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Ultrastructure of bovine *in vitro*-produced blastocysts cryopreserved by vitrification

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Summary

The objective of this study was to examine ultrastructural aspects of bovine *in vitro*-produced blastocysts associated with cryopreservation by vitrification. Morphologically good embryos were used and treated with ethylene-glycol-based vitrification solution (VS). The untreated embryos had conventional fine structure. The post-warming embryos treated with direct exposure to VS (one-step procedure) showed cellular damage structurally by cryopreservation, which included loss of microvilli, disruption of the plasma membrane, mitochondrial changes and swelling of the endoplasmic reticulum. However, nuclei and junctional regions seemed to be resistant to cryoinjury. In contrast, the post-warming embryos pre-equilibrated with 10% ethylene glycol for 5 min and subsequent exposure to VS (two-step procedure) showed less damage than those treated by the one-step procedure. Post-warming embryos treated by the two-step procedure were cultured *in vitro* for 18 h. Some embryos survived and their structures re-formed to the former state, while other embryos showed serious injuries and could not reconstitute the blastocoele. Three post-warming embryos treated by the two-step procedure that survived after *in vitro* culture were transferred to three recipients and one of these resulted in pregnancy. These results indicate that cryopreservation by vitrification can damage membranous structures of the cells of bovine embryos, the extent and nature of this damage being dependent on the vitrification procedure.

Keywords: Blastocyst, Bovine, IVF, Ultrastructure, Vitrification

Introduction

Procedures for successful cryopreservation by vitrification of mammalian embryos have been the subject of intensive investigation since Rall & Fahy (1985) demonstrated that mouse embryos could be successfully cryopreserved by vitrification. Scheffen *et al.* (1986) reported the survival of mouse embryos after vitrification using a simple vitrification solution containing two permeable cryoprotectants – glycerol and propylene glycol – in which embryos were equilibrated in two steps. Complete vitrification of mouse blastocysts, with the absence of both intra- and extracellular ice crystals, was confirmed by cryoelectron microscopy (Valdes *et al.*, 1990). In contrast, bovine blastocysts did not survive after vitrification employing the method used for mouse embryos, although morulae and early blastocysts did survive (Massip *et al.*, 1986; Douchi *et al.*, 1990). Recently, Kuwayama *et al.* (1992, 1994) reported high survival rates of *in vitro*-produced bovine blastocysts after vitrification using a 16-step method. The embryos were completely vitrified and the ultrastructural damage to the plasma membrane was minimised when a solution of glycerol and propylene glycol was used.

Successful vitrification of bovine blastocysts obtained from *in vitro* culture of oocytes matured and fertilised *in vitro* was reported by a simple and rapid procedure (Tachikawa *et al.*, 1993). Mahmoudzadeh *et al.* (1995) showed that embryos at the blastocyst or expanded blastocyst stage on day 7 of *in vitro* culture

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can be cryopreserved effectively by vitrification when EFS, which was first described by Kasai *et al.* (1990) for mouse embryos, was added in two steps to the embryos as compared with one-step addition. Similar results were obtained in our previous study (Ohboshi *et al.*, 1997*b*). Although Mohr & Trounson (1981) observed that cryoinjury of bovine blastocysts was due to loss of membrane integrity, the precise nature of freeze-thaw and/or vitrification-warming damage leading to poor development of cryopreserved bovine blastocysts produced *in vitro* is not fully understood. Therefore, the present study was conducted to elucidate the changes due to cryopreservation by vitrification with ethylene-glycol-based solution, using transmission electron microscopy. Furthermore, warmed embryos were transferred to recipient heifers and the *in vivo* development of warmed embryos was examined.

Materials and methods

Production of *in vitro*-produced blastocysts

Bovine blastocysts derived by *in vitro* maturation and fertilisation were produced as described previously (Ohboshi *et al.*, 1997*b*). Briefly, immature oocytes were collected from small follicles (1–8 mm in diameter) of ovaries obtained from a local slaughterhouse. The maturation medium was SFRE199–2 (Sigma, St Louis, MO) supplemented with 10% calf serum (CS; Gibco, Grand Island, NY), 5 μg/ml insulin (Sigma), 0.01 AU/ml FSH (Denka Pharmaceuticals, Kanagawa, Japan), 1.5 IU/ml hCG (Denka Pharmaceuticals), 1 μg/ml estradiol–17β (Sigma) and 50 μg/ml gentamicin sulphate (Sigma). Immature oocytes surrounded by cumulus oophorus on more than two-thirds of their surface were introduced into 2.5 ml maturation medium in 35 mm culture dishes (100–150 oocytes per dish; Costar, Cambridge, MA) and cultured for 24 h.

