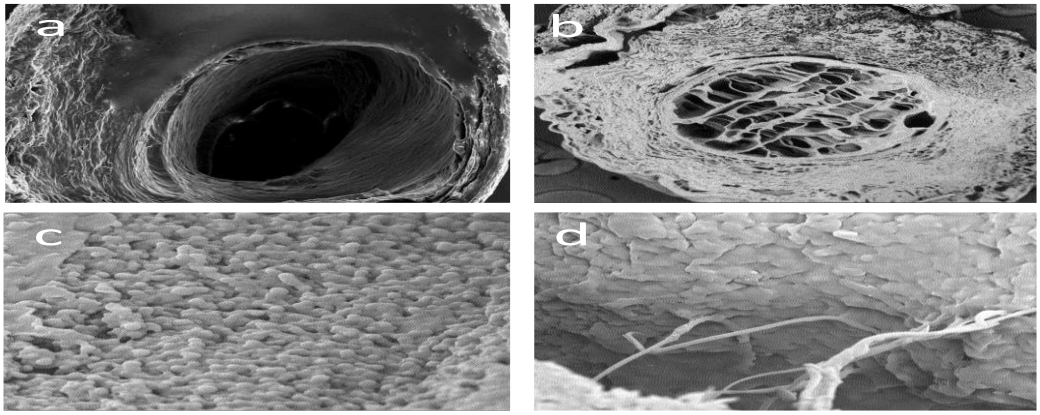


Figure 1: Confirmation of SMA occlusion of vas deferens. (a) Scanning electron microscopic (SEM) image showing patency in the lumen of vas deferens in a sham control rat (200 µm). (b) SEM image showing vas deferens occluded with copolymer SMA (300 µm). (c) SEM images showing coating of copolymer SMA (10 µm). (d) Spermatozoa trapped in the copolymer SMA coated vas lumen (10 µm).

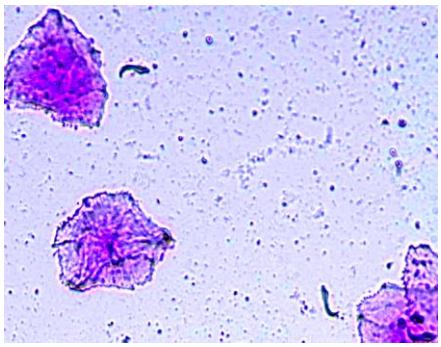


Fig 2. Vaginal smears of female rats (mated) showed the presence of detached sperm heads along with vaginal exfoliated cells

