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A light and electron microscopy study of the yak placentome

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Abstract

In order to clarify and reveal the morphological characteristics of yak placentomes, placentomes obtained from 151 to 180 days of pregnant yaks were observed using light microscopy and scanning and transmission electron microscopy. The results indicated that sessile, dome-shaped yak placentomes seemed to have a relatively complex villous-crypt architecture pattern. There was a straight maternal plate beneath the placentome. Plentiful uterine glands and a dense cellular layer were present in the endometrium lamina propria close to the maternal plate. Trophoblast giant cells appeared to have similar ultrastructure features to these in other ruminants, including abundant mitochondria, an extensive array of rough endoplasmic reticulum, advanced Golgi complex and many specific secretory granules. Trophoblast giant cells could also secrete neutral and acid glycoconjugates. Furthermore, numerous glycoconjugates were distributed in the connective zones between mononuclear trophoblast cells and crypt epithelial cells as well as in maternal connective tissues. Mononucleate trophoblast cells which had abundant microvilli that interdigitated with the corresponding microvilli arising from the crypt epithelial cells, had numerous mitochondria and vesicles, but did not exist glycoconjugates.

Key words: Yak; placentome; microstructure; ultrastructure; histochemistry

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The placenta is the link between the mother and fetus during pregnancy. It is not only a place for material and gas exchange between the mother and fetus but also a temporary organ with multiple functions (Qian, 2003). The normal development of placental morphology and function during pregnancy is closely related to the survival and development of the fetus (Farin et al., 2001; Schlafer et al., 2000), and placental abnormalities are often associated with abortion and perinatal mortality.

Yaks (Bos grunniens) are essential livestock for people living in high altitude areas in Central Asia. They are well adapted to the harsh environment at high altitude, low temperature and hypoxia. However, the reproductive efficiencies of yaks are low. They reach puberty late, at a mean age of 33.4 ± 6.0 months, and most yak cows calve only once every 2 years. Yaks are seasonally polyestrous animals. The breeding season starts from July to October, and the calving season occurs from April to July, with the duration of pregnancy ranging from 248 to 258 days (Yu et al., 1993). The mean gestation lengths are obviously shorter when compared with other bovids, such as 343 days for African buffalo (Syncerus caffer) (Knechtel, 1993), 315 days for water buffalo (Bubalus bubalis) and 280 days for cattle (Bos taurus) (Hafez and Hafez, 2000). With regard to the reproductive organs, some differences are evident between yak and dairy cow. In yak, for example, there are three transverse circles on the uterine cervix, consisting of many tight folds, and the body of the uterus is rather short (Cui and Yu, 1999).

There is, however, no report on the microscopic and ultrastructural structure of the yak placentome. In the present study, the placentomes from six pregnant yaks were collected to investigate the microscopic and ultrastructural characteristics. The distribution pattern of the glycoconjugates was determined by histochemical techniques. Meanwhile, the authors try to show whether the morphology of the yak placentome is similar to that of other bovid genera or not.
1 Materials and methods

1.1 Animals

Six female yaks from 6 to 7 years of age, with the breeding season ranging from July to August, were included in the investigation. The yaks were purchased from an abattoir in Xining City of Qinghai Province as approved by the Welfare Authority of the local government. The experimental yaks were slaughtered at the abattoir; immediately after collection, placentomes were chosen and fixed. The fetal crown-rump lengths of the six yaks were 42.0 cm, 42.3 cm, 44.0 cm, 44.7 cm, 45.6 cm and 47.8 cm, respectively.

1.2 Light microscopy

Three placentomes of the same area of the pregnant horn (close to the fetus) from each yak were randomly selected and further processed for histological and histochemical analysis. The samples were cut into pieces no larger than 1 cm³, fixed in 4% neutral paraformaldehyde phosphate buffer (PH 7.4) for 24 h, dehydrated in graded alcohols, and embedded in paraffin wax. Vertical sections 4-μm thick were cut and stained using a standard hematoxylin and eosin (HE) protocol, periodic acid Schiff (PAS) reaction, Alcian blue (AB, pH 2.5) and AB-PAS (pH 2.5). Light microscopy observations and section image acquisition were carried out using an Olympus DP71 Light Microscope (including DP control and Image-Pro Express, Japan).

