

Development of spermatogonial stem cell niche and immunoexpression of vimentin filaments in the testes of prenatal and postnatal Indian buffalo (*Bubalus bubalis*)

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Abstract: In this study, we established the components of the buffalo spermatogonial stem cell (SSC) niche in prenatal (38.5–280 days of gestation), neonatal, prepubertal, and adult testes histochemically and immunohistochemically. Immunohistochemically, the changes in vimentin expression were evaluated. During prenatal life, the niche of primordial germ cells was formed by the surrounding mesenchymal cells and fibroblasts. The basement membrane (BM) was not organized at this stage. At 7.6 cm crown-rump length, testicular cords with a thin BM were seen. At the late gestational period, testicular cords with well-organized BM were seen. At the neonatal stage, the testicular cords were lined with distinct BM, while during the prepubertal period, distinct BM with peritubular myoid cells (PMCs) were seen. In adult testes, the seminiferous tubules were surrounded by a thick BM surrounded by PMCs. PM cells were only single-layered as seen in H&E-stained paraffin sections, picosirius red-stained, and Masson's trichrome-stained paraffin sections. Collagen fibers were seen to increase quantitatively to provide strength and cushioning to the developing niche of spermatogonial stem cells in the testes. The expression of vimentin increased to a certain extent until adult life, indicating its significant role as an intermediate filament during development.

Key words: Buffalo, spermatogonial stem cell niche, prenatal, postnatal, testis, vimentin

1. Introduction

Spermatogonial stem cells (SSCs) are the foundation of spermatogenesis and male fertility. They are tissue-specific stem cells committed to the establishment and maintenance of spermatogenesis with the abilities of both self-renewal and generation of large numbers of haploid cells. The perpetuation of stem cells (SCs) is one of the main factors for the maintenance of tissue homeostasis in mammals and other vertebrates. The microenvironment that regulates the plasticity and fate of stem cells is known as the "SC niche", and SSCs are located in a dynamic microenvironment that influences all aspects of SC function, including homing, self-renewal, and differentiation [1]. The niche determines cell fate by providing various crucial factors including physical support, nutrients, and hormonal and paracrine signals, all of which are essential for successful spermatogenesis [2]. The SSC niche is located at the basal layer of the seminiferous epithelium and is composed mainly of Sertoli cells and a basal lamina covered by so-called peritubular myoid cells (PMCs). Leydig cells, a few stroma cells of mesenchymal origin, a soft extracellular matrix, and lymphatic and blood capillaries occupy the

interstitial space among seminiferous tubules (STs). All these components contribute together to create the SSC microenvironment, which regulates many aspects of stem cell functions, such as self-renewal, differentiation, and apoptosis [3]. PM cells together with the basement membrane (BM) constitute a physical barrier that provides structural support of the STs [4]. One of the main components of this physical barrier is vimentin [5].

There are three main kinds of cytoskeletal filaments in eukaryotic cells: microfilaments, microtubules, and intermediate filaments. Intermediate filaments help to provide structure to cells and are involved in cell movement. Vimentin, having 466 amino acids, is a 57-kDa class III intermediate filament protein encoded by the *VIM* gene generally present in mesenchymal cells. Vimentin plays a significant role in holding cellular structures of the organelles in the cytosol. This protein has a flexible nature, allowing it to respond to mechanical stress. It interacts with other structural proteins, like microtubules, to make the cell rigid and sturdy. Vimentin is attached to the nucleus, endoplasmic reticulum, and mitochondria, either laterally or terminally. As an organizer of a number

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of critical proteins, vimentin is involved in attachment, migration, and cell signaling [6].

Any disturbance of the SSC microenvironment can lead to the loss or reduction of fertility, and the dysfunction of any of its components can lead to the loss of tissue homeostasis and subsequently to different pathological conditions, ranging from degenerative diseases to cancer [4].

Thorough knowledge of vimentin in the male germ line SC microenvironment during prenatal and postnatal life will help us better understand the stem cell regulation in testes, which will be useful in enhancing male fertility, a model for human stem cell biology and for the protection of wild bovids threatened by extinction. Keeping in view the importance of the niche of SSCs in buffalo, the present research work was aimed at the characterization of vimentin in the SSC niche in prenatal and postnatal buffalo testes. In the investigation, observations were recorded on vimentin to answer one main question: Does the quantity or expression of vimentin in the microenvironment/niche of SSCs change during its development? The present study was designed to answer the pertinent question and will contribute to the understanding of the role of vimentin in SSC biology throughout the testicular developmental process in mammals.