Fertilisation *in vitro* was carried out using frozen-thawed semen obtained from a Holstein bull. The medium for fertilisation *in vitro* was modified HEPES-Tyrode's medium, supplemented with 10 μg/ml heparin (Sigma), 5.0 mM theophylline (Sigma) and 5 mg/ml bovine serum albumin (BSA fraction V; Sigma). Final sperm concentration was adjusted to 1×10^7 spermatozoa/ml. The spermatozoa and the oocytes that had been subjected to maturation culture were co-incubated in 100 μl drops for 6–7 h. This insemination day was defined as day 0.

The presumptive zygotes were transferred to 500 μl droplets of the culture medium for embryonic development *in vitro*. The basal culture medium was modified synthetic oviduct fluid medium (mSOFM; Ohboshi *et al.*, 1997*b*; Takahashi & First, 1992) supplemented with 50 μM β-mercaptoethanal (β-ME, Sigma). Zygotes were cultured as follows: mSOFM supplemented with 1% CS and 0.5 mM glucose between day 0 and day 2; that supplemented with 5% CS and 0.5 mM glucose between day 2 and day 5; and that supplemented with 5% CS and 2.0 mM glucose between day 5 and the end of culture. The embryos were freed from surrounding cumulus cells by gentle pipetting on day 2, and denuded embryos were then cultured in the medium described above. All incubations were performed in a humidified atmosphere of 5% CO_2 in air at 39 °C. Morphologically excellent or good blastocysts at day 7 were selected for subsequent experiments according to the classification criteria described previously (Ohboshi *et al.*, 1997*b*).

Vitrification solution and vitrification procedures

The vitrification solution (VS; Ohboshi *et al.*, 1997*b*) used in this study consisted of 40% (v/v) ethylene glycol (Tokyo Chemical Industry, Tokyo, Japan), 6% (w/v) polyethylene glycol (MW 8000; Sigma) and 0.5 M sucrose (Sigma) in modified Dulbecco's phosphate-buffered solution supplemented with 3 mg/ml BSA (Sigma) and antibiotics (DPBS).

Embryos were cryopreserved in 0.25 ml plastic straws (IMV, L'Aigle, France) by vitrification, in accordance with the one-step protocol, i.e. direct exposure to VS for 1 min on ice, or two-step protocol, i.e. pre-equilibration to 10% ethylene glycol for 5 min at room temperature (20–24 °C) followed by exposure to VS for 1 min on ice (3–4 °C) as described previously (Ohboshi *et al.*, 1997*b*). After cryostorage in liquid nitrogren for between 10 and 40 days, cryopreserved embryos were warmed rapidly by immersing straws in water at 20–24 °C and diluted by 0.5 M sucrose solution and DPBS for 5 min each at room temperature. *In vitro* culture of the post-warming embryos was performed as reported previously (Ohboshi *et al.*, 1997*b*), using tissue culture medium-199 (Gibco) supplemented with 10% fetal calf serum (Gibco) and 100 μM β-ME.

Preparation of embryos for transmission electron microscopy

The embryos for microscopic observation were treated as follows: untreated control embryos (fresh blastocysts), post-warming embryos treated with one- or two-step vitrification protocols and post-warming embryos treated with the two-step protocol followed by 18 h *in vitro* culture. These embryos were immediately fixed, dehydrated and infiltrated with epoxy resin (Ohboshi *et al.*, 1995). The embryos were serially cut into semi-thin and thin sections with glass knives. The semi-thin sections were stained with 1.0%

toluidine blue (Kanto Chemical, Tokyo, Japan) and observed under a light microscope. The thin sections were stained with uranyl acetate and lead staining solution, and examined by transmission electron microscopy.