1.3 Transmission electron microscopy

For transmission electron microscopy, tissue blocks of approximately 1 mm³ were taken from the center of each placentome, then immersed in 2.5% glutaraldehyde in neutral phosphate buffer (pH 7.4, at 4 °C) and post-fixed in 1% osmium tetroxide for 2 hours. Samples were dehydrated in a graded series of ethanol and embedded in Epon812. Semithin sections (1 μm) were cut and stained with toluidine blue. Suitable regions of interest were chosen, and
ultrathin sections (60-80 nm) were cut on a Leica EM-UC6 ultramicrotome, mounted on G200 grids, stained with uranyl acetate and lead citrate and viewed with a JEOL 1230 electron microscope at 120 kV.

1.4 Scanning electron microscope

For scanning electron microscopy, single villous trees were carefully separated from the chorionic plate and further processed for SEM to obtain the triaxial image of villous trees. The samples were immersed in 2.5% glutaraldehyde in neutral phosphate buffer (pH 7.4, at 4 °C) for a minimum of 24 h and post-fixed in 1% osmium tetroxide for 1.5 h. Then, the samples were dehydrated in a graded series of ethanol, dried in a critical point drying apparatus using liquid CO₂, coated with gold, and imaged using a JEM-100CX scanning electron microscope.

2 Results

2.1 Anatomical observation of yak placenta

Visually, the yak placenta was multi-cotyledonary, including multiple independent placentomes and an interplacentome. The placentomes were round, oval or irregular, arranged into three to four columns in the uterine horn, and the placentomes at the center of the uterus were well developed (Fig. 1A). The placentome was divided into uterine caruncles and cotyledons. Uterine caruncles had a convex dome shape with neatly arranged caruncular crypts. Cotyledons were clustered near the fetal villi, which protruded within the corresponding caruncular crypts (Fig. 1B). From the longitudinal section of the placentome, the fetal tissue formed a convex chorion plate (fetal plate), which covered the maternal caruncle like a hat. The maternal tissue had a relatively straight substrate (maternal plate), which was located under the caruncle. Since the maternal plate of the placentome was relatively straight, no caruncle stalk was observed (Fig. 1C).
Fig. 1. Anatomical observation of yak placenta during gestation. A. The yak placenta was composed of multiple separate placentomes (P) and interplacentomes (IP). B. The placentome was composed of fetal chorioallantois (FC) and maternal caruncles (MC); the cotyledonary villi (white arrow) were within the corresponding caruncular crypts (black arrow). C. The vertical section of the placentome; the dome-shaped placentome rested on a straight maternal plate (white arrows), and the chorionic plate (black arrows) covered the convex caruncle like a cap.

2.2 Histology of the yak placentome

Yak placentomes were composed of chorioallantois and uterine caruncles. The chorionic plate vertically projected the primary chorionic villi, and the latter divided into secondary branches. Then, divisions from secondary villi into tertiary villi could also be observed. The uterine caruncles were divided into many compartments (crypts) due to the invasion of chorionic villi. The invading chorionic villi inserted into the corresponding caruncular crypts, an interdigitation occurred (Fig. 2AB). The inner layer of the chorionic villi was fetal mesenchyme, which had scattered mesenchymal cells and blood vessels. The outer layer was lined with a trophoblastic epithelium, which consisted of two different cell types: mononuclear trophoblast epithelial cells and trophoblast giant cells. The mononuclear epithelial cells were irregular or cuboidal in shape and predominated in the trophoblastic epithelium, whose nuclei were oval or rather uneven in shape. Trophoblast giant cells were typically oval and appeared in different positions among mononuclear epithelial cells. One nucleus or two nuclei were
usually visible in a single trophoblast giant cell. The nucleoli were distinct, and the cytoplasm was abundant and stained intensely (Fig. 2C).