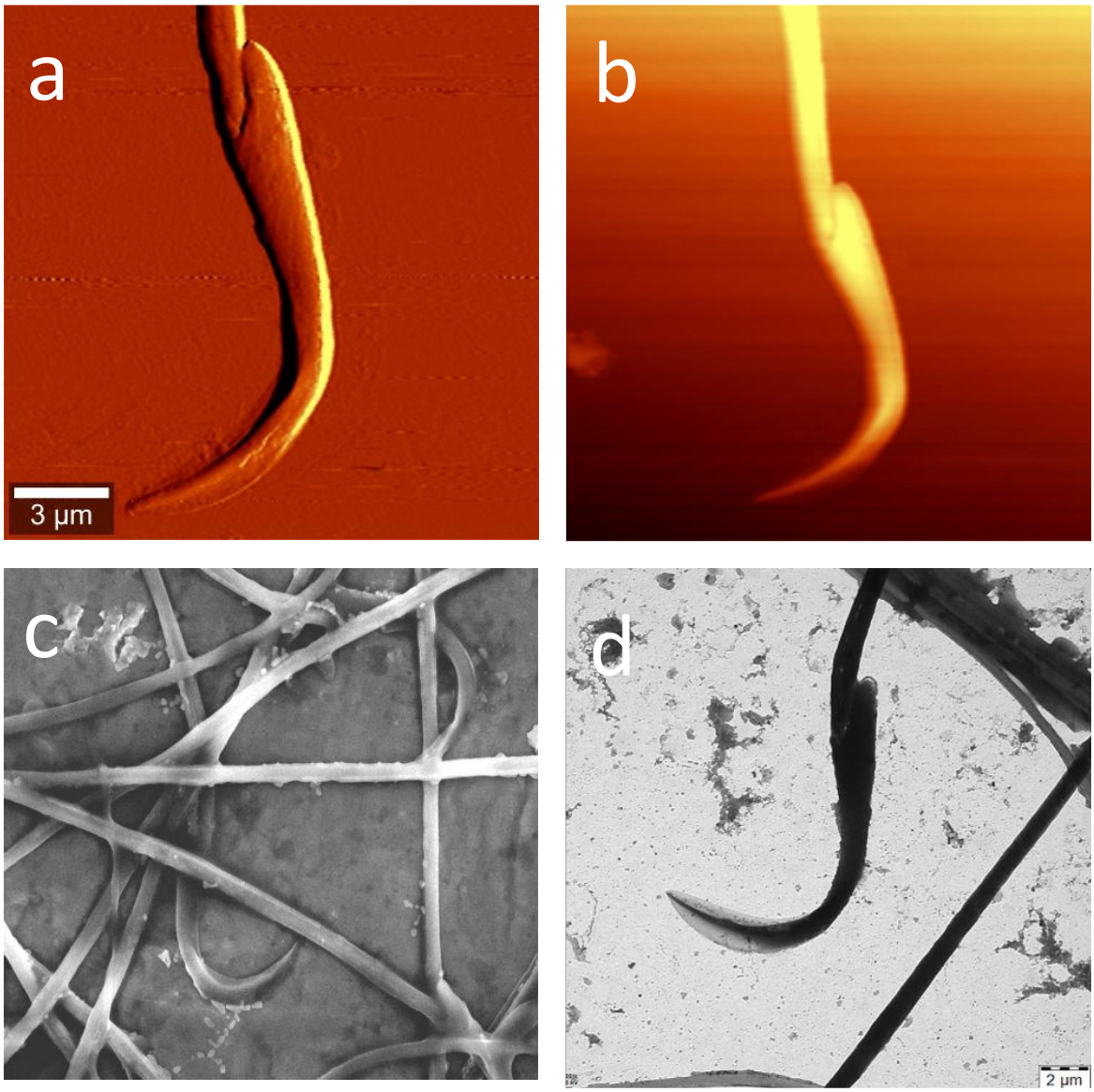


Figure 3: Ultrastructural changes in the spermatozoa. (a-b) Atomic force microscopy (AFM) images of 2D image (a) of normal head and mid piece of sperm cell recovered from rat cauda epididymis (5 µm) and 3D image (b) of normal head and mid piece of sperm cell recovered from rat cauda epididymis (3 µm). (c) Scanning electron microscopy (SEM) image of normal head with distinct acrosomal envelop, mid piece, and tail region of spermatozoa recovered from rat cauda epididymis(5 µm). (d) Transmission electron micrograph (TEM) of rat spermatozoa recovered from cauda epididymis showing normal morphology of head, mid piece, and tail (2 µm).

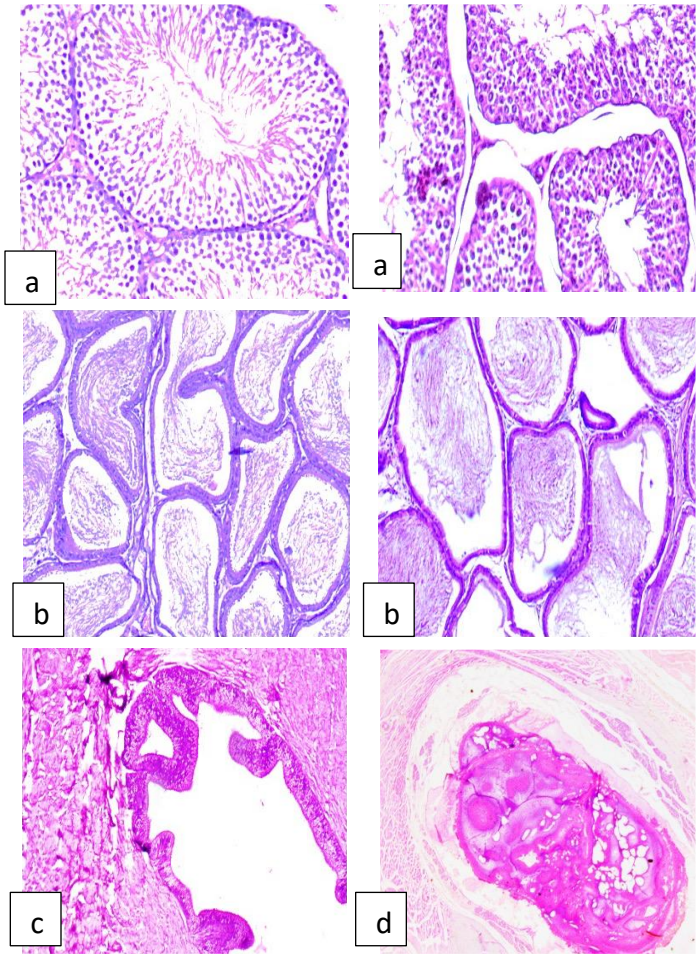


Figure 4: Histological images of reproductive tract showing the testicular section with normal seminiferous tubules (100 µm) containing Sertoli cells and germ cells at different stages of spermatogenesis(a), epididymis section showing normal epididymal ductular lumen containing sperm cells (50 µm)(b), and vas deferens showing lumen patency and normal morphology of mucosal folds in the sham control group of rats (c) and occlusion in the SMA group rats (50 µm)(d) .

