ULTRASTRUCTURAL COMPARISON OF BOVINE BLASTOCYSTS DEVELOPED *IN VIVO* AND *IN VITRO*

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Summary

The ultrastructures of *in vitro*-derived bovine blastocysts have been compared with those of blastocysts obtained from a superovulated cow. *In vivo* blastocysts obtained from the uterus showed well-differentiated features, while *in vitro*-derived embryos, which were developed from *in vitro* fertilized ovum, showed insufficient cellular organizations. *In vitro*-derived embryos contained many undefined cellular organizations in the perivitelline spaces compared with *in vivo*-derived blastocysts. Other features of *in vivo* and *in vitro* blastocysts were characterized by differential development of microvilli projection into blastocoele from the surface of the trophoblast cells. The conceivable reason for the difference between *in vivo* and *in vitro* developments of bovine embryos is that it is likely that *in vitro* culture system adopted in the present experiment may not be sufficient for better embryonic development.
(**Key Words** : Ultrastructure, Bovine, Blastocyst)

Introduction

Since an *in vitro* culture system has been established to mature *in vitro* bovine embryos derived from *in vitro* fertilized oocytes (Xu et al., 1987, Goto et al., 1988), many systems for culturing preimplantation cattle embryos have been reported, pregnancies or offspring being obtained after transfer of those embryos to recipients. In general, it has been suggested that the percentage reaching blastocyst stage and pregnancy rate of bovine embryos obtained from *in vitro* fertilization were still low, but much progress of early embryonic development has been reported.

The ultrastructural aspects of bovine early embryonic development have been studied (Mohr and Trounson, 1981; Brackett et al., 1980; Linares and Ploen, 1981; Massip et al., 1981). Some comparison of structural characteristics of mouse (Mcreynolds and Hadek, 1972) and rabbit (Van Blerkom et al., 1973) embryos developed *in vivo* and *in vitro* has been accomplished, and their achievements contributed to a better understanding of the development of mammalian early embryos. However, there are no reports of detailed comparisons between *in vivo* and *in vitro* development of bovine embryos. The present study was, therefore, undertaken to investigate cellular characteristics of bovine blastocysts developed *in vivo* and *in vitro*.

Materials and Methods

Collection of blastocysts developed *in vivo*

A Japanese black cow (28 months old) was stimulated with 20 AU(Armor Unit) of FSH-P (Antrin; Denka Pharmaceuticals, Japan) administered twice daily in decreasing doses over a 3-day period (5, 3 and 2 AU, respectively). Approximately 50 hr after the initial gonadotrophin injection, luteolysis and heat were induced by the injection of 3 ml of prostaglandin $F_2\alpha$-analogue (Syncrocept; Dainihon Pharmaceuticals, Japan). Heat was checked twice a day. Artificial inseminations were made with frozen-thawed semen from a Japanese black bull. In this experiment, we examined the fertilizing ability of frozen-thawed cattle semen, finding that Japanese black bull has rather higher fertilizability than those of dairy cattle bull. Embyos were collected nonsurgically on the eighth day after standing heat. The medium for flushing the uterus was Dulbecco's phosphate buffered saline (DPBS; Gibco, USA) with 100 iu/ml penicilline (Meiji Seika,

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Japan) and 4 mg/ml BSA (Sigma, USA). After collection, the embryos were washed twice with DPBS supplemented with 10% calf-serum (CS; Gibco, USA). Then, all of the embryos were fixed immediately for transmission electron microscopy (TEM).

Development of embryos cultured *in vitro*

The ovaries were obtained at a local abbatoir and were transported in saline (8.5 g NaCl/l) at 32-35℃ to the laboratory within 2 hr. The cumulus oocyte complexes (COCs) were aspirated from follicules of 1-8 mm in diameter. Only oocytes surrounded with cumulus oophorus were cultured in a 30 mm plastic dish (40-60 COCs/dish; Coaster, USA) containing 2.5 ml of SFRE-2 (Sigma, USA) supplemented with 50 μg/ml gentamicin sulfate and 10% CS at 39℃ for 24 hr in 5% CO_2 in air. The medium was also supplemented with 0.5×10^6/ml granulosa cells. Granulosa cells were collected from the remnant of aspirated follicular fluid by centrifuging at 250 $\times$ g for 5 min to remove supernatant. Then, 2.0 ml of 0.25% trypsin (Difco, USA) in CMF-DPBS was added and reacted for 5 min at 37℃ and washed twice with DPBS supplemented with 4 mg/ml BSA by centrifuging at 750 $\times$ g for 5 min.