2. Material and methods

2.1. Materials

2.1.1. Collection of embryos/fetuses and estimation of age

Embryos/fetuses of different gestational ages were collected from buffaloes presenting with dystocia at the Teaching Veterinary Clinical Complex of Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, Punjab, and abortion cases from livestock farm of the university and different farms in and around Ludhiana. Ages of fetuses were recorded either by the history of insemination/natural service or by measuring the curved crown-rump length (CVRL). The fetal body was measured with a calibrated inelastic thread as a curved line along the vertebral column between the most anterior parts of the frontal bone to the rump at ischiatic tuberosity and designated as CVRL. After measuring the CVRL the approximate ages were calculated by using the formula suggested earlier [7]:

$$Y = 28.660 + 4.496 X \text{ (when CVRL is } < 20 \text{ cm),}$$

$$Y = 73.544 + 2.256 X \text{ (when CVRL is } > 20 \text{ cm),}$$

where Y is age in days and X is CVRL in cm.

2.1.2. Grouping of embryos/fetuses

Embryos and fetuses were classified into three groups based on the CVRL measurements representing early, mid, and late gestational stages:

Group I: Embryos and fetuses of up to 20 cm CVRL,

Group II: Fetuses of more than 20 cm up to 40 cm CVRL,

Group III: Fetuses of more 40 cm CVRL.

2.1.3. Collection of testes from postnatal life and grouping

The testes of buffaloes of all other age groups consisting of neonatal, prepubertal, and adult buffaloes were collected from M. K. Overseas, Dera Bassi, the local abattoir at Bareilly, and from buffaloes presenting with dystocia at the Teaching Veterinary Clinical Complex of Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, Punjab. They were grouped into neonatal, prepubertal, and adult animals. The testes were utilized for histomorphological, histochemical, and immunohistochemical studies. Testes were located either in the inguinal area or in the scrotum. The scrotum or inguinal canal was opened to collect the testes from neonatal calves and the scrotum was opened to collect the testes from prepubertal calves and pubertal adult buffalo bulls. Animals aged more than 20 months were tentatively classified in the adult stage, which was confirmed later by a histological picture of the testis with the presence of active spermatogenesis.

2.2. Methods

2.2.1. Histomorphological studies

The embryos/fetuses and testes of buffaloes were fixed in 10% neutral buffered formalin and processed for paraffin sectioning by dehydration in ascending grades of alcohols and acetone, and were then cleared in benzene and infiltrated and embedded in paraffin [8]. Sections were cut at 4–5 μ m thickness for histological study and were subjected to hematoxylin and eosin staining for histomorphological details and Masson's trichrome staining for collagen fibers [8]. Sections were also stained for collagen fibers by picosirius red [9].

2.2.2. Immunohistochemical studies

The polymer-based horseradish peroxidase method was used for immunostaining as described earlier [10]. Sections in duplicate were mounted on Super Frost positively charged slides (Fisher Scientific). After dewaxing and rehydration, heat-induced antigen retrieval was done in citrate buffer (AR 3 solution, BioGenex) with heating in a microwave oven at 95 °C for 10 min and 98 °C for 5 min. Slides were then left for 30 min in the hot buffer and washed in 0.1 M phosphate-buffered saline (pH 7.4). The endogenous peroxidase activity was blocked by immersing the sections in 3% (v/v) H_2O_2 in methanol for 20 min followed by washing in 0.1 M phosphate-buffered saline (pH 7.4). To prevent nonspecific binding of antibodies, sections were blocked with normal horse serum (Vector's Laboratories, USA). The sections were incubated with primary antibodies (antivimentin by

BioGenex Laboratory) at 4 °C overnight in a staining box. After washing in 0.1 M phosphate-buffered saline (pH 7.4), the sections were incubated with a universal secondary antibody (Vector Laboratories, USA). The chromogen used was 3,3'-diaminobenzidine tetrahydrochloride (DAB) (Vector Laboratories, USA) with Gill's III hematoxylin counterstaining. The sections were washed in running tap water, dehydrated, cleared, and mounted with DPX.

2.2.3. Microphotography

For each section photomicrographs were taken at different magnifications by bright-field microscope with an attached camera and photography unit (Eclipse 80i, Nikon, Japan).

