UTTAR PRADESH JOURNAL OF ZOOLOGY

43(7): 52-59, 2022
ISSN: 0256-971X (P)

SHORT-TERM EFFECT OF *Naja naja* SHED SKIN EXTRACT ON HISTOPATHOLOGY OF TESTIS, EPIDIDYMIS AND VAS DEFERENS OF SWISS ALBINO MICE

RAJARSHI GHOSH [a*], SOUBHIK DAS [a], DEEPSHIKHA MITRA [a], PRADIP BHATTACHARJEE [a] AND SUBIR CHANDRA DASGUPTA [a]

[a] Post-Graduate Department of Zoology, Maulana Azad College, 8 Rafi Ahmed Kidwai Road, Kolkata - 700013, West Bengal, India.

AUTHORS' CONTRIBUTIONS

This research work has been carried out in collaboration among all authors. Author RG designed the study, wrote the protocol and first draft of the manuscript. Authors SD, DM and PB performed the experiments and interpreted the data. Author PB managed the literature survey. Author SCD has made substantial contributions to the final checking of the manuscript. All authors read and approved the final manuscript.

Article Information

DOI: 10.56557/upjoz/2022/v43i72999

Editor(s):
(1) Dr. Villagomez Cortes Jose Alfredo Santiago, University of Veracruz, Mexico.
Reviewers:
(1) Amina Abd Al-Rahman Fayad, University of Baghdad, Iraq.
(2) Said Ahmed Mohamed Hassan, Suez Canal University, Egypt.
(3) Gunasekar Manoharan, New Jersey Bio Science Centre, USA.

Received: 01 March 2022
Accepted: 04 May 2022
Published: 09 May 2022

Original Research Article

ABSTRACT

Ancient literature reveals that snake shed skin had been frequently used to treat several diseases like glaucoma, hernia, psoriasis, hemorrhoids, etc. Previously, it was reported that *Naja naja* shed skin extract causes temporary cessation of the estrous cycle and altered female reproductive hormones in rodents, and the probable bioactive molecule behind such alterations was also identified. Based on this information, the present study was designed to establish the effect of *Naja naja* shed skin extract on the male reproductive system of Swiss albino mice. The extract was prepared with physiological saline and was injected intraperitoneally in the male mice for 7 consecutive days @ 10 mg kg^{-1} body weight. The short-term histopathological studies were performed by Haematoxylin and Eosin staining and the sperm morphology was analyzed by using bright-field microscopy. The snake skin extract markedly changed the gross histological architecture of the seminiferous tubules including disorganization of germinal epithelial cells, accumulation of exfoliated cells in the lumen, tailless sperm in the epididymis, disorganized basal cells in vas deferens as well as marked alteration of sperm morphology. The present work with snake shed skin strongly indicates that it is not simply a biological waste, but it might be a treasure house of many bioactive compounds.

Keywords: *Naja naja*; histopathology; male reproductive system; shed snake skin extract.

**Corresponding author: Email: 1977rajarshighosh@gmail.com;*

1. INTRODUCTION

Out of a million venomous animals, the snakes are the most feared but, at the same time, the most worshipped living creatures on earth. Different body components of the snakes including fat, bile, and shed skin have been used in folk and ethnomedicine of various cultures since ancient times [1,2,3]. Skin shedding or ecdysis is a dynamic event that occurs throughout the lifetime of a snake [4]. A healthy snake sheds its skin in a single piece, like an inverted sock. As soon as a shedding cycle is complete, a new cycle begins, and it is repeated throughout the life of the snake [5,6]. The use of snake shed skin (SSS) in different pathophysiological conditions such as wound healing, burn, glaucoma and eczema has been mentioned in folk medicine [7]. In the traditional medicine concept of China, the SSS has long been used in various diseases such as furuncles, carbuncles and mammary abscesses and was also reported to be useful in curing the inguinal hernia [1]. Based on the concept of traditional healers and ancient medicinal practices of using SSS in reproductive disorders in the South-Eastern part of Asia and India, it could be stated that the SSS could be associated with alteration of the reproductive functions [8].