Sperm preparation was conducted according to the method of Tsuzuki et al. (1991) with slight modification. Briefly, frozen semen was thawed and washed in modified HEPES-TALP (Brackett and Oliphant, 1975). The sperm suspension was diluted with mHEPES-TALP containing 10 μg/ml heparin (Katayama Chemicals, Japan), 5.0 mM theophylline (Sigma, USA) and 5 mg/ml BSA. The final sperm concentration was adjusted to 10×10^6 cell/ml. After the maturation culture, COCs were introduced into 100 μl droplets of the sperm suspension (11-20 COCs/drop) and co-cultured at 39℃ for 6 hr under mineral oil (Katayama Chemicals, Japan) in 5% CO_2 in air. After insemination, the COCs were washed twice in modified SOF (mSOF; Takahashi and First, 1992; added 0.5 mM glucose) supplemented with 1.0% CS and cultured at 39℃ for 42 hr. The cumulus cells surrounding embryos were completely removed and the culture medium was replaced by fresh mSOF added 5.0% CS and the embryos were co-cultured for an additional 8 days with cumulus cells attached onto the bottom of the dish. The culture medium was exchanged with fresh medium every 48 hr. The embryos developed to the blastocyst stage at 144-216 hr after insemination were fixed for TEM.

Preparation of embryos for TEM

The embryos were fixed in 0.8% paraformaldehyde and 2.5% glutaraldehyde in phosphate buffer solution (pH 7.4) at 4℃ for overnight and rinsed three times in PBS for 30 min, post-fixed in 1.33% osmium tetraoxide, dehydrated in a graded series of ethanols, and finally embedded in epoxy resin. The embryos were serially cut into semi-thin and thin sections. The semi-thin sections were stained with 1.0% toluidine blue and observed under a light microscope. The thin sections were stained with uranyl acetate and lead staining solution (Sato, 1968), and examined under a Hitachi H-300 TEM.

Results

Seventeen embryos were recovered from a superovulated cow. Seven of these embryos were blastocysts of good quality. On the other hand, nineteen embryos of blastocysts were obtained from *in vitro* culture system.

In the *in vivo* blastocysts of good quality, the perivitelline space was very narrow containing no cell debris (figure 1), but in some of the *in vivo* blastocysts, degenerated cells were observed in the perivitelline space (figure 2). In all the *in vivo* blastocysts, the flattened trophoblast cells were observed, contacting closely with one another (figure 4). In contrast, most of the *in vitro* blastocysts contained some undefined cellular organizations in the perivitelline space (figure 3).

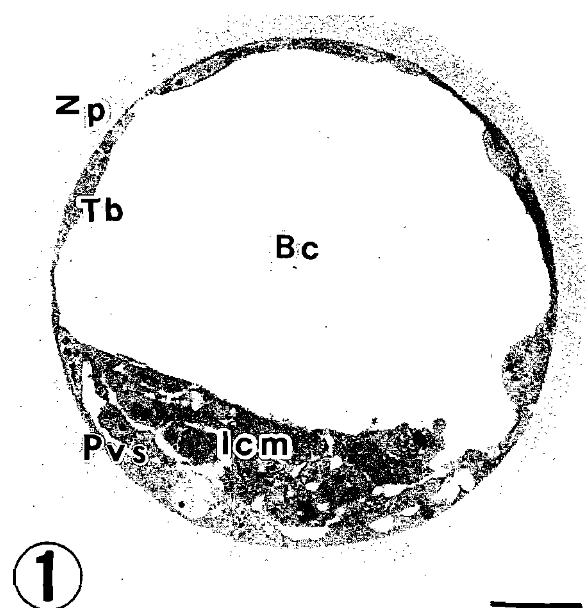

Figure 1. A blastocyst obtained from the uterus, classified as an excellent embryo. A trophoblast cell (Tb) tightly apposed to the zona pellucida (Zp), and inner cell mass (Icm) and blastocoele (Bc). Bar = 20 μm.

