Aspects of placentation systems in the scalloped hammerhead shark, *Sphyrna lewini*, and the manta ray, *Manta birostris*

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Abstract

We investigated yolk sac placentations with umbilical cord specimens of pregnant scalloped hammerhead sharks (*Sphyrna lewini*) from Okinawa and Taiwan histologically and immunohistochemically. The placental tissues were positive for PAS, cytokeratin, hCG, hPL, SPI, PLAP and AFP. Moreover, electron microscopic study of the umbilical cord revealed that the endothelium of ductus vitellointestinalis had ciliar filaments arising from the surface of cylindrical, mucin-positive epithelial cells. In the gravid uterus of a manta ray (*Manta birostris*) specimen with an unborn pup from Okinawa, vascularized villous strings with filaments showing crypt formation termed trophonemata were observed. The stroma had secretory vacuoles and granules of protein and lipid in the uterine gland, in addition to Golgi apparatus, mitochondria and rough rER. Thus, we hypothesize that secretions of lipid or protein granules might be released from the epithelial surface.

Introduction

This paper describes the immunohistochemical and electronmicroscopic findings of yolk sac placenta with umbilical cord in the scalloped hammerhead sharks and structures of the gravid uterus in the manta ray. Although most sharks are oviparous, some species are viviparous including members of *Sphyrna* and Carcharhinidae. We investigate placentas of two specimens of pregnant scalloped hammerhead sharks (*Sphyrna lewini*) obtained by one Okinawa Churaumi Aquarium and one from Suao, Taiwan, and one specimen of a pregnant manta ray (*Manta birostris*) unborn premature pup obtained by Okinawa Churaumi Aquarium. There are over 400 species known most sharks are oviparous, and the remainder, viviparous. Viviparous placental sharks utilize three methods of fetal nutrition to nourish their developing young: villogenesis, histotroph secretion and placentation (Hamlett, 1989). Viviparity can be divided into aplacental viviparity including yolk dependency, oophagy and placental analogues and yolk sac placentation (Wourms, 1981). The yolk sac placentation consists of a modified embryonic yolk sac which is closely appeared to the uterine epithelium. The length of gestation for scalloped hammerhead sharks is about 1 year long, and they typically bear about 20 pups. Until now, very little is known about the immunologic aspects of shark placenta. Only one immunological study has been made on yolk sac placenta in shark, a study by Haines (2006) on the Atlantic sharpnose shark, *Rhizoprionodon terraenovae*. In some

rays, there is a large yolk sac, but no specialized yolk sac attachment. The uterine wall in highly villous and glandular, and its secretion is supposed to be absorbed by the greatly hypertrophied external gill filaments of the fetus (Mossman, 1987).

Materials and Methods

Placental tissue of two pregnant scalloped hammerhead sharks one from Okinawa and one from Taiwan were examined. The shark from Okinawa was provided by Okinawa Churaumi Aquarium. It was caught in a set-net, but died before giving birth. The another hammerhead shark sample was obtained by courtesy of Suao fishing market, Taiwan. Histopathology: The shark placental tissues were stained with PAS (Periodic acid Schiff), cytokeratin, hCG (Human chorionic gonadotrophin), hPL (Human placental lactogen), SP1 (Specific pregnancy glycoprotein 1), PL.Alphos (Placental alkaliphosphatase) and AFP (alpha-feto proten).

In October, 2008, a female manta ray (*Manta birostris*) weighing 963.5kg was captured by dry-net. She died 3 days and the immature baby was removed from uterus by dissection. The uterine epithelium of the manta ray was investigated by immunohistochemical and electronmicroscopic observations. The gravid uterine tissues from the manta were stained with human placental proteins, Sudan III, Prolactin and Mucin.

Scanning electron microscopy (SEM): The frozen dehydrated specimens were observed with a Hitachi S-4200 under accelerator voltage at 10KV following osimium coating.

Transmission electron microscopy (TEM): The sections were made after double staining with uranyl acetate solution and lead nitrate and observed with a JEM1200 EXII (JEOL) electron microscope at 80KV.

Results

In hammerhead sharks, numerous pups each with an umbilical cord and thin membraneous placenta can be seen (Fig.1). Each placenta was connected to the uterine wall. An egg-case membrane exists between maternal and fetal sides.

