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Original Article

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Ultrastructural, Histochemical, and Cytological Study of Testis of Human Fetuses of Various Gestation Periods with Future Implications in Orchidectomy / Orchidopexy in the Patients with Seminoma and Interstitial Cell Tumors of Testis

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Abstract

***Background & Aims*:** The immunohistological and ultrastructural features of the human testis with emphasis upon the process of spermatogenesis and the cytology of the Leydig cells were reviewed in this study. The present study also has its future implications in staging of cancer metastasis in the patients with Seminoma Testis and Leydig Cell Tumors and also in future xenografting of testicular tissue from an infant human donor.

***Materials & Methods*:** The testis tissue samples from aborted human fetuses of various weeks of gestation were taken and then subjected to immunohistochemistry by Ki-67 antibodies and also to Scanning Electron Microscopy and Transmission Electron Microscopy.

***Results*:** In the ultrastructural study, it is shown that the seminiferous epithelium is structurally partitioned by the Sertoli cells into basal and adluminal compartments via the specialized tight junctions between the Sertoli cells. The Leydig cell cytoplasm contains an abundant supply of smooth endoplasmic reticulum and mitochondria with tubular cristae, both features being characteristic of steroidogenic cells.

***Conclusion*:** The detailed ultrastructural study can help the surgeons in the future xenografting processes of testicular tissue from an infant human donor to increase sperm maturity because of highly vascular testicular tissue.

***Keywords*:** Leydig Cells, Sertoli Cells, Spermatogonia, Steroidogenic Cells, Xenografting

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Introduction

Earlier studies of the organization of the human seminiferous epithelium showed that germ cells at different developmental stages formed identifiable collections termed cell associations or stages, but since several stages were seen in a single tubule cross-section

giving the impression of an extremely irregular pattern of spermatogenic development. Earlier studies by researchers showed found pyknotic and severe depletion of Leydig cells following treatment by anabolic androgenic steroids. A few researchers also showed that presence of cytoplasm vacuolization, vesicular-like crista of the mitochondria, numerous lipid droplets and lysosome and phagolysosome in Sertoli cells were characteristic of exogenous stimulants like drugs and toxicants which activate the Fas signaling system causing sloughing of germ cells from seminiferous epithelium and also by enhanced production of Reactive Oxygen Species (ROS) causing apoptosis.

The ultrastructural features of the human testis are reviewed with emphasis upon the process of spermatogenesis and the cytology of the Leydig cells. The seminiferous epithelium is structurally partitioned by the Sertoli cells into basal and adluminal compartments via the specialized tight junctions between the Sertoli cells (1). The intertubular tissue of the human testis is composed of loose connective tissue containing blood vessels, occasional lymph capillaries, macrophages, mast cells, and the Leydig cells which occur either as single cells or form small clusters. The Leydig cell cytoplasm contains an abundant supply of smooth endoplasmic reticulum and mitochondria with tubular cristae, both features being characteristic of steroidogenic cells (2). Human Leydig cells contain large Reinke crystalloids of variable size and number, but their function remains obscure. The frequent occurrence of paracrystalline inclusions within the cytoplasm of the human Leydig cell suggests that these elements are precursors of the Reinke crystalloids (3). The fine structure of primary spermatocytes is described together with the complex transformation of the spermatids into spermatozoa during the process of spermiogenesis. Earlier studies of the organization of the human seminiferous epithelium showed that germ cells at different developmental stages formed identifiable collections termed cell associations or stages, but since several stages were seen in a single tubule cross-section, this gave the impression of an extremely irregular pattern of spermatogenic development (4). Spermatogonia reside in the basal compartment and produce the primary spermatocytes via a series of cell divisions, which move into the adluminal compartment at the commencement of their development, and thus the lengthy process of meiotic maturation is initiated (5).

The present study is done with an aim of its future implications in staging of Cancer Metastasis in the patients with Seminoma Testis and Leydig Cell Tumors and also in future xenografting of testicular tissue from an infant human donor. When the topographic arrangement of germ cells was re-examined with the aid of computer modeling, a highly ordered distribution was revealed, conforming a helical pattern based on the geometry of spirals.

Materials & Methods

10 testis tissues of aborted human fetuses of 1st, 2nd, and 3rd Trimesters of Gestation which did not show any evidence of external morphological abnormality like testicular agenesis, cryptorchism, etc. were collected from the Department of Gynaecology and Obstetrics, All India Institutes of Medical Sciences, Bhubaneswar, after therapeutic abortion from 2017 to 2018, which was used in this study after approval of the Institutional Ethical Committee of Human Research (IEC) and Institute Research Board (IRB) of AIIMS Bhubaneswar. Undescended testis was excluded from the study. They were collected after written informed consent of the legal guardian accorded with institutional guidelines, which is maintained in Fetal Death Register in the Department of Anatomy, AIIMS Bhubaneswar.