The SSS, thought to be a biological waste initially, could be a potential source of bioactive molecules [8]. Limited scientific data are available to establish the therapeutic uses of such moleculesobtained from SSS in health and diseases [2,9,10]. Though largely used by traditional naturopaths for treating various female reproductive disorders, the role of aqueous shed skin extract of Indian cobra, *Naja naja* on the estrous cycle of female mice was well documented [7]. It caused a cessation of the estrous cycle, especially at the diestrus phase. From SSS extract, a small crystalline peptide was isolated as bioactive molecule from *Naja naja* shed skin called NNSS2 which probably was responsible for the estrous cycle inhibition [8]. Further extensive studies have been carried out to investigate the effects of SSS extract on the female reproductive system in mice. But there is no scientific evidence regarding the bioactivity of the SSS on the male reproductive system. Therefore, the present study is targeted to evaluate the role of the aqueous extract of *Naja naja* shed skin on the histopathology of the male reproductive system in Swiss albino mice.

2. MATERIALS AND METHODS

2.1 Specimen Collection and Maintenance

Male Swiss albino mice of 10-12 weeks old were purchased from the approved animal breeders of Maulana Azad College, Kolkata. They were acclimatized for 7 days in polypropylene cages at 25 ± 2°C in a light-controlled environment (12 h in light and dark conditions alternatively). All the animals were fed a standard laboratory diet and had free access to food and water *ad libitum*.

2.2 Collection of *Naja naja* Shed Skin and Maintenance

Fresh *Naja naja* shed skin of both sexes were collected from North 24-Parganas District of West Bengal, India through field collection as per the permission granted by the office of the Principal Chief Conservator of Forests (Wildlife) and Chief Wildlife Warden, West Bengal, India (memo no. C-28011/11/2020, dated 17.01.2020). The skins were identified by the Zoological Survey of India, Kolkata. The shed skins were stored in desiccators at room temperature and an aqueous extraction was made as per requirement.

2.3 Preparation of Snake Shed Skin Aqueous Extract and Estimation of Protein

Freshly powdered shed skin of *Naja naja* was soaked in physiological mammalian saline (0.9 % NaCl solution) overnight at 4°C and it was then centrifuged at 5000 rpm at 4°C for 20 min. The supernatant was collected and expressed in terms of protein content [11].

2.4 Experimental Design

For the study with an aqueous extract of SSS, the male Swiss albino mice were randomized into 2 groups (n=5). One group was considered as control where mice were injected with normal mammalian physiological saline and to the mice of the experimental group, the SSS extract (10 mg kg^{-1} body weight) was administered through the intraperitoneal route for 7 consecutive days (total of 7 exposures). The dose of the SSS extract in this present work has been standardized following the previous experiment which was performed on female Swiss albino mice for investigating the effects of SSS of *Naja naja* on histopathology of ovary and uterus [7]. After 24 h of the last dose being injected, male mice were sacrificed by cervical dislocation. For histopathological analysis of the male reproductive system, testes, epididymis and vas deferens were dissected carefully and freed from adhering tissues.

2.5 Histopathological Studies

The testis, vas deferens and epididymis from control and SSS-treated male mice were isolated, washed in

saline, and fixed in 10 % buffered formalin for 18 h. Then, tissues were dehydrated in upgraded alcoholic series, immersed in cedar wood oil followed by embedding in molten paraffin (melting point: 56-58°C). Serial paraffin thin sections (5 µm) were cut using a rotary microtome. The tissue sections were stained with Haematoxylin and Eosin (HE) following standard protocol and mounted in Dibutylphthalate Polystyrene Xylene (DPX). Finally, the stained tissue sections were observed under a bright-field microscope.

2.6 Sperm Morphology Analysis

The epididymis from the control and SSS-treated male mice was finely minced by anatomical scissors in 1 mL of isotonic saline solution kept in a Petri dish. It was completely squashed with the help of tweezers for 2 min. Then it was incubated for 4h at room temperature to provide the migration of all spermatozoa from epididymal tissue to fluid. The inner content of epididymis was taken out in saline and the material was thoroughly shaken to suspend the sperm in saline solution. The sperm suspension was filtered with the help of cell strainer (PluriSelect, USA; mesh size: 40 µm) to remove the debris and the filtrate was collected in a graduated tube; more saline was added to make the volume 10 mL. The sperm suspension, thus collected, was put in the centre of the clean slide over which 0.02 mL methanol was added, air-dried overnight at room temperature. A drop of diluted Giemsa stock solution (6:1) was put on the material. The material was covered with a cover glass sealed temporarily for observation under bright-field microscope as per the routine procedure.

