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An Anatomical, Histological and Ultrastructural Study on the Placenta of the Red-shanked Douc Langur (Pygathrix nemaeus)

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Original Article

An Anatomical, Histological and Ultrastructural Study on the Placenta of the Red-shanked Douc Langur (*Pygathrix nemaeus*)

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Abstract

A red-shanked douc langur (*Pygathrix nemaeus nemaeus*) is currently enlisted as an endangered primate species. This study was undertaken to describe the gross anatomy, histology and ultrastructural morphology of the douc langur term placenta by light, scanning, and transmission electron microscopy. The primary disc of bidiscoidal placenta was separated to 4-6 small cotyledonary subdivisions and was attached by an umbilical cord. The chorion frondosum of placenta was classified as villous, haemomonochorial and deciduate type. The interhaemal area was composed of a layer of syncytiotrophoblast that occasionally clustered as the syncytial knot, fetal capillary endothelium and its basement membrane. The intervillous mineralized fibrinoid mass and syncytial knot were prominent features in term placenta of the douc langur. The chorion laeve consisted of an avascular layer of amniotic cells, the extravillous cytotrophoblast layer and the endometrial decidual cells layer. The allantoic duct was presented between the umbilical vessels in the umbilical cord. Ultrastructurally, the surfaces of placental chorionic villi were covered by numerous microvilli. The prominent indentations and entrapped maternal red blood cells were observed at the tip of the terminal villi. The syncytiotrophoblasts contained densely clumps of chromatin within the nucleus and dark-staining vesicles in their cytoplasm whereas the cytotrophoblast had clumps of chromatin distributed at the periphery of the nucleus. The result of this study is the biological data for further conservation studies of this species.

Keywords: douc langur, placenta, anatomy, histology, electron microscopy

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บทคัดย่อ

ลักษณะทางมหกายวิภาค และ จุลกายวิภาคของรก ค่างห้าสี

ดำริ ดาราวิโรจน์ [1] ณัฐญา ทั่วประโคน [2] ยงชัย อุตระ [2]*

ในการศึกษาลักษณะทางกายวิภาค จุลกายวิภาค และระดับจุลทรรศน์อิเลคตรอนของรกค่างห้าสี (*Pygathrix nemaeus nemaeus*) ซึ่งเป็นสัตว์ที่จัดอยู่ในสถานภาพใกล้สูญพันธุ์ พบว่ามีลักษณะของรกเป็นสองพู (disc) โดยพูหลักจะมีขนาดใหญ่กว่า และมีสายสะดือมายึดเกาะ ในแต่ละพูจะมีร่องแบ่งเป็น 4-6 พูย่อย รกมีน้ำหนักรวมตั้งแต่ 94-135 กรัม เมื่อจำแนกทางจุลกายวิภาค พบว่า chorion frondosum ของรกค่างห้าสีเป็นแบบ villous, haemomonochorial และ deciduate โดยเนื้อเยื่อส่วนลูกมีการยื่นออกเป็น villus ซึ่งมีการแตกแขนงออกทุกทิศทางคล้ายต้นไม้ และแช่อยู่ในเลือดของแม่ โครงสร้างที่กั้นระหว่างเนื้อเยื่อแม่และลูก คือ syncytiotrophoblast, ผนังหลอดเลือดฝอย และเยื่อบุฐานของลูก syncytiotrophoblast มีการเรียงตัวเป็นเซลล์ชั้นเดียว และในบางบริเวณมีการรวมนิวเคลียสเข้าด้วยกันเป็น syncytial knot เพื่อให้เกิดบริเวณที่เลือดของแม่และลูกสัมผัสกันได้มากขึ้น นอกจากนี้ยังพบการสะสมของเกลือแร่ในบริเวณที่เคยมีก้อน fibrinoid มาก่อน ซึ่งทั้งสองโครงสร้างนี้เป็นลักษณะเด่นที่พบได้ในรกของค่างห้าสี และ primate อื่นบางชนิด ส่วน chorion laeve ของรกประกอบด้วย ชั้นของ amniotic cells, the extravillous cytotrophoblast และชั้น decidual cells และพบส่วนที่หลงเหลืออยู่ของ allantoic duct ในสายสะดือโดยอยู่ระหว่างหลอดเลือด umbilical ลักษณะพื้นผิวทางด้านที่สัมผัสกับเลือดแม่ของวิลไลถูกปกคลุมด้วยไมโครวิลไลจำนวนมาก ซึ่งเป็นส่วนยื่นของเซลล์ syncytiotrophoblast และพบการเว้าเป็นแอ่งบริเวณส่วนปลายของวิลไลได้เด่นชัด ซึ่งมักพบว่ามีเม็ดเลือดแดงของแม่มาติดอยู่ด้วย เซลล์ syncytiotrophoblast ประกอบด้วยนิวเคลียสที่มี การจับกลุ่ม ของโครมาตินอย่างหนาแน่น และมีไซโตพลาสมซึ่งเต็มไปด้วยถุงน้ำที่ย้อมติดสีเข้ม ในขณะที่ cytotrophoblast มีการจับกลุ่มโครมาตินกระจายอยู่ตามขอบของนิวเคลียส การศึกษาครั้งนี้จะเป็นข้อมูลทางชีววิทยาพื้นฐานสำหรับการอนุรักษ์และขยายพันธุ์ค่างห้าสีในอนาคตต่อไป

