Indian Journal of Animal Sciences **90** (4): 553–556, April 2020/Article

Fetal Leydig cells in buffalo testis: Light and electron microscopic study

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Received: 12 March 2019; Accepted: 22 August 2019

ABSTRACT

The present study was conducted on testis of 20 buffalo fetuses ranging from 2.5 cm to 20 cm curved crown rump length (CVRL). The tissues were processed for light and transmission electron microscopic study. The inter-tubular tissue was filled with primitive mesenchymal cells, developing Leydig cells and blood vessels. At 7.5 cm CVRL, some of the interstitial mesenchymal cells started developing into foetal Leydig cells and were androgen positive. These cells were characterized by their polygonal shape, eosinophilic cytoplasm and darkly stained spherical nucleus with prominent nucleoli at 9.5 cm CVRL. At 15.5 cm CVRL, the fetal Leydig cells were present in groups around the interstitial blood vessels, which increased in number and size at 20 cm CVRL and decreased afterwards. By TEM, three types of Leydig cells were observed at 14.5 cm CVRL as stem Leydig cells (Leydig cell A), progenitor Leydig cells (Leydig cell B) and immature Leydig cells (Leydig cell C). Leydig cell A had elongated nucleus with a very thin rim of cytoplasm. Leydig cell B had oval nucleus with comparatively larger amount of cytoplasm. Leydig cell C had adult Leydig cell like features and had round to oval nucleus with lager amount of cytoplasm. The cytoplasm of these cells contained mitochondria with elongated cristae, Golgi apparatus and very few lipid inclusions. The number and size of Leydig cells increased during early prenatal life.

Keywords: Buffalo fetus, Electron microscopy, Histology, Leydig cells

Depending upon morphological and functional aspects, two types of Leydig cells are present as fetal and adult cells in testis of developing rats (Kuopio *et al.* 1989) and human (Codesal *et al.* 1990). These cells are referred as fetal and adult Leydig cells, because they differentiate and start hormone production during fetal life and before and after puberty, respectively (Kuopio *et al* 1989). In literature, some studies have been made on the differentiation of fetal Leydig cells in rat, pigs and human at 1 week of fetal age, but in bovines at cm CRL (Sinowatz *et al.* 1987, Rüsse 1991 and Orth 1993). The morphological studies have been conducted on the fetal Leydig cells in bovines (Abd-Elmaksoud 2005) and buffalo (Kaur 2006), but the detailed information on the histology and ultrastructure of fetal Leydig cells during early fetal life is still lacking. So the present study was undertaken to observe the formation of fetal Leydig cells in buffalo testis.

MATERIALS AND METHODS

The present study was conducted on right and left testis of 20 buffalo fetuses ranging from 2.5 cm to 20 cm curved crown rump length (CVRL). The samples were collected from pregnant non-descript buffaloes slaughtered at slaughter house and from the Veterinary Clinics GADVASU, Ludhiana. The age of fetuses was determined by measuring the CVRL as a curved line in cm using an inelastic thread along the vertebral column between the most anterior parts of frontal bone to the rump at ischiatic tuberosity as described by Edward (1965). The approximate age of the fetuses was calculated by using following formula given by Soliman (1975).

$$Y = 28.66 + 4.496\ X \quad (CVRL < 20\ cm)$$

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

Immediately after collection, both the right and left testes were fixed in 10% neutral buffered formalin (10% NBF) and were processed as per routine acetone benzene technique (Luna 1968). The paraplast embedded blocks were prepared, sections of 5 µm thickness were obtained on clean glass slides and these sections were stained with hematoxylin and eosin stain for histological studies. The sections were also obtained on poly-L-lysine coated slides, which were subjected to immunostainiing procedures to localize the androgen receptors. For electron microscopy, small tissue samples were fixed in Karnovsky's fixative (2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer solution) for 8–12 h followed by secondary fixation in 2% osmium tetraoxide for 2 h. After dehydration, the tissue was embedded in an Epon-Araldite mixture. The ultrathin sections of 70–90 nm thickness were cut and collected on uncoated copper grids. These grids