3. RESULTS AND DISCUSSION

3.1 Effect of Snake Shed Skin (SSS) Extract on the Testis

The histological structure of the normal testis treated with physiological saline showed the usual characteristics; the seminiferous tubules exhibited spermatogenic activity with successive stages of sperm development, healthy germinal epithelium, and presence of interstitial cells of Leydig in between the tubules (Fig. 1A). Significant alterations were observed in the histoarchitecture of the testes treated with SSS extract. The degenerative changes in the seminiferous tubules were not uniformly distributed. Within the same section, some tubules were found to have a greater extent of disturbances than others. The diameter of the tubules significantly decreased, and the interstitial space became wider as compared to the control (Fig. 1B). The germinal epithelial cells became reduced in height, showed increased disorganization with the accumulation of cellular debris in the lumen. As a result, the epithelial covering of the seminiferous tubules became thinner and resulted in the arrest of spermatogenesis (Fig. 1C). Few of the interstitial cells underwent cellular necrosis (Fig. 1D). The necrotic effect on the spermatogenic cells was evident in the sections with elevated cellular congestion and hemorrhage. This is one of the major reasons for testicular weight loss [12, 13,14]. In some sections, the presence of giant cells containing abnormal spermatid nuclei was evident (Fig. 1E). This type of cell is suggested to be formed from multiple nuclear divisions without cytoplasmic separation of the cells [15]. An increased accumulation of cells in the interstitial space was also noticed in the treated mice testis (Fig. 1F). In general, the affected seminiferous tubules showed degeneration of the epithelial layer, intraepithelial vacuolation, degradation of the sperm cells, and the presence of a mixed type of spermatogenic cells in different stages of spermatogenesis.

3.2 Effect of Snake Shed Skin (SSS) Extract on Caput Epididymis

The epididymis of the control mice was shown to have normal histological features; it consisted of several tubules lined with pseudostratified columnar epithelium having stereocilia and the lumen was filled with sperm bundles. The tubules were separated by connective tissue having blood vessels (Fig. 2A).The caput epididymis was taken for comparison from the SSS-treated mice. The tubular diameter increased, although not so significantly. There were few tubules without any sperm in the lumen (Fig. 2B). This might be due to the partial arrest of spermatogenesis after treatment with SSS extract which led to the loss of spermatogenic cells from tubular epithelium or hormonal impairment [16,17]. The most prominent effect of SSS extract in the caput epididymis was the dislodging of the stereocilia (Fig. 2C). The lumen was often found to contain exfoliated germ cells that are the resultants of the loss of premature cell adhesion to the processes of Sertoli cells [17]. These caused the accumulation of germ cell elements into the lumen of seminiferous tubules [18].

3.3 Effects of Snake Shed Skin (SSS) Extract on Vas Deferens

In control mice, the vas deferens consisted of three distinct muscular layers, viz. the outer longitudinal, middle circular and inner longitudinal layers. The lamina propria was also prominent in between the

longitudinal muscle layer and pseudostratified epithelial cells having stereo cilia (Fig. 3A). Basal cells are regularly arranged in the endothelium. The epithelial layer was folded so as to form a stellate lumen containing the sperm bundles (Fig. 3C). On the contrary, the endothelium showed swelling in SSS-treated mice as compared to the control. The cellular height of the epithelial cells did not change much, but it showed nuclear pyknosis, especially in the regions of folds (Fig. 3B). The regular arrangement of the basal cells was affected in the treated mice. The flattening of the endothelial lining consisting of remnants of stereo cilia was also visible along with some endothelial lesions (Fig. 3D).The atrophy in the tubular epithelial cells and loss of proper spermatogenic activities might be due to the functional interruption of the Sertoli cells [19]. This study implied the involvement of hormonal misbalance especially testosterone due to the application of SSS extract into mice [20,21,22,23].