A) A pregnant scalloped hammerhead sharks with unborn pups at Okinawa Churaumi Aquarium: The unborn pups measured about 30cm long and umbilical cord were 90cm long with two braided vessels. The yolk sac placental epithelium had clear cylindrical cells containing blood capillaries and included a mass of nucleated red cells encircled by a red-colored egg-case membrane (Fig.2 A, B). These epithelial cells were stained positively with PAS, cytokeratin and PL.alphos (Fig3). Furthermore, the epithelial cells showed positive staining with hCG,hPL and SP1 just as human placental proteins do (Fig.4). In addition, AFP also showed positive stain in the epithelial cells. From these results showing epithelial cells of scalloped hammerhead shark placenta react to human placental proteins, it can be postulated that the shark placenta produces glycoproteins just as human trophoblasts do (Soma,2007).

B) The pregnant scalloped hammerhead shark examined at Suao, Taiwan: A pregnant scalloped hammerhead shark measuring 150cm long had 20 pups. Each pup measured 42cm long.

The thin yolk sac placenta was 10x10x0.9cm and the umbilical cords were 25cm long. The umbilical cords had one artery, one vein, a ductus vitellointestinalis and an extraembryonic coelom (Fig.5). The endothelial

surface of the ductus vitellointestinalis was covered with seaweed-like ciliated branches and positively stained with mucin (Fig.6, 7). In the ductus vitellointestnalis, a bundle of ciliar filaments protruded from the surface of cylindrical epithelial cells as dynein-forming microtubules (Fig. 8). In chorionic epithelial tissue, columnar epithelial cells with dilated blood capillaries including nuclear erythrocytes were observed. These epithelial cells have nuclei and organellae similar to human cytotrophoblasts, although the development is poor compared with human trophpblasts (Fig. 9).

C) A pregnant manta ray (*Manta birostris*) at Okinawa Churaumi Aquarium: Manta ray reproduction features a uterus with thick villous strings called trophonemata which nourish the unborn pup (Fig. 10). Proliferation of villous epithelium containing mononuclear cells and uterine vessels including a mass of nucleated red cells was observed (Fig. 11). In immunohistochemical studies, these epithelial cells stained positively with PAS and cytokeratin. Also, these epithelial cells stained positively with hCG,hPL, SP1,PL.alphos and AFP (Fig. 12). In addition, Sudan III, Nile blue and prolactin stains were also positive in these cells (Fig. 13). SEM observations showed villous strings with flat surfaces include crypt formation,

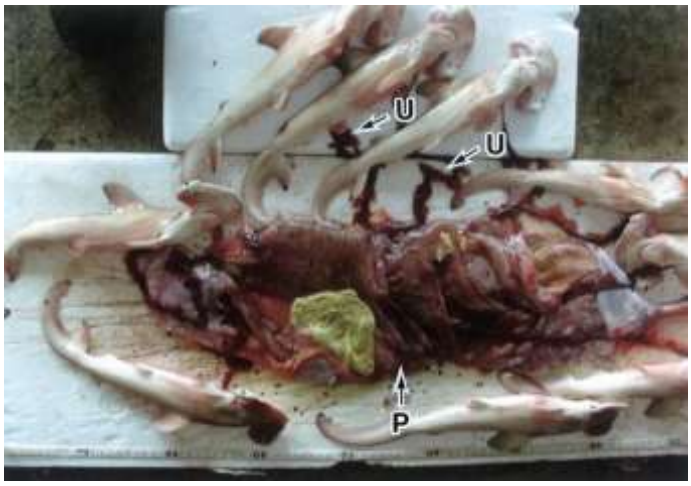

Figure 1. Nine pups dissected from a pregnant scalloped hammerhead shark, showing umbilical cords (U) and thin placental membrane (P).