3. Results and discussion

Results of the present investigation were divided into two major developmental stages, namely prenatal and postnatal, to emphasize the changes in the microenvironment of the germ cells through all stages.

3.1. Histomorphology and immunohistochemistry

3.1.1. Prenatal life

This study was conducted on fetuses from 38.5 days to 280 days of gestation to analyze the development of testicular cords and their components. Upon opening the fetuses, testes were located in different areas in fetuses of various age groups.

3.1.1.1. Early prenatal life (CVRL 1.7 to 20 cm)

During early prenatal development (in 2.2 cm, 2.7 cm, 3.7 cm, and 4 cm CVRL fetuses) the primordial germ cells (PGCs) were surrounded by the mesenchymal cells from the gonadal ridge, developing fibroblasts and nucleated RBCs (Figures 1A–1D). Few PGCs were seen in between the germinal epithelium as they migrated to the gonadal ridge. At these developmental stages, the germ cells did not show permanent contact with the other cells. In bovines the forming testicular cords are rapidly surrounded by a marked basal lamina and a layer of peritubular cells in 3.5 cm CVRL fetuses, and these newly differentiated testicular cords are lined by two types of cell populations: a large number of dark polygonal cells with irregular nuclei, pre-Sertoli cells, and a small number of large, light, round cells with relatively round nuclei, the PGCs [11].

At 5.2 cm CVRL, the testicular cords were not formed but were in a state of formation. The germ cells and Sertoli cells were seen in the forming testicular cords (Figure 1E). These cords were surrounded by a thin layer of BM that consisted of collagen fibers as stained by picrosirius red (Figure 1F). Similar findings of fine wavy collagen fibers were observed by Basavaiah [12] between tubules at 57 days of gestation in sheep. Immunoeexpression of vimentin was observed in the BM (Figures 1G and 1H). A few cells in the testicular cords were also positive for vimentin. These cells could be the Sertoli cells.

At 7.6 cm CVRL, the PGCs were seen in the developing testicular cords (Figures 1I). At this stage, these cells were surrounded by the Sertoli cells. The niche was provided by Sertoli cells, which in turn were in the cords and were confined by the BM from the connective tissue and blood vessels present in the space between the testicular cords. At places around the BM, PMCs were seen. Similar findings were reported by Kaur et al. [13] and Singh et al. [14] in buffalo, in goat [15], and by Basavaiah in sheep [12] at 69 days of gestation. The BM consisted of mainly collagen fibers (Figures 1J and 1K). Immunohistochemical localization of vimentin was observed in the BM and also in some of the Sertoli cells (Figure 1L).

At 9.5 cm CVRL, fully formed testicular cords are visible surrounded by a distinct BM and PMCs as seen in Figure 1M. The mediastinum testis can be seen at this stage as a structure of the testes devoid of cord formation. The size of the cords was increased and gonocytes were seen at this stage in the center of these cords. The gonocytes were surrounded and supported by Sertoli cells, which were present on the BM. Basavaiah [12] reported that spermatogenic cells in the sheep fetus were located on the BM of tubules and consisted of spherical and heterochromatic nuclei, and that the spermatogonia present towards the lumen were larger in size with spherical nuclei that were euchromatic as compared to the basal spermatogonia. The BM and developing tunica albuginea were made up of collagen fibers (Figure 1N). Immunoeexpression of vimentin is observed in the BM and cytoplasm of Sertoli cells.

At 19 cm CVRL, organized testicular cords are seen. The BM surrounding the cords was seen to have developed in thickness and collagen content, which was seen when stained by picrosirius red. Immunopositive reaction to vimentin antibodies was seen expressed by the BM, PMCs, and Sertoli cells.

3.1.1.2. Mid prenatal life (CVRL 20 to 40 cm)

At 20 cm CVRL, gonocytes were seen in the center and towards the periphery of organized seminiferous cords, which were increased in size. The BM was surrounded by a layer of PMCs. Development of the collagen system of the BM, PMCs, and testicular parenchyma was seen with picrosirius red stain. Immunopositive reaction to vimentin antibodies was expressed by the BM, PMCs, and Sertoli cells.