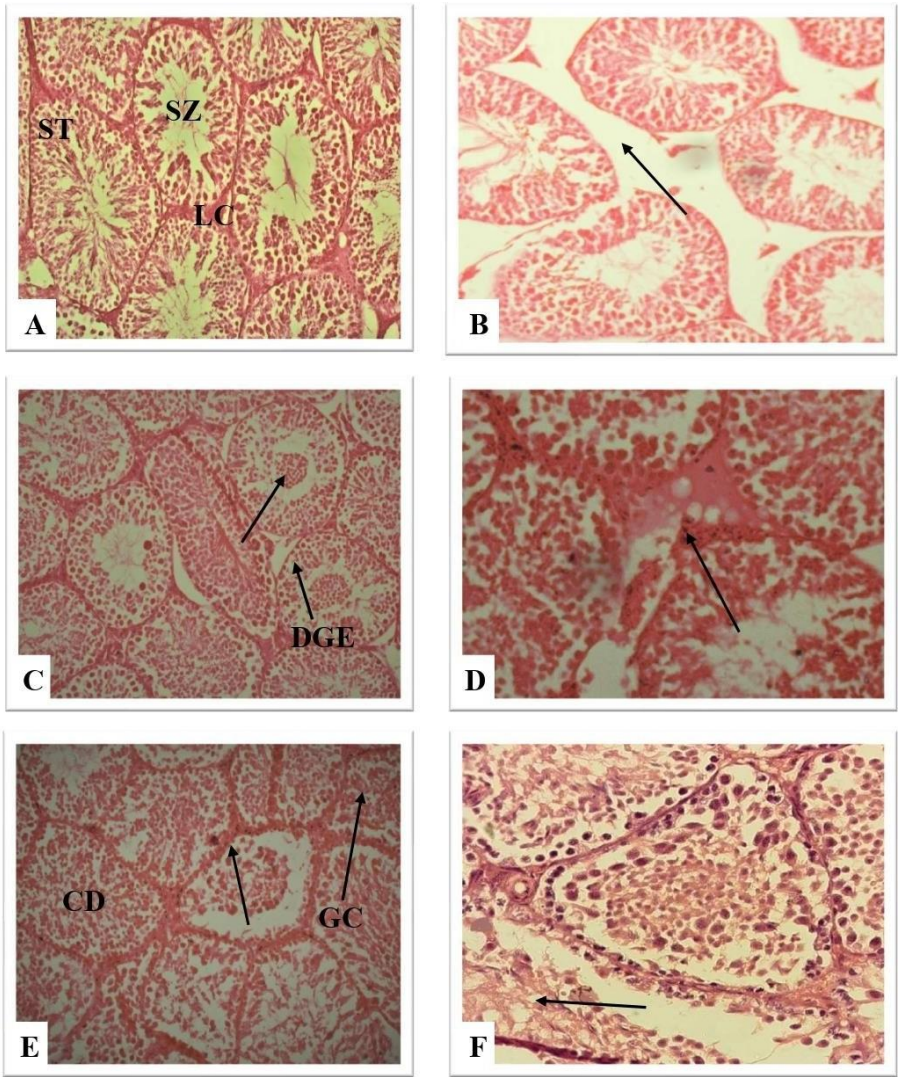

Fig. 1. Photomicrographs of histopathological analysis of testes; [A] The sectional view of testis from control Swiss albino mice showing seminiferous tubules (ST), interstitial cells of Leydig (LC) and spermatozoa (SZ), HE staining (200X); [B] testes of SSS-treated mice showing an enlargement of the interstitial space between the tubules as indicated by the black arrow, HE staining (100X); [C] Testes of SSS-treated mice showing denudation of the epithelial cells into the lumen as indicated by black arrow and disarrayed germinal epithelium (DGE), HE staining (200X); [D] Initiation of cellular necrosis in interstitial spaces, indicated by the black arrow, HE staining (400X); [E] cellular debris in the lumen (CD) and degradation of the spermatogenic cells due to arrest of spermatogenesis (black arrow) and presence of giant cell (GC), HE staining (200X); [F] Increased cellular accumulation in the interstitial space, indicated by the black arrow, HE staining (400X)