Immunohistochemical Study:

For immunohistochemical study, 3mm sections of testis tissues were fixed in 10% Neutral Buffered Formalin (NBF) for 7 hours and then subjected to routine processing and paraffin embedding. Antigen Retrieval from those tissues was performed by heating unstained sections which were immersed in DAKO Target Retrieval Solution. Deparaffinized sections were incubated with 3% hydrogen peroxide for 5 min, which block and inhibit the endogenous enzymes to avoid

producing non-specific binding of antigen in the cell surface, followed by sequential 10 min incubations with xylene and alcohol. It was put in a pressure cooker for 20 minutes (Heat-Induced Epitope Retrieval, HIER) to unmask the antigen epitopes in order to allow the antibodies to bind, followed by biotinylated link antibody and peroxidase-labeled streptavidin, which augmented the antigen expression on the tissue surface. A modified labeled avidin-biotin immunohistochemical staining was performed using antibody Ki-67 by Immunoperoxidase Kit (DAKO) on DAKO Auto Stainer. Staining was completed after a 10 min incubation with DAKO 3,3'-diaminobenzidine (DAB) chromogen, which was used as a signal enhancer. All cases were coded and the grading of the immunostaining was performed on a sliding scale of 1+ to 4+, according to the percentage of reactive cells (0= no staining, 1+= 1% to 25%, 2+= 26% to 50%, 3+= 51 to 75%, 4+>76%).

Ultrastructural study:

Processing for SEM:

The testis tissue was cut in 3 mm size and was washed with three changes of 0.1M Phosphate Buffer 15 min each at 4°C and then the section was flooded with 1% osmium tetroxide (OsO4) to postfix for 2 hours at 4°C. Then it was dehydrated with three changes of acetone 15 min each and then 2 changes in dry acetone 15 min each and was transferred to liquid CO2 in a chamber that is cooled and put under pressure. When acetone has been completely removed and the tissues were impregnated with liquid CO2, the chamber was warmed up to critical point 31.5°C where the density of drying medium is same in both liquid and gas phase at 1100 p.s.i. (Critical Drying Point).

After drying, the specimens were mounted on aluminium stubs with conductive paint (silver or copper paint) with the area of interest exposed towards the surface and then coating was done with gold and flooding argon gas in the chamber for 1 min; ultimately a uniform thin layer was deposited on the specimen (Sputter Coating). After metal coating, the specimen was ready for observation under Scanning Electron Microscope.

Processing for Transmission Emission Microscopy (TEM):

The brain tissue was cut in 2 x 2 mm size and was put in Primary Fixative (Karnovsky's fixative) with 2.5% glutaraldehyde + 2% Paraformaldehyde in 0.1 M PB (pH 7.4) for 12 hr at 4 ^{0}C followed by Secondary fixation by 1% OsO4 for 1 hr at 4 ^{0}C. The tissue was then dehydrated in ascending grades of alcohol at 4 ^{0}C and kept in acetone at room temperature followed by processing with xylene. Epoxy Resin Infiltration was done with descending toluene and ascending Resin ratio for better infiltration of tissue. The resin block was then cut by Knife Boat into 50-70nm sections by Ultramicrotome. Double Staining was done by aqueous uranyl acetate & alkaline lead citrate. The slide was then viewed under TEM and photomicrographs were taken.

Results

Immunohistochemical Study as seen in Fig 1 to Fig 4:

In the 1st week of gestation, Leydig cells formed broad cellular sheets between spermatic cords. They showed strong cytoplasmic positivity. Immunoreactivity was also shown within tubular cells. Testis deep surface showed immunoreactivity which was particularly visible in the apical cytoplasm of the epididymis cells. Testis deep surface showed cytoplasmic immunoreactivity of convoluted seminiferous tubules and Leydig cells. Some of them also showed nuclear labeling. Magnification of tubular cells showed immunocytochemical labeling weaker than fetal Leydig cells. Immunoreactivity was very strong so that both of germ cells and Sertoli cells made dark-colored cord-like structures. Numerous T-positive cells in the testis are immune positive could be seen in Figure 1 and Figure 2.