REFERENCES

1. Amory, J.K. (2004). Testosterone/progestin regimens: a realistic option for male contraception? Curr Opin Investig Drugs. **5:**1025–30.
2. Bozzola, J.J. and Lonnie, D.R. (1999). Electron microscopy: principles and techniques for biologists. Jones and Bartlett Learning. Published by Jones and Bartlett, eBook.
3. Campos-Pereira, F.D., Franco, L.S., Alves, G.A., Goncalves, L., Curtolo, R.F., Hara, R.V., Dolder, M.A.H., Marin-Morales, M.A. and Severi-Aguiar, G.D.D. (2015). Evaluation of diamine putrescine effects on epididymal sperm count and transit in Wistar rats. Toxicol Lett. 238: S121-S121.
4. Dostal, L.A., Faber, C.K., and Zandee, J. (1996). Sperm motion parameters in vas deferens and cauda epididymal rat sperm. Reprod Toxicol. 10: 231-235.
5. England, G.C. (1997). Effect of progestogens and androgens upon spermatogenesis and steroidogenesis in dogs. J Reprod Fertil Suppl. **51**:123–38.
6. Fahim, M.S., Fahim, Z. and Harman, J.M. (1982). Chemical sterilization in the male part I: rats. Arch Androl. **9**:261–5.
7. Fahim, M.S., Wang, M., Sutcu, M.F., Fahim, Z. and Youngquist, R.S. (1993). Sterilization of dogs with intra-epididymal injection of zinc arginine. Contraception. 47:107–22.
8. Freeman, C. and Coffey, D.S. (1973). Sterility in Male Animals Induced by Injection of Chemical Agents into Vas-Deferens. Fertil Steril. **24:** 884-890.
9. Garcia Romero, G., Valiente, C., Aquilano, D., Corrada, Y. and Gobello, C. (2009). Endocrine effects of the GnRH antagonist, acyline, in domestic dogs. Theriogenology. **71**:1234–7.
10. Guha, S.K., Ansari, S., Anand, S., Farooq, A., Misro, M.M. and Sharma, D.N. (1985). Contraception in male monkeys by intra-vas deferens injection of a pH lowering polymer. Contraception. 32: 109-118.
11. Guha, S.K. (1996). Contraceptive for use by a male. US Patent No. 54880705.

12. Guha, S.K. (2007). Biophysical mechanism-mediated time-dependent effect on sperm of human and monkey vas implanted polyelectrolyte contraceptive. Asian J. Androl., 9 (2): 221-227.
13. Kumar, S., Chaudhury, K., Sen, P. and Guha, S.K. (2005). Atomic force microscopy: a powerful tool for high-resolution imaging of spermatozoa. J Nanobiotechnology. 3: 9.
14. Lacoste, D., Dube, D., Trudel, C., Bélanger, A. and Labrie, F.(1989). Normal gonadal functions and fertility after 23 months of treatment of prepubertal male and female dogs with the GnRh agonist GnRH ethylamide. J Androl. **10:**456–65.
15. Lohiya, N.K., Manivannan, B. and Mishra, P.K. (1998). Ultrastructural changes in the spermatozoa of langur monkeys Presbytis entellus entellus after vas occlusion with styrene maleic anhydride. Contraception. 57: 125-132.
16. Lohiya, N.K., Suthar, R., Khandelwal, A., Goyal, S., Ansari, A.S. and Manivannan, B. (2010). Sperm characteristics and teratology in rats following vas deferens occlusion with RISUG and its reversal. Int J Androl . 33: 198-206.
17. Manivannan, B., Bhande, S.S., Panneerdoss, S., Sriram, S. and Lohiya, N.K. (2005). Safety evaluation of long-term vas occlusion with styrene maleic anhydride and its non-invasive reversal on accessory reproductive organs in langurs. Asian J Androl. 7: 195-204.
18. Misro, M., Guha, S.K., Singh, H., Mahajan, S., Ray, A.R. and Vasudevan, P. (1979a). Injectable non-occlusive chemical contraception in the male-I. Contraception. 20: 467-473.
19. Misro, M., Kaur, H., Mahajan, S. and Guha, S.K. (1982). An intravasal non-occlusive reversible contraceptive device in rats. J. Reprod. Fertil., **65:** 9-13.
20. Sharma, R.S., Rajanna, A., Singh, B.K., Mathur, A.K. and Mukherjee, A.K. (2008). Current status of development of RISUG: an intravasal injectable male contraceptive. In: Sharma, R.S., A. Ranjanna, M. Rajalakshmi, (eds.). Proceedings of the conference on recent advances and challenges in reproductive health research. New Delhi: Indian Council of Medical Research,135-149.
21. Varisli, O., Agca, C. and Agca, Y. (2013). Short-Term Storage of Rat Sperm in the Presence of Various Extenders. J Am Assoc Lab Anim. 52: 732-737.