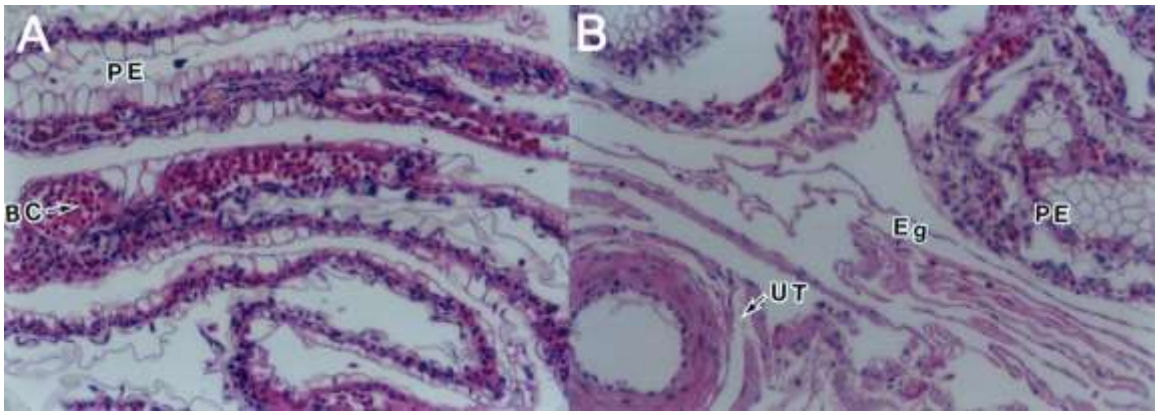

Figure 2. A) Scalloped hammerhead placental epithelium (PE) with clear cylinder cells containing blood capillaries (BC) including many nucleated red cells. HE stain (x100); B) Chorionic villi (PE) and uterine tissue (UT) were intervened by egg-case membranes (Eg). HE stain (x200).

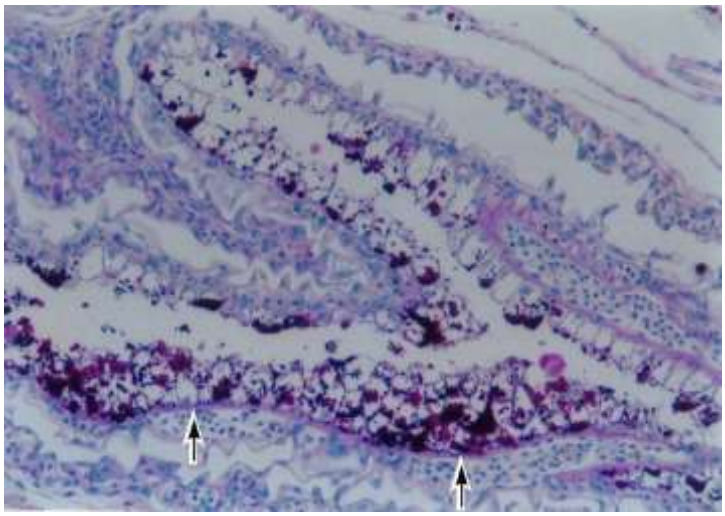

Figure 3. Scalloped hammerhead chorionic epithelial cells positively stained with PAS (↑). PAS stain (x100).

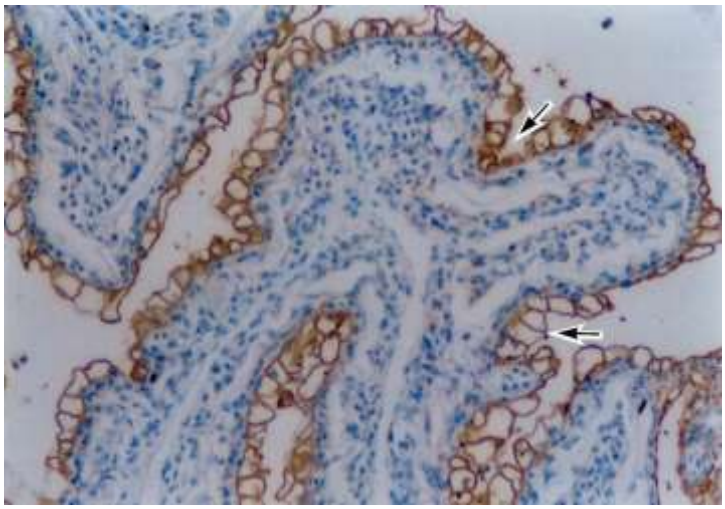

Figure 4. Scalloped hammerhead epithelial cells showed positive staining with hCG (↓). hCG stain (x100).