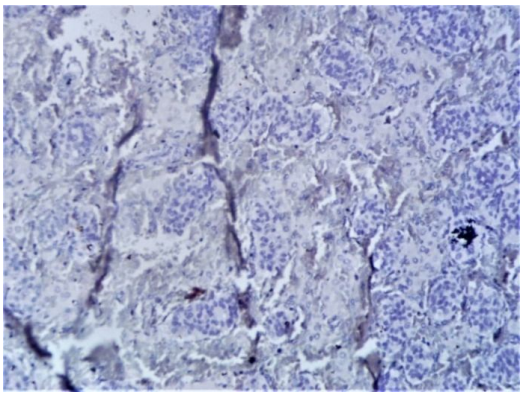

Fig. 1. 10X showing expression in fetal epididymal body at 20 weeks. Testis deep surface showing Ki-67 immunoreactivity which is particularly visible in the apical cytoplasm of the epididymis cells.

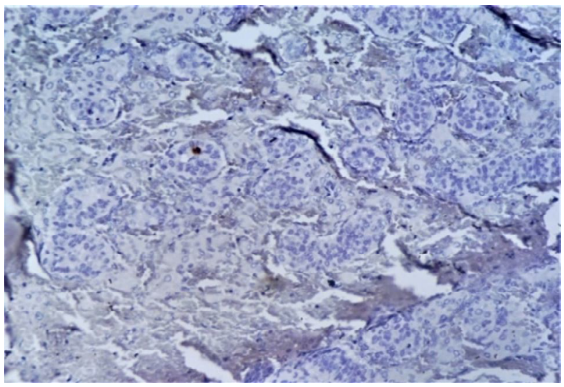

Fig. 2. 20X showing testis deep surface showing cytoplasmic immunoreactivity of convoluted seminiferous tubules showing strong immunocytochemical labelling than weaker labelling in Leydig cells.

In the 2nd week of gestation, most of the Leydig cells became rounded and strongly immunoreactive, although unlabeled cells were visible. There was weaker immunoreactivity in the convoluted seminiferous tubules. Immunoreactive cells appeared to be less numerous, probably because the interstitial tissue/convoluted seminiferous tubules ratio had changed as seen in Figure 3 and Figure 4.

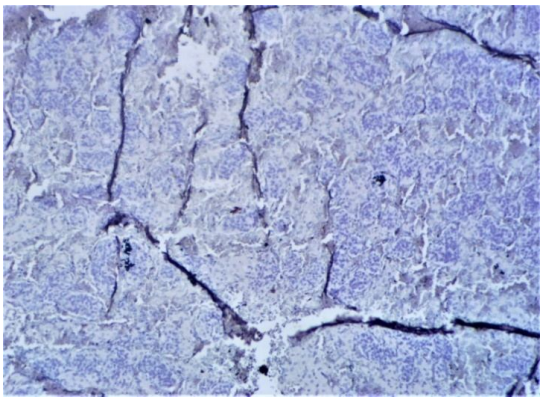

Fig. 3. 10X showing most of the Leydig cells become rounded and strongly immunoreactive, although unlabelled cells are visible with weaker immunoreactivity in the convoluted seminiferous tubules.

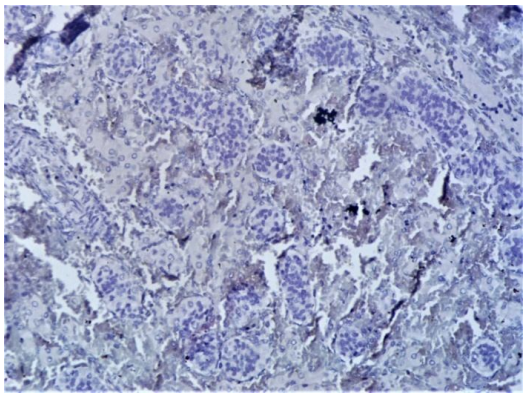

Fig. 4. 20X showing Ki-67 labelling is mainly localized in the cytoplasm, although nuclear staining is also visible

In the 3rd week of gestation, many Leydig cells were slightly flattened because of the reduced space within the interstitial tissue. High magnification of deep surface was seen and the epididymis tubular cells in the fetal membrane showed strong immunoreactivity. High magnification of deep surface and the Leydig cells have regressed. Some immature cells show weak immunoreactivity. Leydig cells and regressed Leydig cells were negative for calretinin as seen in Figure 5 and Figure 6.