3.1.1.3. Late prenatal life (CVRL >40 cm)

At 8 and 8.5 months of gestation, well-organized and tightly placed seminiferous cords are seen in the parenchyma of testes (Figure 2A). A clear demarcation between the testicular parenchyma and mediastinum testis can be seen at this stage, which is seen more clearly with picrosirius red stain (Figures 2B and 2C). A strong immunopositive reaction for vimentin is seen at this stage in the BM, PMCs,

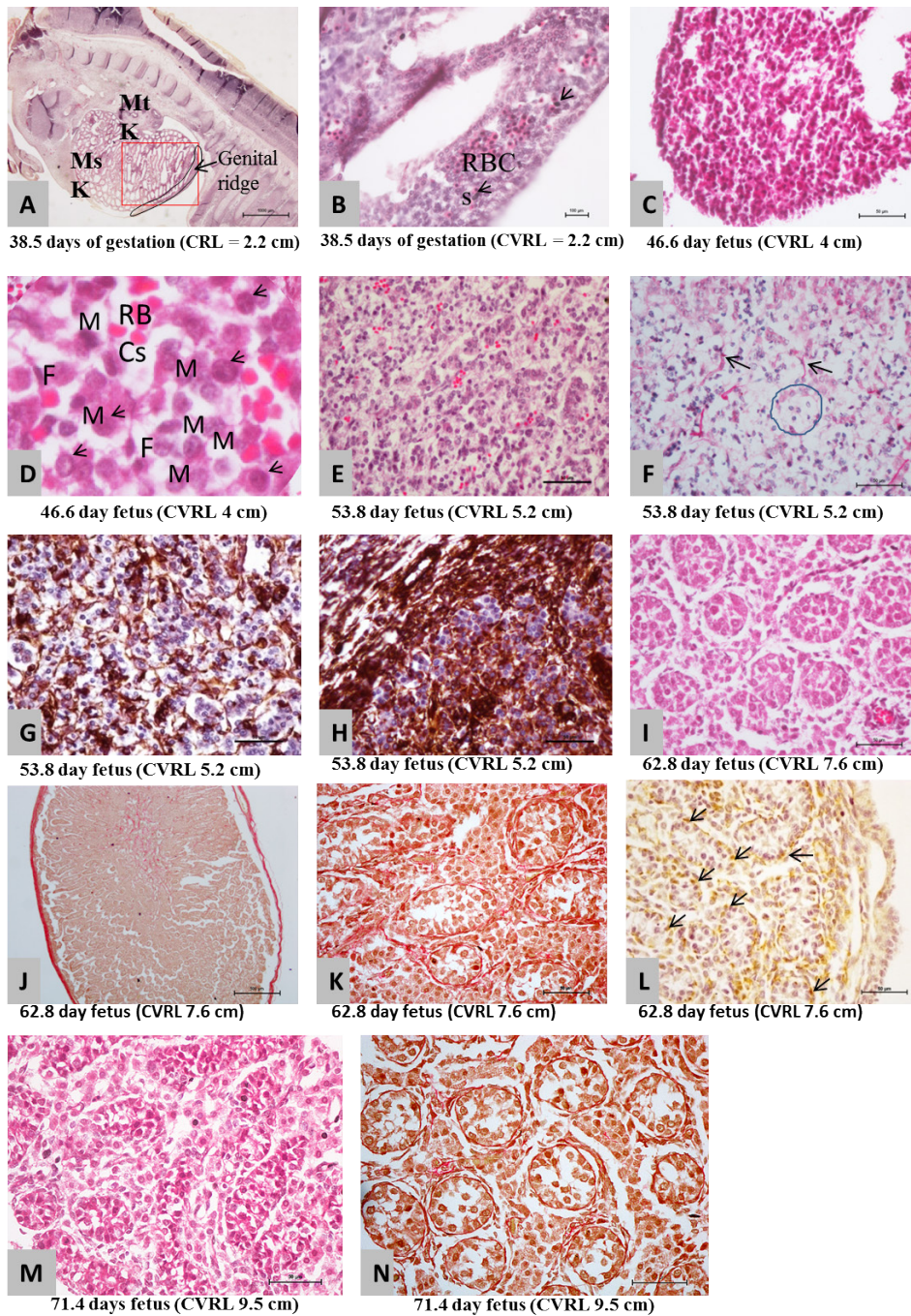


Figure 1. A) Sagittal section of approximately 38.5-day-old fetus (CVRL 2.2 cm) showing largely developed mesonephros (Ms K) and smaller undeveloped metanephros (Mt K). Genital ridge (outline) is located on the ventromedial aspect of the mesonephros. H&E, 40 \times . B) 38.6-day-old fetus (CVRL 2.2 cm) showing genital ridge comprising mesenchymal cells, immature RBCs, differentiating fibroblasts, and PGCs (arrow). H&E, 400 \times . C) Cross-section of indifferent gonad from the fetus at 4 cm CVRL. H&E, 400 \times . D) Magnified image of 1C showing the presence of mesenchymal cells (M), fibroblasts (F), immature RBCs, and PGCs (arrows). E) 53.8-day-old fetus (CVRL 5.2 cm) showing the different cells forming the niche during prenatal life. H&E, 400 \times . F) Formation of circular patterns (outline) had begun by this stage of prenatal life with the help of collagen fibers (arrow). Picosirius red, 400 \times . G) Immunostaining with vimentin showing intense positive staining and elucidating the beginning of the formation of cords, 400 \times . H) Developing tunica albuginea along with tunica vasculosa stain intensely positive for vimentin, 400 \times . I) CVRL 7.6 cm fetus showing well-organized tubules with defined BM. H&E stain, 400 \times . J) CVRL 7.6 cm fetus showing the presence of collagen fibers in tunica albuginea. K) Distinct BM is seen, which is composed of collagen fibers (CVRL 7.6 cm). Picosirius red, 400 \times . L) Immunostaining for vimentin gives a positive reaction in the BM and cytoplasm of cells located on the BM. These cells may be possible Sertoli cells (arrow); 400 \times . M) 71.4-day-old fetus (CVRL 9.5 cm) showing organized testicular cords with PGCs (arrow) localized in the center of cords while a few gonocytes are seen towards the periphery. H&E, 400 \times . N) 71.4-day-old fetus (CVRL 9.5 cm) showing organized testicular cords surrounded by BM composed of collagen fibers. Picosirius red, 400 \times .

and Sertoli cells. Prenatal expression of vimentin has also been established in fetal human testes and confirmed as a marker of Sertoli cells [16].

3.1.2. Postnatal life

3.1.2.1. Neonatal stage

