Effects of Cytochalasin-D on the Maintenance of Blastocoels of Bovine Blastocysts Produced In Vitro

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Abstract: *In the present study, we investigated the role of actin filaments in the blastocoel (BC) of bovine blastocysts produced in vitro by using cytochalasin-D (CD), an inhibitor of actin polymerization. Blastocysts classified as good or poor based on their morphological normality were exposed to 2 μM CD for 1.5–2 hr. After incubation, the presence or absence of BC was observed, and the re-expansion rate was assessed after transferring CD-treated blastocysts with collapsed BC to a non-CD medium. Ultrastructural observation was also undertaken using a transmission electron microscope (TEM). The remaining blastocysts were immersed in a hypotonic solution of sodium citrate so that the cells could be counted to confirm the classification grade of blastocysts in this study. The percent of maintained BC under the presence of CD in the good group was significantly lower (P<0.05) than that in the poor group (3.3% versus 27.7%, respectively). The average cell number of blastocysts in the good group was significantly more (P<0.05) than that in the poor group. In addition, when the blastocysts with shrunken BC in both groups were cultured, re-expansion rates in the good and poor groups were 83.3 and 75.0%, respectively, and no significant difference was observed between groups. Based on observation of the ultrastructure with TEM, the microvilli on the surface of some trophoblast cells of some blastocysts in the poor group in the presence of CD showed a translucent matrix, and their electron density was low compared with that of trophoblast cells of blastocysts in the good and non-treated (control) groups. However, the electron density of microvilli after removal of CD in the poor group increased to a level comparable to those of the good and control groups. These results suggest that polymerizing actin may be required to sustain the blastocoel and microvilli of blastocysts produced in vitro. However, in poor grade blastocysts, the polymerization ability of actin present in the filamentous form in the microvilli in some cells might be lower than that in good grade blastocysts.*

Key words: *Blastocyst, Blastocoel cavity, Actin, Cytochalasin-D*

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Introduction

Embryos produced by *in vitro* fertilization can be developed to the blastocyst stage in cattle [1, 2]. At that stage, a cavity is formed within the embryo [3].

The blastocoel (BC) of mammalian embryos is formed by the action of the Na^+ /K^+ pump at the morula stage. This pump is located among the trophoblast (TB) cells, and it transports some ions across the cell membranes into the center of the ball of compact blastomeres. Osmotic action transports fluid to the interior of the morula, resulting in expansion of the embryo due to hydrostatic pressure [4, 5]. After formation of the BC, the cavity is maintained by the tight junction permeability seal of the TB cells [6].

Actin is an important skeletal element in the development of mammalian oocytes and embryos [5]. In the mouse, actin filament may play a crucial role in the process of blastocyst hatching [7]. In the pig, actin distribution in embryos is an important factor of embryonic quality [8]. When the polymerization of actin filament was prevented, 2- to 4-cell porcine embryos produced *in vitro* did not develop to the blastocyst stage [9]. In bovine blastocysts produced *in vitro*, actin was located beneath the plasma membrane of the TB cells [10]. Despite these reports, the role of actin filament in

the polymerization of bovine blastocysts has not yet been fully clarified.

We undertook an assessment of the effect of cytochalasin-D, an inhibitor of actin polymerization, on the maintenance of BC and the ultrastructure of bovine blastocysts produced *in vitro*.

Materials and Methods

In vitro maturation of oocytes

Bovine ovaries from Japanese black or Holstein cows and heifers were obtained from a slaughterhouse and brought to our laboratory in physiological saline (NaCl, 0.9%). Oocytes were aspirated from superficial follicles (2–6 mm in diameter) and washed four times with Hank's salt balanced TCM-199 (Gibco BRL Products, MD, USA) supplemented with 98 U/ml penicillin and 98 μg/ml streptomycin (Katayama Chemical Co., Osaka, Japan). Oocytes surrounded by more than one layer of cumulus cells were selected and cultured in 25 mM Hepes buffered and Earl's salt balanced TCM-199 (Gibco BRL Products) supplemented with 0.12 U/ml follicle stimulating hormone from pig pituitary (No. F-2293, Sigma, MO, USA), 50 μM dimethyl-sulfoxide (DMSO, No. 134-07, Nacalai Tesque, Kyoto, Japan, [11]), 5% (V/V) calf serum (CS, Gibco BRL Products), and antibiotics (98 U/ml penicillin, 98 μg/ml streptomycin, Katayama Chemical Co. and 100 μg/ml dibekacin sulfate, Meiji Seika Co., Tokyo, Japan) for 23–24 hr under 5% CO_2, 95% air and 100% humidity.

