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# GROSS AS WELL AS MICROSCOPIC ANATOMY AND PHYSIOLOGICAL FUNCTIONS OF FETAL PLACENTA IN JAFFRABADI BUFFALOES

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## ABSTRACT

The present study was conducted to know the gross morphology and histo-morphological structure of fetal placenta in Jaffrabadi buffaloes. Parameters like calf weight, placental weight, numbers, and size of cotyledons were observed in Jaffrabadi buffaloes and morphologically, fetal cotyledons were convex and non-pendunculated in Jaffrabadi buffaloes were found. Histological studies of small and large cotyledons showed extensive branching of secondary and tertiary villi that were longer, slender, and well developed in Jaffrabadi buffalo. A less developed basal lamina was seen in small cotyledon whereas developed basal lamina with numerous capillaries and connective tissue were observed in the large cotyledon. The diameter of trophoblast giant cells (TGC) in larger cotyledons were significantly ($P<0.05$) than the small cotyledons in expelled placenta at full term in Jaffrabadi buffaloes. A distinct distribution of carbohydrate and lipids in cotyledons were observed between large and small cotyledons as evident by acid mucopolysaccharides, neutral polysaccharides, and sudanophilic staining. Specific staining for calcium with Alizarin red stain showed that calcium is not present in a noticeable amount in small and large cotyledons. Isolation and culture of Jaffrabadi placental cells in M-199 medium with antibiotics and 2% FBS results in the efficient production of progesterone, estrogen, and testosterone. This study has shown that trophoblast cells are the actual sites for steroid hormone production. These cultured placental cells ($1x10^6$ cells/ ml) produce Progesterone, Estradiol-17β and Testosterone in the range of 1.72 to 2.12, 16.03 to 19.51 and 0.51 to 0.58 ng/ml, respectively in Jaffrabadi buffalo.

**Keywords**: *Bubalus bubalis*, buffaloes, gross-morphology, histo-morphometry, trophobalst, hormones, placenta, cotyledon, Jaffrabadi buffalo

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## INTRODUCTION

India has a huge population of river type, swamp type as well as wild Asian buffaloes. Hence, India has a great biodiversity of buffalo germplasm, including the renowned Jaffrabadi buffaloes, famous for high milk fat. Around 97% (195 million) of the total global buffalo population (201 million) resides in Asia and only 3% in rest of the world (Hedge, 2019). India alone have more than 50% of world's buffalo population with an annual increase of about 1% (Kumar and Singh, 2010). Buffaloes have been the backbone of the White Revolution in India and have a major contribution to the national economy.

Recently, placentology and placental pathology are getting significant importance. The bovine placental structure and development can help veterinarians, pathologists, and researchers to understand several economically important infectious abortifacient diseases and pregnancy failure (Manzoor *et al*., 2018).

Placental chorionic villi are lined with a layer of irregularly arranged trophoblast epithelial cells and a vascularized mesenchyme. Underneath, fetal capillary loops, also called intraepithelial capillaries, form an undulating basement membrane. The binucleate cells (BNC; giant cells) are scattered in uniformly distributed uni-nucleate trophoblast epithelial cells (TEC) (Bjorkman, 1969; Al-Ramadan, 2014). The TEC are believed to be involved in nutrient exchange. Hence, numerous microvilli, apical vesicles along with numerous mitochondria are present in mono-nucleated TEC. On the other hand, binucleate cells produce hormones such as placental lactogen and progesterone (Duello *et al*., 1986; Igwebuike, 2006). Carvalho *et al.* (2006) studied placental binucleate trophoblast giant cells (BNC) of the water buffalo, *Bubalus bubalis*, highlighting the synthesis of BNC specific proteins. The frequency of BNCs was 20% of the trophoblastic cells in placentas during the first trimester which increased to 27% in the later stages. Microscopically, a prominent granular endoplasmic reticulum and Golgi body were observed in binucleate cells suggesting active protein synthesis. Therefore, this study was carried out to show different anatomical, physiological, and cellular structures and numbers in the fetal cotyledon of Jaffrabadi buffaloes.

## MATERIALS AND METHODS

This work was performed at Veterinary College, Junagadh and the samples were collected from Jaffrabadi buffaloes reared at Cattle Breeding farm, Junagadh. A total of ten full term expelled placenta was collected at time of natural parturition in Jaffrabadi buffaloes. The newborn calf weight, placenta weight, size and number of placental cotyledons were recorded, and cotyledons were categorized into small, medium, and large size based on cotyledon diameter.

### Histological examination

For histology, the placental cotyledonary tissues were collected in 10% neutral buffered formalin and kept for at least 1 to 2 days for fixation. Further, these tissues were dehydrated in graded alcohol, cleared in xylene, and embedded in paraffin. Tissue sections of 5 to 6 µm thicknesses were prepared and routinely stained Hematoxylin and Eosin staining method and different special staining method, PAS- Alcian Blue at pH 2.5 for Acid and neutral mucopolysaccharides, Oil Red O (Luna, 1968) and Sudan Black B (Chayen *et al*., 1969) for lipid and Alizarin Red S (Prophet *et*

*al*., 1994) for calcium distribution. The sections were observed under a light microscope and the histological alterations in the tissue were recorded. Micrometry was done by Carl Zeiss Zen 2 (blue edition) microscopic image analysis software and ImageJ software (Schneider *et al*., 2012).

