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Insemination-driven epithelial remodeling and TLR4-mediated immune activation underlie sperm storage tubule maturation in young female kadaknath chickens

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Long-term sperm storage in the avian utero-vaginal junction (UVJ) is essential for reproductive efficiency, yet the developmental status of sperm storage tubules (SSTs) and their immunological regulation in young laying hens remains poorly understood. This study examined whether SSTs are functionally competent prior to insemination and how insemination influences their maturation in the indigenous Kadaknath chicken breed. Forty, six-month-old with no prior exposure to natural mating or artificial insemination hens were assigned to either non-inseminated controls or artificially inseminated groups. UVJ tissues were collected at 0, 1, 6, and 20 hours post-insemination and analyzed using histological and immunohistochemical techniques, with a focus on Toll-like receptor 4 (TLR4) expression. In non-inseminated hens, SSTs were sparse, structurally immature, and indicative of a non-functional state. Artificial insemination induced a highly organized, epithelial remodelling process involving invagination of ciliated mucosal folds, gradual loss of cilia, SST separation, lumen formation, and eventual establishment of mature, non-ciliated SSTs. Concomitantly, insemination led to a marked upregulation of TLR4 expression in the UVJ mucosa, whereas negligible expression was observed in non-inseminated hens. This insemination-dependent activation of innate immune signalling highlights a coordinated interaction between epithelial differentiation and immune responsiveness during SST maturation. Overall, the findings demonstrate that SSTs in young Kadaknath hens are developmentally primed but require insemination as a physiological trigger for structural and immunological maturation, offering valuable insights for fertility management and conservation of indigenous poultry breeds.

Keywords: Artificial Insemination, Hen, Sperm Host Glands, Toll Like Receptors , Utero-Vaginal Junction

Asian poultry breeds such as the Kadaknath chicken, native to central India especially the districts of Jhabua, Morena, and Sheopur in Madhya Pradesh[1] are valued for their resilience to environmental stress, making them well suited for sustainable poultry production in tropical regions[2,3]. Kadaknath, a rare indigenous Indian breed known for its black meat and medicinal significance[4], has gained renewed importance in backyard farming[5] due to its reproductive persistence, comparatively higher egg production[6], low egg cholesterol[7], and antioxidant-rich meat[8], all of which enhance its productivity and consumer appeal.

Despite the reproductive advantages of sperm storage in birds, the microanatomy and immune regulation of the oviduct in indigenous breeds remain poorly understood. The avian oviduct consists of five distinct regions (infundibulum, magnum, isthmus, uterus or shell gland and vagina) and uniquely supports long-term sperm storage through sperm storage tubules (SSTs) located at the utero-vaginal junction, which are critical for sustained fertility[9]. However, the developmental maturity and functional status of SSTs in young, non-mated or non-inseminated laying hens are largely unknown, and detailed histological characterization of sperm storage tubule (SST) development has been hindered by the thickness and opacity of the oviductal walls. Immune regulation in the female reproductive tract is crucial after insemination, with the innate immune system particularly Toll-like receptors playing a key role in balancing defense[10,11] and reproductive function. Toll-like receptor-4 (TLR4) a transmembrane pattern recognition receptor is especially important in initiating immune signalling in response to physiological challenges such as insemination and may influence sperm survival, epithelial remodeling, and the functional activation of sperm storage tubules in the utero-vaginal junction[12].

This study addresses the lack of information on the developmental status of sperm storage tubules (SSTs)

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and their immune characteristics in young laying hens, particularly in the indigenous Kadaknath breed. It aims to assess the histological and functional status of SSTs and associated Toll-like receptor 4 (TLR4) expression in non-inseminated hens at the onset of egg laying, and to determine whether artificial insemination can stimulate the development and functional activation of previously non-functional SSTs in the utero-vaginal junction.

Materials and Methods

Experimental design and animal husbandry

A total of 40 (n=40) healthy, six-month-old female Kadaknath chickens, at the initial stage of their egg-laying phase, were selected for the experiment. Importantly, all females had no prior exposure to natural mating or artificial insemination, ensuring assessment of SST status before any sperm-related stimulation. Additionally, five, one-year-old healthy male Kadaknath chickens were used solely as semen donors.