Sperm preparation

Frozen semen obtained from one Japanese black bull was thawed in a water bath (37–39°C) for 1 min. The thawed semen were diluted with BO solution [12] by 2, 4, 8, 16 and 32 times, 2 min each time in a stepwise manner, to remove glycerol. After dilution, the semen was centrifuged for 8 min (750 $\times$ g, RT) and the concentration of sperm cells was adjusted to 1,800–1,900 $\times$ 10^4 cells/ml by dilution with an equal volume of BO solution supplemented with 10 mM caffeine (No. C-4144, caffeine sodium benzoade, 50:50 (w/w) mixture, Sigma), 20 μg/ml heparin (No. 411210010, Acros Organics, Geel, Belgium), and 15 mg/ml bovine serum albumin (No. 01-2030, Fraction V, Katayama Chemical Co.). The matured oocytes were transferred to 100 μl insemination drops (20–40 oocytes/drop) and incubated for 6 hr. After insemination, the oocytes were transferred to 100 μl drops of 25 mM Hepes buffered TCM-199 supplemented with 1% CS, 50 μM DMSO, 5 mM sodium lactate, and 0.4 mM sodium pyruvate. On the 3rd day after insemination, the oocytes were removed from the cumulus cells layer with a mouth-operated pipette and cultured for an additional 7 days.

Cytochalasin-D treatment

Blastocysts obtained at the 7–10th day after insemination were classified into 2 groups (good and poor) based on their morphological aspects [13]. Good grade blastocysts were characterized by more than 80% of all blastomeres having a regular shape, and poor grade blastocysts in the range from 25 to 80% of all blastomeres having a regular shape. Blastocysts in both groups were exposed to TCM-199 supplemented with 2 μM cytochalasin-D (CD, No. C-8273, Sigma) and incubated for 1.5–2 hr under 5% CO_2 and 95% air with 100% humidity. After incubation, the presence or absence of the BC was observed by an inverted light microscope (IMT, Olympus).

Re-expansion test

Blastocysts in which the BC was shrunken were washed twice with Hank's salt balanced TCM-199 and introduced into TCM-199 supplemented with 15% CS, 5 mM sodium lactate, 0.4 mM sodium pyruvate, and 1 mg/ml glucose and antibiotics. The blastocysts were then incubated for 2.5–6 hr under paraffin oil.

Analysis of cell numbers

The blastocysts in each group were introduced into 0.5% sodium citrate solution for 60 min, prefixed with methanol-acetate solution for 3–5 min (RT), mounted on a glass slide, and stained with Giemsa solution (No. 9203, Merk, Darmstädt, Germany) for 4 min (RT).

Transmission electron microscopy

Blastocysts, treated with or without CD, were fixed with 3% glutaraldehyde (TAAB Lab. Equip. Limited, Berkshire, UK) in 0.1 M phosphate buffer (pH 7.4) supplemented with 5 mM D-glucose for 2–3 hr (4°C). After a washing with the buffer, the blastocysts were post-fixed with 1.33% osmium tetroxide (No. 300, Nisshin-EM, Tokyo, Japan) in 0.1 M phosphate buffer (pH 7.4) for 1–1.5 hr (4°C), dehydrated through an acetone series, and then embedded in Quetol-812 (No. 340, Nisshin-EM). The thick (Ca. 1 μm thickness) or thin (Ca. 60–80 nm thickness) sections were made with a glass knife attached to a microtome (MT-2B, Sorvall). Thick sections were stained with 0.5% toluidine blue for a few seconds and then observed with a light microscope. The thin sections were stained by uranyl acetate and lead citrate solution [14] for 5–10 min (RT)