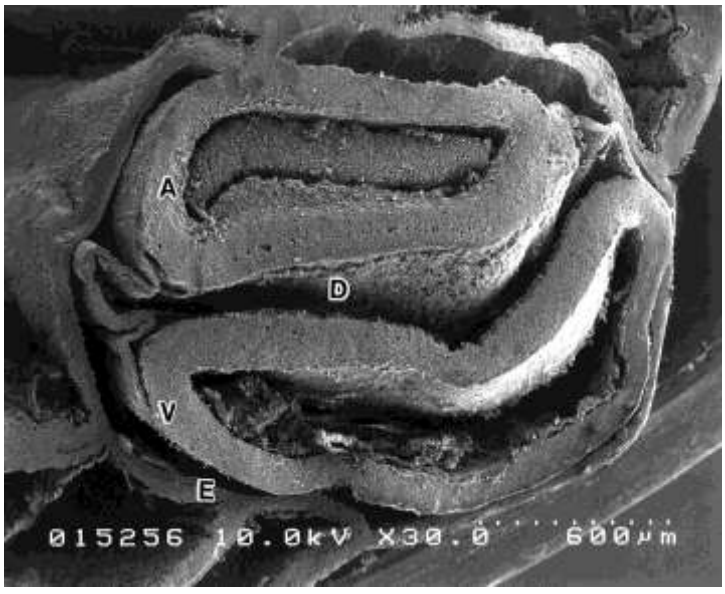

Figure 5. Scanning electron-micrograph of the umbilical cord of a scalloped hammerhead shark: A:Umbilical artery, D: Ductus vitelloinguinalis, V: Umbilical vein, E: Extraem bryonic coelom. (x120).

Figure 6. The endothelial surface of ductus vitellointestinalis covered with seaweed-like ciliated branches (↑). (x7200).

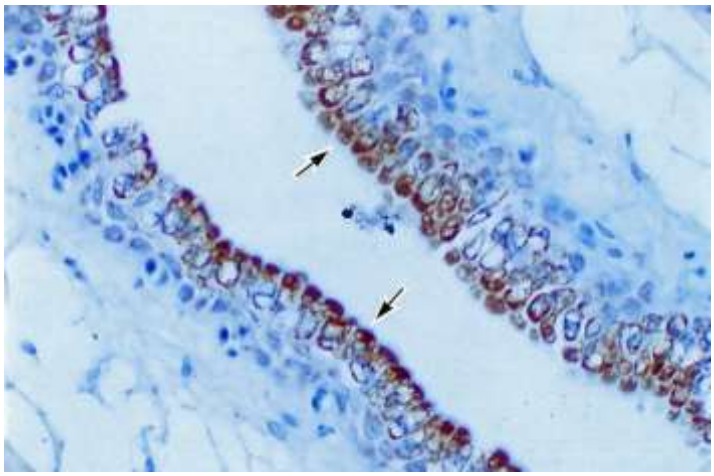

Figure 7. Endothelium of ductus vitellointestinalis positively stained with mucin2. Mucin stain. (x200).

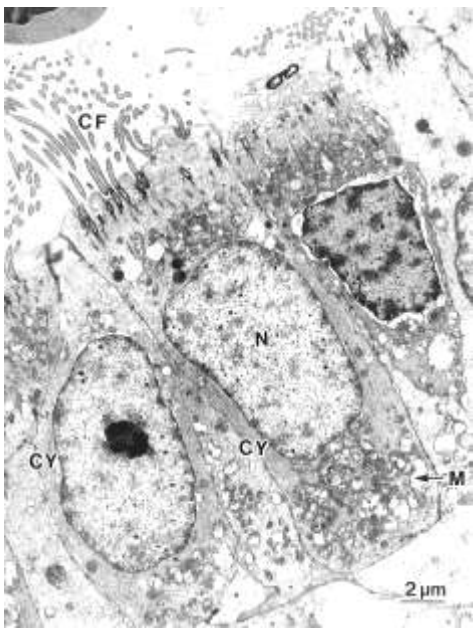

Figure 8. In the fine structure of ductus vitellointestinalis, bundles of ciliar filaments (CF) protrude from the surface of cylinder epithelial cells (CY) as microtubules. Nucleus (N), Mitochondria (M). (x5000).