คำสำคัญ: ค่างห้าสี, รก, กายวิภาค, จุลกายวิภาค, จุลทรรศน์อิเลคตรอน

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Introduction

A red-shanked douc langur (*Pygathrix nemaeus nemaeus*) is a member of the 3 subspecies of douc langurs (*Pygathrix nemaeus*) which are the old world monkeys found only in eastern Indochina. It is currently enlisted as an endangered species on the IUCN Red List of Threatened Species 2010 (http://www.iucnredlist.org/). In Thailand, most of the captive douc langurs are not found in nature. They were obtained from Vietnam, Laos or Cambodia. Due to a declining number of this species, many aspects of reproductive biology have been researched including comparative studies of placental morphology and physiology. The placenta is a temporary feto-maternal organ responsible for the nutrient and gas exchanges during a gestation period. In primates, 2 different types of the placenta have been reported; epitheliochorion and hemochorion. The lower primate such as a lesser bush baby (*Galago senegalensis*) has a diffuse, non-deciduate and epitheliochorion placenta (Njogu et al., 2006) whereas the higher primate such as the macaque (De Rijk and Van Esch, 2008) has discoidal, deciduate and hemochorion type similar to that in human placenta. Numerous studies have been accorded to the old world monkey placentation (Enders et al., 2001; Wei et al., 2004; Benirschke, 2008) but that of the red-shanked douc langur placenta is apparently rarely available. The present study was undertaken to describe the gross anatomy, histology and ultrastructural morphology of the red-shanked douc langur placenta by light, scanning, and transmission electron microscopy.

Materials and Methods

Description of the macroscopic anatomy and measurement of the relevant structures were conducted on 7 red-shanked douc langur normal term placentas obtained from Dusit Zoo. All specimens were weighed before being preserved in 10% buffer formalin, and conventional dissecting was subsequently performed. For histological study, placental tissues incised from primary and secondary

lobes and umbilical cords were embedded in paraffin and stained with H&E for light microscopic evaluation. For transmission electron microscopy (TEM), fresh placental tissues from chorion frondosum were immersed in 2.5% (v/v) glutaraldehyde in 0.1M phosphate buffer for 48 hours. Specimens were placed in 1% OsO_4 in 0.1M phosphate buffer pH 7.2 and were then dehydrated in a graded series of ethanol followed by immersion in propylene oxide. Placental tissues were subsequently infiltrated with Spurr resin in propylene oxide and incubated at 70^0C for 10 hours. After that, the ultrathin sections (90 nm) were made and stained with uranyl acetate and lead citrate for study of cell structures by TEM (JEOL, JEM-2100). For scanning electron microscopic (SEM) study, small pieces of tissue were cut and fixed in 2.5% glutaraldehyde in 0.1M phosphate buffer. Then, the tissue were post-fixed in 2% OsO_4 and were dehydrated by ethanol and isoamylacetate, respectively. The specimens were mounted on aluminum stubs and coated with gold and were visualized by SEM (JEOL, JSM 5410LV).