The crypts of the yak placentome consisted of an inner layer of maternal connective tissues and the outer crypt epithelium. The crypt epithelium predominantly contained the uninucleate cryptal epithelial cells, and among the uninucleate cells, there were a few cryptal giant cells. The uninucleate epithelial cells were generally cuboidal in shape. The rounded or ovoid nuclei were situated in the center of the cells. The nucleoli were rather indistinct, and the cytoplasm was stained lightly. Cryptal giant cells were larger than uninucleate epithelial cells, and their shapes were usually ovoid or irregular; they contained three or even more nuclei that almost filled the cells, so the cytoplasm was scarce. The maternal connective tissues were stained intensely and had abundant blood vessels (Fig. 2C). The straight maternal plate was located beneath the placentome, and there were plentiful uterine glands, blood vessels and a dense cellular layer in the endometrium lamina propria close to the maternal plate. The glandular epithelium was simple columnar or cuboidal, occasionally there existed secretory products in the glandular cavity.

2.3 Histochemical observations of the yak placentome

Paraffin sections of paraformaldehyde-fixed material were stained with PAS for neutral mucopolysaccharides and muco- and glycoprotein or were stained for acid mucopolysaccharide with Alcian blue (AB, pH 2.5). The AB-PAS (pH 2.5) reaction was used to separate acid glycoconjugates from neutral types. Fetal mesenchyma was essentially negative to the PAS reaction; positive Alcian blue staining is indicated by a blue or light blue stain, and after the reaction with both dyes, the fetal mesenchyma appeared blue (Fig. 3ABC).

Trophoblast giant cells were strongly positive after the PAS reaction, and red granules were observed after incubation with the Schiff reagent. They were also stained light blue with AB
(pH 2.5) and stained intensely reddish-purple or purple in the AB-PAS (pH 2.5) reaction. The positive staining was either distributed in the entire cytoplasm of TGCs or restricted to one side of the cell (Fig. 3ABC). Mononuclear trophoblast epithelial cells and uninucleate crypt epithelial cells remained unstained with PAS, AB (pH 2.5) or AB-PAS (pH 2.5). Strongly positive staining (red) with the PAS reaction was observed in the junction regions between trophoblasts and the crypt epithelium, and light blue staining with AB (pH 2.5) or intensely reddish-purple or purple staining with AB-PAS (pH 2.5) were also found. The staining properties of the basement membranes of both trophoblasts and the crypt epithelium were similar to that of the junction regions. Maternal connective tissues were stained red in the PAS reaction, light blue in the AB (pH 2.5) reaction and light reddish-purple in the AB-PAS (pH 2.5) reaction (Fig. 3ABC).

Fig. 2. Histological section (H&E) of yak placentome showing that the chorionic plate projected the primary villi (PV), secondary villi (SV) and tertiary villi (TV) into the corresponding caruncular crypt (CC). There were characteristic trophoblast giant cells (Rectangles) and crypt giant cells (Circle) in the trophoblast epithelium (TE) and crypt epithelium (CE). CT: connective tissue. Me: Mesenchyme.

Fig. 3. Distribution of glycoconjugate in yak placentome. A. The PAS reaction indicated that the red positive granules (Arrowheads) filled the trophoblast giant cells (TGCs), and the
positive reaction also appeared in the contact areas (Triangles) between the crypt epithelium (CE) and trophoblast epithelium (TE) as well as in the basal lamina (Asterisks) and maternal connective tissue (CT), Me: Mesenchyme. CGC: Crypt giant cell. B. AB (pH 2.5) staining of the yak placentome had similar results to those of the PAS reaction; the positive areas appeared light blue. C. The intensely reddish-purple or purple positive areas were observed following the AB-PAS reaction.

2.4 TEM observations of the yak placentome

2.4.1 Mononuclear trophoblast epithelial cells

Electron micrographs showed that the mononuclear trophoblast epithelial cells were usually columnar or irregular. The cytoplasmic membrane had a relatively smooth surface and was attached closely to the adjacent uninuclear trophoblast epithelial cells through tight junctions and desmosomes. The apical border had abundant microvilli interdigitating with microvilli of the corresponding uninucleate cryptal epithelial cells, forming the fetal-maternal contact zone. A great number of intracellular vesicles were present in the apical cytoplasm of the trophoblastic cells; sometimes, a few vesicles were also observed wrapped in the network of the microvilli. Their nuclei presented ovoid or irregular shapes and appeared either in a basal or central position. Abundant intranuclear euchromatins were distributed diffusely over the whole nuclear region, while a relatively few heterochromatin were organized in clumps mostly attached to the nuclear membrane. The cytoplasm contained regular organelles including mitochondria and endoplasmic reticulum. Most of the round or ovoid mitochondria were mainly concentrated in the apical and supranuclear cytoplasm, but a few were found beneath the nucleus. The rough endoplasmic reticulum (RER) was dispersed throughout the cytoplasm.
At the bottom of the cells, a well-developed basement membrane was distinctly seen, under which a fetal mesenchyme rich in capillaries was observed (Fig. 4A).