**Isolation and culture method**

For the isolation and culturing of placental cells, fetal cotyledons were collected immediately after the parturition from animals in the phosphate buffer saline (PBS, supplemented with 100 IU/ ml Penicillin G, 100 µg/ml Streptomycin and 100 µg/ml Gentamicin) solution and transfer into the laboratory. Fetal cotyledon cut and half sections were minced with scissors and dissociated with 0.125% trypsin in phosphate buffer saline (PBS) for 30 minutes at 37°C. The minced tissues and cells were centrifuged (500 g / 2000 rpm for 10 minutes) and resuspended into serum-free M-199 media (supplemented with Hank's salts and L-glutamine and antibiotics 1% penicillin/ streptomycin 10,000 IU/ 10,000 µg/ml). The tissue and cells were washed three times with serum-free M-199 medium and filtered with 4 layers of cheesecloth. The trophoblast cells were collected by centrifugation (500 g / 2000 rpm for 10 minutes) from the filtrate. The cells seeded at a final density of $1x10^6$ cells/ml by manual counting through the Neubauer chamber. This standard protocol provided a successful non-contaminated healthy growth of placental cells. These cells of density at $1x10^6$ cells/ml were resuspended in M-199 medium with 2% fetal bovine serum (FBS) in 6 well plates for 24 h with controlled atmosphere $CO_2$ incubator at 37°C. After incubation of 24 h, the supernatant culture medium was collected and stored to estimate different hormone ($P_{4,}$ $E_{2,}$ and Testosterone) secretion by standard ELISA kits.

**Data recording and analysis**

The statistical evaluation of obtained results/data was performed by assessment of the mean values (x) and standard error of the mean (SEm) in each monitored placenta of cows and buffaloes. The significance (P) of differences in the mean values of corresponding treatments given to placental cell indicators was carried out by two-way analysis of variance (ANOVA) (Snedecor and Cochran, 1994). The relationship between the monitored indicators was evaluated by the significance of the correlation coefficient.

## RESULTS AND DISCUSSIONS

The gross, histological, and micrometrical observations on small and large fetal cotyledons of and Jaffrabadi buffalo were recorded to find out the structural changes between small and large size cotyledons.

**Gross observation**

Full term expelled placenta (n=10) was collected from Jaffrabadi buffaloes for gross observations. The mean weight of newborn calf of Jaffrabadi buffaloes was 34.80±0.79 kg.

A total of 10 normally expelled fetal placenta from buffalo was collected and found average calf birth weight (34.80±0.79 kg) and male to female ratio (sex ratio) of newly born calves was 0.67 (4/6) in Jaffrabadi buffalo. Morphologically, the cotyledons were observed convex and non-pendunculated in Jaffrabadi buffaloes (Figure 1A). Similar morphological features were reported by Mossman (1987) and Anderson (1927) in the exotic cattle breeds. The mean weight of the expelled fetal placenta in Jaffrabadi buffalo was recorded 3.37±0.10 kg. Sarvaiya *et al.* (1990); Murugeppa *et*

*al*. (1998) recorded lower fetal membrane weight in Surti buffaloes. Surti buffaloes are smaller in size than the Jaffrabadi buffaloes. A positive correlation of placental weight and calf birth weight was observed in the species. Similar findings were observed in triple cross cattle (Padodara and Arya, 2014) and swamp buffaloes of Assam (Bhuyan *et al*., 2016).

The number of cotyledons in the expelled fetal placenta in Jaffrabadi buffalo in the study ranged between 72 to 138 and the mean was 102.45±4.00. Earlier, the number of placenta reported by in cattle (Adeyinka, 2012) and buffalo (Raja Ram, 1982) were 82.0±19.5 and 77 to 90, respectively. Carter (2019) opined that the number of cotyledons in the placenta ranges from 50 to 175 and a significant variation in cotyledon numbers is observed within and between species.

The fetal cotyledon in buffalo was divided into three categories (Table 1, Figure 1B) based on mean diameter namely, small (3.76±0.09 cm), medium (6.03±0.06 cm) and large (9.02±0.08 cm). The cotyledon diameter on uterine horn of non-descript breed of buffalo was reported as 8.64±0.58 cm (Ranjan and Singh, 2013) whereas a diameter of 10.8±2.06 cm was reported by Raja Ram (1982). Similarly, the mean cotyledon length of African buffalo (Schmidt, 2005) and Cameroon cattle (Kouamo *et al*., 2018) is reported as 6.4 to 7.2 cm and 7.9±0.7 cm, respectively. Altered physiology and variable gestation length could be the probable reason for the difference in size and number of cotyledons. A long gestation period in buffalo might justify the need of larger and numerous placental cotyledons. However, we could not find a significant correlation between the number and size of cotyledons in Jaffrabadi buffaloes.