Neonatal testes were composed of freely spaced seminiferous cords placed in connective tissue with Leydig cells, surrounded by tunica vasculosa and tunica albuginea. The seminiferous cords were encompassed by a distinct BM, on which the germ cells and Sertoli cells rested. The BM was surrounded by very fine collagen fibers as seen with Masson's trichrome staining (Figure 2D). A layer of PMCs was also seen surrounding the BM and the fibers around it. These PMCs were observed to be located near the Sertoli cells and germ cells as they support ST development and function, including the deposition of the BM of the tunica propria. Staining of sections with picosirius red stain confirmed the presence of collagen fibers and also outlined the wavy structure of the BM. Collagen fibers were also seen stained in the intertubular tissue. Immunostaining for antivimentin (Figures 2E and 2F) gave strong positive results in the BM, Sertoli cells, and intertubular tissue, indicating the role of the protein in stabilizing cytoskeletal interactions as the testes develop.

3.1.2.3. Prepubertal stage

Formation of the lumen is noticed to have started by this stage, indicating that the animal is about to attain puberty and that spermatogenesis will start soon. The seminiferous cords are now called STs as they attain the tube-like structure into which spermatozoa will be released after the completion of each spermatogenesis cycle. STs are defined by their BM, which was surrounded by a layer of PMCs. These cells were located at places near Sertoli cells and SSCs, just as in neonatal stage (Figure 2G). Intertubular tissue at this stage was well developed and the STs were placed tightly next to each other in the connective tissue as compared to neonatal life. The capillary plexus, blood vessels, and lymph vessels were also well developed in the intertubular tissue in this stage. Leydig cell density was increased to some extent in this stage. Presence of collagen is confirmed by brightly stained wavy BM and connective tissue as seen when a section is stained with picosirius red stain (Figure 2H), while the cells present in the STs show slight staining indicating the need of collagen in the BM to maintain the integrity of the tubules. Staining with Masson's trichrome stain (Figure 2I) further confirmed the presence of collagen fibers around the BM. Immunostaining with antivimentin yields positive results in Sertoli cells, PMCs, and BM of the prepubertal testicular tissue section. A positive result is also seen in the lining of blood vessels and capillaries (Figures 2J–2L). Expression of vimentin was weaker when compared to the

intense positive results in the prenatal and neonatal stage. Vimentin has been reported to be immunolocalized in perinuclear positions in pre-Sertoli cells of neonatal and prepubertal (testes of 4–20 weeks in bovines) [17].