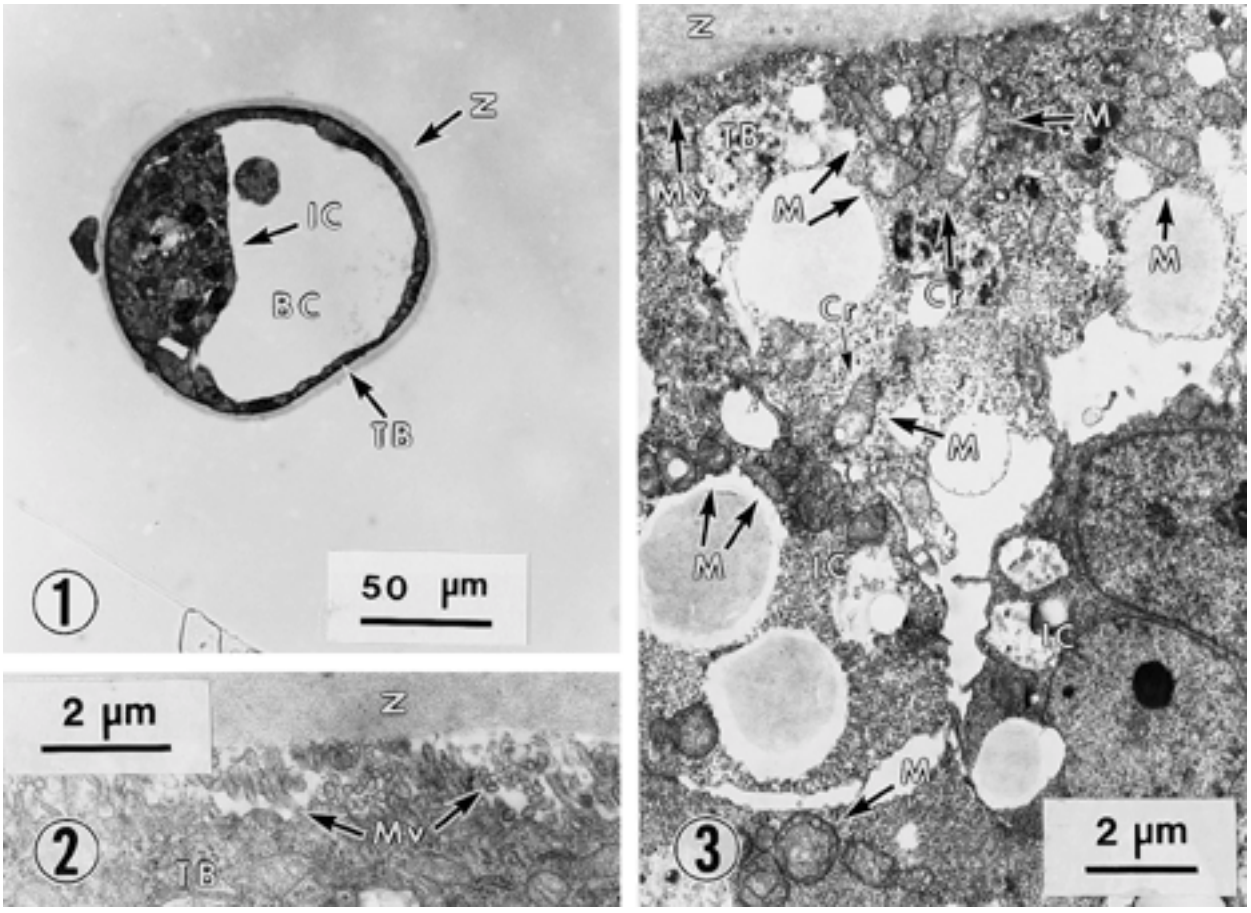

Fig. 1. Cross section of a control blastocyst observed by light microscopy. Cells of the inner cell mass (IC) and trophoblast (TB) are clearly distinguished. Blastocoel (BC) is seen within the embryo. Z: zona pellucida.

Fig. 2. Section observed by transmission electron microscopy. Microvilli (Mv) are observed on the outer surface of a TB cell of a control group. Z: zona pellucida.

Fig. 3. Section observed by transmission electron microscopy. Mitochondria (M) with transversed cristae (Cr) are dispersed within the cytoplasm in IC and TB cells. Mv: microvilli. Z: zona pellucida.

each and then observed with a transmission electron microscope (H-800, Hitachi, Tokyo, Japan).

Statistical analysis

The percentage of blastocysts in which the BC was maintained was statistically analyzed by chi-square test [15]. The cell numbers of blastocysts in each group were subjected to Duncan's new multiple range test.

Results

The percentages of blastocysts (Fig. 1) in which the BC was maintained are shown in Table 1. The good grade group treated with CD showed a much lower percentage of blastocysts in which the BC was maintained ($P<0.05$) compared with that in the control group (non-CD treated group). This value was also significantly lower ($P<0.05$) than that in the CD-treated poor grade group (Fig. 4).