**Histological comparison**

While comparing small and large cotyledon, a visible basal lamina was lacking in small cotyledon which together with the varying forms and shape of trophoblast epithelial cells (TEC). This made it difficult to assign the trophoblast epithelial to a specific epithelial type. More numerous capillaries with the connective tissue were observed in large size cotyledon to indent the basal lamina and adopted an inter-epithelial position, displacing the trophoblast epithelial cells (TEC) in the process in comparison to small size cotyledon (Figure 2A and 2B). Histologically, extensive branching of the fetal villi continued and found terminal branches became extremely fine. Secondary villi were generally longer when compared to corresponding branches in the buffaloes (Figure 2A and 4B). The endometrium was well developed with a vascular covering of the caruncle and presenting gaps. The cotyledon had villous covered by trophoblast uninucleated and binucleated giant cells. The mucosal epithelium was simple prismatic and corium glands in the limit of the myometrium presented a tortuous appearance. Caruncle and cotyledons were covered respectively by simple cubic epithelium and trophoblast giant binucleate cells (Oliveira *et al*., 2010).

In H and E stained sections, the bizarre shape of secondary and tertiary villi made it difficult to accurately describe the regular trophoblast epithelial cells (TEC). The TEC was comprised of two types of cells, namely uninucleated cells (UNC) and binucleated cells (BNC). Uninucleated trophoblast epithelial cells were found in great variety, size, and shapes but BNCs were generally larger and ovoid with two nuclei. Nuclei and cytoplasm of BNC were more intensely stained

than UNC (Figure 4B). A similar observation was made by Carvalho *et al*. (2006) that the cuboidal mononucleate cells predominate in number but smaller than BNC, and no secretory granules were visible in the cytoplasm of these cells. However, Raja Ram (1982) observed cuboidal and columnar cells of epithelium stained darker and contains light to darkly stained nuclei. Few cells showed extensive vacuolation in their cytoplasm where most histochemical reactions in these cells were either negative or occurred in traces. The giant cells generally stained darkly eosinophilic than other cells and revealed a darker supranuclear cytoplasm with H and E staining.

Masson's Trichrome stains revealed the core of the fetal villi and ground substance at the stromal line homogenously stained mesenchymal cells and other tissues. Distribution of the collagen fibers in the tertiary villi arranged and stained more in the small size cotyledon compare to large size cotyledon (Figure 3A and B).

**Micrometrical comparison**

In the present study, the mean length of the tertiary villi (µm) of the small cotyledon and large cotyledons were 56.00±2.15 and 114.71±5.36 in Jaffrabadi buffaloes. But between small and large cotyledon it was found highly significant difference ($P<0.05$). Most studies on bovine placentas have been focused on the fine structure of the cotyledons. There is a general agreement that the placenta is primarily an epithelio-chorial organ but also possesses small regions of a syndesmochorial character. Baur (1972) compared the overall villous surface of the cattle and human placenta and observed it is roughly 10 times larger than that of the human placenta. Hradecky *et al*. (1988) measured the length of villi in several artiodactyl cotyledons and reported that the villi length in cows were 17 mm with extensive branching. Furthermore, the villi were more compact and covered with single-layered flat to cuboidal epithelium in the placentas of older animals. Raja Ram (1982) observed villous epithelium appeared cytotrophoblastic to syntrophoblastic and measured 30 to 60 µm in height in buffaloes. The cells varied from simple cuboidal to the column with frequent occurrence of giant cells and epithelium generally stained darker.

The mean diameter of trophoblast epithelium cells (TECs) measured for small and large cotyledon in Jaffrabadi buffaloes were 7.19±0.32 and 7.59±0.33 µm, respectively (Table 2). The mean diameters of TECs did not differ significantly ($P>0.05$) in Jaffrabadi buffalo. The mean nuclear diameter of TECs (µm) in small and large cotyledon was 3.44±0.66 and 4.44±0.46 in Jaffrabadi buffalo, respectively. The mean nuclear diameter of trophoblast epithelium cells of large cotyledon was significantly ($P<0.05$) larger than the small cotyledon. But the picture of trophoblast giant cells (TGCs) was found to be different than the TECs. The mean diameter of TGCs (µm) of small and large cotyledon in the Jaffrabadi buffalo, was 13.89±0.37 and 16.57±0.54, respectively. The mean diameter of TGC of small and large cotyledon differs significantly ($P<0.05$) in Jaffrabadi buffalo. The mean nuclear diameters of TGCs in small and large cotyledons were 12.28±0.66 and 14.28±0.75 in Jaffrabadi buffalo, respectively. The mean nuclear diameter of trophoblast giant cells showed a significant ($P<0.05$) difference between small and large size cotyledon.

The size of a fully mature, granulated BNC ranges between 30 to 50 µm (Duello *et al*., 1986; Wooding, 1992). The epithelium is simple with prominent lateral cell membranes between crypt epithelial cells. Generally, crypt epithelial cells are large cuboidal cells (12 to 18 µm) which

are pale in appearance with one or more nuclei in the basal or central location in the cell (Schmidt, 2005). Ranjan and Singh (2019) observed that the size of the trophoblastic epithelium, cryptal epithelium, and nucleus diameter (µm) were 21.32±1.57, 7.51±0.21, and 7.75±0.36 respectively in the buffaloes. However, the nucleus of the trophoblastic epithelium was found darkly stained with the eosinophilic cytoplasm as seen in their study.