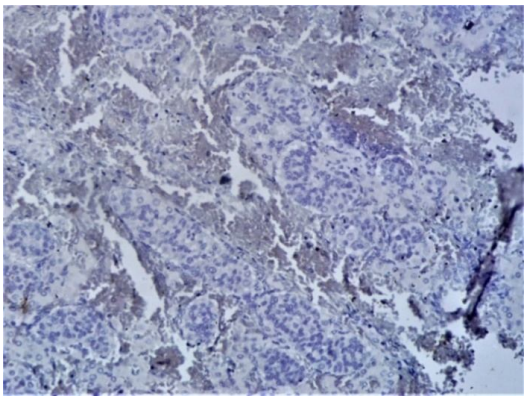

Fig. 5. 40X showing testis deep surface: Ki-67-immunoreactive cells appear to be less numerous, probably because the interstitial tissue/convoluted seminiferous tubules ratio has changed. Many Leydig cells are slightly flattened because of the reduced space within the interstitial tissue.

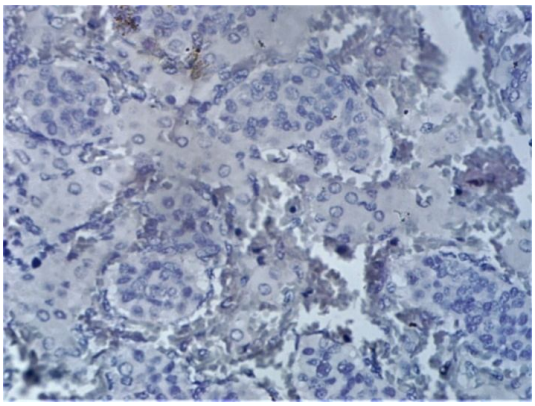

Fig. 6. 40X showing High magnification of deep surface: the epididymis tubular cells in the fetal membrane show strong Ki-67 immunoreactivity.

The testis of aborted human fetuses showed increased expression in later stages of gestation as compared to the initial weeks of gestation showing immuno-reactivity.

Ultrastructural Study as seen in Fig 7 to Fig 20:

In the 1st week of gestation, interstitial cells showed a fibroblast-like pattern besides other interstitial cells with darker cytoplasm. The nucleus was similar to that of myofibroblasts or somewhat heterochromatic, but the cytoplasm had more abundant microfilaments. Cytoplasm of fetal Leydig cell showed abundant smooth endoplasmic reticulum and mitochondria with tubular cristae, a little rough endoplasmic reticulum, and electron-dense bodies. Degenerating fetal Leydig cell showed its dark cytoplasm with scant smooth endoplasmic reticulum, residual bodies, and lipid droplet accumulation were seen. Interstitial cells showing irregularly outlined nuclei and cytoplasmic areas with abundant smooth endoplasmic reticulum and lipid droplets could be seen. The mitochondria have parallel cristae. Some rough endoplasmic reticulum (rER) cisternae were continuous with the perinuclear cisterna. Some rER cisternae were markedly enlarged and contain an amorphous substance and others display ribosomes only on one surface. Testicular interstitium showed abundant fetal Leydig cells, an infantile Leydig cell with a multilobed nucleus and cells with a fibroblast-like pattern. Scanty involuting fetal Leydig cells with abundant lipid, infantile Leydig cells with multilobed nuclei, and fibroblast or myofibroblast-like cells could be seen. Numerous fibroblastic cells, many of which were elongated and were in a peritubular location, and large Leydig cells could be seen. The gonocyte from the testes of developing fetus showed round nucleus contains a centrally located, prominent nucleolus, and the chromatin was finely dispersed. The cytoplasm displayed a distinct Golgi apparatus, many mitochondria, and polyribosomes as well as a microfilament-rich zone beneath the cell membrane. Chromatid bodies in an intermediate cell from the testis were composed of finely granular material, which were surrounded by larger, electron dense granules as seen in Figure 7 to 10.

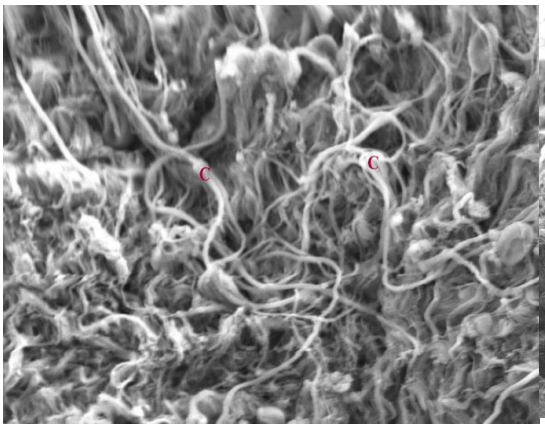

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Fig. 7. C-Surface of Seminiferous Tubules