All birds were housed at the Livestock Farm, Faculty of Veterinary and Animal Sciences, Banaras Hindu University under standard management conditions. Female and male birds were maintained separately and allowed a three-week acclimatization period prior to the commencement of the experiment. After acclimatization, the females were randomly divided into four experimental groups (n = 10 per group). One group served as the control and remained non-inseminated to evaluate the baseline structural and immunological status of SSTs. The remaining three groups were subjected to artificial insemination and sampled at different post-insemination intervals (1, 6, and 20 hours) to examine insemination-induced changes in SST development and immunological responses.

Semen collection and artificial insemination

Semen was collected from five male Kadaknath chicken using the abdominal massage technique. Briefly, gentle pressure was applied to the abdominal region and cloaca to obtain ejaculates, which were collected in sterile containers. Immediately after collection, semen was diluted in a 1:1 ratio with sterile normal saline to ensure uniformity and viability. Artificial insemination was performed on 30 experimental females via the vaginal route using freshly diluted semen. The control group (n = 10) was not inseminated and served to assess the inherent, unstimulated condition of SSTs in young laying hens.

Tissue collection and fixation

The experimental protocol was approved by the Institutional Animal Ethics Committee (Ref. no.: IAEC/RGSC-BHU/2020-21/35), and all procedures were conducted in accordance with prescribed ethical guidelines. Tissue samples from the utero-vaginal junction (UVJ), the region known to harbor SSTs, were collected from birds at 0 hour (control, non-inseminated), and at 1, 6, and 20 hours post-insemination. These time points were selected to capture early histological and immunological changes associated with insemination-induced stimulation of SSTs. Collected tissues were immediately fixed in 10% neutral buffered formalin for further processing.

Histological processing and staining

Fixed tissue samples was processed using the standard alcohol–xylene–paraffin embedding protocol. Transverse sections of 5 µm thickness were cut using a rotary microtome. Sections were stained with Hematoxylin and Eosin (H&E) for general histological evaluation and Masson's trichrome stain[13] to highlight connective tissue components and overall histoarchitectural organization, with particular emphasis on the morphology, distribution, and developmental status of SSTs.

Slide preparation for immunohistochemistry

Microscope slides with frosted ends (Borosil, India) were thoroughly cleaned using a detergent solution followed by triple rinsing in distilled water. Slides were air-dried overnight under dust-free conditions and subsequently coated with a 2% solution of (3-aminopropyl) triethoxysilane prepared in acetone for 30 minutes to enhance tissue adhesion. After a brief acetone rinse, slides were dried at 37°C for 24 hours and stored until use.

Immunostaining procedure

Immunohistochemistry was performed[14] to assess the immunological status of the utero-vaginal junction, particularly focusing on Toll-like receptor 4 (TLR4) expressions as a marker of immune responsiveness potentially associated with SST activation.

Fixation

Tissue samples were fixed in 10% neutral buffered formalin for 72 hours.

Processing

Tissues were dehydrated, cleared, and embedded in paraffin using the alcohol–xylene–paraffin method.

Sectioning

Paraffin blocks were sectioned at 5 µM thickness and mounted on adhesive-coated slides, which were kept overnight at room temperature (37°C).

Deparaffinization

Sections were deparaffinized in three changes of xylene (3 minutes each).

Rehydration

Slides were passed through graded alcohols (absolute, 90%, and 80%) and finally rinsed in distilled water.

Antigen Retrieval

Heat-induced antigen retrieval was performed in citrate buffer (pH 6.0) using a microwave oven at 700 W for 20 minutes. (approximately 100°C).

Primary Antibody Incubation

Sections were incubated overnight at 4°C with rabbit anti-TLR4 primary antibody (Sigma-Aldrich, USA) diluted 1:200.

Detection

Immunoreactivity was visualized using an Anti-Rabbit HRP-DAB detection kit (R&D Systems, Minneapolis, MN, USA), following the manufacturer's instructions.

Counterstaining

Sections were counterstained with Meyer's hematoxylin, dehydrated, cleared, and mounted.

This immunostaining approach enabled the evaluation of insemination-induced immunological changes in the UVJ and provided insights into the potential role of immune signaling in the functional maturation of SSTs.