In the present study, the mean count of TECs per $mm^2$ in the small and large cotyledons were 316.80±16.74 and 241.50±16.65 in Jaffrabadi buffalo, respectively. However, the mean count of TGCs per $mm^2$ in the small and large cotyledons were 117.80±5.34 and 76.40±4.70 in Jaffrabadi buffalo, respectively. A marked significant ($P<0.05$) difference was observed in the mean count of TECs and TGCs per $mm^2$ in the small and large cotyledons in this species. The ratio between TECs and TGCs in small and large cotyledons in both the species showed no significant ($P>0.05$) difference. Also, in the present study, around 75% (72 to 77) TECs and 25% (24 to 27) TGCs were observed. Similarly, researchers have reported that the fetal trophoblast epithelium covering the chorionic villi consists of roughly 80% uninucleate trophoblast cells (UTCs) and 20% binuclear trophoblast giant cells (TGCs) in the bovine (Polei *et al*., 2020; Peter, 2013; Wooding, 1992) and 20 to 28% BNCs were distributed in the trophoblastic layer of buffalo placenta (Carvalho *et al*., 2006).

A non-significant ($P>0.05$) correlation between size and number of TGC was found in in small ($r = -0.232$) and large ($r = -0.203$) cotyledon of Jaffrabadi. This can be explained by the fact that an increase in the size of TGC compensates for the decrease in the number of Giant cells. Interestingly, a significant ($P<0.05$) positive correlation was found between the size of TGC and the number of TEC in small cotyledon in Jaffrabadi ($r = 0.299$) but in large size cotyledon any significant correlation was not found. This relationship indicates that the size of TGC compensates for the decrease in the number of TEC for the involvement in endocrine functions. The length of tertiary villi of cotyledon was found to have a positive correlation to the size of TGC and TEC in both cotyledons (small and large) but was found negatively correlated with the size of TEC. The reason is obscure and needs to be addressed in the future to find the scientific reason that makes difference between both the species.

**Histochemical distribution**

The distribution of neutral and acid mucopolysaccharides in the small and large cotyledon of Jaffrabadi buffalo was demonstrated by Periodic Acid Schiff (PAS) and Alcian Blue (AB), respectively (Figure 5A and 5B). Strongly PAS-positive cytoplasmic granules were found in the trophoblastic epithelium of the villous arcade area in the large cotyledon of Jaffrabadi buffaloes compared to the small-sized cotyledons. A strong reaction of neutral mucopolysaccharides showed for tunica intima of blood vessels was observed. A weak alcinophilic reaction was found at the basement membrane of the villous and in the center of the villi in the large cotyledon of both the species compared to the small one. High activity of neutral mucopolysaccharides indicates it containing glycogen thus confirming that a high level of metabolism would be expected in placental trophoblasts where considerable activity for transport, degradation, and synthesis of materials is taking place. A similar reaction of PAS was observed during the mid and late phase of pregnancy and some of the migrating giant cells showed alcinophilic granules at mid-phase in buffaloes

(Ranjan *et al*., 2013) and acid mucopolysaccharides (glycosaminoglycans, GAGs) content increased in term placenta than young placenta in human (Lee *et al*., 1973). The BNCs, characterized by a hyper-chromatic cytoplasm with active ribosome and mature Golgi-bodies, are distributed randomly throughout the trophoblast (Wango *et al*., 1990). The BNCs, when stained with PAS stain show large membrane-bound granules produced by Golgi bodies in almost half of the cells (Rodriguez *et al*., 2004).

The histochemical investigation of calcium was demonstrated by Alizarin Red stain, but calcium could not be found deposited in any parts of the small and large fetal cotyledon of Jaffrabadi buffalo (Figure 6A and 6B). Although very few and little globular pattern calcium deposits were observed only in the large-sized fetal cotyledon. Although, the parathyroid hormone and calcitonin are not permeable to placenta but the fetus is involved in large quantities of calcium. However, 25-hydroxy vitamin D crosses the placenta spontaneously but the permeability of 1, 25 $(OH)_2$ D is uncertain. The fetus receives ionized calcium from mother at a rate of approximately 50 mg/day and 330 mg/day at 20 and 35 weeks, respectively. At birth, the placental source of calcium ends abruptly and the serum calcium level declines, possibly provoked by hypoparathyroidism and/or hyper-calcitonemia residual from fetal life (Kumar and Kaur, 2017). This could be the reason for the absence of calcium in the full-term expelled placental cotyledons. However, in disagreement with our findings, few researchers have reported the presence of calcium deposits in the placenta at various gestational stages. The probable explanation for this is explained by Wallingford *et al.* (2018), who stated that the deposition of calcium in the placenta can be an indicator of viral infection. However, the exact pathophysiology of placental calcification is poorly understood. Similarly, Agababov *et al.* (2007) hypothesized that the increased placental calcification at early embryonic life might be caused due to nanobacterial infection, and it is named pathological placental calcification (PPC).