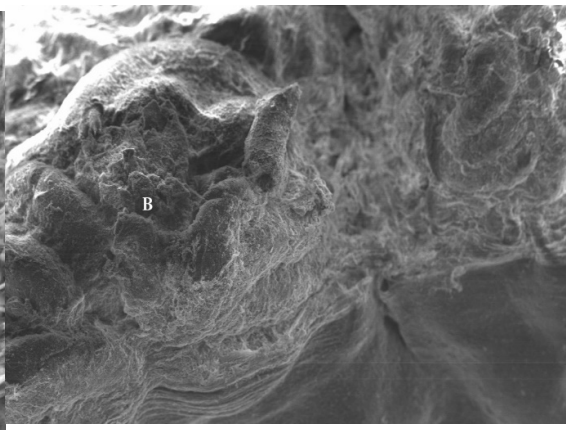

Fig. 8. B-Seminiferous Tubules

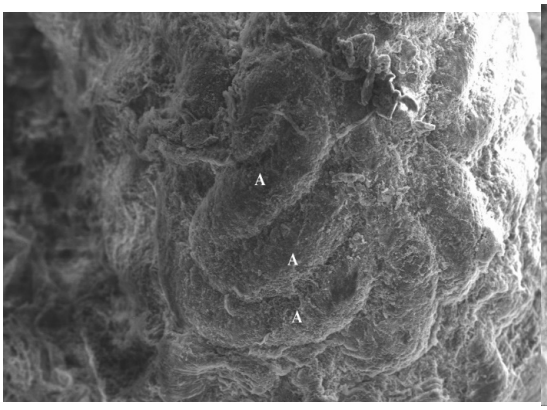

Fig. 9. A-Hollow tubular like structures of the seminiferous tubules

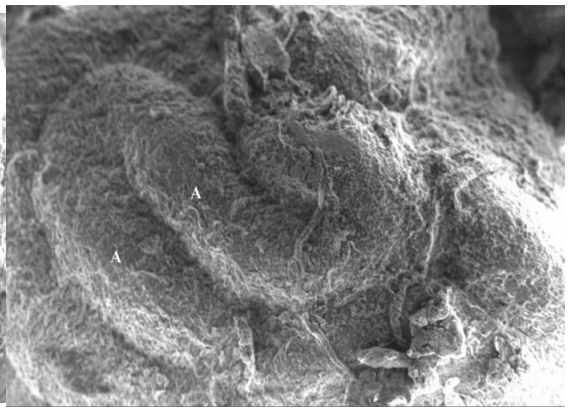

Fig. 10. A-Hollow tubular like structures of the seminiferous tubules

In the 2nd week of gestation, only blood vessels in the testis interstitium were positive. Only peritubular Follicular lymphoma (FL) cells, some primitive spermatogonia and immature Sertoli cells manifested immunopositivity. The basement membrane of the cords showed profound immunopositivity. A strong positive reaction could be observed in blood vessels of the testis interstitium. In the testicular cord, the interstitium was composed of fusiform peritubular cells around the testicular cord. In the center, the Leydig cells were large and numerous, their cytoplasm was dark or light, and contained many lipid vacuoles. In the intermediate area, there were many fusiform or polygonal mesenchymal cells and immature Leydig cells could be seen. The peritubular cells are arranged in one or two layers. Leydig cells generally had a dark cytoplasm and were clearly separated from each other. The mesenchymal cells had a large cytoplasm and the histiocytes could be seen. No immature Leydig cell were visible. The voluminous nucleus gives the undifferentiated triangular mesenchymal cell its shape; it was surrounded by a thin border of dense cytoplasm poor in organelles. The cell has a long thin cytoplasmic expansion as seen in Figure 11 to Figure 15.

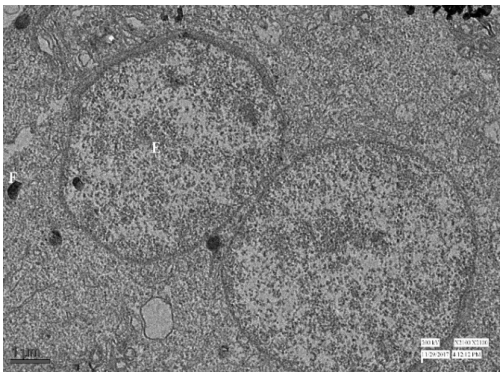

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Fig. 11. E-Nucleus of Sertoli Cells in normal mitochondria of Sertoli cells with intact nuclear Membrane, F-Dense body in cytoplasm of spermatid cells

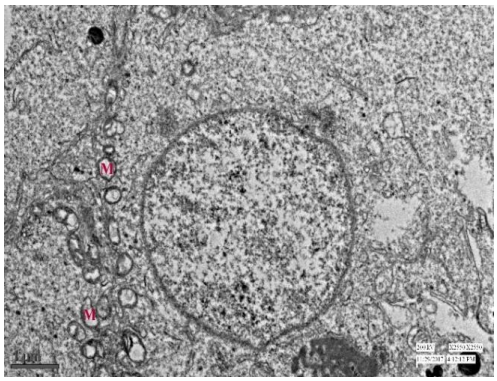

Fig. 12. M-Normal Leydig cells with no cytoplasmic vacuolation.

