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Microscopic Study of the Pig Peri-implantation Embryos

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전자현미경에 의한 착상 전후 돼지수정란의 형태학적 변화에 관한 연구

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요 약

전자현미경에 의해 자궁부착 전후의 돼지 수정란의 형태형성 및 분화에 따른 배발생 과정을 검토하였다. 돼지 초기배는 자궁이주후 균일하게 자궁에 배분되기전 약 2~3일간은 자궁각의 proximal portion에 존재하며, 임신 4일째에 할구와 할구의 경계를 상실하는 tight한 gap junction을 가진 상실배로 발달한다. 배반포를 형성하는 시기에 estradiol 17-β 는 compact한 상실배를 cavitated blastocyst로 발달을 촉진시키면서, steroid hormone이 이후의 배발생을 지배한다. Hatching의 시기는 교배후 6~7일경 zona pellucida을 둘러싸고 있는 glycoprotein의 thinning과 lysis에 의해 이루워지는데, hatching 과정은 embryo의 세포수와 무관하였으며, 이때의 embryo의 직경은 0.5~1.0 mm 인 것을 본 실험에서 확인하였다. 12일경부터는 embryo는 prostaglandins, IGF-binding protein, retinol binding protein, plasminogen activator등의 단백질이 풍부해 이들 인자가 elongation 개시 후보로 고려될 수 있었다. 또한 이 시기의 embryo는 embryonic disc로 발달시 progesterone과 estrogen을 estradiol 17β 로 전환할 수 있으며, 이러한 변화와 함께 spherical stage로 부터 tubular 혹은 filamentous form으로 변형되었다. Estrogen이 임신을 통해 prostagladins의 분비를 uterine lumen에 지시하는지는 알 수 없으나 13일 경을 전후해 conceptus estrogen이 uterine arterial blood flow, uterine vasular permeability을 증가시키는 것으로 나타났으며, 자궁에서 protein과 calcium, $PGF_2\alpha$, plasminogen inhibitor를 증가시키는 것으로 나타났다. 이 시기의 자궁 변화와 함께 embryo의 attachment는 trophoblast와 uterine membrane사이의 느슨한 결합에 의해 개시되었으며, 18일경 uterine과 trophoblastic microvilli의 interdigitation에 의해 완성된다. 이 시기에 conceptus attachment를 위해 필요한 uterine microvilli에서의 glycocalyx의 형성과 endometrial epithelium의 erosion을 야기하기 위해 plasminogen activator을 분비하였으며, 반면 자궁에서 plasminogen 역할을 하는 것은 estrogen이며, blastocyst cell 표면의 lectin binding이 attachment에 중요한 역할을 한다. 이상과 같은 일련의 과정을 거친 초기배는 성공적인 임신으로 유도된다고 본다. 따라서, 본 연구는 이상과 같이 착상을 전후한 시기의 배를 전자현미경에 의해 형태형성의 변화를 특히 착상을 전후해 배 취사율이 높은 시기를 대상으로 분석하였다. 이 분석 시기중 성공적인 착상성공율은 56%(71/126)였다.

(Kew words : blastocyst, ultrastructure, pig, implantation)

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Ⅰ. INTRODUCTION

Ultrastructure studies of peri-implantation stages in the large domestic mammals are very limited and most of them are cleavage and early blastocyst stages (Norberg, 1973; Calarco & McLaren, 1976; Brackett et al., 1980; Linares & Ploen, 1981; Massip et al., 1981; Mohr & Trounson, 1981, 1982). Winterberger-Torres and Flechon (1974) and Geisert et al. (1982) described the ultrastructure of sheep and pig blastocysts, respectively. The works on the pig focused mainly on the structural changes related to the elongation of the blastocyst from 11th day of pregnancy. The pig blastocyst *in vivo* loss its zona pellucida in the uterus on the 6th day (Hunter, 1977). At day 10 the spherical blastocysts grow approximately 0.25 mm/hr in a diameter up to 9 mm, after which a very fast increase in length takes place of 30~45 mm/hr (Geisert et al., 1982). This fast growth explains the presence of spherical and tubular blastocysts within one uterine horn at day 11 of gestation (Perry & Rowlands, 1962; Anderson, 1978). The disappearance of Rauber's layer in blastocyst about 2 mm in diameter was found by Geisert et al. (1982), who described an 'elongation zone' in tubular and filamentous blastocysts from day 10 onward running from the area of the embryoblast to the tips of the growing blastocyst.