The cell numbers in each group are shown in Table 2. Good grade blastocysts showed a greater number of cells ($P<0.05$) than did the poor grade group.

The percentages of the re-expanded blastocysts with re-expanded cavity in the good and poor (Fig. 6) grade groups were 83.3% (10/12) and 75.0% (3/4), respectively. However, no significant difference was observed between the two groups ($P>0.05$).

On observation with TEM, in the non-treated blastocysts, cuboidal and large nuclei were present in the cells of the inner cell mass (IC) and the trophoblast

Table 1. Percentage of blastocysts with blastocoel

Grades of blastocysts	No. and (%) of blastocysts with blastocoel			
	N	Treated with CD	N	Non-treated with CD
Good	32	1 (3.1)[b]	24	24 (100.0)[c]
Poor	47	13 (27.7)[a]	24	23 (95.8)[c]

Values with different superscripts are significantly different ($P<0.05$). CD: 2 μM cytochalasin-D.

Table 2. Cell numbers of blastocysts treated with and without CD

Grades of blastocysts	Mean cell numbers of blastocysts			
	No. of CD-treated blastocysts observed	Mean cell number	No. of non-CD treated blastocysts observed	Mean cell number
Good	48	103.2 ± 4.7^{a}	14	102.5 ± 10.9^{a}
Poor	47	61.9 ± 6.2^{b}	14	59.9 ± 5.3^{b}

Data are means ± SE. Values with different superscripts are significantly different ($P<0.05$). CD: 2 μM cytochalasin-D.

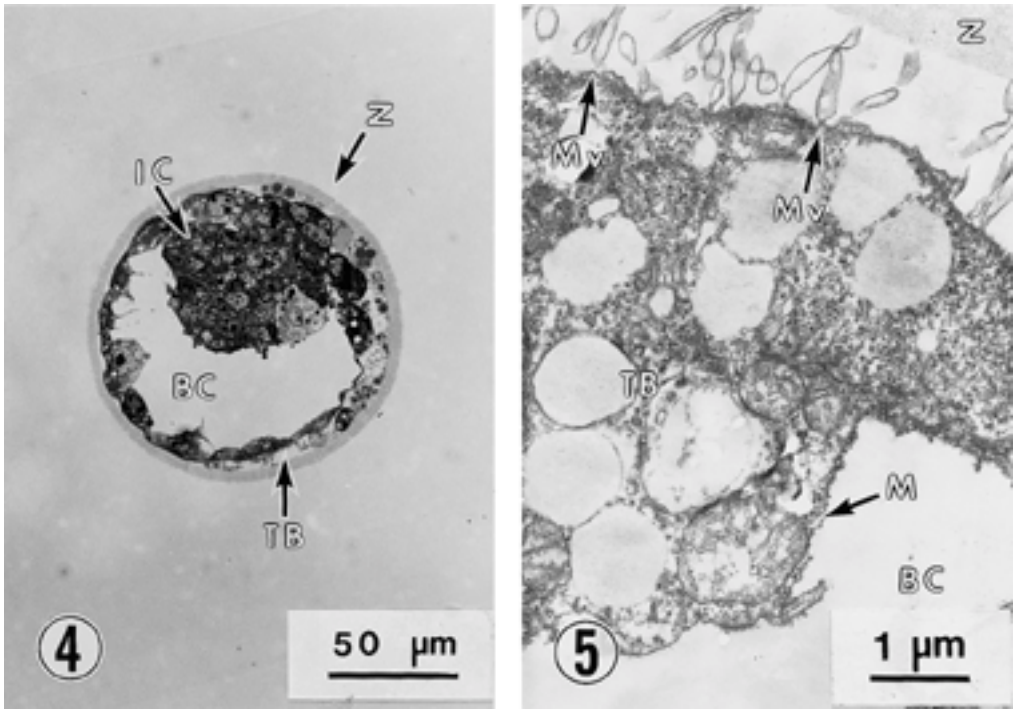

Fig. 4. Cross section of a blastocyst in the poor grade group, treated by cytochalasin-D, observed by light microscopy. IC and TB cells are distinguished. There is a BC within the embryo. Z: zona pellucida.

Fig. 5. Section of a blastocyst treated with cytochalasin-D, observed by transmission electron microscopy. Microvilli (Mv) with a translucent matrix are seen on the outer surface of a TB cell. M: mitochondria. Z: zona pellucida. BC: blastocoel cavity.