Embryo transfer

In the embryo transfer experiment, blastocysts that had originated from oocytes collected from either Holstein or Japanese black cows were used. Embryos had been cryopreserved in the vitrification solution by the two-step procedure and judged viable after approximately 18 h of post-warming culture *in vitro* according to the method described above. They were nonsurgically transferred to Holstein heifers on day 7 or 8 of oestrus, day 0 being the day of onset after treatment with $PGF_{2\alpha}$ analogue. Individual embryos were transferred to each recipient, unilaterally into the luteal side of the uterus horn. The pregnancy of recipients was confirmed by rectal palpation and ultrasonography at day 60.

Results

Microscopic observation of embryos

In this study, 29 blastocysts produced *in vitro* were examined, and all were classified as excellent or good under an inverted microscope before treatment. The numbers of embryos in each treatment group were as followed: 6 untreated control embryos (fresh blastocyst), 7 post-warming embryos treated by the one-step procedure, 7 post-warming embryos treated by the two-step procedure, and 9 post-warming embryos treated by the two-step procedure followed by *in vitro* culture.

The fresh blastocycsts serving as controls (Fig. 1) were spherical and surrounded by a zona pellucida. The trophoblast cells were flat and elongated, and were tightly packed on the internal face of the zona pellucida. The inner cells mass cells were polyhedral and were separated by intercellular spaces. The post-warming embryos treated by the one-step procedure (Fig. 2) had markedly different features from control embryos. The embryonic cells were intensively swollen and partial efflux of the cytosol was observed, especially in the trophoblast cells. The post-warming embryos treated by the two-step procedure (Fig. 3) had similar features to those treated by the one-step procedure, and showed cellular swelling and efflux. However, the damage in the post-warming embryos treated by the two-step procedure was less severe than that in those treated by the one-step procedure. After *in vitro* culture of the post-warming embryos treated by the two-step procedure,

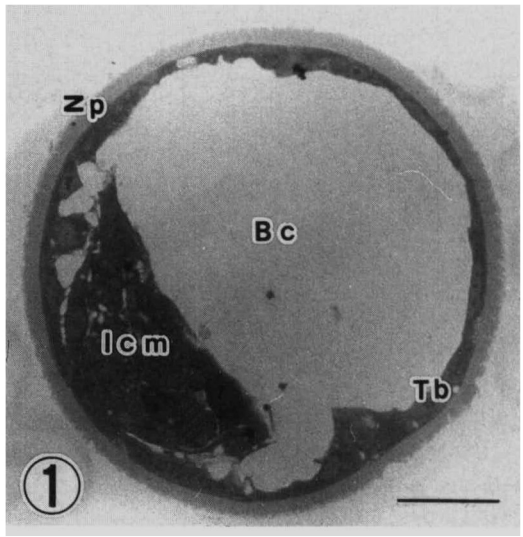

Figure 1 Light micrograph of a fresh (control) *in vitro*-produced blastocyst with well-developed trophoblast cells (Tb) and inner cell mass (Icm). Zona pellucida (Zp) had a normal shape and the blastocoele (Bc) developed well. Scale bar represents 20 μm.

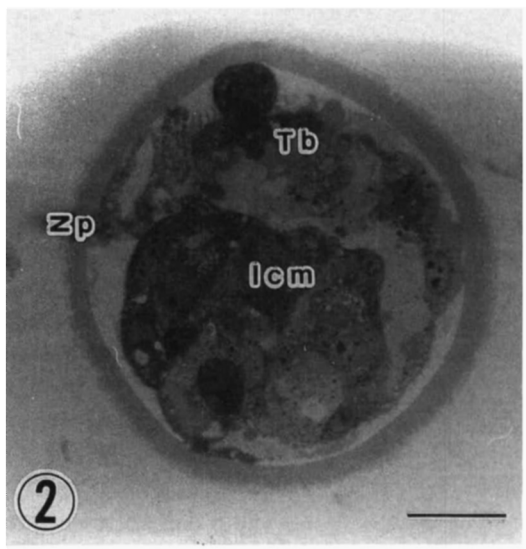

Figure 2 Light micrograph of a post-warming embryo prepared by the one-step procedure. Note the partially broken trophoblast cells (Tb) and markedly swollen inner cell mass cells (Icm). Scale bar represents 20 μm.

the embryos showed various degrees of cryoinjury. Six of these embryos had completely reformed blastocoele cavities at the end of the culture period (Fig. 4). Three embryos that showed severe damage preventing re-expansion of the blastocoele were judged as degenerated (Figs. 5, 6). The debris from cryoinjured cells was excluded from the blastocoele (Figs. 5, 6).