3.1.2.4. Adult stage

Adult or pubertal testes were characterized by the physiologically active STs and were histologically characterized by the presence of cells in different stages of spermatogenesis. A thick well-developed basement was seen surrounding the tubules, which in turn were surrounded by a layer of PMCs that had morphologically developed and were located near Sertoli cells and SSCs (Figure 3A). Three to five layers of partially overlapping myofibroblasts covered on both sides by an inconstant basal lamina were reported by Abd-Elmaksoud [11]. Picosirius red staining (Figure 3B) showed that the BM comprises mainly collagen fibers, which give it a wavy appearance and stain the cytoplasm of PMCs also. The same was confirmed by staining with Masson's trichrome (Figures 3C and 3D). Immunostaining for vimentin (Figures 3E and 3F) gave positive results in Sertoli cells, PMCs, and BM. It has been established that there is cross-talk between the extracellular matrix and adjoining cells (germ cells and Sertoli cells) for the spermatogenetic process [18]. Similar expression of vimentin has been observed in the testes of bovines [17], and in stallions, differential expression was related to tubular degeneration [19]. Presence of collagen and vimentin in the BM has also been correlated with spermatogenesis in the humans and their absence has been reported in azoospermic men [20].

3.2. Summary and conclusions

At an early stage of development (5.2 cm CVRL), the niche of PGCs was formed by the surrounding mesenchymal cells with fibroblasts developing into PMCs. The BM was not organized at this stage. At further development, at 7.6 cm CVRL, testicular cords with a thin BM were seen. The gonocytes were surrounded by pre-Sertoli cells. At the late gestational period (8 months of gestation), testicular cords with well-organized BM were seen. The gonocytes were seen mostly at the center of the cords surrounded by the Sertoli cells. At the neonatal stage, the testicular cords were lined with distinct BM. Gonocytes were located mostly in the center of the cords. During the prepubertal period, STs with distinct BM with PMCs were seen. The gonocytes were surrounded by the Sertoli cells and BM.

In adult testes, the STs were surrounded by a thick BM, wavy in appearance, which in turn was surrounded by PMCs. PMCs were only single-layered as seen in H&E-stained paraffin sections, picosirius red-stained, and Masson's trichrome-stained paraffin sections. Collagen fibers were seen to increase quantitatively to provide strength and cushion the developing niche of SSCs in the