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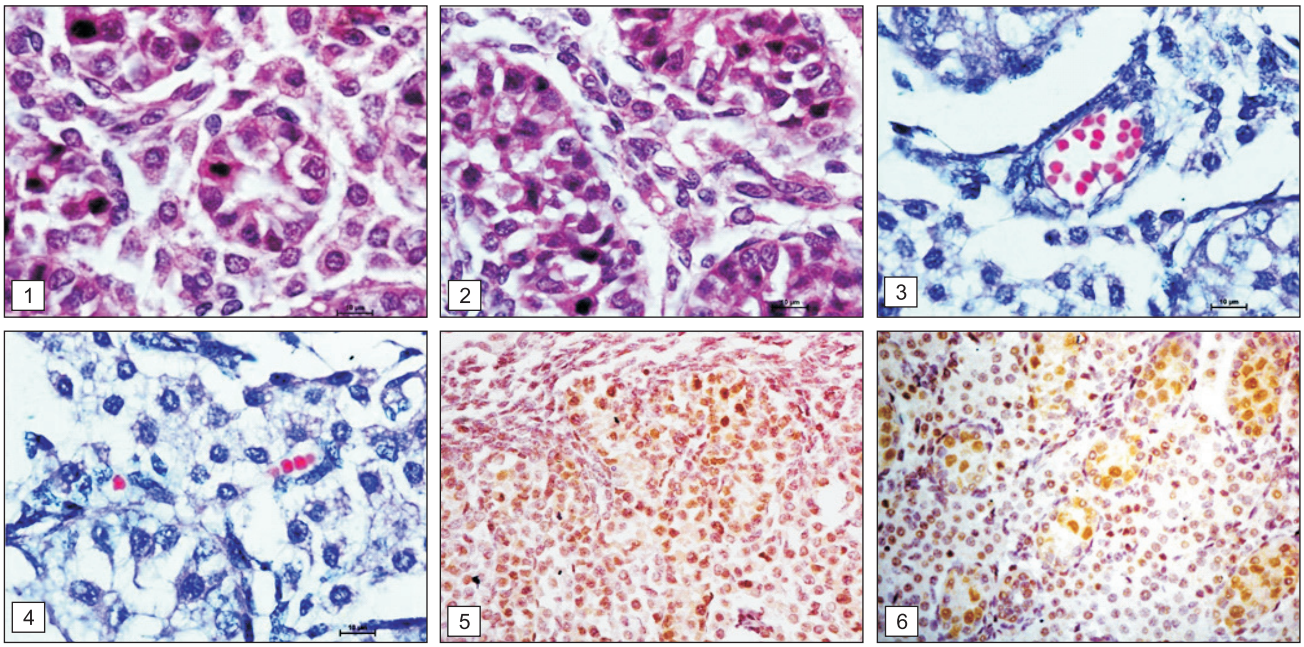


Figs 1–6. **1.** Paraffin section of buffalo foetal testis at 7.5 cm CVRL showing formation of spermatic cords (SC) and foetal Leydig cells (LC) in the intertubular area. Haematoxylin and eosin stain ×1000. **2.** Paraffin section of buffalo foetal testis at 9.5 cm CVRL showing increase in number and size of foetal Leydig cells (LC) in the intertubular area. Haematoxylin and eosin stain ×1000. **3.** Paraffin section of buffalo foetal testis at 15.5 cm CVRL showing Type A (LCA), Type B (LCB), and Type C (LCC) foetal Leydig cells in the intertubular area. Haematoxylin and eosin stain ×1000. **4.** Paraffin section of buffalo foetal testis at 20.0 cm CVRL showing increase in number and size of foetal Leydig cells (LC) in the intertubular area. Haematoxylin and eosin stain ×1000. **5.** Buffalo fetal testis at 7.5 cm CVRL immunostained for Androgen receptor Poly HRP, Original magnification ×400. **6.** Buffalo fetal testis at 9.5 cm CVRL immunostained for Androgen receptor Poly HRP, Original magnification ×400.