Results

Gross Structural Observations

The avian oviduct in Kadaknath chickens was clearly differentiated into the infundibulum, magnum, isthmus, uterus (shell gland), and vagina. The utero-vaginal junction (UVJ), situated at the interface between the uterus and vagina, displayed a distinct gross appearance. While both the uterus and vagina exhibited a comparatively darker coloration, the UVJ appeared conspicuously lighter, allowing for clear anatomical demarcation of this specialized region (Fig. 1 and Fig. 2).

Histological Findings

Histological examination of the utero-vaginal junction revealed the characteristic organization of a tubular organ composed of four well-defined layers (Fig. 3)

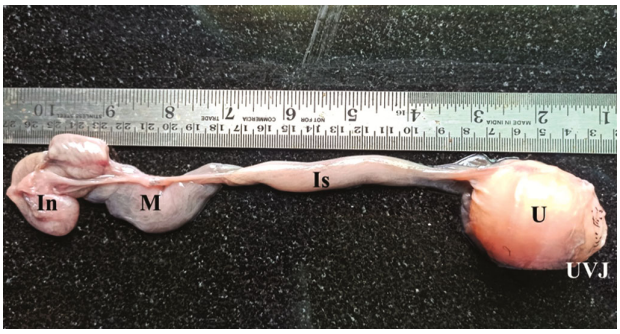

Fig. 1 — Photograph showing the different parts of the oviduct in Kadaknath Chicken. The labeled parts include the infundibulum (In), magnum (M), isthmus (Is), uterus or shell gland (U), and uterovaginal junction (UVJ).

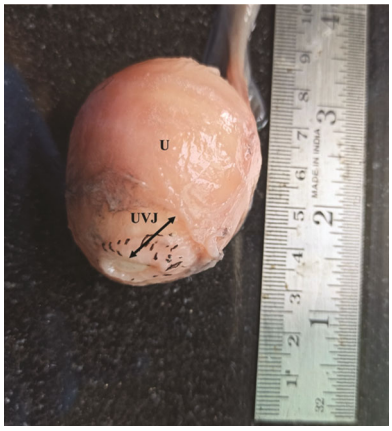

Fig. 2 — Photograph showing the uterovaginal junction in Kadaknath Chicken. The labeled parts include uterus or shell gland (U) and and uterovaginal junction (UVJ).

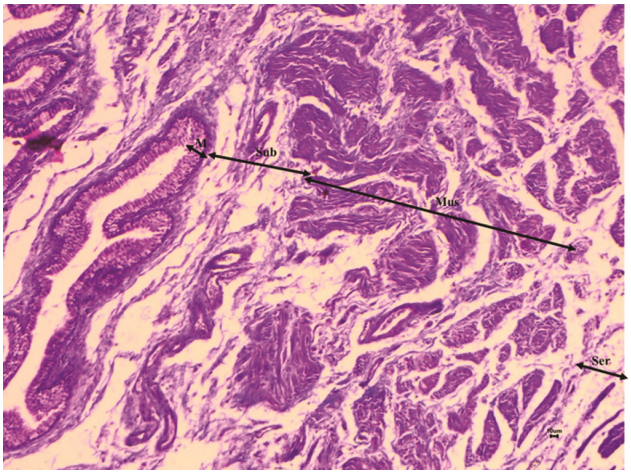

Fig. 3 — Light micrograph showing the different histological parts of the uterovaginal junction in the Kadaknath Chicken. The labeled parts include mucosa (M), submucosa (Sub), muscularis (Mus) and serosa (Ser). 20 hours 10 x masson`s trichome

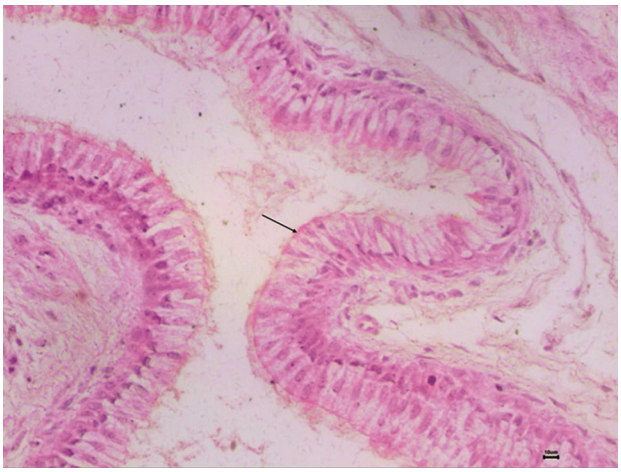

Fig. 4 — Light micrograph showing the histology of mucosa (ciliated pseudostratified columnar epithelium) at uterovaginal junction in the Kadaknath Chicken, the mucosal epithelium shown by arrow. 6 hours 40 x (Hematoxylin and Eosin)