Additional information on the morphology was obtained by transmission electron microscopy. The

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fresh embryos had well-developed microvilli on the surface of trophoblast cells, projecting into the perivitelline space, and few cellular fragments and/or pieces of debris were observed in the perivitelline space. Adjacent trophoblast cells formed junctional regions characterised by tight junctions along the external surface followed by desmosomes. Entangled cytoplasmic projections were observed between trophoblast cells and inner cell mass cells (Fig. 7*A*).

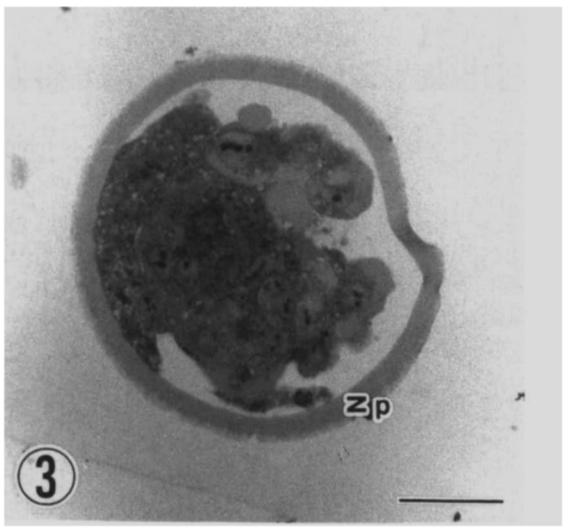

Figure 3 Light micrograph of a post-warming embryo prepared by the two-step procedure. The blastocoele was not clearly observed and all cells were aggregated. Zona pellucida (Zp) was partially deformed. Scale bar represents 20 μm.

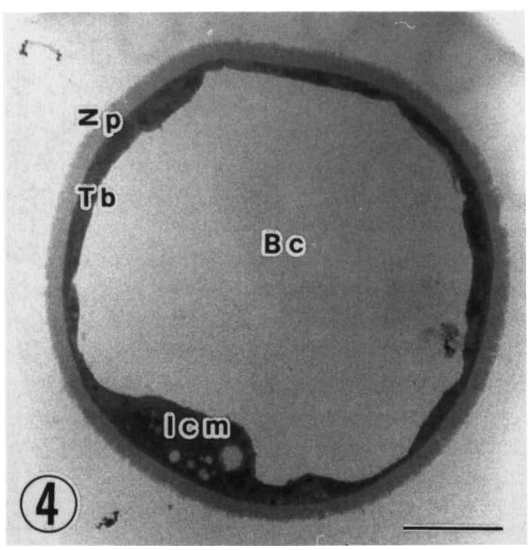

Figure 4 Light micrograph of a post-warming embryo prepared by the two-step procedure followed by 18 h *in vitro* culture. Note the well-developed inner cell mass (Icm), and expanding blastocoele (Bc). Trophoblast cells (Tb) tightly apposed to the zona pellucida (Zp) were observed. Scale bar represents 20 μm.

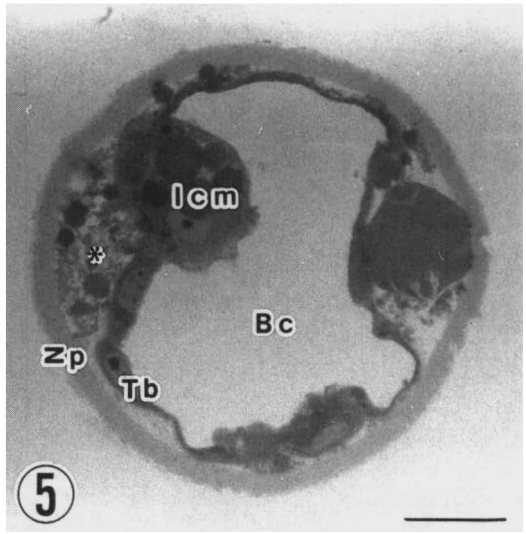

Figure 5 Light micrograph of a post-warming embryo prepared by the two-step procedure followed by 18 h *in vitro* culture. Note the insufficiently organised embryonic cells with inadequate inner cell mass (Icm) and trophoblast cells (Tb), resulting in the distorted blastocoele (Bc). Cellular debris (asterisk) was seen in the perivitelline space. Scale bar represents 20 μm.