### 2.4.2 Trophoblast giant cells

The ovoid TGCs usually contained a smooth cell membrane and ovoid or round nuclei. The nuclei were generally located in the center of the cells, sometimes situated in lateral cytoplasm. The heterochromatins of TGCs were organized in clumps mostly attached to the nuclear membrane, while a few were situated centrally in the nucleus. Furthermore, abundant euchromatins were distributed in the nucleoplasm (Fig. 4B). The cytoplasm of TGCs was stained intensely and contained abundant organelles, other than lipid droplets, that were characterized by the presence of many secretory granules, which were round or ovoid in shape, either distributed in the whole cytoplasm or concentrated toward one side of the cell. These secretory granules were completely surrounded by a membrane, grainy and of variable electron density. Some of them contained microvesicles of variable numbers (Fig. 4BCD). Cytoplasmic components also included abundant round or ovoid mitochondria. Many mitochondria were usually concentrated close to the endoplasmic reticulum and lipid droplets, and the others were distributed diffusely over the whole cytoplasm (Fig. 4D). In addition, the TGC cytoplasm also contained rough endoplasmic reticulum grouped in parallel arrays. Polyribosomes were observed on the surface of the RER or distributed throughout the cytoplasm, and free ribosomes were abundant through the cytoplasm (Fig. 4E). Additionally, the Golgi apparatus was well developed, arranged parallel to each other, and many vesicles and vacuoles of varying size were around each Golgi (Fig. 4F).
2.4.3 Uninucleate cryptal epithelial cells

The uninucleate cryptal epithelial cells were usually cuboidal in shape and had a smooth cytomembrane. Their nuclei appeared round or slightly irregular and were usually located in the basal zone of the cells, but some were situated in the middle of the cell. Many euchromatin were seen in the whole nucleoplasm, while heterochromatin were mostly attached to the nuclear membrane. The cytoplasm contained mitochondria, which were round or ovoid in shape and mainly distributed in the supranuclear cytoplasm. The cytoplasm also contained rough endoplasmic reticulum, free ribosomes, and polyribosomes. The Golgi apparatus was poorly developed and rarely observed. Several lipid droplets of different sizes were occasionally found in the supranuclear cytoplasm. The apical part of the uninucleate cryptal epithelial cells contained numerous microvilli, forming a contact zone with mononuclear trophoblast epithelial cells. However, there was no microvillar junction between the uninucleate cryptal epithelial cells and TGCs. These cells were based on well-developed basement membranes that were closed to the maternal connective tissues (Fig. 4A).

Fig. 4. TEM micrographs of yak placentome. A. The trophoblast and crypt epithelial cells (TEC and CEC) were connected via a microvillar junction (MVJ) and rested on a well-developed basement membrane (B). There were mitochondria and vesicles within the sub-microvillous cytoplasm of TECs. Some lipid droplets could be seen within the cytoplasm
of the CEC; NU. Nuclei; Me. Mesenchyme; CT. Connective tissue (×4000); Inset: Showing MVJ and vesicles (×15000). B. There were numerous cytoplasmic granules within the trophoblast giant cells (TGCs) (×5000). C. Many granules of the TGCs contained microvesicles (×15000); D. TGC, showing mitochondria (M) and lipid droplet (LD) (×12000). E. TGC, showing rough endoplasmic reticulum (RER) (×15000); F. TGC, showing Golgi complex (×20000).