Results

Gross anatomy: The placentas were macroscopically round or ovoid. A bidiscoidal placenta of the douc langur was composed of a primary disc to which the umbilical cord was attached and a slightly smaller secondary disc (Fig 1 & 2). However, a placenta with a very small secondary disc was also observed. The average weight of placentas including fetus was 112.6 gram and the size of the term placentas was 15x8x3 cm, approximately. The average length and width of the primary disc were 8.64 and 7.2 cm whereas those of the secondary disc were 6.2 and 5.1 cm, respectively. Details of placental size and weight are shown in Table 1. The umbilical cord was spiral and contained 3 umbilical blood vessels; two umbilical arteries and one vein. The average length of the cord was 30.15 cm. Further out the cord, the arteries were branched to supply on the fetal surface of placenta which was roughly separated to 4-6 small cotyledonary subdivisions (Fig 1). Immediately after attachment to the primary disc, fetal blood vessels ramified over the disc and at least 4 vessels coursed over the placental membrane to the secondary disc.

Microscopic morphology: The histological study of the douc langur placenta demonstrated a characteristic of hemochorial placenta with distinctive trabecular network resulted from the connection between branched villi. The primary villi were branched in all directions, and both free and anchoring villi were bathed by the maternal blood in the intervillous space (Fig 3A). The types of chorionic villi ranged from a simple slender with a few branches to a complexly branched form arising from the main stem. The chorionic villi were composed of mesenchyme or connective tissue core, fetal blood

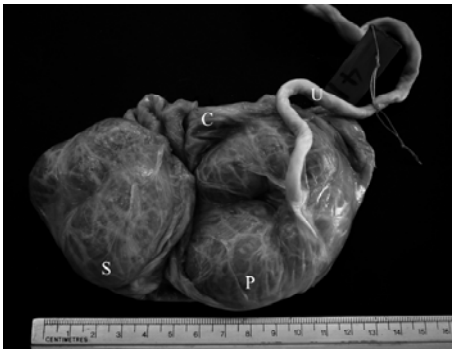

Figure 1 Macroscopic anatomy of a red-shanked douc langur placenta. On the fetal surface, umbilical cord (U) was attached to the center of a primary disc (P) of the bidiscoidal placenta. A smaller secondary disc (S) and the chorion laeve free membrane (C) was also identified.

Figure 2 On the maternal side, the primary (P) and secondary (S) discs were covered by deciduated tissue. The chorion laeve (C) had several small folds on the maternal surface. U: umbilical cord.

Table 1 Structural parameters of red-shanked douc langur termed placentas

Sample	Weight (gram)	Primary disc		Secondary disc		Umbilical cord
		Length (cm)	Width (cm)	Length (cm)	Width (cm)	Length (cm)
D22	97	10	8	4.5	3	39
D28	99.2	8.9	7.2	7	5.1	22
D31	94	7	6.5	7.5	6.2	27
D32	131	10.5	7.6	7.5	6	31
D41	135	8.1	7	6.2	6.2	32
D42	102	8	6.8	6.8	6.2	30
D46	130	9	7	4	3	22.5

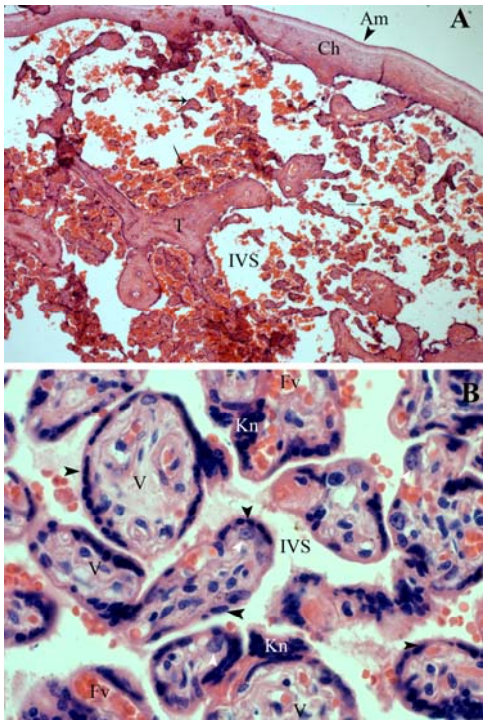

Figure 3 A micrograph of douc langur placenta A) the chorionic villi protruding from fetal side were connected to other villi as trabecular arrangement (T). Terminal villi (arrow) were distributed and bathed in the maternal blood in intervillous space (IVS). Fetal side was composed of a single layer of amnion (Am), chorion (Ch) and villi. OM: 4x. B) A single layer of syncytiotrophoblast (arrow head) encircled the connective tissue and fetal vessels (Fv) of the terminal villi (V), and formed the characteristic of syncytial knot (Kn). This haemomonochorial placenta was termed after a single layer of trophoblasts covering the terminal villi. OM: 40x