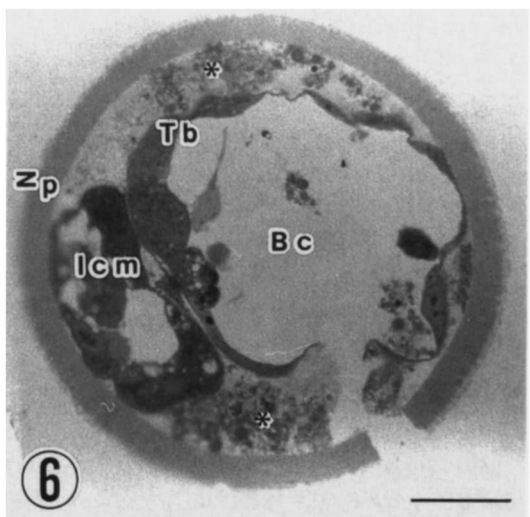

Figure 6 Light micrograph of a post-warming embryo prepared by the two-step procedure followed by 18 h *in vitro* culture. Note the inadequately organised embryo with collapsed inner cell mass (Icm) and detached trophoblast cells (Tb), leading to cellular debris (asterisk) and the distorted blastocoele (Bc). The zona pellucida (Zp) was partially broken. Scale bar represents 20 μm.

Nuclei were somewhat flattened in trophoblast cells and spherical in inner cell mass cells, and contained well-developed nucleoli (Fig. 7*B*). Large numbers of mitochondria with several transverse cristae were observed, and these were cylindrical, spherical or oval in shape (Fig. 7*A*). Rough endoplasmic reticulum

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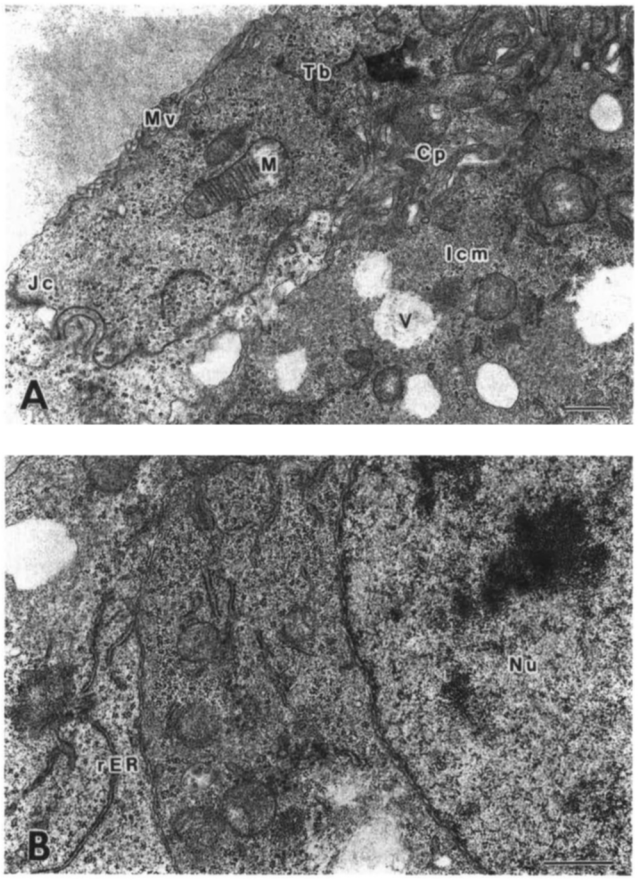

Figure 7 Electron micrographs of control *in vitro*-produced embryos. (*A*) Note the well-developed microvilli (Mv), the junctional complex (Jc) with high electron density and vacuoles (V) of various sizes and shapes. Cytoplasmic projections (Cp) were entangled between trophoblast cells (Tb) and inner cell mass cells (Icm). Mitochondria (M) showing transverse cristae were also seen. Scale bar represents 1 μm. (*B*) Note the rough endoplasmic reticulum (rER) in the cells. Normal nucleus (Nu) was clearly observed. Scale bar represents 1 μm.