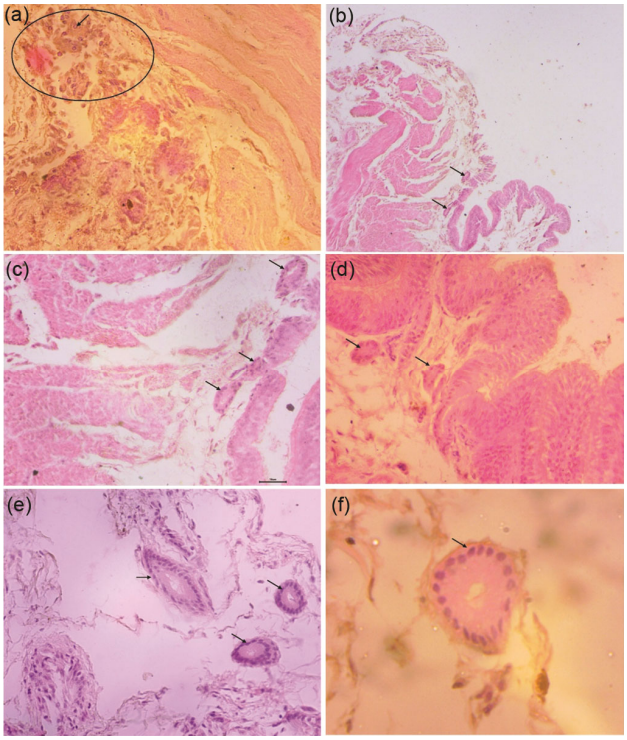

Fig. 5 — Light Micrograph showing sequential development of sperm storage tubules (hematoxylin and eosin stain) **a:**40x control birds very small size of sst inside the (circle and arrow) **b**:10x 20h chain of sst developing from mucosa (arrows) **c:** same as **b** but at 40x **d:**40x 20h sst without canal (arrows) **e:**40x 1h sst with canal **f:** 100x 1h sst (arrow)

- ***Mucosa***

Lined by pseudostratified ciliated columnar epithelium interspersed with secretory (goblet) cells (Fig. 4).

- ***Submucosa***

Contained developing sperm storage tubules (SSTs), lined by simple columnar epithelium with a narrow or absent lumen.

- ***Muscularis and Serosa***

Formed the outer structural layers of the UVJ.

In the non-inseminated group, SSTs were poorly developed and sparsely distributed, becoming discernible only under higher magnification (≥40X). In contrast, inseminated birds exhibited a noticeable increase in SST prominence and structural differentiation, although spermatozoa were not observed within the SST lumina at the examined time points.

Sequential Development of Sperm Storage Tubules

A stepwise transformation of the mucosal epithelium into mature, non-ciliated sperm storage tubules was documented in inseminated birds. Five distinct developmental stages were identified (Fig. 5, Diagram 1)

Mucosal Fold Stage

The mucosa consisted of ciliated pseudostratified columnar epithelium forming prominent mucosal folds.

Invagination of Mucosal Fold

Localized epithelial invagination occurred, marking the initiation of SST development.

Formation of SST Attached to Mucosal Fold

A nascent SST structure emerged, accompanied by progressive loss of cilia and epithelial differentiation.

Separation of SST without Lumen

The SST detached from the mucosal fold and adopted a circular profile; however, a central lumen was not yet evident.

Lumen Formation (Canalization)

Development of a distinct lumen completed the transformation into a mature, functional, non-ciliated SST.

These sequential changes indicate that artificial insemination acts as a stimulus for morphological maturation of SSTs in young laying hens.