The trophoblast is the first cell layer to differentiate in the blastocyst of the mammal, as indicated by the presence of many lysosomes and pinocytotic activity already in 0.3 mm blastocyst, and by the absence of yolk granules used for metabolic purpose. Pig trophoblast cells of all blastocysts studied appeared to take up even parts of dead uterine epithelial cells, as indicated by the presence of cilia within some lysosomes. At this times, the presence of many mitochondria is an indication of a high metabolic activity, first developing in the trophoblast and later also in the hypoblast and embryoblast. The mitochondria resumbled those found in steroid-producing cells (Belt & Pease, 1956; Enders, 1973; Gemmell et al., 1974). Enders (1973) showed that steroid secreting cells is not present in the trophoblast cells of pig blastocysts up to 11 days of gestation. Neither Sasaki et al. (1979), who studied 13 day pig blastocyst nor Geisert et al. (1982) mentioned the presence of abundant rough endoplasmic reticulum (RER) in trophoblast or hypoblast. Therefore, estrogen which might play some role in the maternal recognition of pregnancy may be derive from maternal steroid precursor as was suggested by Perry et al. (1973). In species with a relatively long preimplantation period, the role of the trophoblast prior to implantation is like to be largely determined by the food requirement of the embryo. Embryonic loss in pig is up to 40% of ovulated oocytes and the most embryos die during the first 25 day of gestation.

In present study, our attention was taken to examine the indications of morphological changes of pig blastocyst around the hatching and peri-implantition which result in most embryonic death.

Ⅱ. MATERIALS AND METHODS

1. Recovery of embryos

Pigs were bred at estrus (day 0) after two estrous cycles of normal duration (17~22 day) were observed. Twenty-nine Landrace sows were sacrified from day 6 to day 18 of gestation (day 0 is the day estrus). Preimplantation blastocysts were flushed from the uterus with 25 ml of NCSU-23 medium according to the methods described by Kim & Petters (1994).

2. Electron microscopy

For scanning electron micrograph, embryos were fixed immediately in a medium containing 3% glutaraldehyde in 0.1 M carcodylate buffer (pH 7.2) with 1% glucose for 1 hr at 0℃, as described by Vaccaro & Brody (1981). The tissues were fixed in 5.0% glutaraldehyde plus 1.0% ruthenium red (RR) in carcodylate overnight at 4℃. The blocks of both embryos and tissues were washed with fresh buffer containing 1% RR, and post-fixed for 2 hr in 1.0% osmium tetroxide in containing 0.75% RR.

Ⅲ. RESULTS

1. General observation

To standardize results, only the similiar shape or morphogy embryos were studied. Morphological features of the interaction between the hatching blastocyst and implantation in pig were studied by electron microscopy. The observations extended from the late blastocyst stage to the completion of trophoblastic erosin of the epithelium and early decidual transformation of the epithelium and early decidual transformation of the stromal cells. At the cleavage stage, blastomeres rapidly were devided but there was little or no net increase in the volume of the embryo because blastomeres size became progressively reduced. After shedding of the zona pellucida, the blastocyst established the tips of microvilli and with bleb-like cytoplasmic protrusions of the epithelial cells. In pig, close apposition to the uterine wall begins at about 12 days and then attachment occurred during the afternoon of the 16 or 18th day post coitum (p. c). Between day 7 and 17 of pregnancy, 90 blastocysts from 0.3 to 12 mm in diameter were flushed from the uterine horns of Landrace pigs. The ultrastructure of embryos was similiar at all ages studied.