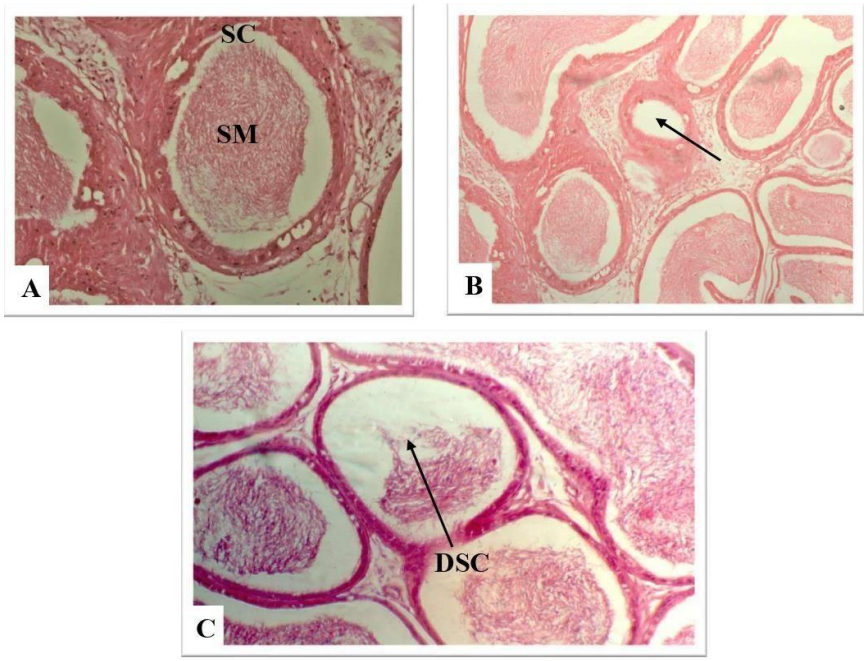

Fig. 2. Photomicrographs of histopathological analysis of caput epididymis; [A] The control caput epididymis showing the usual arrangement of stereo cilia (SC) and spermatozoa (SM), HE staining (200X); [B] Tubule without sperm in SSS-treated mice as indicated by the black arrow, HE staining (100X); [C] Epithelial layer of treated caput epididymis with dislodged stereo cilia (DSC) and debris of spermatozoa and abnormal sperm cells as indicated by black arrow, HE staining (200X)