Immunohistochemical Analysis

Immunohistochemical evaluation demonstrated strong positive expression of Toll-like receptor 4 (TLR4) in the mucosal epithelium of the utero-vaginal junction in all inseminated birds (Fig. 6). In contrast,

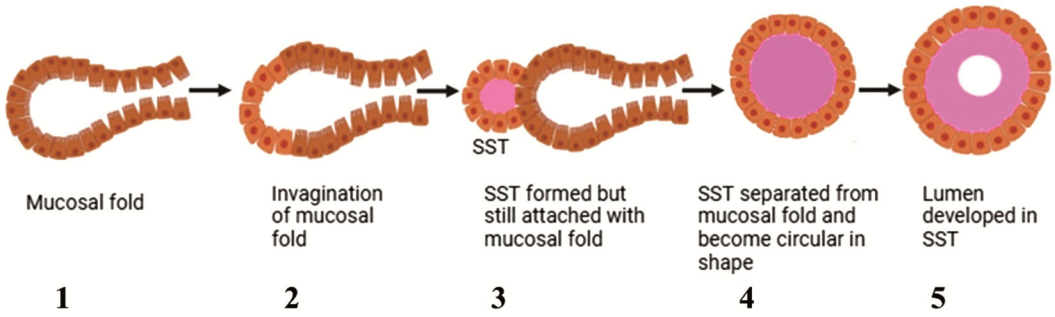

Diagram. 1 A stepwise transformation of the mucosal fold into mature, non-ciliated SSTs: 1. Formation of mucosal fold 2. Invagination of Mucosal Fold 3. Formation of SST (Attached to the Mucosal Fold) 4. Separation of SST without lumen 5. Lumen Formation in the SST (canalization of SST)

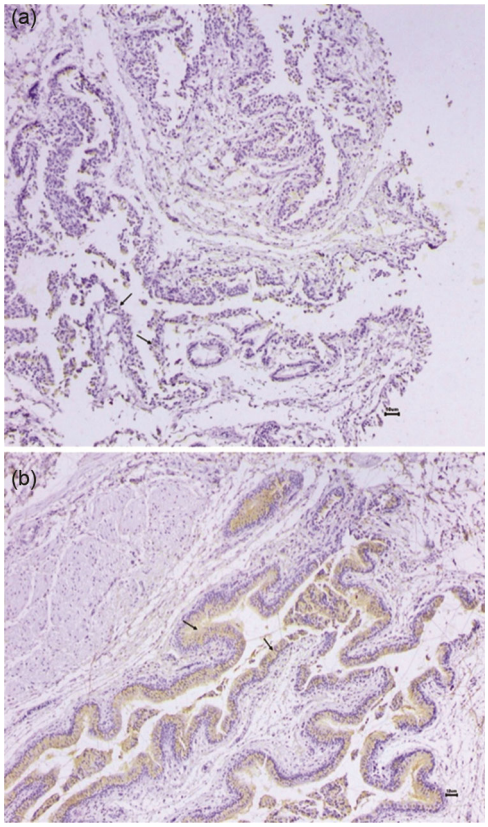

Fig. 6 — Light Micrograph showing expression of TLR4 at the mucosa of uterovaginal junction in the Kadaknath Chicken. a:10x control, TLR4 negative (arrows) b:10x 20h, TLR4 positive (arrows)

negligible to absent TLR4 immunoreactivity was observed in the non-inseminated group. This differential expression suggests insemination-induced activation of innate immune signalling pathways within the UVJ.

Discussion

The present study provides compelling evidence that sperm storage tubules (SSTs) in young Kadaknath chickens at the onset of egg laying, these structures are structurally immature and functionally inactive before inseminationn. Artificial insemination initiates a coordinated sequence of epithelial remodeling and innate immune activation, culminating in the formation of mature SSTs capable of supporting sperm storage. This dual structural–immunological response represents a critical preparatory phase in avian reproductive physiology, consistent with recent advances highlighting the dynamic plasticity of the avian female reproductive tract in response to reproductive cues[15,16].

The SSTs in non-inseminated birds were sparse, underdeveloped, and detectable only at high magnification (40X and higher) suggests that SST formation in young hens is not fully autonomous. Instead, SSTs appear to exist in a primed but quiescent state, awaiting physiological stimulation. This finding extends earlier observations that SSTs can be present in prepubertal pullets as epithelial invaginations[17], but may lack functional competence until reproductive activation[18,19]. Recent ultrastructural and transcriptomic studies further support this concept, demonstrating that SST epithelial differentiation and secretory activity are significantly enhanced only after exposure to semen or mating stimuli.[20,21]