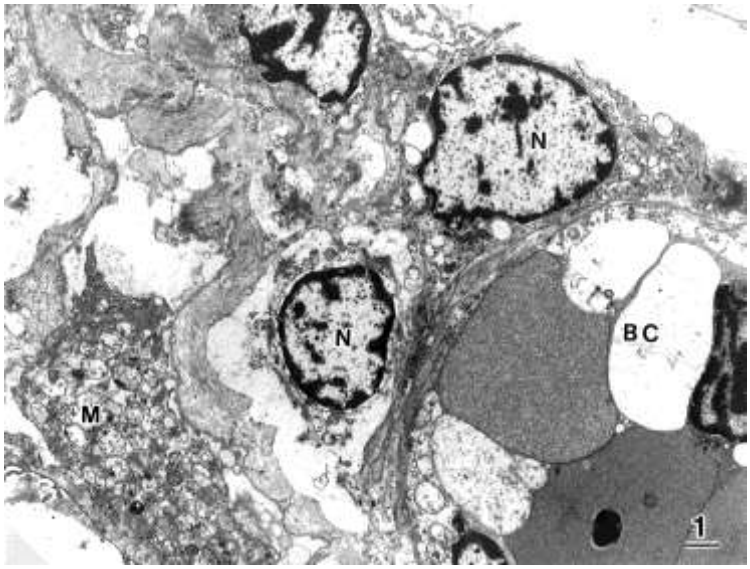

Figure 9. Ultrastructure of columnar epithelial cells, adjacent to dilated blood capillary (BC), showing these cells contain large nucleus (N) with mitochondria (M) and organellae like human trophoblasts. (x5000).

Figure 10. Ciliar villous strings (VS)(↑) called trophonemata from the gravid uterus of the manta ray.

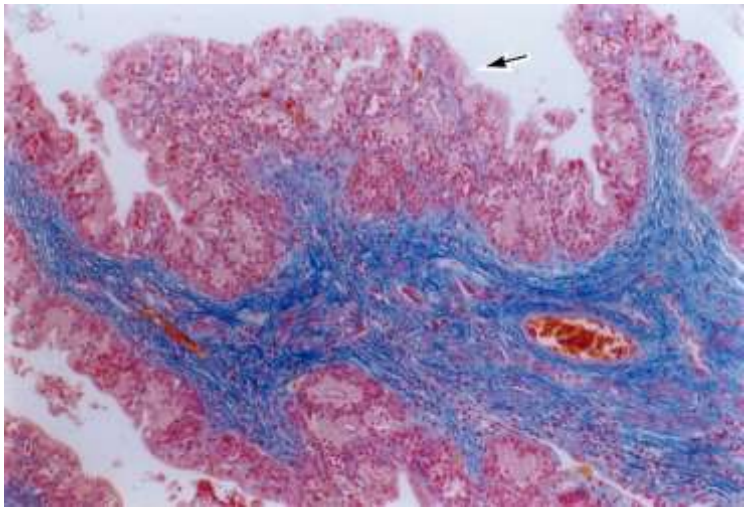

Figure 11. Proliferation of villous epithelium(↑) containing mononuclear cells and uterine vessels within the gravid uterus of a manta ray. Azan stain. (x100).

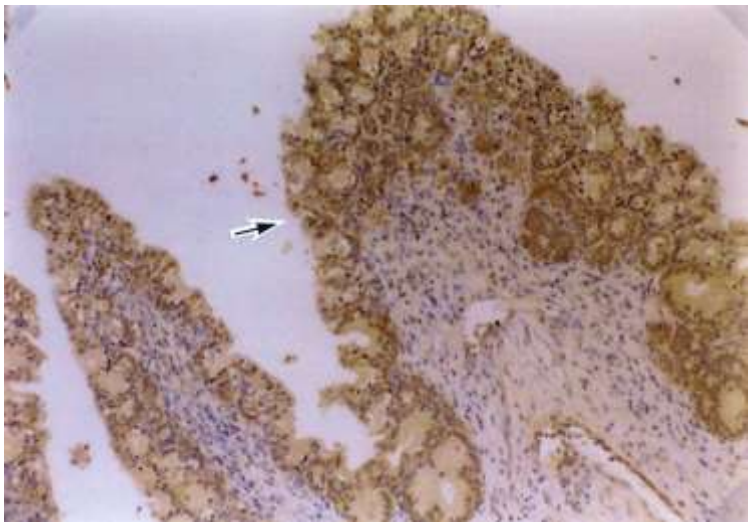

Figure 12. Villous epithelium(VE)(↑) stained with hPL within the gravid uterus of a manta ray. hPL stain. (x100).