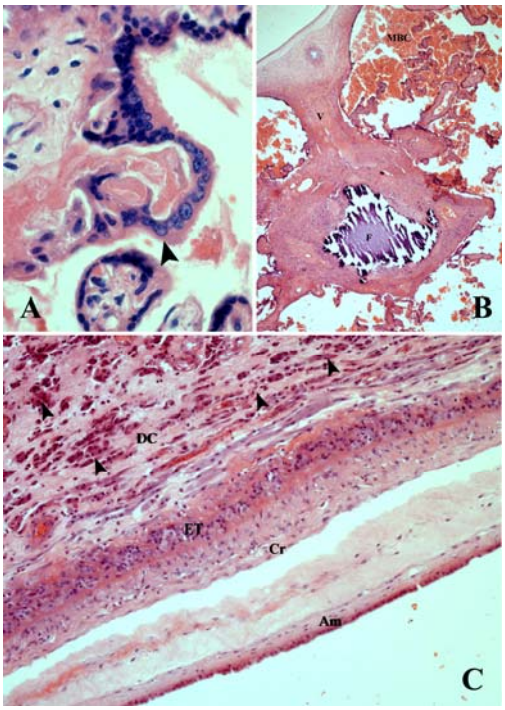

Figure 4 A) The intravillous cytotrophoblasts (arrowhead) were presented in some parts of chorionic villi. Microvilli were also found at the apical surface of these trophoblasts. MO: 40x. B) A large mineralized fibrinoid structure (F) was observed in the primary villus (V), MBC: maternal blood cells. MO: 4x. C) A chorion laeve of douc langur placenta was composed of a single cellular layer of amnion (Am), chorion (Cr), extravillous cytotrophoblasts (ET), decidual layer (DC) containing maternal decidual cells (arrowhead). MO: 10x.

vessels and a layer of different trophoblast types (Fig 3B). The outermost cell layer containing numerous microvilli was the syncytiotrophoblast which was the major type and became more syncytial whereas a few underneath trophoblasts enclosing the mesenchyme were the cytotrophoblast. The prominent syncytial character of the syncytiotrophoblasts was demonstrated by the clustering of a large number of nuclei within one cell. Syncytium was remarkably found around the anchoring and the terminal villi. Interestingly, we also found a continuous layer of cytotrophoblast at the tip of villi in some areas (Fig 4A). A number of chorionic villi from fetal side attached to decidua basalis mostly occurred at the periphery of placenta.

The syncytiotrophoblast was a small cell with a typically dark blue-stained nucleus and acidophilic cytoplasm whereas the cytotrophoblast was a larger cell with a large euchromatic nucleus, a prominent nucleolus and pale cytoplasm. The maternal blood space was lined by a thin layer of syncytiotrophoblast. Moreover, the segregation of the trophoblast nuclei into clusters called syncytial knots were observed on a large number of terminal villi within the intervillous space (Fig 3B). The interhaemal area of douc langur placenta is shown in Fig 4A-B. We also found that not only the syncytial knots causing the thinner syncytial trophoblast lining the maternal blood spaces but also the indentation of the trophoblast by fetal capillaries reduced the distance between the maternal blood and the fetal vessels. In addition, the mineralized structures or previous fibrinoid material were extensively observed (Fig 4B). They were found as a purple clump in the core of both primary and secondary villi. The intervillous mineralized fibrinoid mass and syncytial knot were prominent features in term placenta of the douc langur.

The fetal side of the douc langur placenta was covered by the amniotic membrane which was composed of a single layer of pink, cuboidal cells and the underneath ill-stained connective tissue layer. The amnion was avascular whereas the underlying chorion carried the large fetal vessels. The next layer of the fetal part was layers of mesenchymal cells, trophoblasts and large fetal blood vessels that further became the component of chorionic villi. The fetal side was thinner than the maternal side. Fetal capillaries were close to the trophoblast and often bulged into it. The size of fetal red blood cells was not different from that of the maternal blood cells.