2. Embryo stages distribution

Total 362 ova were recovered from 29 pigs with mean ovulation rate of 14±1.7 corpora lutea. Cell stages of embryos recovered on each of the collection days are shown in Table 1. On the day 7, 11, and 13, 114, 69, and 94 morphologically normal embryos were recovered from 9, 7, and 13 pigs, respectively. Typically, morulae to blastocyst were collected on the day 6, blastocyst to hatched blastocyst on day 7, and hatched disc on the day 13, respectively. Forty five (35.7%) of 126 embryos failed to hatching and implantation.

3. Ultrastructure of preimplantation blastocyst

Blastocyst obesrved in utero during this period were surrounded by the zona pellucida, which was moderately electron-dense and composed of fine interfaced filamentous elements. Fig. 3A shows a presentative blastocyst morphology. The blastocyst at day 7 of gestation were free-floating in the lumen or tenuously adherent to the uterine epithelium. In most of the pig blastocysts we have examined, the trophectoderm was well differentiated and surrounded the blastocyst cavity. There is no report about identical twin in pig. As shown in Fig. 1, we identified spontaneous twin embryos. It seems that identical twin are produced by the separation or even by the separation of the inner cell mass into two regions within the same blastocyst. In blastocyst of the pre-implantation, supernumerary spermatozoa were observed engulfed in trophoblast cells as shown in Fig. 2C. Similar supernumerary sperm inclusions have been found in implanting blastocysts. Furthermore, these sperm tail sometimes were shown in a trophoblast cell during the early attachment stage. Althou-

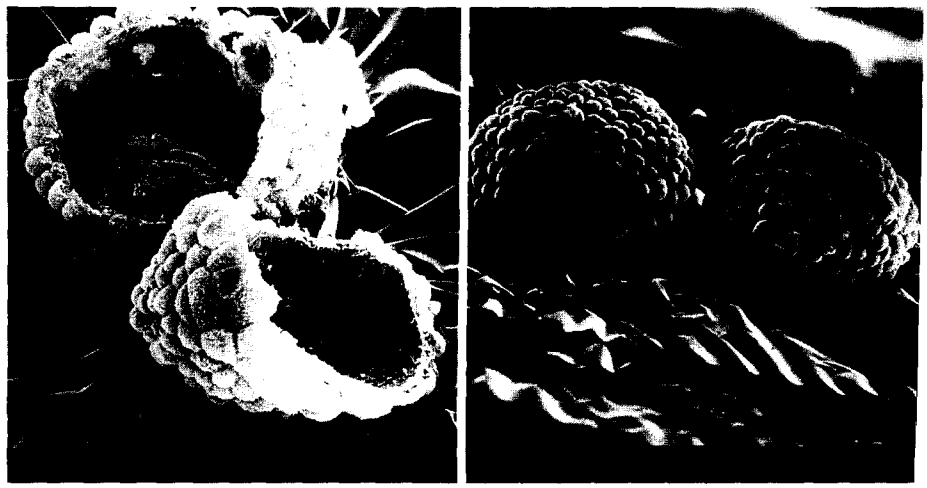

Fig. 1. Two views of identical twin blastocyst. Splitting occurs during hatching, so each twin will have its own chorion and amnion. A) shows internal surface of blastocyst, B) shows individual cells.

