Effect of *Naja naja* Laurenti shed skin extract on estrous cycle, hormone - cytokine profiles, histopathology of ovary and uterus of Swiss albino mice

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The snake shed skin though considered as biological waste products have been mentioned in folk and traditional medicine for treatment of ailments like skin disorders, parturition problems etc. Shedded skin extract (5 mg.kg\(^{-1}\), sc) did not produce any change in the estrous cycle of normal cycling female mice. However in 10 mg.kg\(^{-1}\), sc dose, the extract caused a temporary cessation of the estrous cycle at diestrous phase in normal cycling female mice for 10 days. SSAE (10 mg.kg\(^{-1}\), sc) caused a significant change in the level of LH, FSH, progesterone, estradiol, IL-1\(\beta\), IL-6 and TNF-\(\alpha\).  Histopathology of uterus and ovary showed structural disorientation in both. The results substantiate the influence of snake shed skin in mice reproductive cycle.

**Keywords:** Endocrine profile, Estrous cycle, *Naja naja*, Shed snake skin

Snakes and its body parts (fat, bile, shed skin etc.) have been used in folk and ethno medicine of various cultures since ancient time\(^1\). The shedded skins are a treasure house of bio-active molecules and have been used in Chinese and Levantine medicinal systems for treating glaucoma, eczema, hemorrhoids, wound healing, psoriasis. The ash of shed snake skin has been used in the treatment of inguinal hernia\(^3\). In Indian traditional medicinal system, ash of shed snake skin is used for inducing labour.

Estrous cycle of mice is marked by 4-5 days. The ovarian cycle is divided into estrous or ovulatory phase with 12 h duration, metestrous or luteal phase is of 24 h. Diestrous or the follicular phase is of 1 day duration and is known as D1 phase or sometimes it continues for 2 days and is known as D2 phase. Proestrous marks the end of the follicular phase and is of 12 h duration\(^4\). The whole of the estrous cycle or the ovarian cycle is under the feedback regulatory mechanism of hypothalamic pituitary gonadal axis involving gonadotropin releasing hormone (GnRH), luteinizing hormone (LH), follicle stimulating hormone (FSH), and the ovarian hormones, estrogen and progesterone.

Cytokines are perceived to have an immune-regulatory property and are closely associated with different phases of reproductive cycle like embryogenesis, implantation, maternal recognition of pregnancy, lactation, labor induction\(^5\). The immune system plays a critical role in maintaining the reproductive function and it is coordinated by the interaction between the cytokine and the endocrine system. Cytokines play an important role in the regulation of ovarian function, gonadal steroid secretion, corpus luteum function, embryo development and implantation\(^7\). The cytokines synergistically operates in a highly complex integrated network that has both stimulatory and antagonistic interaction with the endocrinal system of the body. Infact the whole of the estrous cycle is under the regulation of the various interplays between cytokines and the hormones, and the inflammatory dysfunction underlies the complication associate with the estrous cycle\(^7\).

Based on concept of traditional healers and ancient medicinal practices of the South Eastern part of Asia and India it could be stated that the snake shed skin may be associated with altering the reproductive function. Keeping this in view the present study has

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been aimed to assess the effect of shed skin extract of snake (*Naja naja* Laurenti) on the estrous cycle, hormone profiles, cytokines, and histopathology of ovary and uterus of Swiss albino mice.

**Materials and Methods**

*Chemicals and reagent*—The following chemicals were used: Bovine serum albumin (Sigma, USA), Copper sulphate (E. Merck, India), DPX (Qualigen, India), eosin (Sigma, USA), estradiol, ethanol, folin ciocalteau (SRL, India), formaldehyde (Qualigen, India), FSH, haematoxylin (Qualigen, India), IL-1β (R & D, USA), IL-12 (R & D, USA), IL-6 (R & D, USA), LH, paraffin wax (56–58 °C) (E. Merck, India), progesterone, sodium–potassium tartarate (E. Merck, India), sodium bicarbonate, TNF-α (R & D, USA) and xylene (E. Merck, India).

*Experimental animals*—Female Swiss Albino mice (10-12 weeks old) were purchased from M/s. B.N Ghosh and Co., Kolkata, India. They were housed in polypropylene cages at 26±2 °C in light controlled environment (12 h light/dark) with free access to food and water. Estrous cycle was tracked by carrying out vaginal smears daily. Vaginal lavage was performed using approximately 30 µL of 0.9% NaCl, which was dispensed in the vagina through glass tip applicator. Cells were collected and studied under microscope to confirm each stage of estrous cycle (diestrous, metestrous, proestrous and estrous). Only the mice showing two consecutive 4-5 days cycle were used for the experiments. Regulations of the Animal Experimental Ethics Committee were followed, and were in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animal (CPCSEA), Government of India.

*Collection of shed snake skin*—Fresh *N. naja* shedded skins of both sexes were collected from North 24-Parganas of West Bengal, India through field collection as per the permission granted by the Ministry of Forests & Wild Life, Govt. of West Bengal, India (2105/WL/4R-1 (PI-IX)). The skins were identified by the Zoological Survey of India.