2.5 SEM observations of the yak placentome

The scanning electron microscopy showed that a single villous tree of the yak placentome displayed conical or triangular shapes and could be described as a Christmas tree. A wider base of the Christmas tree close to the chorionic plate was observed but gradually became slender along its course. Additionally, at the villous tip, the stem villus frequently divided into two or more parallel, vertical terminal branches, which were generally thinner in diameter than the villus of origin (Fig. 5A). Yak chorionic villi branched to the third order. The stem villi, which were in the central part of the Christmas tree or close to the chorionic plate, projected relatively long and large secondary villi at approximately right angles, and most secondary villi were leaf-like and arranged in layers. Then, the secondary villi also projected numerous long and slender tertiary villi (Fig. 5B), which were covered with trophoblast cells (Fig. 5C). In contrast to the villus in the central part or close to the chorionic plate, the simple appearance of secondary and tertiary villi could be seen at the tip of the yak villous tree (Fig. 5D). The shorter and smaller secondary villi were generally projected at approximately right angles or slightly acute from each primary villus, and simple and bulbous tertiary villi covered with trophoblast cells was observed (Fig. 5EF).
Fig. 5. SEM micrographs of yak placentome. A. SEM micrograph of a single villous tree in yak. B. Enlargement of the central part of the villous trees shown in A. Secondary villi branches from the stem villi are arranged in layers. C. Note the long and slender tertiary villi that are covered with trophoblast cells. D. Enlargement of the tip of the villous trees shown in A. A single villous tree dividing into several stem villi. E. Note that the simple (bulbous) appearance of secondary and tertiary villi. F. SEM micrograph of simple tertiary villi in yak.

3 Discussion

From this investigation, we concluded that the villous-crypt architecture pattern of placentomes was basically similar to different bovid genera. However, some obvious differences in the shape of a single villous tree and their ramification pattern were observed in each species. First, similar to cattle, yaks have a conical or Christmas tree-like shape villous tree (Leiser et al., 1997; Leiser et al., 1998). However, the villous trees of African buffalo and water buffalo placentomes were often described as a “Tuscany-cypress” shape due to their slender form and rudimentary branching (Schmidt et al., 2006; Abd-Elnaeim et al., 2003). Secondly, villous branching patterns in African buffalo were distinctly less complex than that
in cattle and yak and more closely resembled features of the water buffalo. In African buffalo and water buffalo, there were two types of villous trees, a rough type and a smooth type (Abd-Elnaeim et al., 2003; Schmidt et al., 2006). However, the smooth type of villous tree was not found in the placentomes of yak and cattle (Leiser et al., 1997; Schmidt et al., 2006). Furthermore, the secondary and tertiary villi of African buffalo and water buffalo appeared simple and bulbous; in contrast, the secondary villi of yak and cattle were long and slender and equipped with distinct, pointed tertiary villi (Leiser et al., 1997; Abd-Elnaeim et al., 2003; Schmidt et al., 2006). Stem villi projected villi of denser arrangements at approximately right angles or slightly acute, which resulted in the maximal functional surface area of villous tissue in a given space (Leiser et al., 1997). Thirdly, our present study found that at the villous tip, the stem villi frequently divided into two or more parallel, vertical terminal branches. Schmidt et al. (2006) reported similar results in African buffalo and indicated that the division from the villous tip into several terminal branches occurred frequently in African buffalo but seldom in cattle. Additionally, Miglino et al. (2007) carried out a study on cloned placentae in cattle and found that one villous tree projected more than one primary villus occupying each crypt, as opposed to usually one primary villus of the cotyledon in each primary crypt of the caruncle in non-cloned placentae. The total volume of terminal villi, the “working part” of villous trees, distinctly increased because the stem villi might contribute to enhancing the efficiency of transplacental diffusional exchange (Leiser et al., 1997).