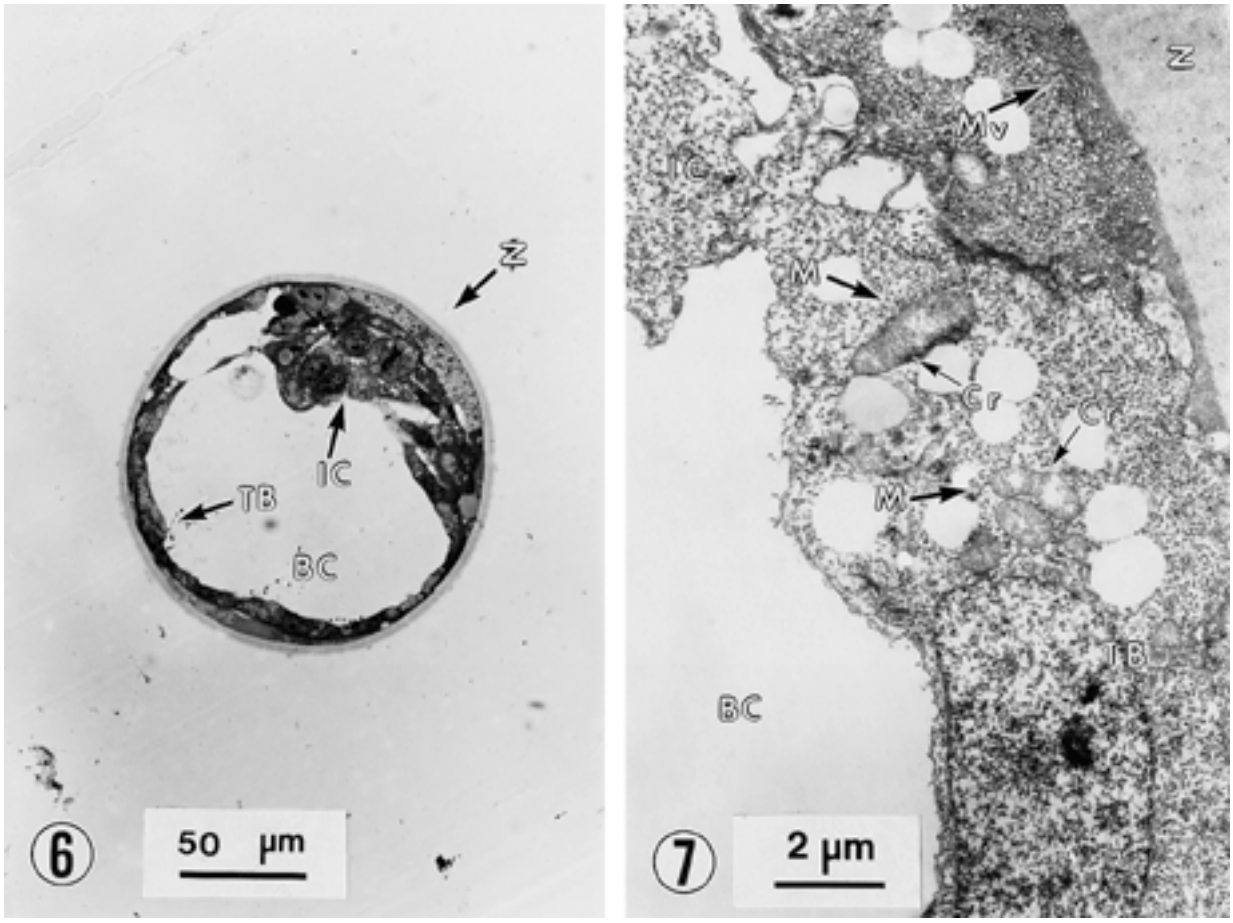

Fig. 6. Cross section of a blastocyst in the poor grade group, removed from cytochalasin-D, observed by light microscopy. IC and BC are surrounded by TB cells. Z: zona pellucida.

Fig. 7. Section, removed from cytochalasin-D, observed by transmission electron microscopy. Microvilli (Mv) with high electron density on the outer surface of TB cells. M: mitochondria. Cr: cristae. BC: blastocoel.