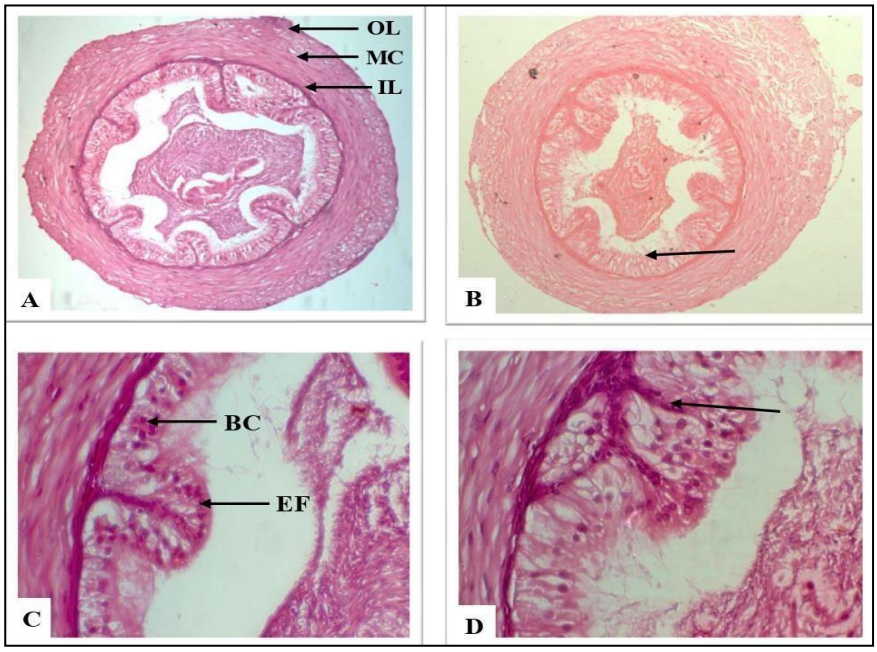

Fig. 3. Photomicrographs of histopathological analysis of vas deferens; [A] Vas deferens in control mice showing three layers of muscles viz. outermost longitudinal (OL), middle circular (MC) and innermost longitudinal (IL) layers, HE staining (200X); [B] Swelling of the endothelium with remnants of stereocilia in treated mice, indicated by black arrow, HE staining (200X); [C] Normal vas deferens showing a regular arrangement of basal cells (BC) within the basement membrane and epithelial fold (EF), HE staining (400X); [D] Irregularly distributed basal cells as indicated by black arrow and disturbed epithelial fold, HE staining (400X)

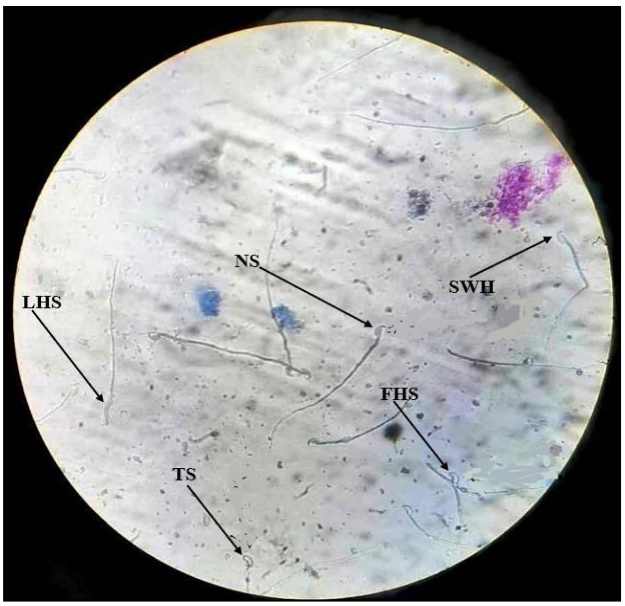

Fig. 4. Changes in sperm morphology in mice treated with snake shed skin (SSS) extract. Normal sperm with hook and tail (NS), long-headed sperm (LHS), tailless sperm (TS), forked-headed sperm (FHS) and sperm without hook (SWH) were also observed when treated with SSS, Giemsa staining (400X)

3.4 Changes in Sperm Morphology

The direct effects of SSS extract on sperm morphology are quite evident from the obtained data. Certain types of abnormal sperm morphs were visible following the treatment of SSS extract in mice. A normal motile sperm contains a marked hook and a distinct tail which is a unique and important characteristic to predict the fertilization capacity [24]. When the SSS extract was administered to the mice, the number of abnormal sperms including long-headed and tailless sperm increased significantly (Fig. 4) which reduced sperm motility to a greater extent resulting in a decreased fertilizing capacity of mice [25]. A number of forked-headed sperm was also observed in treated mice groups. It was also observed that SSS extract could lead to the production of sperm without any hook (Fig. 4). Reduced sperm motility associated with morphological changes might be due to low levels of ATP that drive its movements [26,27] and abnormalities in male gonadotropins [19,28,29,30]. The extract possibly had direct effects on Sertoli cells of seminiferous tubules, the principal player in spermiogenesis [31]. Any disorganization in the epithelial layer or testicular tubular atrophy can alter Sertoli cell functions and thereby decrease normal and healthy sperm production [32].

4. CONCLUSION

The present study reflected the effects of aqueous extract of *Naja naja* shed skin on the histological characteristics of the male reproductive system in mice. It showed marked alterations in the gross histopathology of the overall reproductive organs including degeneration of epithelial cells of the seminiferous tubules, accumulation of cellular debris in the lumen, temporary arrest of spermatogenesis, tubules without sperm, abnormal and reduced sperm motility, degradation of the stereo cilia in the epididymis, disorganized basal cells and eventually reduced fertility.