Two different cell types had been recognized in the trophectoderm of the placenta of ruminant animals: mononucleate trophoblast cells and trophoblast giant cells (Klisch et al., 1999; Schlafer et al., 2000). Similar results were found in the present study. Trophoblast giant cells of yak villus appeared typically oval and usually contained one or two nuclei; TGCs with more than two nuclei described by Klisch et al. (1999) in cattle but absent in yak might suggest that serial sections should be further examined. In his fundamental work on TGC development, Wimsatt (1951) describes binuclear TGCs that developed from mononuclear
TGCs by acytokinetic mitoses in which mitotic figures were much larger than those in mitoses of normal mononuclear trophoblast cells. This is an early hint that TGC nuclei may be polyploid because the size of the mitotic figures depends on the number or size of the chromosomes they contain. The functional significance of polyploidization is supposed to be an increased synthetic capacity of TGCs, which results from the elevated number of gene copies available for transcription. In multinuclear cells, the transport between nucleus and cytoplasm could be facilitated by a higher ratio of nuclear surface to nuclear volume (Klisch et al., 1999). For example, the mouse giant cell trophoblast layer is adjacent to the decidua zone, where the trophoblast giant cells are P-TGCs (parietal trophoblast giant cells), which are the largest mononuclear polyploid cells, and the number of ploidy can reach 1024 n (normal tissue cell are biploid, which is 2n, Simmons et al., 2007). Their volume is approximately 16 times that of sponge trophoblast cells and more than 100 times that of glycogen cells (Lee et al., 1998), and they can secrete vascular endothelial growth factor (VEGF) and proliferin (Plf) to promote vascular hyperplasia and AM (Yotsumoto et al., 1998), NOS (Gagioti et al., 2000) and anticoagulant factor (Weiler-Guettler et al., 1996) to expand blood vessels and promote maternal blood flow to the placenta.

The ultrastructure features of TGCs of yak placentomes were similar to those in cow, sheep, goats, deer, African buffalo and Tragulidae (Wooding, 1992; Schmidt et al., 2006; Wooding et al., 2007), including abundant mitochondria, rough endoplasmic reticulum, well-developed Golgi bodies and many characteristic membrane-bound granules. According to the excellent research published in recent years (Wooding, 1992; Yamada et al., 2002; Wooding et al., 2005), these granules contain numerous protein and glycoprotein constituents, such as placental lactogen, prolactin-related protein I and pregnancy-associated glycoproteins. The contents of the TGC granules are delivered into the maternal compartments following migration and fusion of TGCs with maternal endometrial epithelial cells (Wooding, 1992). In addition, TGCs are thought to produce steroid hormones such as estradiol and progesterone (Wango et al., 1991;
In yaks, TGCs were characterized by the presence of many lipid droplets, serving as a store of low-density lipoprotein cholesterol, which was the principle substrate for the biosynthesis of steroid hormones (Njogu et al., 2006).

Noticeably, the granules of TGCs in yaks were positive to periodic acid schiff (PAS, appeared red) staining and Alcian blue (AB, appeared blue) staining, showing that they contained neutral and acidic glycoconjugates; similar chromogenic reactions were discovered in the special granules of other ruminants (Wooding, 1992; Yamada et al., 2002; Miglino et al., 2007). In yaks, the positive reactions also presented in the microvillar junction regions between trophoblasts and the crypt epithelium and in the maternal connective tissues. Interestingly, the distribution patterns of positive reactions to the two dyes in yaks were very consistent with the distribution of pregnancy associated glycoproteins (PAGs) in cows studied by Wooding et al. (2005). According to their research, the more modern PAGs were expressed primarily in trophoblast binucleate cells and seen in the stromal layer within the maternal caruncles, whereas the more ancient PAGs were localized largely in the contact regions of the trophectoderm and uterine epithelium. The PAGs expressed in yaks should be further studied using relevant techniques in the future.

Electron microscopy examination in our present study on yak placentomes showed that mononucleate trophoblast cells had morphological characteristics relating to specialized functions primarily involved in substance exchange, including the presence of numerous mitochondria, abundant microvilli and large numbers of vesicles in the apical cytoplasm of the cells. The microvilli interdigitated with the corresponding microvilli arising from the crypt epithelial cells, forming the feto-maternal contact zone (Bjorkman, 1969; Guillomot and Guay, 1982), which could provide an increased surface area for the absorption of materials. Plentiful vesicles in the apical cytoplasm of the cells might indicate pinocytotic activity delivering exchange material, and the mitochondria appeared to be related to substance exchange (Bjorkman, 1969; Guillomot and Guay, 1982).
In conclusion, the morphological structures of yak placentomes were similar to those of other bovid genera; however, certain differences were observed. These findings might provide morphological evidence for evolutionary relationships between different bovid genera. The presence of a relatively complex villous-crypt architecture pattern, special absorptive cells and characteristic endocrine TGCs in yak placentomes are indispensable for fetal survival and development during gestation.

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