(TB) cells. TB cells displayed an abundance of microvilli on their outer surface facing the perivitelline space (Fig. 2). Many vesicles and lipid droplets were dispersed within the cytoplasm (Fig. 3). Junctional complexes, consisting of a tight junction and desmosome, were formed between the TB cells. Filamentous fibers were observed near the junctional complexes. Mitochondria were prolated or cuboidal in shape, and were dispersed within the cytoplasm. Lysosome-like complexes were spherical or oval in shape and were dispersed throughout the cytoplasm (Fig. 3).

On observation of the IC cells, lysosome-like complexes were seen in their cytoplasm. Mitochondoria dispersed though the cytoplasm were spherical and oval in shape, and were involved in traversed cristae (Fig. 3). The cytoplasmic density of the IC cells was dramatically higher than that of the TB cells.

A large proportion of the cell organelles treated with CD in both good and poor (Fig. 5) groups were identical to those in the control group regardless of shrinking or non-shrinking of their BC. However, some embryos in the poor grade group showed microvilli with a translucent matrix in some TB cells (Fig. 5). On the other hand, CD-treated blastocysts in the poor group showed the same electron density in microvilli when they were removed from the medium containing CD (Fig. 7).

Discussion

In the present study, almost all of the blastocysts showed shrinkage of BC when they were treated with CD, suggesting that the BC of the bovine blastocyst

may be sustained by the polymerization of globular actin (G-actin) into filamentous form (F-actin). Based on observation with a transmission electron microscope, junctional complexes formed between TB cells in the *in vivo* bovine blastocysts, had some skeletal network [16]. Yotsutani *et al.* [10] reported that filamentous actin stained with phallacidin was observed in junctional sites formed between blastomeres in bovine embryos produced *in vitro*. They suggested that actin might participate in the process of blastocyst formation. It is well known that phallacidin can stain only polymerized actin (F-actin), not G-actin [18]. On the other hand, in the mouse, BC is easily collapsed by the addition of cytochalasin-B, an inhibitor of polymerization of actin [17]. This indicates that F-actin may play an important role in maintaining the BC of blastocysts in mouse.

Cytochalasin-D is another inhibitor of the polymerization of actin in cells [18]. Compared to cytochalasin-B, Type-D has no inhibitory effect on the transport of glucose [19]. In the mouse, transportation of glucose at the blastocyst stage might be related to the capacity of filamentous actin to form and maintain the BC [20]. In the rat, glucose absorption and utilization of embryos was associated with microvilli at the somite stage [21]. Considered in light of these reports, our results suggest that the BC of the bovine blastocysts produced *in vitro* might not be related to glucose transportation via the microvilli.

Jiang *et al.* [22] reported that morphologically excellent blastocysts showed a significantly greater number of the cells than that of morphologically lower quality embryos. It is widely accepted that cell number is one of the crucial criteria for evaluating embryo quality [4]. Our results indicate that morphologically good grade blastocysts have larger numbers of cells than poor grade blastocysts, and that our classification of blastocysts into good and poor grades might be feasible.

Poor group blastocysts did not maintain their BC as well as those of the good group under the CD treatment condition. Microvilli in some TB cells of some blastocysts in the poor group treated with CD, though not in the good group, showed a translucent matrix. Gallicano [5] indicated that two types of F-actin may be present in mouse embryos: one sensitive to CD treatment, the other insensitive to it. F-actin that is sensitive to CD is located in the cytoplasm of TB cells facing BC in mouse blastocysts [17]. Contrarily, F-actin that is non-sensitive may be sited in the cortical region including microvilli of 8-cell mouse embryos [5, 23]. It is well known that F-actin forms the core of microvilli in mammalian oocytes [24]. With these facts in mind, our results suggest that the F-actin in the microvilli of some blastocysts in the poor group may be sensitive to CD, differing from that of the good group. In the case of actin filament that is insensitive to CD, this may be presented in the peripheral cytoplasm of TB cells facing BC of poor grade blastocysts. However, when blastocysts with shrunken BC in both groups were incubated after removal of CD, the re-expansion rate did not differ significantly between groups. This finding suggests that polymerization ability might be the same for both grades of blastocysts if they are sensitive to CD.

In conclusion, maintenance of the BC of bovine blastocysts produced *in vitro* might be related to the polymerization of actin filament, and some percentage of lower grade blastocysts may have CD sensitive actin filaments in microvilli and CD insensitive actin filaments in the peripheral cytoplasm of TB cells facing BC.

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