The necrotic changes and reduced spermatogenic activities that occurred followed by the administration of the SSS extract might have caused a reduction in the weight of testes. The direct effect on the functional integrity of Sertoli cells was reflected in the arrest of spermatogenesis and degradation of spermatogenic cells. The increased cellular debris in the interstitial space was a clear indicator of the fact that testosterone secretion was being affected in the treated mice. Testosterone is crucial for the maintenance of the interstitial cells of Leydig, which in turn is important for the structural integrity of the seminiferous tubules and proper spermatogenetic activities. Decreased sperm motility, as in the case of tailless sperm observed in SSS- treated samples may be associated with infertility in male mice. There might be several reasons behind alteration of the sperm morphology and its effects on fertilization ability. Altered enzymatic activities of oxidative processes and subsequently low levels of ATP content could be the major issues determining sperm motility. The sperm count, on the other hand, was

also crucial for usual fertility, which was affected after SSS extract injection.

The overview of the SSS extract on the male reproductive system and the possible reasons behind them clearly indicate that the SSS is a potential source of active biomolecules with therapeutic advantages. Future works are required to evaluate the hormonal and biochemical changes due to SSS extract administration into male mice and to investigate the nature of active biomolecules present in the shed skin of *Naja* and other snakes.

ETHICAL APPROVAL

The experimental protocols used for this study were approved by the Institutional Animal Ethical Committee of Maulana Azad College and followed strictly.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Lev E. Traditional healing with animals (zootherapy): Medieval to present-day Levantine practice. Journal of Ethnopharmacology. 2003;85:107. DOI: 10.1016/s0378- 8741(02)00377-x
2. Costa-Neto EM. Animal-based medicines: biological prospection and the sustainable use of zootherapeutic resources. Anais da Academia Brasileira de Ciencias. 2005;77:33. DOI: 10.1590/s0001-37652005000100004
3. Soewu DA. Wild animals in ethnozoological practices among the Yorubas of southwestern Nigeria and the implications for biodiversity conservation. African Journal of Agricultural Research. 2008;6:421.
4. Kardong KV. Vertebrates: Comparative Anatomy, Function, Evolution (5th ed.). New York: McGraw Hill. 2009;216-217. ISBN: 978-007-127033-5.
5. Mattison C. The new encyclopedia of snakes. Princeton: Princeton University Press;2007.
6. Parker HW. Snakes- A natural history talks. London: Cornell University Press; 1977.
7. Mukherjee S, Dasgupta SC, Gomes A. Effect of *Naja naja* Laurenti shed skin extract on estrous cycle, hormone- cytokine profiles, histopathology of ovary and uterus of Swiss albino mice. Indian Journal of Experimental Biology. 2013;51:235-240.
8. Mukherjee S, Dasgupta SC, Gomes A. Estrous cycle cessation caused via activating the apoptotic pathway by NNSS2 isolated from *Naja naja* shedded skin. Journal of Toxins. 2016;3(1):3.
9. El-Kamali HH. Folk medicinal use of some animal products in Central Sudan. Journal of Ethnopharmacology. 2000;72:279-289. DOI: 10.1016/s0378-8741(00)00209-9
10. Adeola MO. Importance of wild animals and their parts in the culture, religious festivals, and traditional medicine, of Nigeria. Environmental Conservation. 1992;19:125-134. DOI:https://doi.org/10.1017/S0376892900030605
11. Lowry OH, Rosenburg NJ, Farr AL, Roudall R. Protein measurement with the Folinphenol reagent. Journal of Biological Chemistry. 1951;193:265-275.
12. Lue Y, Sinha HAP, Wang C, Leung A, Baravarian S, Reutrakul V, Sangsawan R, Chaichana S, Swerdloff RS. Triptolide: a potential male contraceptive. Journal of Andrology. 1998;19(4):479-486.
13. Takihara H, Cossentino MJ, Sakotoku J, Cockett ATK. Significance of testicular size measurements in andrology II. Correlation of testicular size with testicular function. Journal of Urology. 1987;137:416-419. DOI: 10.1016/s0022-5347(17)44053-5
14. Udoh P, Kehinde A. Studies on anti-fertility effect of pawpaw seeds (*Carica papaya*) on the gonads of male albino rats. Phytotherapy Research. 1999;13(3):226-228. DOI: 10.1002/(sici)1099-1573(199905)13:3<226::aid-ptr396>3.0.co;2-e
15. Mishra RK, Singh SK. Effect of aqueous leaf extract of *Azadirachta indica* on the reproductive organs in male mice. Indian Journal of Experimental Biology. 2005;43: 1093-1103.
16. Creasy DM. Pathogenesis of male reproductive toxicity. Toxicologic Pathology. 2001;29:64-76. DOI: 10.1080/019262301301418865
17. Vidal JD, Whitney KM. Morphologic manifestations of testicular and ependymal toxicity. Spermatogenesis. 2014;4:e979099. DOI: 10.4161/21565562.2014.979099
18. Donmez YB, Kizilay G, Topcu-Tarladacalisir Y. MAPK immunoreactivity in streptozotocin-induced diabetic rat testis. Acta Cirurgica Brasileira. 2014;29:644-650. DOI: 10.1590/S0102-8650201400160004
19. Crews D, Williangham E, Skipper JK. Endocrine disruptors: Present issues, future