The lipids were demonstrated by Oil Red O stain and Sudan Black B in the small and large cotyledon of Jaffrabadi buffalo (Figure 7A and 7B). Both the cotyledonary tissues showed a strong Sudanophilic reaction, and the Oil Red O positive lipids were observed. This positive reaction indicates the probable site for steroidogenesis and the storage of lipid hormone in cotyledonary tissues. According to our findings, a similar reaction was observed in buffaloes by Ranjan *et al.* (2013). They observed strong reactions for lipids at trophoblastic and cryptal epithelium, with advancing gestation the maternal septa and villous core showed moderate activity. Also, traces of lipids were found during early pregnancy in buffalo that increased with gestation. The lipid content was more on the fetal side as compared to the maternal side (Prasanth Babu, 2008). Bjorkman (1954) observed that the cryptal epithelium and the trophoblastic epithelium were rich and poor respectively in fatty materials.

**Hormonal levels in cotyledonary placental cells**

The basal level of $P_4$, $E_2$, and Testosterone (T) was measured from cultured placental cells ($1x10^6$ cells/ml) in the small and large cotyledon of Jaffrabadi buffalo is mentioned in the Table 3. There was no significant difference between the two sizes of the cotyledon. No reference was found for the basal level of T from placental cells in bovine. Although, few workers measured T from placental tissues at different gestation periods and post-partum periods and found 0.15 to 1.74 ng/g

Table 1. Animal and gross morphology of fetal placental characteristics of Jaffrabadi buffaloes (mean ± SEM, n=10).

| Sr. No. | Particulars |
|---|---|
| 1 | Average age (yrs) - 8.40±0.48 |
| 2 | Average gestation period (days) - 317.00±2.48 |
| 3 | Birth calf sex ratio (Male: Female) - 0.67 |
| 4 | Calf birth weight (kg) - 34.80±0.79 |
| 5 | Weight of placenta (kg) - 3.37±0.10 |
| 6 | Total fetal cotyledon (number) - 102.45±4.00 |
| 7 | Diameter of cotyledon (cm) |
| | Small - 3.76±0.09 |
| | Medium - 6.03±0.06 |
| | Large - 9.02±0.08 |
| 8 | Number of cotyledon |
| | Small - 26.1±1.18 |
| | Medium - 62.0±5.44 |
| | Large -19.6±1.19 |

Table 2. Comparative micrometrical parameters of fetal placental villi and cells in the small and large cotyledon of Jaffrabadi buffalo (mean ± SEM).

| Particular | Parameters and unit | Small cotyledon | Large cotyledon |
|---|---|---|---|
| Length of tertiary villi | Length (µm) | $56.00\pm2.15^{a}$ | $114.71\pm5.36^{b}$ |
| Trophoblast epithelial cells (TEC) | Cell diameter (µm) | 7.19±0.32 | 7.59±0.33 |
| | Cell nucleus (µm) | $3.44\pm0.66^{a}$ | $4.44\pm0.46^{b}$ |
| | Number of cells / $mm^2$ | $316.80\pm16.74^{a}$ | $241.50\pm16.65^{b}$ |
| Trophoblast Giant Cells (TGC) | Cell diameter (µm) | $13.89\pm0.37^{a}$ | $16.57\pm0.54^{b}$ |
| | Cell nucleus (µm) | $12.28\pm0.66^{a}$ | $14.28\pm0.75^{b}$ |
| | Number of cells / $mm^2$ | $117.80\pm5.34^{a}$ | $76.40\pm4.70^{b}$ |
| Ratio TEC vs TGC | - | 2.74±0.18 | 3.26±0.27 |
| Total % | TEC | 72.89 | 75.97 |
| | TGC | 27.11 | 24.03 |

Values with different superscripts within a row differ significantly ($P<0.05$).

Table 3. The basal level of $P_4$, $E_2$, and Testosterone from cultured placental cells (ng/ml, mean ± SEM) in small and large cotyledon.

| Hormone | Small cotyledon | Large cotyledon |
|---|---|---|
| Progesterone ($P_4$) | 2.12±0.25 | 2.00±0.36 |
| Estradiol-17β ($E_2$) | 19.51±2.68 | 18.73±2.83 |
| Testosterone (T) | 0.58±0.21 | 0.55±0.19 |

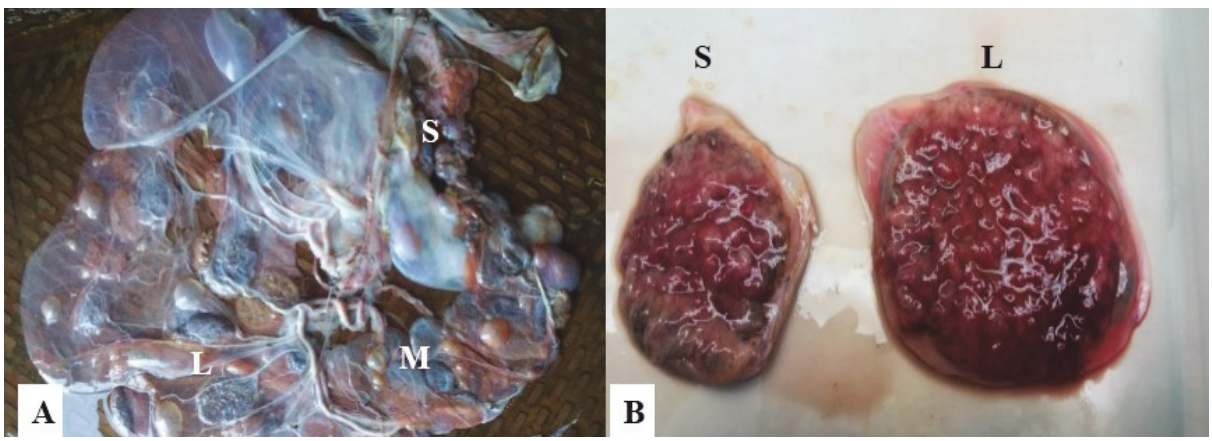


Figure 1. A. Placental Gross Morphology of Jaffrabadi buffalo (L) large size (M) medium size (S) small size cotyledon; B. Feto-maternal interface of a cotyledon characterized by villi and ventral view of the fetal cotyledon (small and large size).