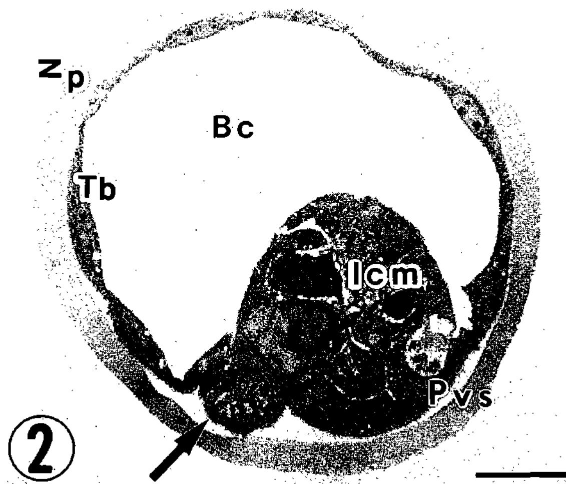

Figure 2. A blastocyst obtained from the uterus, classified as fair quality. Inner cell mass (Icm) was probably under development. Degenerated cell (Dc, arrow) is observed in the perivitelline space (Pvs). Bar = 20 μm.

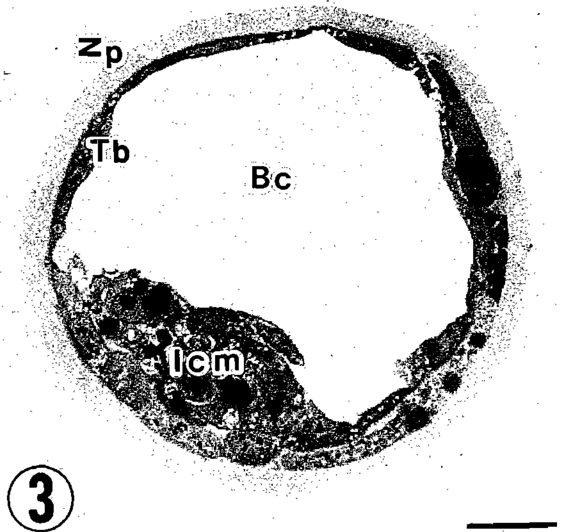

Figure 3. An *in vitro* -derived blastocyst of good quality. Trophoblast cells (Tb) and inner cell mass (Icm) are well developed enough to be transferred. Bar = 20 μm.

In vivo blastocysts demonstrated well-developed microvilli on the external surface of trophoblast cells (figure 6). In some of the embryos with the degenerated cells in the perivitelline space, there were some lacking region of the microvilli, especially adjacent to the degenerated cells (figure 5), though many microvilli were observed on the surface of trophoblast cells. The trophoblast cells were interconnected with a distinct junctional complex in the apical region of the interblastomeric space (figures 5, 6 and 8). Clusters of desmosomes were also clearly recognized along with large bundles of microfilaments (figure 8). The connections between trophoblast and inner cell mass cells were quite different from one another. Not apparent junctional complex and desmosome were observed, but cytoplasmic projections were entangled and electron dense plasma membranes were often seen (figures 4, 6 and 7).

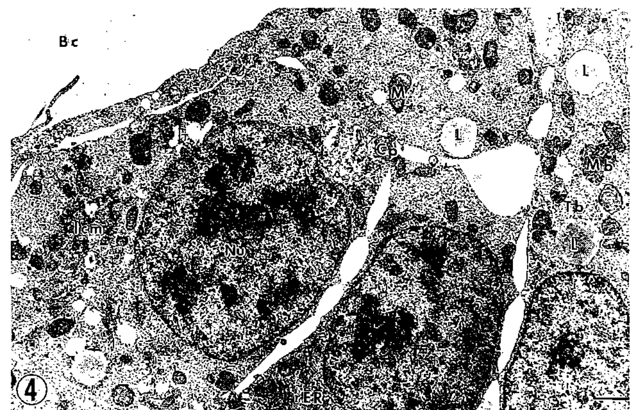

Figure 4. An *in vivo*-derived blastocyst of excellent quality. Many mitochondria (M) and well-developed rough endoplasmic reticulum (rER) are observed. Bar = 2 μm.