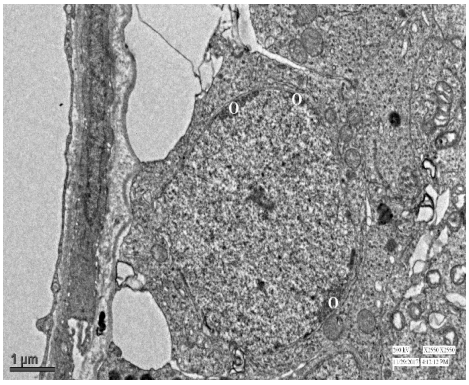

Fig. 13. O-Normal arrangement of peripherally Heterochromatin and Euchromatin

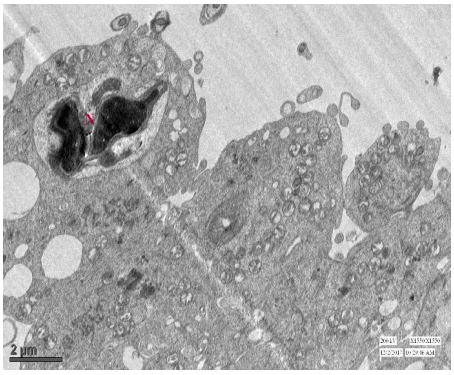

Fig. 14. N-Spermatogonia with round nucleus

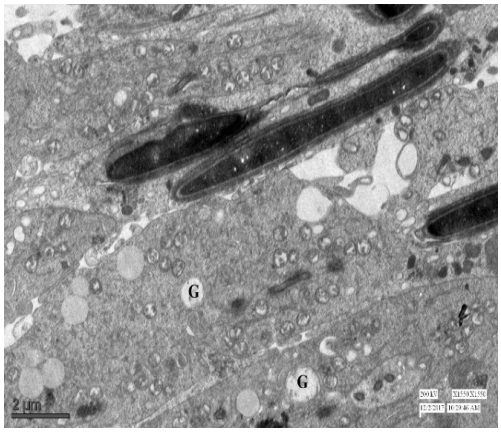

Fig. 15. G-Normal Mitochondria of Sertoli cells

In 3rd week of gestation, the lamina propria consists of one or two layers of flattened peritubular cells. In the tunica propria, predominately elongated ML cells were found. Peritubular cells showed an intensive immune expression in their cytoplasm. Primitive spermatogonia and immature Sertoli cells are seen like Figure 16 to Figure 20. The detailed arranges of the Sertoli and Leydig cells could be better visualized in Transmission Emission Microscopy (TEM) and Scanning Electron Microscopy (SEM).

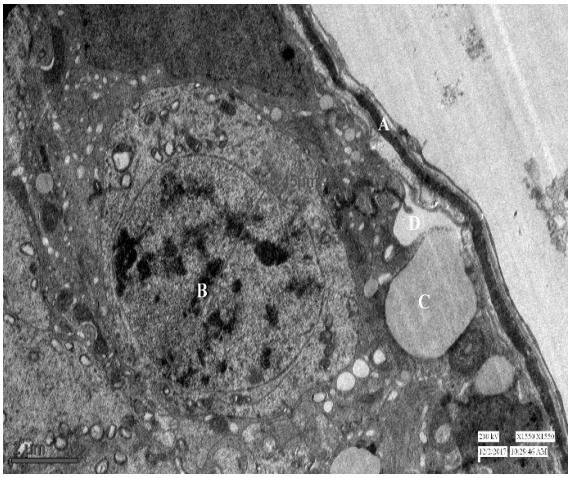

Fig. 16. A-Basement Membrane, B-Sertoli Cells, C-Primary Spermatocytes, D- Empty vacuolar spaces in Sertoli cells