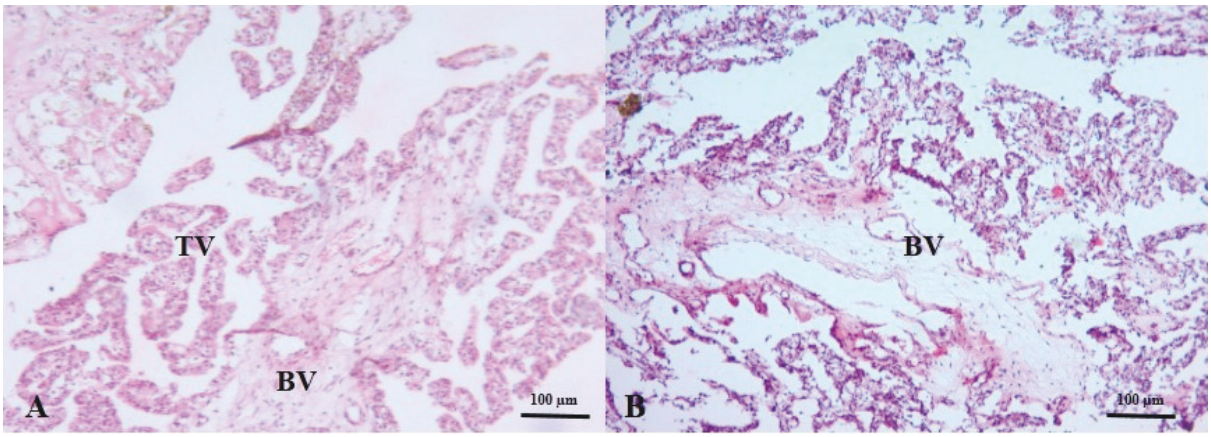


Figure 2. Section of large cotyledon (A) and small cotyledon (B) of buffalo: Showing well developed secondary villi with tropho-epithelial cells in lamina propria and at an outer surface of tertiary villi (TV) and engorgement of a few blood vessels (BV) (H and E X100).

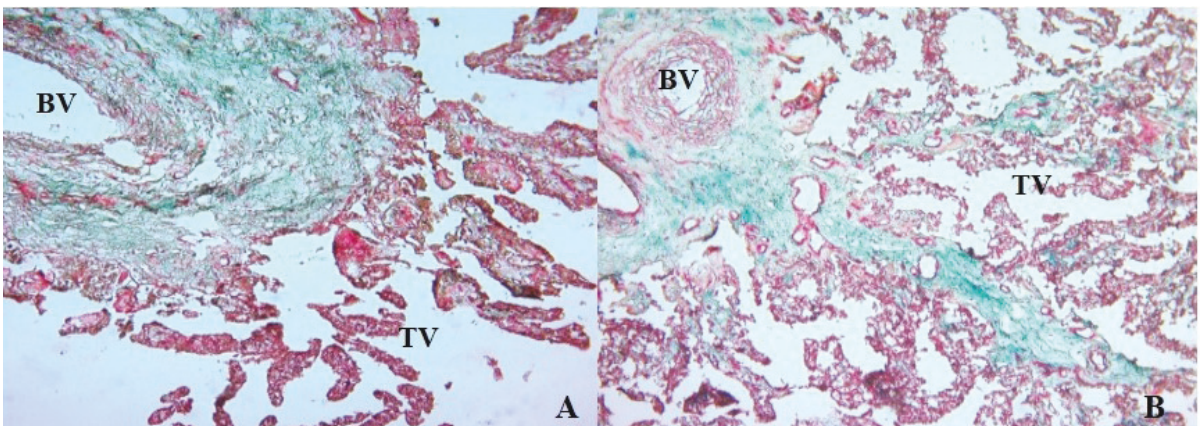


Figure 3. Section of large cotyledon (A) and small cotyledon (B) of Jaffrabadi buffalo: Showing collagen fiber around the lamina propria and few fibers in villous trees and at terminal villi (TV). The center of the villous trees is marked by the large fetal vessels (BV) (Masson's trichrome X 100).