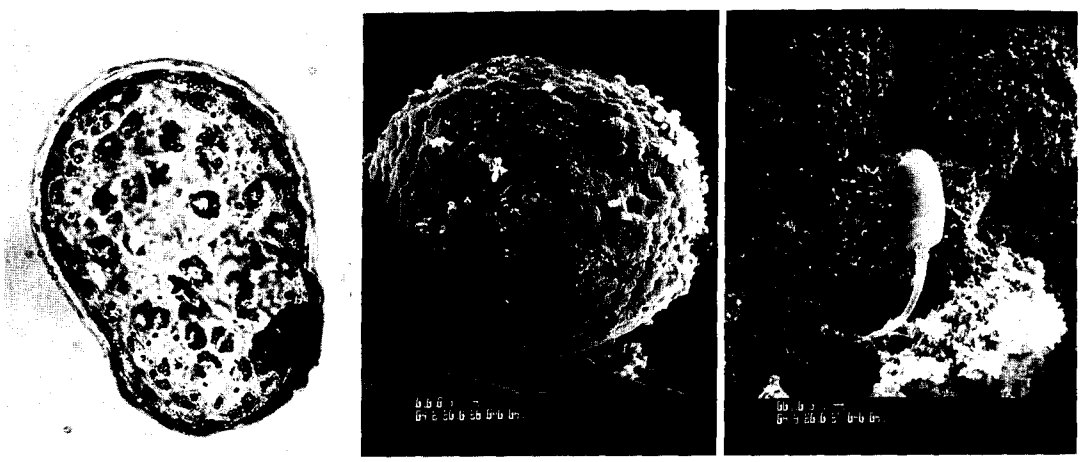

Fig. 2. Scanning electron micrograph of the pole cells of pig hatched blastocyst just prior to completion of cleavage. A. Light microscopy of hatched blastocyst, B) SEM photograph of same hatched blastocyst showing external individual cells (day 7). C) After hatching, sperm attached ZP has complete appearence. Scale bar 5 ㎛.

gh the axial filament complex and peripheal coarse fibres appeared the degenerated form, the tail sheath and longitudinal columns were well preserved. A sperm tail was found within the embryonic cell mass, apparently being phagocytized by an embryonic cell. The serial section shows that the sperm head and tail is partially outside the trophoblast cells.

4. Histological and ultrastructural characteristic in postimplantation embryos

The parietal endoderm is specialized for

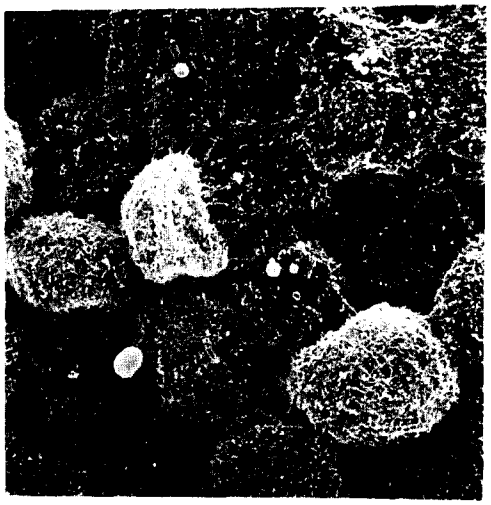

Fig. 3. Scanning electron micrograph of parietal endoderm cells of 13 days p. c. porcine embryo attached to Reichert's membrane.

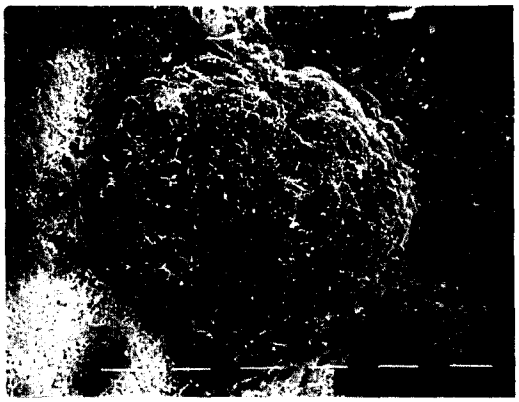

Fig. 4. Inner cell mass increasing by mitosis. Shortly before attachment, inner cell mass in extensively prolifered and blastocyst is trapped in an epithelial crypt.