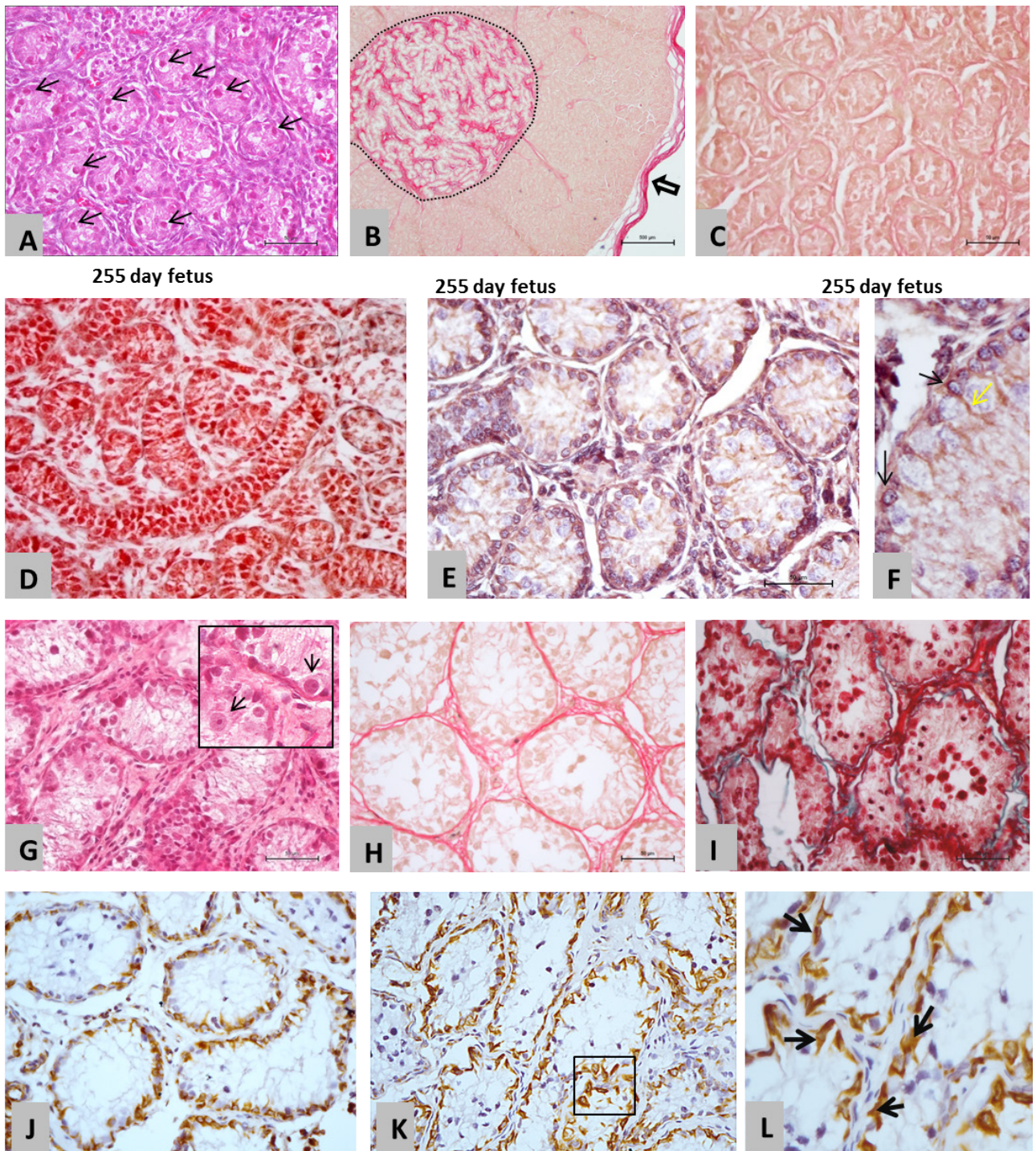


Figure 2. A) 255-day-old fetus showing germ cells in cords lined with BM. H&E, 400 \times . B) Paraffin sections of prenatal buffalo fetus (8.5 months) showing intense staining of mediastinum testis (dotted outline) and tunica albuginea (arrow) rich in collagen fibers. Picrosirius red, 40 \times . C) Distinct BM is seen around the testicular cords. Picrosirius red, 400 \times . D) Collagen fibers are seen in the BM and connective tissue as stained by light green stain in neonatal testis. Masson's trichrome, 400 \times . E) Vimentin is seen in the BM, PMCs, and Sertoli cells in neonatal testis; 400 \times . F) Magnified view of 2E showing the staining of Sertoli cells (arrow) and their cytoplasmic processes (yellow arrow) with vimentin in neonatal testis. G) Large germ cells (arrow) in cords surrounded by BM in prepubertal buffalo testis, H&E 400 \times . H) The BM consists of numerous collagen fibers, which give it a wavy appearance. PMCs are present lining the BM in prepubertal buffalo testis. Picrosirius red, 400 \times . I) Collagen fibers are seen in the BM and connective tissue as stained by light green stain in prepubertal buffalo testis. Masson's trichrome stain, 400 \times . J-K) Immunolocalization of vimentin in Sertoli cells and BM in prepubertal buffalo testis; 400 \times . L) Magnified view of vimentin localization in Sertoli cells.