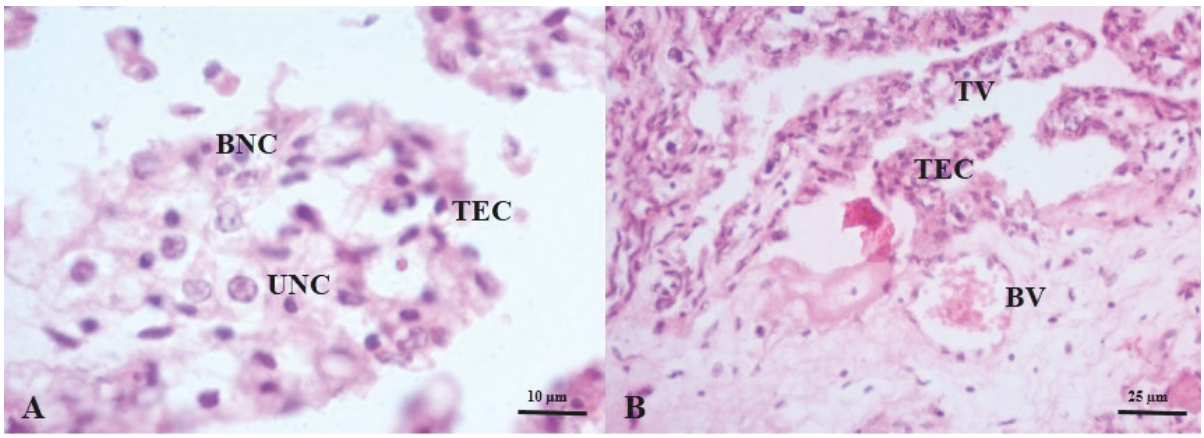


Figure 4. A) Section of large cotyledon: Center of the tertiary villi binucleated trophoblast (BNC) and uninucleated trophoblast cells (UNC) are observed with granule filled cytoplasm and at the surface trophoblast epithelial (TEC) layer is observed (H and E X 1000). B) Section of large cotyledon of Jaffrabadi buffalo: Severe infiltration of blood cells in the vessel (BV) and numerous trophoblast epithelial cells (TEC) are found in the villi, lamina propria is vascularise (H and E X 400).

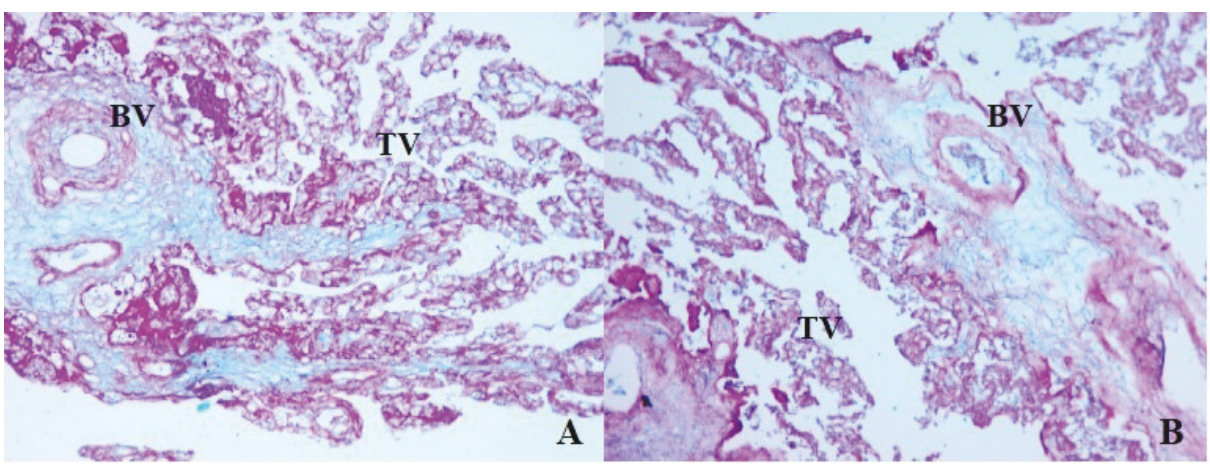


Figure 5. Periodic Acid Schiff's and Alcian Blue staining for NMPS and AMPS demonstration in the A) large cotyledon & B) small cotyledon: Tissues showing the strong reaction of the periodic acid Schiff (red for NMPS and blue for AMPS) in center of the tertiary villous (TV) and the villi (BV-blood vessel) (PAS-AB X 100).