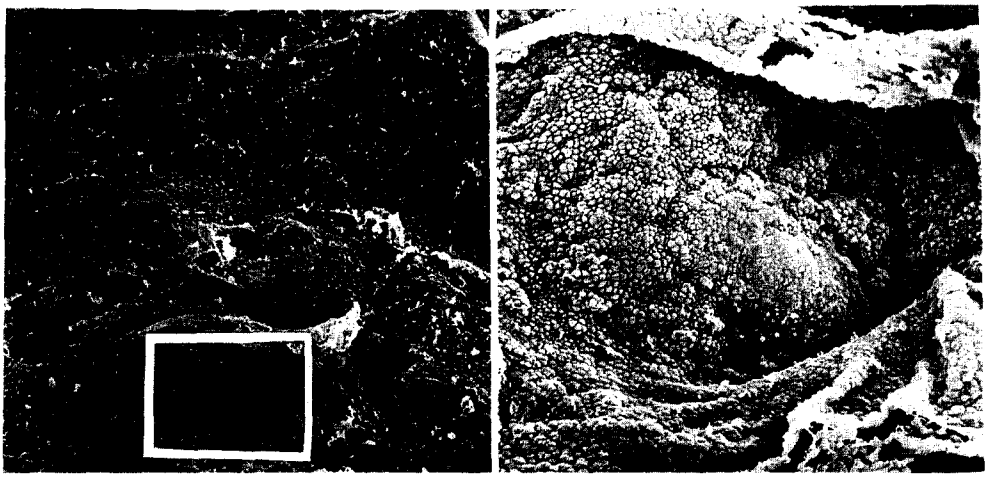

Fig. 5. Scanning electron micrograph of a day 14 $^{1}/_{2}$ porcine embryo showing the embryonic disc exposed through a rupture of Rauber's layer. On the left, a bridge of trophetoderm still traverses the embryonic ectoderm.

synthesizing and secreting a thick basement membrane known as Reichert's membrane between themselves and trophectoderm (Fig. 3). Reichert's membrane is one of the major barrier's between the maternal and fetal environments. However, there is not known about the relationship between the function of Reichert's membrane and its structure and composition.

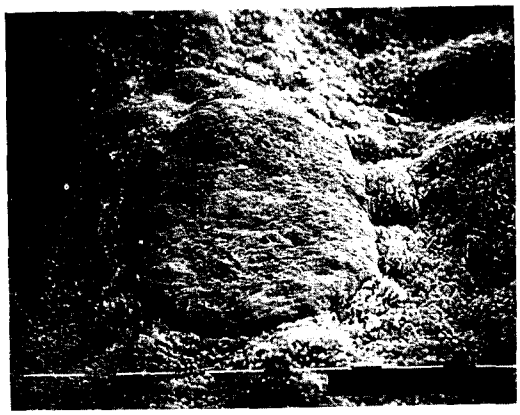

Fig. 6. Section through the uterus of pig at gestational day 18. Arrow indicate the embryos.

Fig. 4 presents a survey picture of an early attachment site, taken at low magnification. The blastocyst was attached to the uterine epithelium at a few points. The microvilli of the uterine epithelium were short, and bleb like structures formed by the epithelial cells which were seen attached to the blastocyst. A large portion of the cytoplasm of both trophoblastic and embryonic cells in the peri-attachment stages was occupied by patches of filamentous mesh or plaques, which gave a translucent appearence to the cells in low electron microscope. At the early attachment stage (14 $^{1}/_{2}$ days after p. c.), contact between uterine epitherial cells and the trophoblast cells was confined to the tips of the microvilli and to the bleb-like cytoplasmic protrusions of the epithelial cells (Figure 4 and 5).