In the maternal side, the outermost was layers of endometrial loose connective tissue with numerous decidual cells (deciduas basalis). A large endometrial vein and spiral arteries were found between the deciduas basalis and the basal plate. The cytotrophoblastic shell obviously observed in the basal plate was continuous, slightly thick and markedly delineated from the underlying endometrium. In the chorion laeve (decidua capsularis) that the placental membrane connects between 2 discs, the tissue arrangement from maternal to fetal side were decidual cell layer, chorion layer comprising of a row of extravillous trophoblast clusters and amnion layer, respectively (Fig 4C). Extravillous trophoblast infiltrating deciduas basalis was found at the placental floor.

The umbilical cords were composed of 2 umbilical arteries and 1 umbilical vein which lied within the embryonic connective tissue (mesenchyme). The remnant of allantoic duct was also presented between the umbilical arteries. At the ultrastructural level, a scanning electron microscopic (SEM) study revealed that the surfaces of placental chorionic villi were covered by numerous microvilli (Fig 5A). The multibranched villi were composed of the ill-defined trophoblasts and their encompassed convoluting vessels. At the tip of the terminal villi, the prominent depressions were clearly visible, and maternal red blood cells were usually trapped in these indentations (Fig 5B). The diameter of the round-shaped terminal villi was approximately 30 μm whereas their length ranged from 50-100 μm.

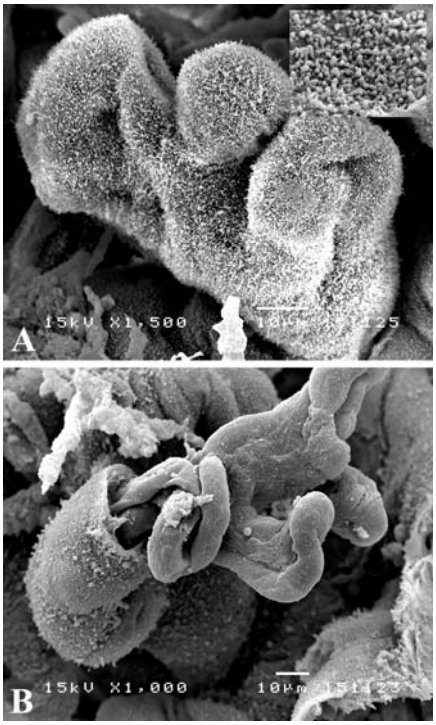

Figure 5 Scanning electron micrographs of terminal villi of douc langur placenta. A) The round or oval shape of terminal villi projected to intervillous space showed the indentation and microvilli (inset) on the surface. Bar: 1 μm. B) The villus having a rupture trophoblast epithelium showed winding fetal blood vessels inside. Bar= 10 μm.

A transmission electron microscopic (TEM) study demonstrated that the villi were mostly covered by syncytiotrophoblasts while cytotrophoblastic cells lied beneath the syncytium and bordered the basement membrane (Fig 6). The syncytiotrophoblasts of term placentas were of low columnar cells arranged either as a simple layer or a cluster called "syncytial knot". The cell size ranged between 10 and 12 μm in diameter. The epithelial trophoblasts contained a densely clump of chromatin within an oval- to irregular-shaped nucleus and dark-staining vesicles in their cytoplasm. At the apical surface of the syncytiotrophoblast, there were numerous microvilli projected outward to the intervillous space. The inner-layered cytotrophoblast had clumps of chromatin distributed at the periphery of the nucleus and a few of electro-lucent vesicles in the cytoplasm. Golgi complexes and ribosomes were also observed in their cytoplasm. The border of the cytotrophoblast was rather smooth and did not exhibit any processes. The endothelium of the fetal capillary rested upon a thin basement membrane was closely surrounded by the cytotrophoblasts. Intercellular junction of adjacent trophoblast cells were connected by either tight junctions or occasional desmosomes.