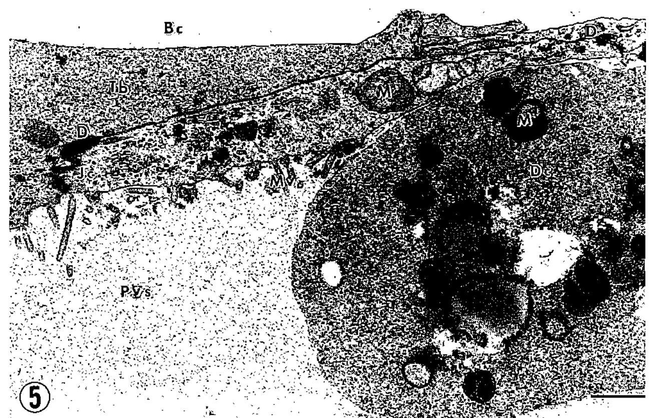

Figure 5. An *in vivo* blastocyst of fair quality. Degenerated cell (Dc) was seen in the perivitelline space (Pvs). Mitochondria (M) with different electron density and the lacking region of microvilli (Mv). The apical region is upper left of the photograph and junctional complexes (Jc) were observed. Bar = 1 μm.

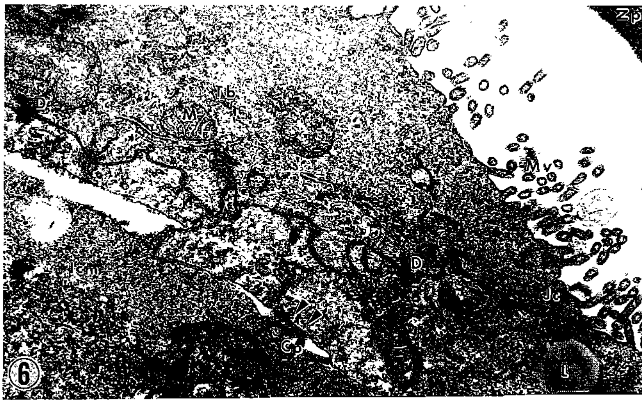

Figure 6. An *in vivo* blastocyst of excellent quality. Difference of cellular adhesion between trophoblast cells (Tb) and an inner cell mass (Icm) are seen. Zone of apposition (arrowheads) with slightly higher election density. The apical region is lower right of the photograph and junctional complexes (Jc) were observed. Bar = 1 μm.

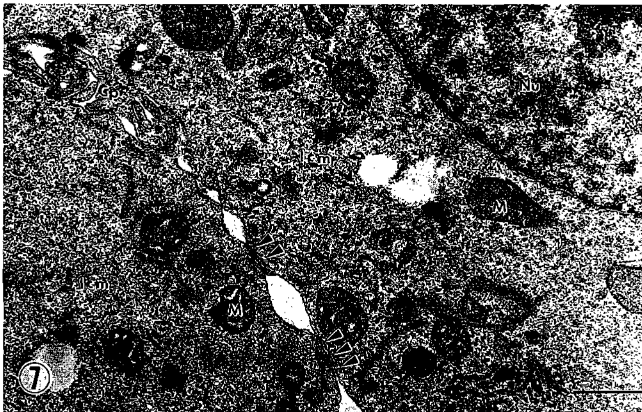

Figure 7. An *in vivo* blastocyst. Connection between inner cell mass (Icm) with narrow appositional zones between the two cells, forming possibly gap junction (arrowheads). Cytoplasmic projections (Cp) are entangled. Bar = 1 μm.

Morphological features of the cytoplasmic inclusions of the trophoblast cells were very close to those of the inner cell mass cells (figure 4). A large number of mitochondria were observed, showing cylindrical, spherical or oval in shape with several transverse cristae (figures 6 and 7). The mitochondria of the blastocysts of fair quality showed high electronic density, indicating poor development of the embryos (figure 5). Rough endoplasmic reticulum (rER) were occasionally observed in the inner cell mass cells (figure 9). Many free ribosomes and polysomes were scattered throughout the cytoplasm (fig. 9).