The outer surface of the ICM remains covered by trophectoderm until about day 12, when embryonic disc was exposed through a hole in Rauber's layer of trophectoderm as hatched blastocyst began to elongate (Fig. 6). At this stages, there were signs of polarization in outer cells of the ICM as they differentiate into embryonic ectoderm, the surface epithelium of the embryonic disc. This differentiation occured just before the ICM was exposed by the loss of Rauber's layer of trophectoderm. As the result, the ICM is seen to comprise more than one layer of cells with a basal lamina between them and en-

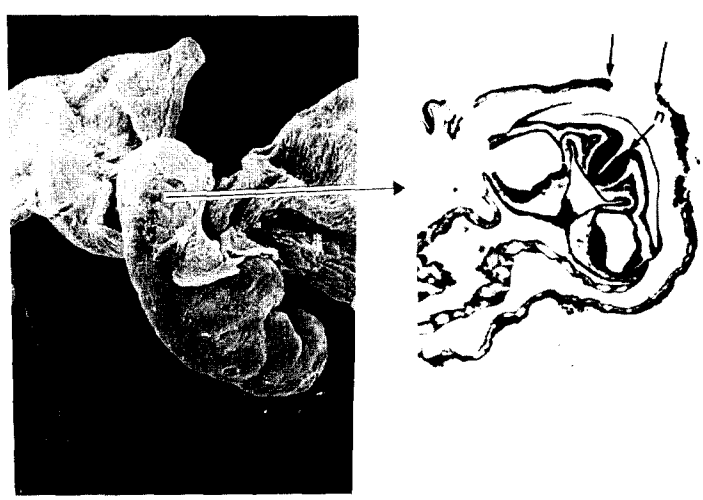

Fig. 7. Representative section through a 4mm pig embryo to illustrate the formation of amnion and chorion. Arrows indicate the chorioamniotic folds. N : Neural tube.

Table 1. Cell stage distribution of embryos recovered surgically from pigs on day 7, day 11, and day 13 of gestation

Day of gestation	Stages									
	D	UFO	C	M	EBL	BL	X	I	H	EB
Day 6	0	6	5	27	57	21	9	–	–	–
Day 7	4	20	18	0	0	9	3	9	48	
Day 13	3	29	0	0	0	10	0	0	21	63

D:degeneration, UFO: unfertilization embryos, C:cleaved embryos, M:morular
EML:early blastocyst, BL:middle blastocyst, X:expanded blastocyst, I:hatching blastcyst, H:hatched blastocyst, EB:embryonic disc.

doderm.

In the pig, amniogenesis occured by the folding of somatopleure (Fig. 7). The amnion arises as two cell layers, an outer layer of mesoderm and an inner layer of ectoderm, that enfold the developing embryo and protect it from mechanical damage. Fusion of the folds was followed by a complete separation of the amniotic and chorionic mesoderm, so that the amnion covered fetus floats free in the extraembryonic coelom. Thus, the allantois may grow into and completely fills the extraembryonic coelom, except for the region of attachment of the yolk sac to the chorion.

Ⅳ. DISCUSSIONS

It is difficult to obtain general picture of developing embryos of domestic animal. In this study, we have investigated the ultrastructural feature of porcine embryos from the time of fixation to the uterine wall until the completion of implantation. The sequence of these events after the shedding of the zona pellucida is generally classified by 1) Pontamine blue reaction, 2) emergence of W-bodies, 3) local oedema of the uterine stroma, 4)histochemically demonstrable increase in endometrial alkaline phosphatase, 5) histological decidualization according to Finn and McLaren (1967). During the early attachment as shown in Fig. 4, we have observed that the bleb-like structures were adherent to the blastocyst during the earliest stage of attachment. The candidates responsible for the initial attachment between the trophoblast and uterine epithelium at implantation has been investigated extensively. These factor such as heparan sulfate proteoglycan (Tang et al., 1987), lactose aminoglycan (Dutt et al., 1987), galactose N-acetyl-glucosamine (Chavez et al., 1991), galactosyl transferase (Sato et al., 1984), lacto-N-fucopentose-1 (Lindenberg et al., 1988), Arg-Gly-Asp-dependent receptors (Carson et al., 1988), and stage-specific glycoproteins (Anderson et al., 1986) have been suggested as candidates in the initial attachment of the trophoblast to uterine epithelium.