The absence of a defined lumen in control group further supports the interpretation that these SSTs are structurally immature. Lumen formation is considered essential for sperm accommodation and maintenance, and its absence likely precludes effective sperm storage[22,23]. Contemporary studies using ultrastructural and immunohistochemistry have emphasized canalization as a hallmark of functional SST maturation, closely associated with changes in epithelial polarity and junctional remodeling.[24]

Artificial insemination triggered a highly ordered morphogenetic sequence transforming ciliated mucosal folds into non-ciliated, canalized SSTs. The progressive loss of cilia during epithelial invagination represents a functional transition from a transport-oriented epithelium to a storage-specialized epithelium. Ciliated cells facilitate sperm movement along the oviduct, whereas non-ciliated SST epithelial cells create a relatively stable microenvironment conducive to prolonged sperm survival.[25,26] Recent molecular analyses indicate that this transition is accompanied by altered expression of cytoskeletal and adhesion-related genes, reinforcing the concept of active epithelial reprogramming during SST formation.[27]

The circular morphology of detached SSTs likely minimizes surface area relative to volume, reducing exposure to luminal stressors and immune effector molecules.[28,29] Such architectural optimization is might be contributed to sperm quiescence and longevity within the female reproductive tract.[30] This interpretation is supported by recent functional studies demonstrating reduced oxidative stress and metabolic activity of sperm within mature SSTs compared with luminal spermatozoa.[31,32]

Although spermatozoa were not detected within SST lumina at early post-insemination intervals, the observed canalization indicates structural readiness for sperm entry and retention. This suggests that SST maturation precedes, and possibly facilitates, stable sperm storage. Similar temporal dissociation between structural maturation and sperm occupancy has been reported in other avian species, suggesting a conserved preparatory mechanism.[33,34] One of the most significant findings of this study is the insemination-dependent upregulation of Toll-like receptor 4 (TLR4) in the UVJ mucosa. TLR4 is a key pattern recognition receptor that detects both microbial ligands and endogenous danger-associated molecular patterns. Its expression following insemination indicates activation of innate immune pathways in response to semen or spermatozoa. Recent studies in avian and mammalian systems have highlighted TLR4 as a critical mediator of post-mating immune modulation rather than classical inflammation.[35,36]

Importantly, this immune activation should not be interpreted solely as a defensive response. Instead, emerging evidence suggests that controlled activation of innate immune signaling within the reproductive tract plays a role in tissue remodeling, immune tolerance, and regulation of sperm–female tract interactions.[33,37] In this context, TLR4 activation may facilitate epithelial differentiation, modulate local cytokine environments, and contribute to the establishment of a balanced immune state that permits sperm survival while maintaining mucosal integrity. Recent work has further demonstrated that TLR4-associated cytokines, such as IL-6 and TGF-β, are directly involved in epithelial repair and differentiation within reproductive tissues.[38,39]

The absence of TLR4 expression in non-inseminated birds underscores the tight coupling between reproductive exposure and immune readiness. Together with histological findings, this suggests that SST maturation is governed by a coordinated interplay between structural remodeling and immune signaling. Such coupling between epithelial morphogenesis and innate immunity has recently been proposed as a conserved reproductive strategy across vertebrates.[40,41]

From an evolutionary and applied perspective, the ability of insemination to activate both epithelial and immune pathways underscores the efficiency of avian reproductive strategies. For indigenous breeds such as Kadaknath, understanding the timing and regulation of SST maturation has practical implications for optimizing artificial insemination protocols, improving fertility outcomes, and designing conservation-oriented breeding programs. Recent advances in indigenous poultry reproductive management further emphasize the importance of aligning insemination timing with reproductive tract readiness to maximize fertility and genetic conservation[42-44].

Conclusion

This study advances the understanding of avian reproductive biology by demonstrating that sperm storage tubules in young Kadaknath chickens are developmentally primed but functionally immature prior to insemination. Artificial insemination acts as a decisive physiological cue that synchronizes epithelial morphogenesis and innate immune activation, establishing a functional sperm storage niche in the utero-vaginal junction.

Funding statement

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Conflict of interest

The authors declare that they have no conflict of interest.

Ethical statement

The necessary approval from the Institutional Animal Ethics Committee (Ref. no.: IAEC/RGSC-BHU/2020-21/35) was obtained prior to the commencement of the experiment, and all applicable guidelines were strictly followed throughout the study.

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