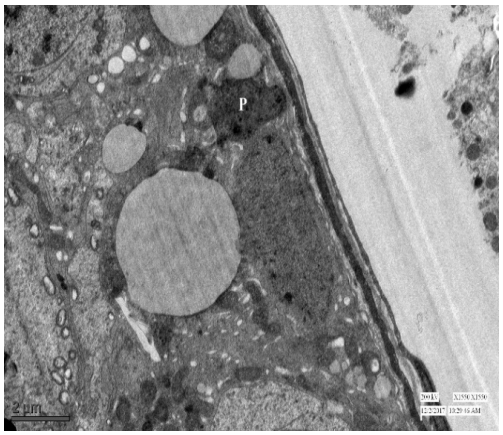

Fig. 17. P-Degenerated cell near Basement Membrane

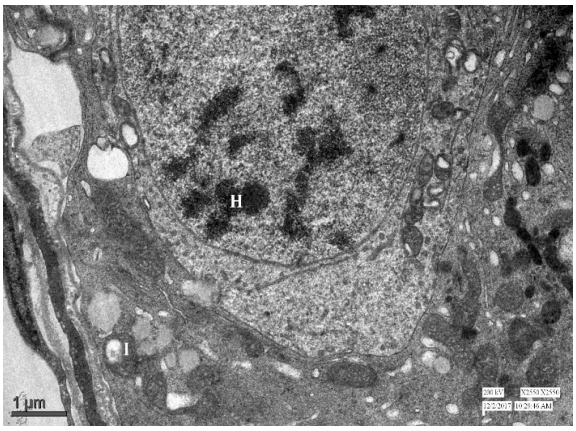

Fig. 18. H-Dense clumped marginal chromatin in primary spermatocytes, I-Vacuoles in Mitochondria of Sertoli cells

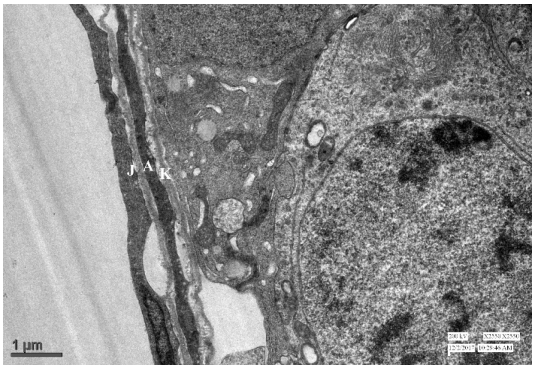

Fig. 19. A-Basement Membrane, J-Lamina Propria, K-Seminiferous Epithelium

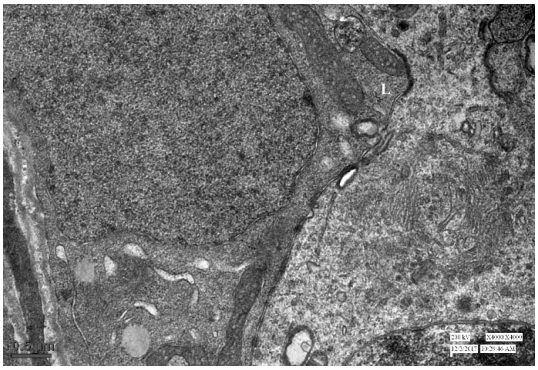

Fig. 20. L-Irregular and Thickened Basal Lamina which may be signs of inflammatory damage of testicular tissues

Discussion

Feinberg et al. (1997) found empty vacuolar spaces between Sertoli cells that are regarded to be the place where spermatogonia and spermatocytes should be located and characteristic of progressive apoptosis to Sertoli cells following treatment of anabolic androgenic steroids (6). Blanco et al. (2002) found pyknotic and severe depletion of Leydig cells following treatment by anabolic androgenic steroids (7). Presence of cytoplasm vacuolization, vesicular-like crista of the mitochondria, numerous lipid droplets as well as lysosome and phagolysosome in Sertoli cells were characteristic of exogenous stimulants like drugs and toxicants, which activate the Fas signaling system causing sloughing of germ cells from seminiferous epithelium and also by enhanced production of Reactive Oxygen Species (ROS) in the cells (7, 8). Moreover, it is considered that LP thickening depends on the length and diameter decrease of the seminiferous tubules and is the result of the disproportionate reduction in the volume of the seminiferous epithelium (10, 11). Close relationship between Leydig cells and blood vessels suggests that these cells are at high risk of exogenous toxicants and multivacuolated Leydig cells are probably a form of cell involution. Some reports have showed that some exogenous stimulants may induce myoid cells to produce more collagen and ECM that are responsible for basal lamina thickness (12,13). Spermatogenesis in the human testis is subjected to a precise regulation in keeping with the ordered arrangement of the germ cells seen in other mammalian species (14).

These were not found in the precent ultrastructural study, so the cellular architectures of the testicular tissues were intact. In our study, the Ki-67 immunoreactivity, which is particularly visible in the apical cytoplasm of the epididymis cells in fetal epididymal body, was found. There was also cytoplasmic immunoreactivity of convoluted seminiferous tubules, showing strong immunocytochemical labeling which was weaker in Leydig cells. Cytoplasmic immunoreactivity of convoluted seminiferous tubules shows strong immunocytochemical labeling than weaker labeling in Leydig cells in our study (15).