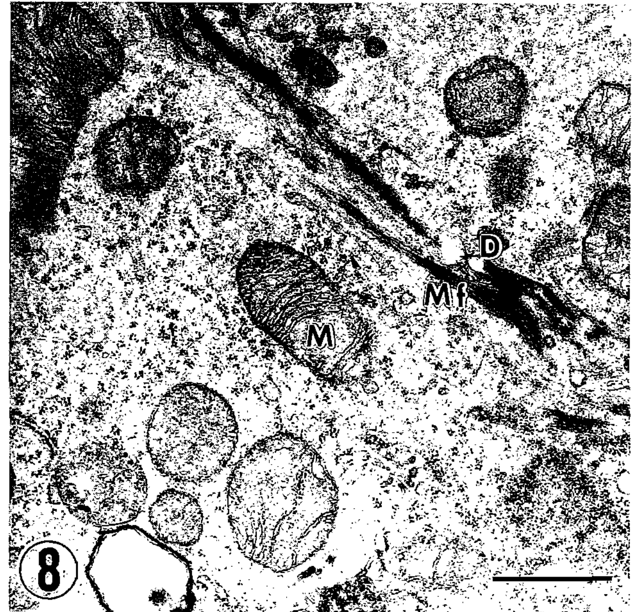

Figure 8. An *in vivo* blastocyst. Clusters of desmosomes (D) between two trophoblast cells (Tb). Desmosomes (D) are arranged in series and microfilaments (Mfs) are apposed. The apical region is upper left of the photograph and junctional complexes (Jc) were observed. Bar = 1 μm.

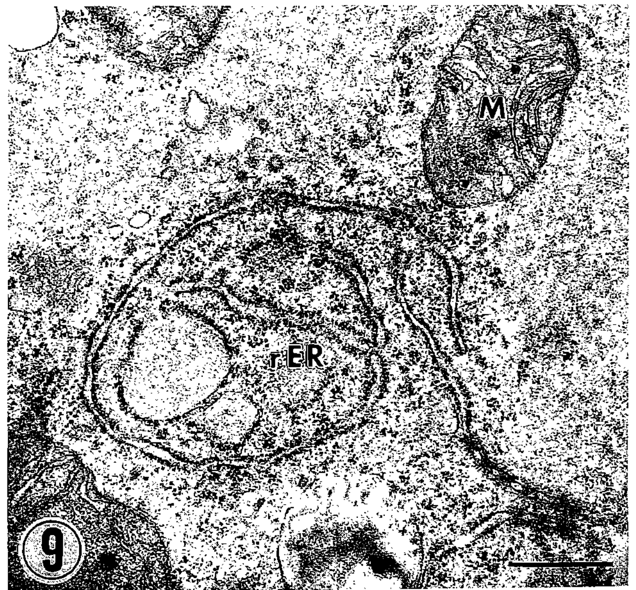

Figure 9. An *in vivo* blastocyst. Well-developed rough endoplasmic reticulum (rER) in inner cell mass (Icm). Mitochondria (M) with well-developed cristae. Bar = 0.5 μm.

Gross appearance of the *in vitro* blastocyst was nearly the same as that of the *in vivo* embryos. In the *in vitro* embryos, cell fragments and/or debris were often observed in the perivitelline space (figure 11), whereas some blastocysts of good quality showed no such cellular features in the corresponding region (figure 13). Well-developed microvilli were observed on the surface of the trophoblast cells of the *in vitro* blastocysts (figures 10 and 11). The junctional complexes and cytoplasmic projections of the trophoblast cells of *in vitro* embryos were similar to those of *in vivo* blastocysts (figures 10 and 12-14). On the other hand, cluster of desmosomes, which were demonstated *in vivo* embryos were not observed in the trophoblast cells of the *in vitro* embryos (figure 13). Morphological characteristics of mitochondria, the shape of the nuclei, and rER of the *in vitro* embryos were not essentially different from those of the *in vivo* embryos (figures 11 and 14). It was extremely conspicuous that irregularly membrane-bounded body, numerous vesicles and lipid droplets in various sizes were recognized in all cells of the *in vitro* embryos (figures 10, 12 and 14). Other cytoplasmic features such as ribosomes, Golgi apparatus, smooth endoplasmic reticulum (sER) were almost the same for both the *in vivo* and *in vitro* blastocysts.

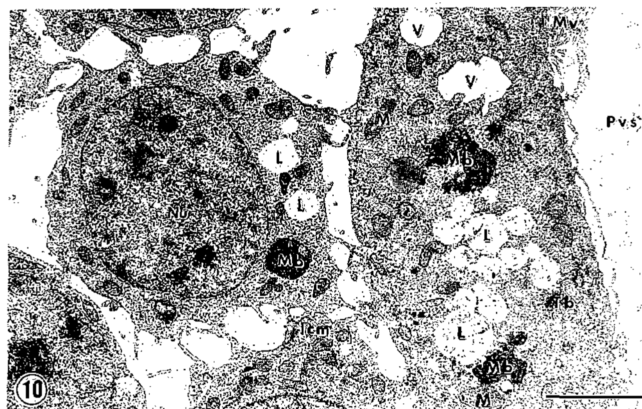

Figure 10. An *in vitro* blastocyst of good quality. Lacking region of microvilli (Mc), and vacuole (V) in various sizes and shapes, lipid droplet (L) and membrane-bounded body (Mb). Bar = 2 μm.