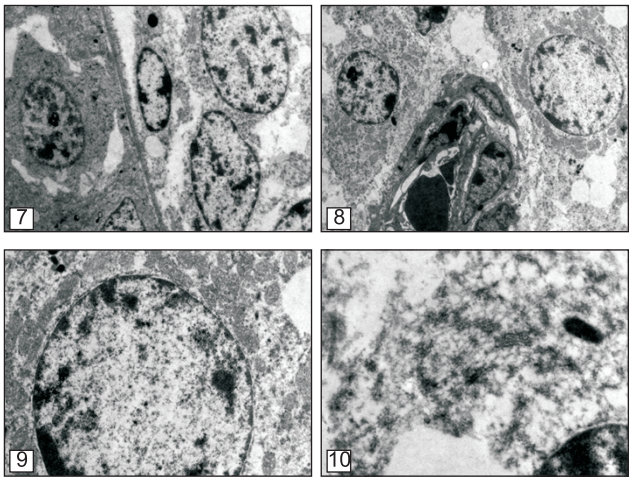
were stained with uranyl acetate for 15 min followed by lead citrate for 10 min and finally examined under transmission electron microscope for detailed study.

RESULTS AND DISCUSSION

Light microscopic studies: At 5 cm CVRL, the interstitial tissue contained mesenchymal cells, differentiating fibroblasts, numerous blood vessels and connective tissue cells. At 7.5 cm CVRL, some of the interstitial mesenchymal cells started developing into foetal Leydig cells (Fig. 1). These cells are characterized by their polygonal shape, eosinophilic cytoplasm and darkly stained spherical nucleus with prominent nucleoli at 9.5 cm CVRL (Fig. 2). Some of the fibroblasts were also observed at different stages of differentiation which would form mature Leydig cells. Moon and Hardy (1973) also postulated that the first mature Leydig cells arise from the mesenchymal cells. These cells were thought to be originated from the tunica albuginea as observed in goat embryos by Singh *et al.* (1979) and Farooqui *et al.* (2012).

At 15.5 cm CVRL, the fetal Leydig cells were present in groups around the interstitial blood vessels (Fig. 3). At this stage, the interstitum increased in size which continued upto 20 cm CVRL (Fig. 4). This increase may be due to the differentiation of the most mesenchymal cells into fetal Leydig cells to constitute large population of these cell types in the interstitum. Orth (1993) also observed that the number of mesenchymal cells decrease with the increase in the fetal Leydig cells. Similarly, the increase in the number of fetal Leydig cells was observed in the testis of buffalo by Baishya and Vyas (1990), in humans by Padmini and Rao (2012), in bovine foetus by Abd-Elmaksoud (2005) and in goat foetus by Farooqui *et al.* (2012). The increase in the number of interstitial endocrine cells may be related with the increase in the testosterone production. This period of increased number of fetal Leydig cells may be called as growth phase fetal Leydig cell population as mentioned earlier by Huhtaniemi and Pelliniemi (1992). The development of Leydig cells may be due to the swelling reaction of the gubernaculum and normal descent of the testis (Hullinger and Wensing 1985). The number of fetal Leydig cells increased from 7.5 to 20 cm CVRL, but decreased with the advancement of fetal age as observed by Kaur *et al.* (2011). This could be due to degeneration of earlier Leydig cells or proliferation of inter-tubular tissue during prenatal development of testis (Wrobel 1990). The increase in their number may be due to their differentiation from mesenchymal cells and their infrequent mitosis.