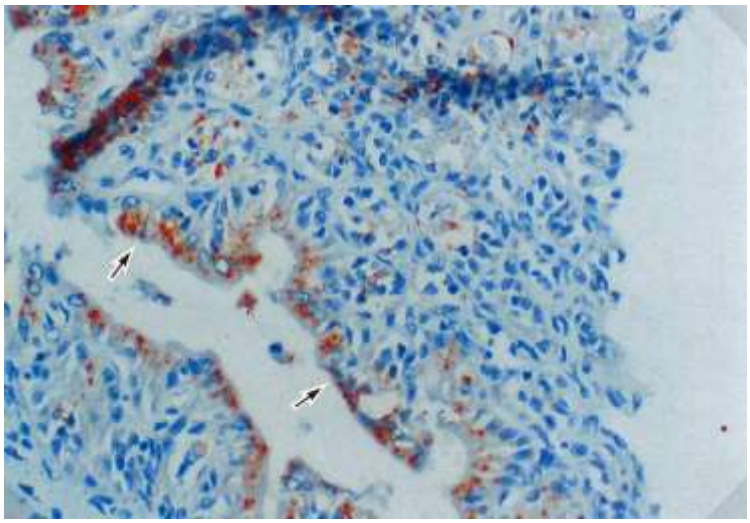

Figure 13. Villous epithelium stained with Sudan III(↑) within the gravid uterus of a manta ray. Sudan III stain. (x200).

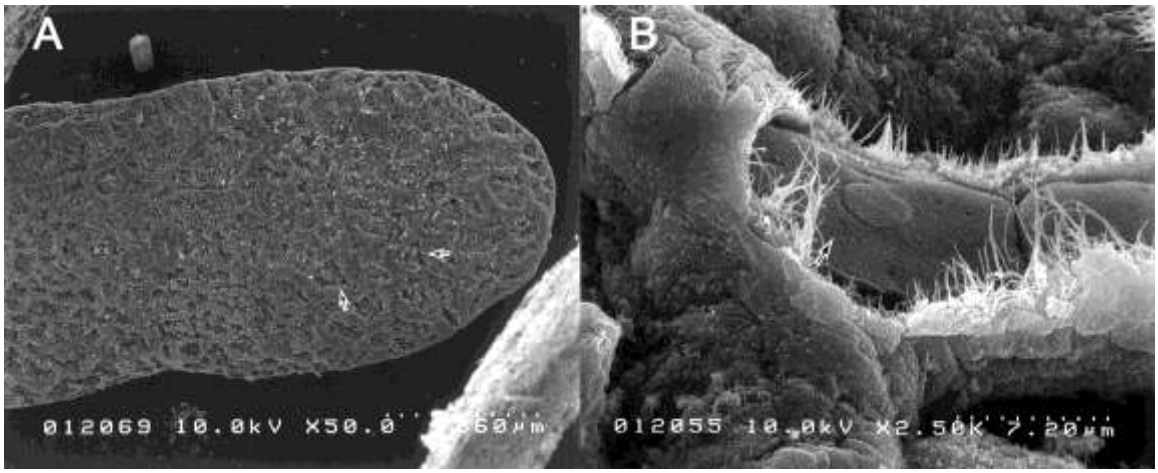

Figure 14. SEM findings: A) Cut surface of a villous string with flat surface showing crypt formation (↑) (x100); B) Nucleated red cells below crypts (NKC)(↑). (x5000).