The major differences between post-blastocyst development *in vitro* and *in vivo* involved the fate of four structures that are not a part of egg cynder itself *in vivo*: these are the mural trophoblast, parietal endoderm, Reichert's membrane and partial endoderm (Snell & Stevens, 1966). *In vivo*, mural trophoblast forms the wall of the blastocoel, which becomes the yolk cavity and is lined by Richert's membrane and parietal endoderm cells. *In vitro*, however, the mural trophoblast does not enclose a three dimensional sheet of giant cells on the coverslips (Cole & Paul, 1965). In pig, close apposition to

the uterine wall begins at about 12 days and then attachment occurred during the afternoon of the 16 or 18th day post coitum. Between day 7 and 17 of pregnancy, 90 blastocysts from 0.3 to 12 mm in diameter were flushed from the uterine horns of Dutch Landrace pigs. The ultrastructure of embryos was similiar at all.

It has been shown that the primitive and extraembryonic ectoderm layers grow and elongate to form the core of the egg cylinder and that the outer endoderm cells differentiate into two morphologically and biochemically distinguishable subpopulations, the visceral endoderm and the parietal endoderm (Leivo et al. 1980). The parietal endoderm cells first appear at the time of implantation when primitive endoderm cells migrate on to the inner surface of the trophectoderm which is covered by a thin basal lamina containing fibronectin and laminin.

V. ABSTRACT

Morphological features of the interaction between the hatching blastocyst and implantation in pig were studied by electron microscopy. The observations extended from late blastocyst stage to the completion of trophoblastic erosion of the epithelium and early decidual transformation of the epithelium and early decidual transformation of the stromal cells. Between day 7 and 17 of pregnancy, blastocysts from 0.3 to 12 mm in diameter were flushed from the uterine horns of Dutch Landrace pigs. On the 7th of development in the pig blastocyst, the blastocyst shedded of the zona pellucida established the tips of microvilli and with bleb-like cytoplasmic protrusions of the epithelial cells. From day 11 on in pig embryo, the bilayered trophoblast undergoes a dramatic phase of elongation so that the initially spherical expanded blastocyst becomes tubular. In pig, close apposition to the uterine wall begins at about 12 $^{1}/_{2}$ days and then attachment occurred during the afternoon of the 16th or 18th day post coitum. At this stage, embryonic loss compared with corpus luteum number is up to 40% of ovulated oocytes. Therefore, the implantation failture of these embryos may be mainly caused by morphological abnormality and failture of zona shedding.

VI. REFERENCES

1. Anderson, T. L., G. E. Oslon and L. H. Hoffman. 1986. Stage specific alterations in the apical membrane glycoproteins of endometerial epothelial cells related to implantation rabbits. Biol. Reprod. 34:701-720.
2. Blet, W. D. and D. C. Pease. 1956. Mitochondrial structure in sites of steroid secretion. J. Biophys. Biochem. Cytol. Spple. 2:369-374.
3. Brackett, B. G., Y. K. Oh, J. F. Evans and W. J. Donawick. 1980. Fertilization and early development of cow ova. Biol. Reprod. 2: 609-622.
4. Calarco, P. G. and A. McLaren. 1976. Ultrastructural observation of preimplantation stages of the sheep. J. Embryol. Exp. Morphol. 36:609-622.
5. Carson, D. D., J. P. Tang and S. Gay. 1988. Collagen support embryo attachment and outgrowth *in vitro*: effects of the Arg-Gly-Asp sequence. Dev. Biol. 127:368-375.
6. Chavez, D. J. 1991. Possible involvement of D-galactose in the implantation process. In:Denker, H, W., J. D. Aplin.(eds). Trophoblast invasion and endometrial receptivity: Novel aspects of the cell biology of embryo implantation. trophoblast research 4, NY. ; 259-272.
7. Cole, R. J. and J. Paul. 1965. Properties of cultured preimplantation mouse and rabbit

embryro, and cell strains derived from them. In:Preimplantation.