Discussion

According to the principle system of placental classification (Ramsey,1982), the douc langur placenta is chorioallantoic, bidiscoidal, deciduate and hemochorial. Similar to other Simians (higher primates), the douc langur has the chorion protrusions occurring only in disc-like areas of the placental surface. The two-disc contact zone or bidiscoidal placenta in the douc langur is normally found in most new world monkeys except the howling monkey (*Alouatta spps*.) (Ankel-Simons, 2007). Although these two discs are structurally similar, the primary disc is usually larger and located close to the embryonic pole of the placenta, whereas the smaller secondary disc develops on the opposite

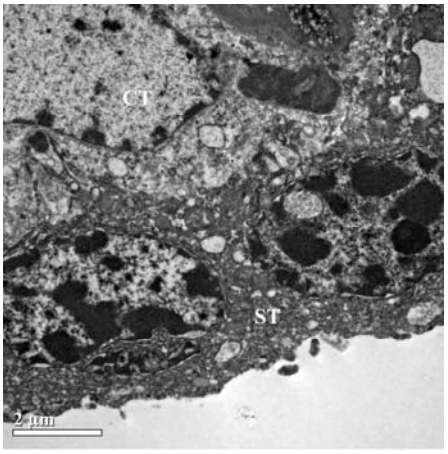

Figure 6 A transmission electron micrograph showing the syncytiotrophoblast (ST) and underlying cytotrophoblast (CT) of a chorionic villus of a douc langur placenta, Bar= 2 μm.

side of the placenta. Although one of the placental samples obtained from a douc langur had a very small secondary disc, the infant was born normally. It possibly suggested that the disc was functionally normal. In most of primates including douc langurs, an umbilical cord is normally attached to the primary disc without branching. However, there were vascular anastomoses within the chorion and 4-6 vessels that connect the two placental lobes. Consequently, the developing fetus was supplied with bidiscoidal placenta connected by umbilical vessels. In pygmy chimpanzee, the apparently long umbilical cord and marginal insertion was reported (http://placentation.ucsd.edu/index.html).

The type of placentation in primates is varied; lorises and lemurs have a diffuse and epitheliochorial type, tarsiers have labyrinthine and haemochorial type whereas the old and new world monkeys have villous and haemochorial type (Carter and Pijnenborg, 2011). The red-shanked douc langur has a haemochorial type which is the most efficient interchange between mother and fetus and has a vascular arrangement so-called trabeculae which is an intermediate between the villous and lamellar type (Carter and Enders, 2004). The development of the old world monkey placenta is comparatively earlier during ontogeny than that of new world monkeys, and the implantation proceed more rapidly. Old world monkeys including douc langur have a large part of blastocyst remaining in the uterine cavity (superficial implantation) and the small area of blastocyst attaches to the endometrium antimesometrially (Ankel-Simons, 2007).

Only in all of the great apes that the interstitial implantation occurred (A.M Carter, 2007). The haemochorion placenta may be referred to as haemotrichorial, haemodichorial or haemomonochorial according to the number of trophoblast layers of the interhaemal area (Enders et al, 1998). In the red-shanked douc langurs, a layer of trophoblast was evident, thus haemomonochorial placenta was termed. Haemochorion placentas were also found in rodents, insectivores, armadillos and hyaenas (Enders and Carter, 2004). The apparent advantage of haemomonochorial placenta was the direct contact of trophoblast to maternal blood.

The proliferation of the trophoblast in douc langurs and other old world monkeys was rapidly developed at the early stage of gestation (Carter, 1999), therefore the branched chorionic villi were presented from a very early stage. On the other hand, in the new world monkeys, arborescent villi were found at a late stage of fetal development. This suggested that syncytial knots commonly found on the villous surface were prominent in term placenta. Numerous anchoring villi were regularly arranged and clearly observed in douc langur placenta. This structure are important in the mechanical stability of maternal-fetal junction (Enders et al., 2001). Trophoblastic shell of douc langur placenta is continuous and thick similar to that of the baboon and macaque. Studies of cytokines and growth factors of this interface of non-human primates were reported (Wei, et al, 2004). The mineralized structures were found inside fetal blood vessels and this calcified mass was an evidence of fibrinoid material and extravillous cytotrophoblast (x-cells) developed during fetal growth and markedly observable in late pregnancy (De Rijk and Van Esch, 2008). It had no pathologic significance. Unlike other primates and human, macrophages (Hofbauer cells) and other leukocytes were rarely found in douc langur chorionic villi stroma.

The ultrastructural structures of non-human primate placentas were reported in several species such as the structure of anchoring villi, trophoblastic shell in the macaque (King and Blankenship, 1994) and baboon (Enders et al, 2001), chorioallantoic structures of the prosimian (Njogu, et al, 2006). The SEM and TEM studies of chorionic villi of the douc langur were firstly reported in this study. However, many aspects of placentation of douc langur such as temporal developmental study and essential molecules regulating the growth of placental cells are awaiting further studies.

Acknowledgements

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