*Preparations of shed snake skin aqueous extract (SSAE)*—Fresh shedded skin of *N. naja* was powdered using mortar and pestle and soaked in physiological saline overnight at 4 °C. It was centrifuged at 5000 rpm at 4 °C for 20 min. Supernatant was collected and expressed in terms of protein content.

*Experimental design*—Swiss Albino mice with normal estrous cycles (diestrous, metestrous, proestrous and estrous,) were selected. The shed skin extract (5 mg.kg⁻¹, sc and 10 mg.kg⁻¹, sc) was administered at estrous or diestrous phase. Vaginal lavage was observed every 24 h for 10 days. On Days 5 and 10 blood was collected (from retro-orbital plexus) and the serum was separated for estimation of hormone (LH, FSH, estrogen and progesterone) and cytokines (IL-1β, TNF-α, IL-6 and IL-12) level. Tissues (uterus and ovary) were collected for histopathological studies.

*Effect of SSAE on serum hormone and cytokine level*—Serum hormones (LH, FSH, estradiol, and progesterone) and cytokines (IL-1β, TNF-α, IL-6 and IL-12) levels were estimated using enzyme-linked immunosorbent assay (ELISA) kits (R & D, USA) according to the manufacturer’s instruction.

*Histopathological studies*—On days 5 and 10 of SSAE treated mice (10 mg.kg⁻¹, sc) ovaries and uterus were isolated, washed in saline and kept in 10% buffered formalin for 18 h. The tissues were dehydrated using graded ethanol (50 - 100%), followed by clearing in xylene (1 h), embedded at 58±1 °C for 4 h and paraffin blocks were prepared. Paraffin sections (5 µm thick) were cut using a rotary microtome (Wesvox, India). The paraffin sections were deparaffinised in xylene and stained with haematoxylin-eosin following standard protocol and mounted in DPX. Histopathological changes were observed with a bright field microscope (Motic, Germany) and photographs were captured with Motic software (Motic Images Plus 2.0 Software).

*Statistical analysis*—The results were expressed in terms of mean ± SE (n=6). The data were subjected to one way analysis of variance (ANOVA) followed by Tukey’s test using GraphPad InStat 3 software to establish statistical significance (*P* < 0.05; **< 0.01; ***< 0.001).

**Results**

The protein content of SSAE was 6 ±1 mg.mL⁻¹.

*Effect of SSAE on the estrous cycle*—SSAE (5 mg.kg⁻¹, sc) did not have significant effect on the estrous cycle, but when administered in 10 mg.kg⁻¹, sc dose, it caused a temporary cessation at the diestrous phase for 10 days.

*Effect of SSAE on the serum hormone level*—SSAE caused a significant increase in the serum LH level on Day 5 as compared with control diestrous group
and returned to normal level at Day 10 (Fig. 1a). SSAE caused a significant increase in the serum FSH level on Day 5 as compared with control diestrous group and returned to normal on at Day 10 (Fig. 1a). SSAE caused a significant increase in the serum progesterone level on Day 5 as compared with control diestrous group which decreased significantly on Day 10 as compared with control diestrous group (Fig. 1b). SSAE did not significantly altered the serum estradiol level on Day 5 as compared with control diestrous group, which increased significantly on Day 10 as compared with control diestrous group (Fig. 1b).

**Effect of SSAE on the serum cytokine level**—SSAE caused a significant decrease in the serum IL-1β level as compared with control diestrous group which decreased significantly on Day 10 as compared with control diestrous group (Fig. 2a). SSAE caused a significant decrease in the TNF-α level on Day 5 as compared with control diestrous group and returned to normal level at Day 10 as compared with control diestrous group (Fig. 2a). SSAE caused a significant decrease in the serum IL-6 level on Day 5 as compared with control diestrous group and returned to normal level on Day 10 as compared with control diestrous group (Fig. 2b). SSAE did not significantly changed serum IL-12 level on Day 5 and Day 10 as compared with control diestrous group (Fig. 2b).

**Histopathological studies**—SSAE (10 mg kg⁻¹, sc) produced loss of uterine glands and structural disorientation of the lumen. The endometrium, myometrium and perimetrium showed constricted structure leading to a complete loss of cellular architecture of the uterus on Day 5 as compared with control diestrous group uterus (Fig. 3a and b). On Day 10 regeneration of the uterine glands and thickening of the lumen was observed. The loss of

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**Fig. 1**—Effect of shed snake skin aqueous extract (SSAE; 10 mg kg⁻¹, sc) on serum hormones (LH, FSH, progesterone and estradiol) on female Swiss albino mice [Values are mean ± SE from 6 experiments each. One-way ANOVA followed by Tukey’s test. p values: * < p<0.05, ** P < 0.01, *** p < 0.001 control vs. Day 5 SSAE, control vs. Day 10 SSAE, SSAE Day 5 vs. Day 10 SSAE].