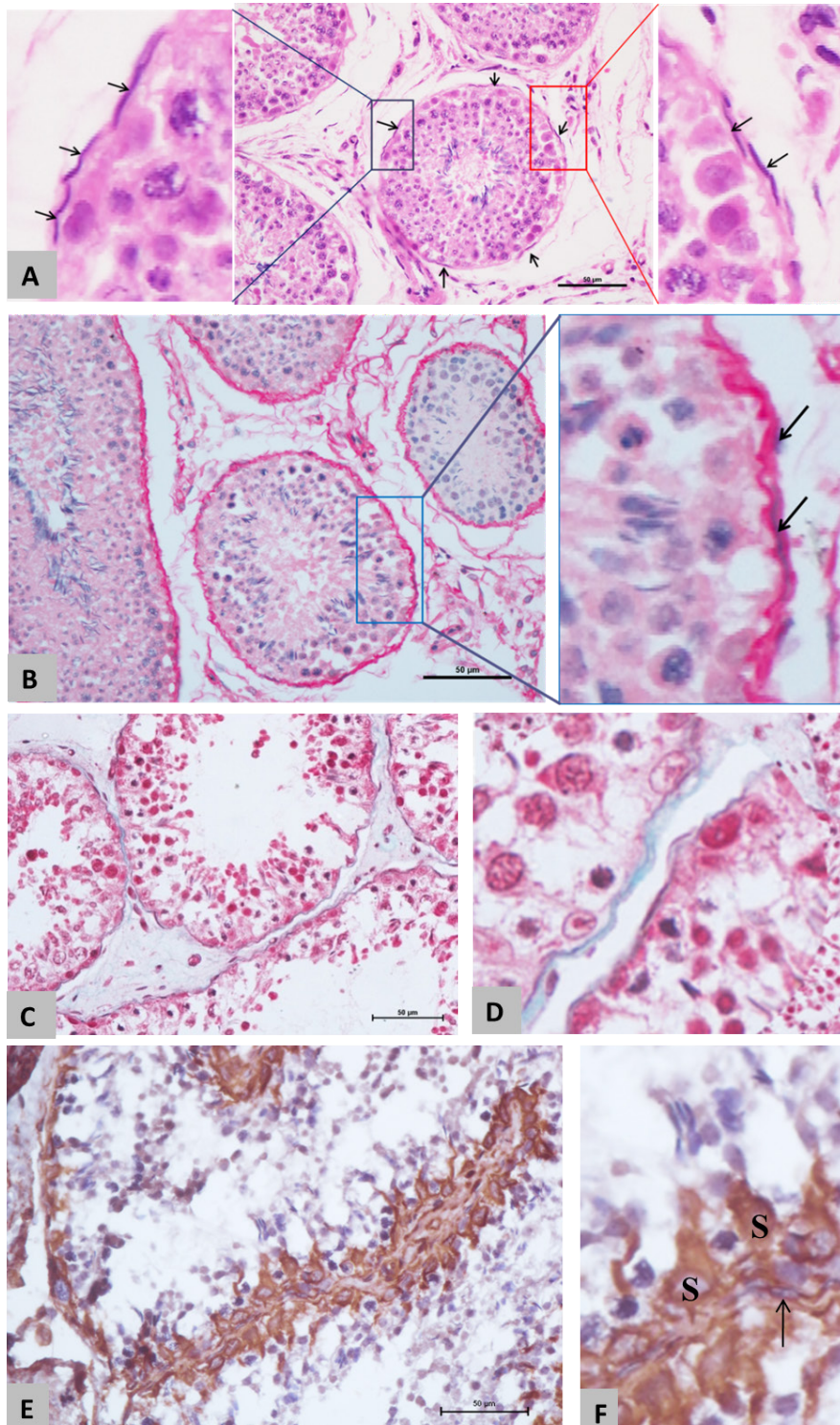


Figure 3. Testis of adult buffalo. A) SSCs are located on the BM, which is lined by PMCs. PMCs (arrows) are elongated cells present in a single layer around the ST. H&E, 400 \times . B) The BM consists of numerous collagen fibers, which give it a wavy appearance (enlarged view in inset). PMCs (arrows) are seen just outside the BM. Picrosirius red, 400 \times . C) Collagen system is seen in the BM and connective tissue as stained by light green stain (enlarged view in inset). Masson's trichrome stain, 400 \times . D) Magnified view from an area of 3C. E) Immunostaining with vimentin shows a strong positive reaction in Sertoli cells, PMCs, and BM and slightly weaker reaction in connective tissue and negative reaction in the rest of the cells of seminiferous tubules; 400 \times . F) Magnified image of 3E showing positive cytoplasmic reaction VIM in Sertoli cells (S) and PMCs (arrow) positive for vimentin.

testes. The expression of vimentin reduced to a certain extent until adult life, indicating its significant role as an intermediate filament during initial development in the organization of a number of critical proteins.

In conclusion, the functional components of the SC niche varied with time and the integrity of functional proteins responsible for the SC niche is a must for active spermatogenesis.

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