8. Wolstenholme, G. E. W. and M. O'Connor, eds. J. and A. Churchill. 1988. Stages of Pregnancy. Ciba Foundation Symposium. London, pp. 82-122.
9. Dutt, A., J. P. Tang, and D. D. Carson. 1987. Lactosaminoglycans are involved in uterine epithelial cell adhesion *in vitro*. Dev. Biol. 119:27-37.
10. Enders A. G. 1973. Cytology of the corpus luteum. Biol. Reprod. 8:158-182.
11. Finn, C. A. and A. McLaren. 1967. A study of the early stages of implantation in mice. J. Reprod. Fert. 13:259.
12. Geisert, R. D., R. H. Renegar, W. W. Thatcher, R. M. Rober, and F. W. Bazer. 1982a. Establishment of the pregnancy in pig: I. Interrelationships between preimplantation development of the pig blastocyst and uterine endometrial secretions. Biol. Reprod. 27:925-939.
13. Geisert, R. D., J. W. Brookbank, R. M. Roberts, and F. W. Bazer. 1982b. Establishment of the pregnancy in pig: II. Cellular remodelling of porcine blastocyst during elongation on day 12 of pregnancy. Biol. Reprod. 27: 941-955.
14. Gemmell, R. T., B. D. Stacy, and Thorbarn. 1974. Ultrastructural study of secretory granules in the corpus luteum of the sheep during the estrous cycle. Biol. Reprod. 11: 447-462.
15. Hunter, R. H. F. 1977. Physiological factors influencing ovulation, fertilization, early embryonic development and establishment of pregnancy in pigs. Br. Vet. J. 133:461-470.
16. Leivo, I., A. Vaheri, R. Timple, and J. Wartiovaara. 1980. Dev. Biol. 76:100-114.
17. Linares, T., L. Ploen. 1981. On the Ultrastructure of seven day old normal and abnormal bovine embryo. Anat. Histol. Embryol. 10:212-226.
18. Lindenberg, S., K. Sundlberg, S. J. Kimber, and A. Lundblad. 1988. The milk oligosaccharide, lacto-N-fucopentaose I, inhibits attachment of mouse blastocysts on endometrial monolayers. J. Reprod. Fertil. 83:149-158.
19. Kim, J. H. and R. M. Petters. 1994. Production of predominant pronuclear for efficient microinjection and embryo transfer in pig. Submit.
20. Massip, A., J. Mulnard, R. Huygens, C. Hanzen, P. van der Zwalmen, and F. Ectors. 1981. Ultrastructural of the cow blastocyst. J. Submicr. Cytol. 13:31-40.
21. Mohr, L. R. and A. O. Trounson. 1981. Struc tural changes associated with freezing of bovine embryos. Biol. Reprod. 25: 1009-1025.
22. Mohr, L. R. and A. O. Trounson. 1982. Comparative ultrastructural of hatched human, mouse and bovine blastocyst. J. Reprod. Fertil. 66:499-504.
23. Norberg, H. S. 1973. Ultrastructural aspects of the preattached pig embryo. Cleavage and early blastocyst stage. Z Anat. Entwicklungsgeschichte 143:95-114.
24. Perry, J. S., and I. W. Rowlands. 1962. Early pregnancy in the pig. J. Reprod. Fertil. 4: 175-188.
25. Sato, M., T. Muramatsu, and E. G. Berger. 1984. Immunological detection of cell surface galactosyltransferase in preimplantation mouse embryos. Dev. Biol. 102:514-518.
26. Snell, G. D., and L. C. Stevens. 1966. Early Embyrology. In: Biology of the Laboratory Mouse. Second ed. Green EL ed. McGraw-Hill. New york. pp. 205-245.
27. Tang, J. P., J. Julian, S. R. Glasser and D. D. Carson. 1987. Heparan sulfate proteglycan synthesis and metabolism by mouse

uterine epithelial cells *in vitro*. J. Biol. Chem. 262:12832-12842.

28. Winterberger, T. S., and J. E. Flechon. 1974. Ultrastructural evulation of the trophoblast cells of the preimplantation sheep blastocysts from day 8 to day 18. J. Anat. 188: 143-153.