**Fig. 2**—Effect of shed snake skin aqueous extract (SSAE; 10 mg kg⁻¹, sc) on cytokines (IL-1β, TNF-α, IL-6, IL-12) on female Swiss albino mice [Values are mean ± SE from 6 experiments each. One-way ANOVA followed by Tukey’s test. p values: * < p<0.05, ** P < 0.01, *** p < 0.001 control vs. Day 5 SSAE, control vs. Day 10 SSAE, SSAE Day 5 vs. Day 10 SSAE].
cellular architecture to some extent was revived with thickening of the endometrial, myometrial and perimetrial lining as compared with control diestrous group uterus (Fig. 3 a and c).

SSAE (10 mg.kg\(^{-1}\), sc) produced a loss of mature follicle with increasing number of immature follicle. SSAE (10 mg.kg\(^{-1}\), sc) treated ovary showed loss of oocyte, zona pellucida layer, thinning of granulosa cell lining and antrum. This lead to a total structurally disoriented follicle and an increasing number of immature follicles on Day 5 as compared with control diestrous group ovary (Fig. 3 d and e). On Day 10 SSAE (10 mg.kg\(^{-1}\), sc) showed a complete loss of matured follicle with an increasing number immature follicles and structurally degenerated ovary as compared with control diestrous group ovary (Fig. 3 d and f).

**Discussion**

SSAE (5 mg.kg\(^{-1}\), sc) did not alter the normal estrous cycle of female Swiss albino mice. In 10 mg.kg\(^{-1}\), sc dose it caused a temporary cessation of the estrous cycle and altered the normal uterine and ovarian function. It caused temporary cessation at the diestrous phase when administered at estrous or diestrous phase for 10 days. SSAE (10 mg.kg\(^{-1}\), sc) significantly increased serum LH, FSH, progesterone on Day 5, which decreased significantly on Day 10. Serum estradiol level did not change significantly on Day 5, which increased significantly on Day 10.

At 10 mg.kg\(^{-1}\), sc dose SSAE significantly decreased IL1-\(\beta\), TNF-\(\alpha\) and IL-6 on Day 5. The decreased level of IL1-\(\beta\), TNF-\(\alpha\) was maintained on Day 10, IL-6 returned to normal level and on Day 10. SSAE (10 mg.kg\(^{-1}\), s.c) did not significantly change serum IL-12 levels on Day 5 and Day 10 respectively.

Histopathology of ovary and uterus showed structural disorientation.

The estrous cycle is mainly under the regulation of two steroidal hormones, estradiol and progesterone\(^9\). In the estrous cycle progesterone increases at two phases: at proestrous (LH dependent) and at diestrous phase which is LH independent. The progesterone is generally secreted from corpus luteum\(^7\) and is also marked by the presence of low level of LH, FSH and a rising estradiol. At the diestrous phase (also known as the follicular phase) the reproductive tract prepares for receipt of the

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Fig. 3—Effect of shed snake skin aqueous extract (SSAE; 10 mg. kg\(^{-1}\), sc) on female Swiss albino mice uterus and ovary. [a: Control diestrous uterus (showing normal uterine architecture), b: Day 5 diestrous uterus (X = absence of uterine glands; Y = distorted luminal structure), c: Day 10 diestrous uterus (U = reappearance of uterine glands; Z = thickened endometrium), d: control diestrous ovary (showing normal ovarian architecture), e: day 5 diestrous ovary: (X = distorted follicular structure; Y = absence of matured follicle; Z = follicles showing phase arrest), f: Day 10 diestrous ovary (U = distorted follicular structure; V = presence of immature follicle); H & E, 65X.
IFN-α is mainly coordinated through a mechanism involving estradiol and progesterone biosynthesis which is proportional to the number of immature follicles and fibrous structures. Both, structural and functional disorientation were observed in both ovary and the uterus. Persistent diestrous phase in SSAE (10 mg.kg⁻¹, s.c) treated mice on Day 5 which subsequently increased on Day 10, suggesting that SSAE might be involved in apoptosis of the uterus. Contrary to normal ovarian histology at the diestrous phase, there were increasing number of immature follicles in SSAE treated mice on Day 5 which subsequently increased on Day 10 suggesting that SSAE might be associated with the apoptogenic activity. Further studies on its detailed mechanism to establish its mechanism of action are in progress.

SSAE caused a temporary cessation at diestrous which delayed the onset of next reproductive cycle hence it may have the property to act as an anti-fertility agent in the near future. Further studies on the nature of the active principles present and its probable mechanism of action need to be studied. To conclude, this study for the first time reports that the shed snake skin (Naja naja) has the ability to interfere with the normal reproductive functions in female albino mice, through hormone/cytokine alteration, establishing the folk concept of using shed snake skin in health and diseases; further work is warranted to establish its anti-fertility property.

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Conflict of interest
The authors declare that there are no conflicts of interest.

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