Immnunohistochemical studies showed that the localization of androgen positive foetal Leydig cells gave brown homogenous staining at 7.5 cm CVRL (Fig. 5), which appeared darker at the periphery and lighter in the centre at 9.5 cm CVRL (Fig. 6). The percentage of androgen positive cells was 24.19±1.54, 34.20±3.23, 47.72±6.42 and 47.68±2.28 at 7.5 cm, 9.5 cm, 17.5 cm and 19.5 cm CVRL, respectively. The present data indicated that the percentage



Figs 7–10. **7.** Transmission electron micrograph of buffalo foetal testis at 17.5 cm CVRL showing peritubular myoid cell, Type A and Type B foetal Leydig cells. Original magnification ×1600. **8.** Transmission electron micrograph of buffalo foetal testis at 17.5 cm CVRL showing Type C foetal Leydig cells near the blood vessel. Original magnification ×1600. **9.** Higher magnification of Fig. 8 showing rounded nucleus and abundance of mitochondria in Type C foetal Leydig cells. Original magnification ×5000. **10.** Higher magnification of Fig. 8 depicting numerous strands of smooth endoplasmic reticulum along with few lipid droplets, and vacuolization in Type C foetal Leydig cells. Original magnification ×6300.

of androgen reactive cells and the intensity of reaction increased with the increase in foetal age at early prenatal life. Similar observations have been reported in buffalo foetuses from 7.5 cm to 98 cm CVRL by Bansal *et al.* (2015).

Micrometrical studies showed that there was an increase in the diameter of foetal Leydig cells from 7.21±0.16 to 10.04±0.21 µm with respect to the age of the buffalo foetus. Similar findings were observed by Kaur *et al.* (2011) as the diameter of fetal Leydig cells remained same in both the right and left testes but there was an increase in the diameter of fetal Leydig cells with fetal age. Our results corresponded well with the findings of Singh *et al.* (1979) and Pachpande *et al.* (2006) who also reported relative increase in the size of Leydig cells with increasing age in goat and sheep embryos, respectively.

Electron microscopy: The transmission electron microscopy showed that the intertubular tissue was filled with primitive mesenchymal cells, developing Leydig cells and blood vessels. The primitive mesenchymal cells had elongated nucleus with thin rim of cytoplasm which extended on either side (Fig. 7). Leydig cells develop from stem Leydig cells (Ledig cell A) which is a mesenchymal cell present in inter tubular tissue through at least two intermediate cells, progenitor Leydig cells (Leydig cell B) and immature Leydig cells (Leydig cell C). At 14.5 cm CVRL, all three types of cells were present (Figs. 7 and 8). Similar observations had been reported by Chen *et al* (2010). Rebourcet *et al.* (2014) studied transgenic mice and established that sertoli cells control peritubular myoid cell fate and support adult Leydig cell development. Leydig cell A had elongated nucleus with a very thin rim of cytoplasm. Leydig cell B had oval nucleus with comparatively larger amount of cytoplasm. Leydig cell C had adult Leydig cell like features and had round to oval nucleus with lager amount of cytoplasm (Fig. 9). The cytoplasm consisted of mitochondria with varying shapes and elongated cristae, numerous strands of smooth endoplasmic reticulum and few lipid inclusions and vacuolization (Figs 9 and 10). The mitochondrial matrix contained intra-mitochondrial granules and collagen fibrils. The Golgi apparatus consisted of flattened saccules and vesicles. The amount of lipid droplets were less but number of vacuoles were seen in abundance. Similar findings had been reported by Kerr and Knell (1988) in rat testis and in bovine testis by Abd-Elmaksoud (2005).

REFERENCES

Abd-Elmaksoud A. 2005. 'Morphological, glycohistochemical and immunohisto chemical studies on the embryonic and adult bovine testis'. PhD Thesis, Munich, Germany.

Baishya G and Vyas K N. 1990. Studies on foetal testicular development in Surti buffalo (*Bubalus bubalis*). *Indian Veterinary Journal* **68**: 556–60.

Bansal N, Pathak D, Uppal V, Gupta A and Singh I. 2015. Androgen receptor in buffalo testis during prenatal life: An Immuno-histochemical study. *Indian Journal of Veterinary Anatomy* **27**(2): 6–8.

Chen H, Stanley E, Jin S and Zirkin B R. 2010. Stem Leydig cells: From fetal to aged animals. *Birth Defects Research Part C: Embryo Today: Reviews* **90**: 272–83.