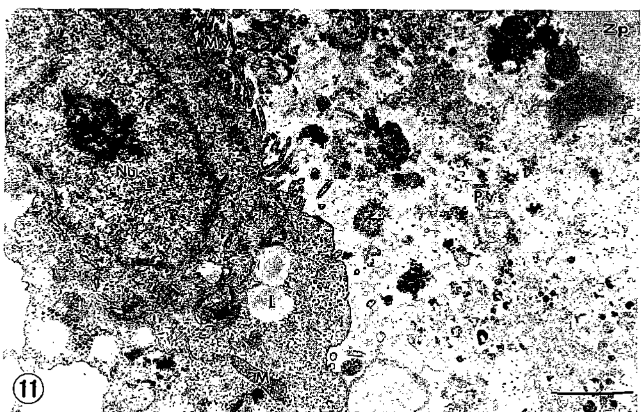

Figure 11. An *in vitro* blastocyst of good quality with lacking region of microvilli (Mv) and scattering many cell debris in the perivitelline space (Pvs). Bar = 2 μm.

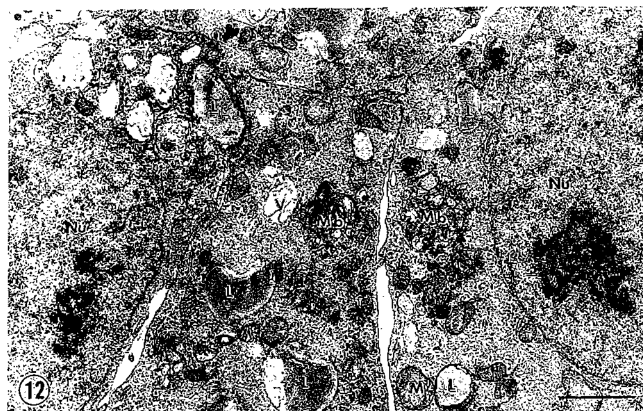

Figure 12. An *in vitro* blastocyst. Well-organized cellular features of inner cell mass with conspicuousness of membrane-bounded body (Mb) and lipid droplet (L) in the cytoplasm. Bar = 2 μm.

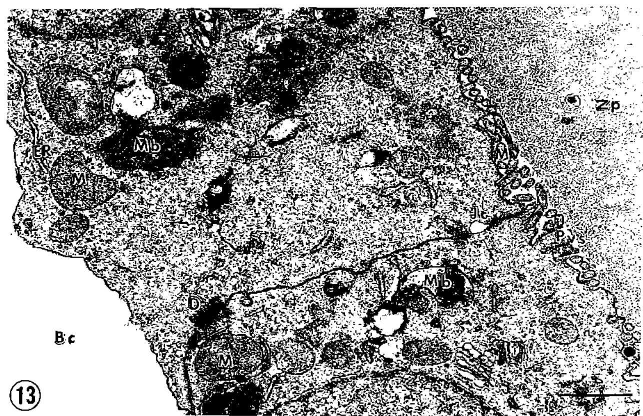

Figure 13. An *in vitro* blastocyst. Junctional region of trophoblast cells (Tb) having well-developed microvilli (Mv) in subzonal space. Junctional complexes (Jc) and large desmosomes (D) are observed. Cytoplasm contains membrane-bounded body (Mb). Bar = 1 μm.

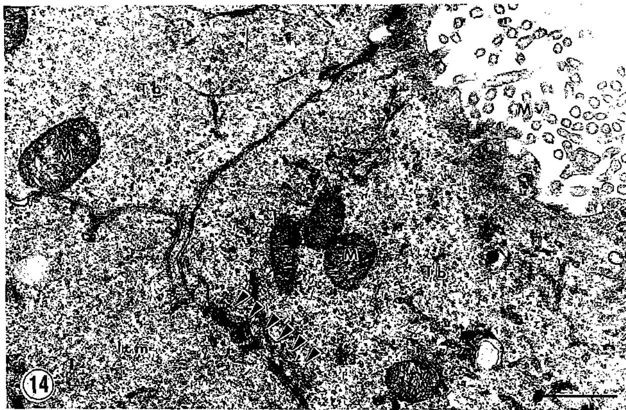

Figure 14. An *in vitro* blastocyst. slightly higher electron density in narrow appositional zone (arrowheads) between trophoblast cell (Tb) and inner cell mass (Icm). Bar = 1 μm.