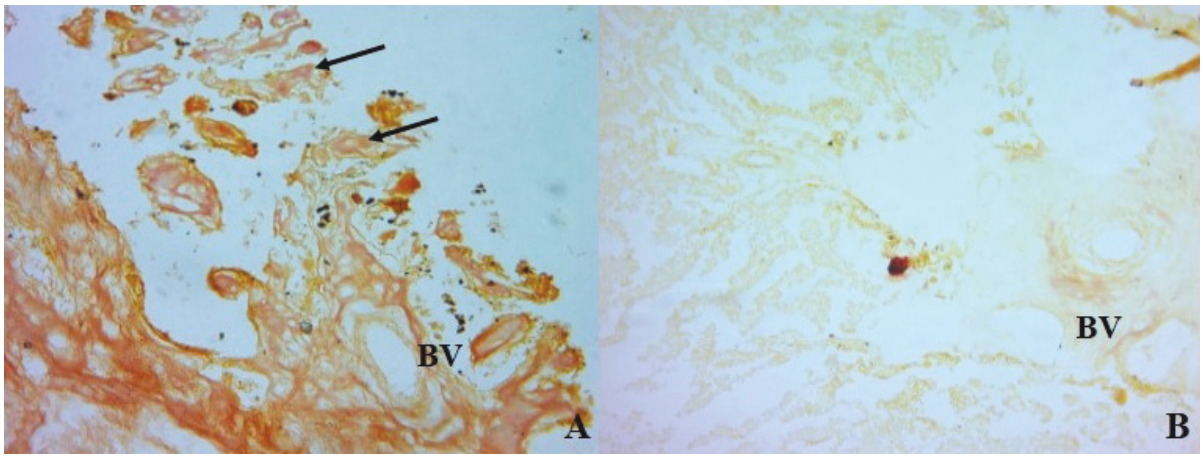


Figure 6. Alizarin Red staining (X 100) for calcium deposition in A) large cotyledon & B) small cotyledon: Globular pattern of very little calcium (arrow mark) deposition found at the boundary of villi in large cotyledon, BV-Blood vessels (Alizarin Red X 100).

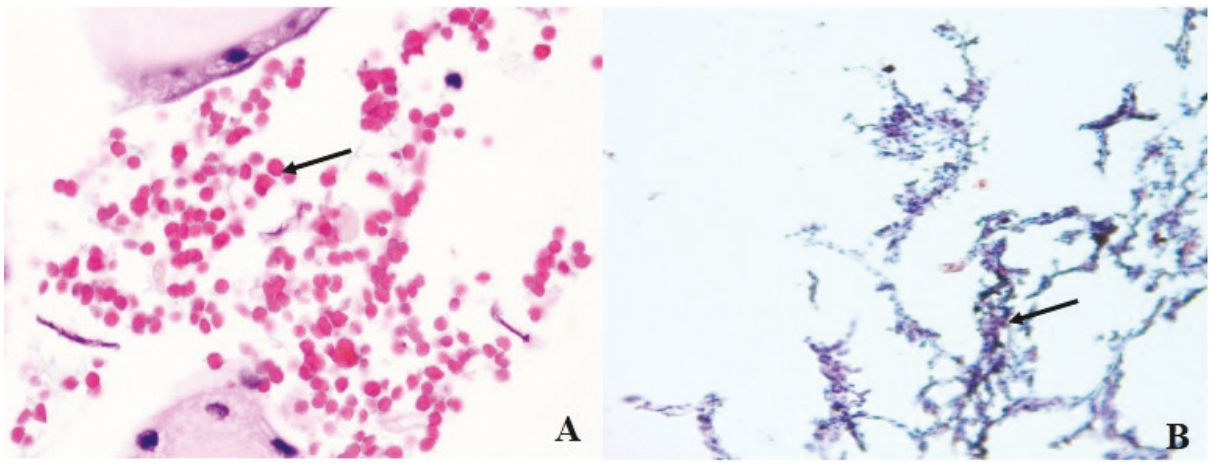


Figure 7. A) Oil Red O staining (X 400) for lipid deposition in cotyledon: Tissues showing strong reaction of the lipid part in middle of the villous (arrow mark) and the villi (Oil Red O X 400); B) Sudan Black B staining (X 100) for lipid deposition in cotyledon: Distribution of lipid content in trophoblastic epithelium (arrow mark) at tertiary villi (Sudan Black B X 100).

during 60 to 272 days of gestation period in cattle (Khatri, 2011) and found the higher level at 272 days than 60 to 220 days of gestation period. The level of T was measured 2.9±0.1 ng/ml (Hong *et al*., 2019) in the term placenta of humans.

## CONCLUSION

Grossly, weight of placenta, number and size of cotyledon, and calf birth weight were observed higher compared to cattle and other breeds of buffaloes. Histo-morphologically, tertiary villi length, cellular and nuclear diameter of trophoblast epithelial cells and trophoblast giant cells are more in large cotyledon than small cotyledon in Jaffrabadi animal. The histochemical investigation of the distribution of neutral and acid mucopolysaccharides in the small and large cotyledon of Jaffrabadi buffalo revealed strong PAS-positive cytoplasmic granules were found in the trophoblastic epithelium of the villous arcade area in the large cotyledon Jaffrabadi buffaloes compare to the small-sized cotyledons. A strong reaction of neutral mucopolysaccharides showed for tunica intima of blood vessels was observed. A weak alcinophilic reaction was found at the basement membrane of the villous and in the center of the villi in the large cotyledon of both the species compared to the small one. High activity of neutral mucopolysaccharides indicates it containing glycogen thus confirming that a high level of metabolism would be expected in placental trophoblasts where considerable activity concerning transport, degradation, and synthesis of materials is taking place. The calcium distribution was demonstrated by Alizarin Red stain, but calcium could not be found deposited in any parts of the small and large fetal cotyledon of Jaffrabadi buffalo. Although very few and little globular pattern calcium deposits were observed only in the large-sized fetal cotyledon. The lipids were demonstrated in the small and large cotyledon of Jaffrabadi buffalo in both the cotyledonary tissues showed a strong Sudanophilic reaction and the Oil Red O positive lipids were observed. Trophoblast cells can be used to produce natural steroid hormones. The physiological role of trophoblast epithelial cells and trophoblast giant cells in terms of peptide, and protein hormones synthesis need to be investigated.

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