Codesal J, Regadera J, Nistal M, Regadera-sejas J and Paniagua R.1990. Involution of human fetal Leydig cells. An immunohistochemical, ultrastructural and quantitative study. *Journal of Anatomy* **172**: 103–14

Edward M J. 1965. Observations on the anatomy of reproductive organs of cows with special reference to those features sought during examination per rectum. *New Zealand Veterinary Journal* **13**(2): 25–37.

Farooqui M M, Chandrapal A and Prakash A. 2012. Histological and histochemcial studies on the prenatal development of testis in goat (*Capra hircus*). *International Journal of Morphology* **30**: 1408–21.

Huhtaniemi I and Pelliniemi L J. 1992. Fetal Leydig cells: Cellular origin, morphology, life span and special functional features. Proceedings of the *Society for Experimental Biology and Medicine* **201**: 125–40.

Hullinger R L and Wensing C J. 1985. Testicular organogenesis in the fetal calf: interstitial endocrine (Leydig) cell development. *Acta Anatomica* **121**(2): 99–109.

Kaur M, Bansal N and Uppal V. 2011. Histogenesis of testicular parenchyma during prenatal life in buffalo. *Indian Journal of Morphology* **29**: 1109–14.

Kaur M. 2006. 'Studies on histogenesis and organogenesis of the testis in buffalo (*Bubalus bubalis*).'M.V.Sc. Thesis, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, India.

Kerr J B and Knell C M. 1988. The fate of fetal Leydig cells during the development of the fetal and postnatal rat testis. *Development* **103**(3): 535–44.

Kuopio T, Tapanainen J, Pelliniemi L J and Huhtaniemi I. 1989. Developmental stages of fetal-type Leydig cells in prepubertal rats. *Development* **107**: 213–20.

Luna L G. 1968. *Manual of histological staining methods of the Armed Forces Institute of Pathology*. 3rd ed. New York, Blakistan Division, Mc-Graw Hill Book Company.

Orth J M. 1993. Cell biology of testicular development in the fetus and neonate. *Cell and Molecular Biology of the Testis*. (Eds) Desjardins C and Ewing L L. Oxford University Press, Oxford.

Pachpande A M, Raut S S, Patil A D, Dhande P L, Gaikwad S A and Yadav S B. 2006.*Histological and Histochemical Study of Testis and Epididymis during Prenatal Development in Sheep*. *Proc XX Convention IA VA*, pp. 65, Jabalpur, India.

Padmini M P and Rao B N. 2012. Prenatal histogenesis of human fetal testis. *International Journal of Basic and Applied Medical Sciences* **2**: 112–16.

Rebourcet D, O'Shaughnessy P J, Pitetti Jean-Luc, Monteiro A, O'Hara L, Milne L, Tsai Y T, Cruickshanks L, Riethmacher D, Guillou F, Mitchell R T, van 't Hof R, Freeman T C, Nef Serge and Smith L B. 2014. Sertoli cells control peritubular myoid cell fate and support adult Leydig cell development in the prepubertal testis. *Development* **141**: 2139–49.

Russe I. 1991. *Lehrbuch der EmbioIogie der Haustiere*. (Eds) Russe I and Sinowatz F. Paul Parey Verlag, Berlin, und Hamburg.

Singh Y, Sharma D N and Dhingra L D. 1979. Morphogenesis of the testis in goat. *Indian Journal of Animal Sciences* **49**: 925–31.

Sinowatz F, Amselgruber W and Russe I. 1987. Prä-und Postnatal Differenzierung der Leydig Zellenbeim Rind. *Acta Europaea Fertilitatis* **3**: 191–96.

Soliman M K. 1975. Studies on the physiological chemistry of the allantoic and amniotic fluids of buffaloes at various periods of pregnancy. *Indian Veterinary Journal* **52**: 111–17.

Wrobel K H. 1990. The postnatal development of the bovine Leydig cell population. *Reproduction of Domestic Animals* **25**(2): 51–60.