Discussion

The ultrastructural features of bovine *in vivo* blastocysts obtained from the uterus have been reported by some researchers (Linares and Ploen, 1981; Mohr and Trounson, 1981; Massip et al., 1981) and the results obtained from the present study supported their evidence in a sense. Distinct features of the blastocysts were observed in the trophoblast cells. The epithelium of the trophoblast cells were characterized by the junctional complexes and well-developed microvilli projecting to the perivitelline space. These morphological features have been considered to play an important role in the formation and expansion of the blastocyst by active fluid transport and the maintenance of preganancy by secreting factor (s) (Linares and Ploen, 1981; Massip et al., 1981). Hence, the blastocysts having the lacking area of microvilli, which were classified morphologically as *in vitro* embryos of fair quality, might not proceed to further development, leading to the failure of pregnancy by embryo transfer (Linares and Ploen, 1981). Ducibella et al. (1975) demonstrated in the mouse that during the final phases of blastocyst expansion, tandem desmosomes with large bundles of filaments develop in trophoblast junctional complex. Massip et al. (1981) and we, in the present study, also observed the same morphological features of cow blastocysts recovered from the uterus in which trophoblast cells are consolidated by numerous desmosomes associated with fibrillar elements.

On the other hand, in the *in vitro* blastocysts, numerous or tandem desmosomes were not observed in the trophoblast cells, though junctional complex was clearly recognized. Without these stronger cell contacts, the hydrostatic pressure thought to accompany with blastocoele expansion would probably disrupt the zonula occludens (Massip et al., 1981), resulting in the inhibition of further embryonic development. Yotsutani et al. (1993) showed that cytoskeletal proteins were observed in *in vitro* bovine embryos, and the junctional complex formation of *in vitro* blastocysts might be somewhat delayed compared with that of *in vivo* embryos.

There was no big difference in the ultrastructural features of the cytoplasmic organelles among the blastocysts, except those of rER. The development of the rER in *in vitro* embryos was not sufficient compared with of the *in vivo* embryos. In this study, the frozen-thawed semen from different breed of cattle was used. Kajihara et al. (1987) reported that the *in vitro* development of interbred crossed embryos is superior to that of purebred embryos. However, advanced rER has not been observed in the *in vitro* blastocysts obtained from the present study. The rER is, in general, considered to be involved in protein synthesis. The *in vitro* blastocysts contained many cell debris and/or degenerated cells in the perivitelline space resulting in fair development of the embryos. The irregularly membrane-bounded body, numerous vesicles and lipid droplets in various sizes were conspicuous in the cytoplasmic inclusions of the *in vitro* blastocysts contrasted to those of the *in vivo* blastocysts. Jung (1989) demonstrated that *in vitro* culture of rabbit blastocysts not only changes protein synthesis but also enhances protein degradation, and apoptotic bodies of dead cell are extruded into blastocyst cavity and perivitelline space or undergo phagocytosis by nearby cells. The membrane-bounded bodies have been thought to be autophagic vacuoles containing waste products of the initial divisions or the process of embryonic metabolism (Linares and Ploen, 1981). Therefore, morphologically fair blastocysts might deviate a part of blastomeres on embryonic development up to blastocyst stage. The reason for these poorly developed blastomeres is, in the case of *in vivo* embryos, probably the abnormalities within the embryos or the abnormal function of the oviduct and uterus, because of superovulation treatment (Linares and Ploen, 1981), while in the case of *in vitro* embryos, fairly developed blastocysts may be due to the deficiencies of culture system.

In conclusion, the present results suggest that the morphological classification of *in vivo* bovine blastocysts could be useful for the estimation of the subsequent embryonic development (Linares and Ploen, 1981). However, the *in vitro* blastocysts obtained in the present study showed some ultrastructural deficiencies such as

poor developments of desmosomes, microvilli, rER and the conspicuousness of vaculoes and membrane-bounded bodies. Therefore, more improvement of *in vitro* culture system of bovine embryos might be required for better embryonic development.

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