directions. The Quarterly Review of Biology. 2000;75:243-260.
DOI: 10.1086/393498

20. Bartke A. Pituitary-Testis relationship. Reproductive Biology. 1991;1:136-152.
21. Sharpe RM, Maddocks S, Kerr JB. Cell-cell interactions in the control of spermatogenesis as studied using Leydig cell destruction and testosterone replacement. American Journal of Anatomy. 1990;188:3-20.
DOI: 10.1002/aja.1001880103
22. Xu Y, Lei H, Guan R, Gao R, Li H, Wang L, Song W, Gao B, Xin Z. Studies on the mechanism of testicular dysfunction in the early stage of a streptozotocin induced diabetic rat model. Biochemical and Biophysical Research Communications. 2014;450:87-92.
DOI: 10.1016/j.bbrc.2014.05.067
23. Kianifard D, Sadrkhanlou RA, Hasanzadeh S. The ultrastructural changes of the sertoli and leydig cells following streptozotocin induced diabetes. Iranian Journal of Basic Medical Sciences. 2012;15:623-635.
24. Aitken RJ, Best FSM, Warner P, Templeton A. A prospective study of the relationship between semen quality and fertility in the case of unexplained infertility. International Journal of Andrology. 1984;5:297-303.
DOI: 10.1002/j.1939-4640.1984.tb00792.x
25. Murugavel T, Ruknudin A, Thangavelu S, Akbarsha MA. Antifertility effect of *Vinca rosea* (Linn.) leaf extract on male albino mice. A sperm parametric study. Current Science. 1989;58(19):1102-1103.
26. Tso WW, Lee CS. Effect of gossypol on boar spermatozoa *in vitro*. Archives of Andrology. 1981;7:85-88.
DOI: 10.3109/01485018109009381
27. Iamsaard S, Burawat J, Kanla P, Arun S, Sukhorum W, Sripanidkulchai B, Uabundit N, Wattathorn J, Hipkaeo W, Fongmoon D, Kondo H. Antioxidant activity and protective effect of *Clitoriaternatea*flower extract on testicular damage induced by ketoconazole in rats. Journal of Zhejiang University-Science B. 2014;15(6):548-555.
DOI: 10.1631/jzus.B1300299
28. Adamkovicova M, Toman R, Martiniakova M, Omelka R, Babosova R, Krajcovicova V, Grosskopf B, Massanyi P. Sperm motility and morphology changes in rats exposed to cadmium and diazinon. Reproductive Biology and Endocrinology. 2016;14:42.
DOI: 10.1186/s12958-016-0177-6
29. Singh SK, Pandey RS. Effect of subchronicendosulfan on plasma gonadotropins, testosterone, testicular testosterone and enzymes of androgen biosynthesis. Indian Journal of Experimental Biology. 1990;28:953-956.
30. Bodding PO. Studies in Santhal Medicine and Connected Folklore. Calcutta: Baptist Mission Press, The Royal Asiatic Society of Bengal; 1925.
31. Bedford JM. Significance of the need for sperm capacitation before fertilization in eutherian mammals. Biology Reports. 1983; 28:108-120.
DOI: 10.1095/biolreprod28.1.108
32. Bardin CW, Cheng CY, Musto NA, Gunsalus GL. The Sertoli cell. In: The Physiology of Reproduction (Ed., Knobil E, Neill J). Raven Press, New York. 1988; 933-974.