The detailed ultrastructural study can help the surgeons in the future xenografting of testicular tissues from infant human donors to increase sperm maturity, because of highly vascular testicular tissue. This study in Eastern India Population will aid the researchers nationwide and worldwide about the cytological architecture of fetal testicular tissue. It will also enrich the clinician in staging of the cancer metastasis in Seminoma and Leydig cell tumor patients.

It is hypothesized from this study that the Fas signaling system assist in the development of Seminoma and Interstitial Cell Tumors of Testis which needs

Orchidectomy/Orchidopexy in such patients, which should be evaluated further by Immunoelectron Microscopy and correlation of DICER1 Gene by Nuclear Genome Sequencing (NGS) along with Mass Spectrophotometry for estimation of the drugs and stimulants involved in the Fas signaling system causing apoptosis of the testicular cells. This study needs further study with Immunoelectron Microscopy and Genetic Analysis.

Human Leydig cells contain large Reinke crystalloids of variable size and number, but their function remains obscure. The frequent occurrence of paracrystalline inclusions within the cytoplasm of the human Leydig cell suggests that these elements are precursors of the Reinke crystalloids. Thus, the functions of Reinke's crystalloids need further study.

Acknowledgments

None declared.

Ethical statement

This study was approved by the Institutional Ethical Committee of Human Research (IEC) and Institute Research Board (IRB) of AIIMS Bhubaneswar.

Conflict of interest

None declared.

Funding/support

None declared.

Data availability

The raw data supporting the conclusions of this article are available from the authors upon reasonable request.

References

1. Riva A, Tandler B, Ushiki T, Usai P, Isola R, Conti G, Loy F, Hoppel CL. Mitochondria of human Leydig cells as seen by high resolution scanning electron microscopy. Arch Histol Cytol 2010;73(1):37-44.
2. Miething A. Arrested germ cell divisions in the ageing human testis. Andrologia 2005;37(1):10-6
3. Miething A. Multinucleated spermatocytes in the aging human testis: formation, morphology, and degenerative fate. Andrologia 1993;25(6):317-23.
4. Jiang H, Zhu WJ, Li J, Chen QJ, Liang WB, Gu YQ. Quantitative histological analysis and ultrastructure of the aging human testis. Int Urol Nephr 2014;46(5):879-85.
5. Johnson L, Petty CS, Neaves WB. Age-related variations in seminiferous tubules in men. A stereological evaluation. 1986 J Androl 7(5):316-22.
6. Feinberg MJ, Lumia AR, McGinnis MY. The effect of anabolic-androgenic steroids on sexual behavior and reproductive tissues in male rats. Physiol Behav 1997;62(1):23-30.
7. Blanco A, Flores-Acuna F, Roldan-Villalobos R, Monterde JG. Testicular damage from anabolic treatments with the β2-adrenergic agonist clenbuterol in pigs: a light and electron microscope study. Vet J 2002;163(3):292-8.
8. Gopalkrishnan K, Gill-Sharma MK, Balasinor N, Padwal V, D'Souza S, Parte P, Jayaraman S, Juneja HS. Tamoxifen-induced light and electron microscopic changes in the rat testicular morphology and serum hormonal profile of reproductive hormones. Contraception 1998;57(4):261-9.
9. Johnson L. Spermatogenesis and aging in the human. J Androl 1986;7(6):331–54.
10. Kerr JB. Functional cytology of the human testis. Baillieres Clin Endocrinol Metab 1992;6(2):235-50.
11. Plas E, Berger P, Hermann M, Pfluger H. Effects of aging on male fertility? Exp Gerontol 2000;35(5):543–51.
12. Richardson LL, Kleinman HK, Dym M. Altered basement membrane synthesis in the testis after tissue injury. J Androl 1998;19(2):145-55.
13. Santoro G, Romeo C, Impellizzeri P, Arco A, Rizzo G, Gentile C. A morphometric and ultrastructural study of the changes in the lamina propria in adolescents with varicocele. BJU Int 1999;83(7):828-3.
14. Haider SG. Cell biology of Leydig cells in the testis. Int Rev Cytol 2004;233(4):181-241.
15. Sato Y, Nozawa S, Yoshiike M, Arai M, Sasaki C, Iwamoto T. Xenografting of testicular tissue from an infant human donor results in accelerated testicular maturation. Hum Reprod 2010;25(5):1113-22.