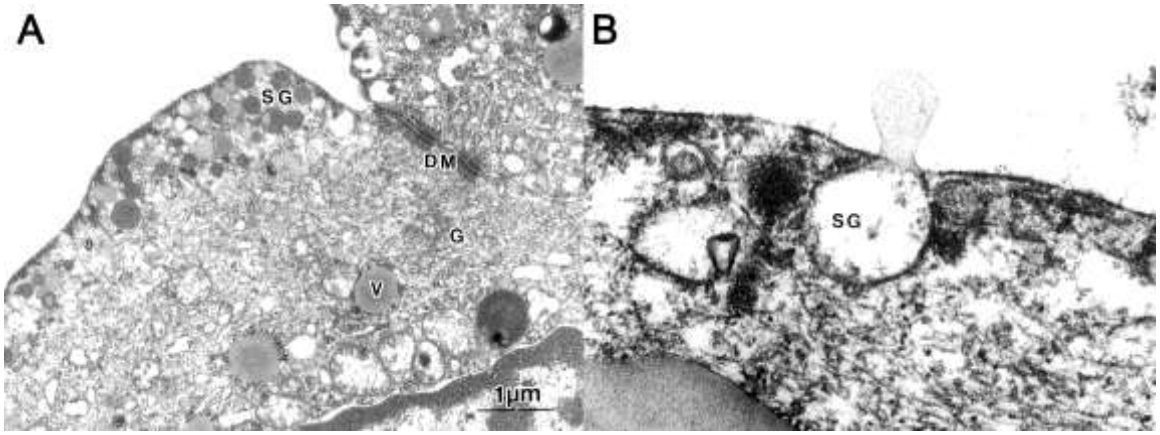

Figure 15. Transmission EM findings: A)Villous strings not only have vacuoles (V) and granules (g) showing secretion of protein or lipid by the uterine gland, but also Golgi apparatus (G), rER and secretory granules (SG) as well as desmosome (DM), (x21000); B)Secretory granules (SG) such as lipids or proteins are excreted from the surface of the epithelium. (x105000).

where blood capillaries including nuclear red cells were found (Fig. 14 A, B). By TEN, they had vacuoles and granules showing secretion of lipids within the uterine gland, which contained Golgi apparatus, mitochondria and rER (Fig. 15 A, B). This demonstrates excretion of lipid or protein granules from the surface of the epithelium.

Discussion

This is the first detailed description of placenta of *Sphyrna lewini* and uterine secretion of *Manta birostris*. This hammerhead shark placenta is found to utilize yolk and nourish many pups during the relatively long gestation period.

Marsupial reproduction is characterized by a relatively short gestation and production of an extremely undeveloped new-born (Freyer, 2007). The placenta is formed of a yolk sac that is attached to the endometrium until birth. Unlike eutherian mammals, in most marsupials and monotremes, the allantois does not contact the chorion to form a placenta.

Scalloped hammerhead shark placenta has unique umbilical cord with appendiculae, chorionic tissue including blood capillaries and uterine tissue even though there is loose contact between chorionic tissue and uterine endometrium. The shark placenta is known to be specialized to have characteristics of steroid－producing tissues (Hamlett,1990).

Chorionic epithelial cells of scalloped hammerhead sharks showed positive staining with human placental proteins such as hCG, hPL, SP1 and PL.alphos. The trophoblast like cells with large nuclei adjacent to dilated capillaries including nucleated red cells were found, though the organelles were not so rich as human trophoblast. The umbilical cord and appendiculae in the Atlantic sharpnose shark have previously been studied (Hamlett, 1993, 2005), but this is the first study of ultrastructure of the umbilical cord of scalloped hammerhead sharks. In addition to typical findings of an umbilical cord consisting of to vessels,

the ductus vitellointestinalis and extraembronic coelom, the ductus vitellointestinalis was covered with seaweed-like ciliated brushes in which ciliar filaments were protruded densely as microtubles forming dynein. The endothelium stained with mucin-like yolk products. Yolk granules are reportedly transported to the fetal gut by the ciliated cell lining the ductus vitellointestinalis (Hamlett, 1989). Ultrastructural observations suggest that yolk metabolic products are transported across the vittelline epithelium through the yolk sac endoderm. The southern stingray, *Dasyatis americana* has placental analogues called trophonemata for uterolactation (Hamlett, 1985, 1996, 2005).

This is the first report of placental analogues in manta ray (*Manta birostris*). The uterus of manta ray with an immature pop included thick villous strings called trophonemata which were stained positive with human placental proteins such as hCG,hPL,SP1,PL.Alphos and AFP, in addition to Sudan III and prolactin. In ultrastructural findings of villous strings, villous stroma with vacuoles and secretory granules suggest secretion of protein and lipid, and in organelles, Golgi apparatus, mitochondria and rough ER were observed. Thus, it can be concluded that lipid or protein granules are excreted from the surface of the uterine epithelium to nourish the baby inside the mother manta.

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