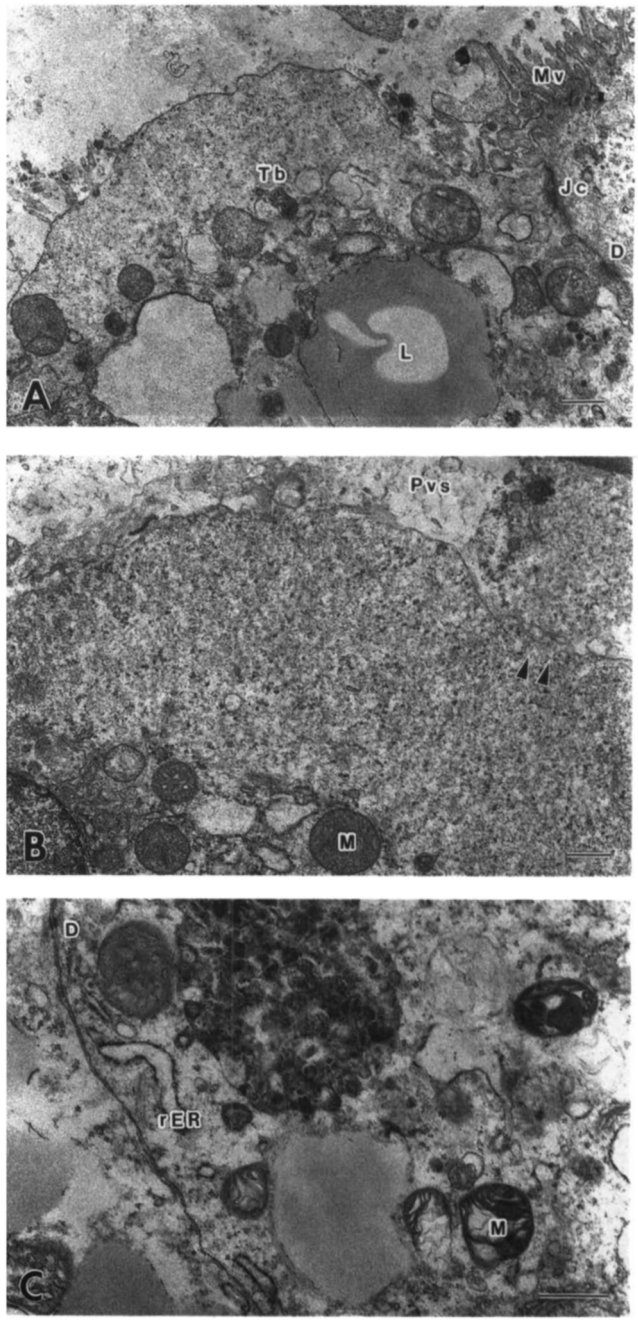

Figure 8 Electron micrographs of post-warming embryos prepared by the one-step procedure. (*A*) Note the disappearance of microvilli (Mv) on the surface of trophoblast cells (Tb). However, junctional complexes (Jc) and desmosomes (D) were observed between two trophoblast cells. A large lipid droplet (L) was seen in a trophoblast cell, showing a curious shape. Scale bar represents 1 μm. (*B*) Note the irregularly shaped trophoblast cell showing evenly dispersed cellular components in the perivitelline space (Pvs) and inactive mitochondria (M). The plasma membrane was partially ruptured (arrow heads). Scale bar represents 1 μm. (*C*) Note the almost completely disorganised cellular components, such as abnormally shaped rough endoplasmic reticulum (rER), and mitochondria (M) with inactive cristae and inner membrane. However, a desmosome (D) with high electron density was observed. Scale bar represents 1 μm.

was often seen, but was less developed (Fig. 7A, *B*). Irregular membrane-bounded bodies, a few lipid droplets and vesicles were also present in the cytoplasm (Fig. 7*A*). Other cytoplasmic organelles included many free ribosomes, smooth endoplasmic reticulum and Golgi apparatus.

The post-warming embryos treated by the one-step procedure clearly showed cellular damage due to cryopreservation. Microvilli on the surface of trophoblast cells were damaged and their plasma membrane was discontinuous, indicating efflux of cytoplasm (Fig. 8*A*, *B*). No cytoplasmic projections were seen, but tight junctions represented by electron-dense areas in the apical region of the interblastomeric space, and desmosomes, were observed, which formed junctional complexes interconnecting with the trophoblast cells (Fig. 8*A*). There was no serious

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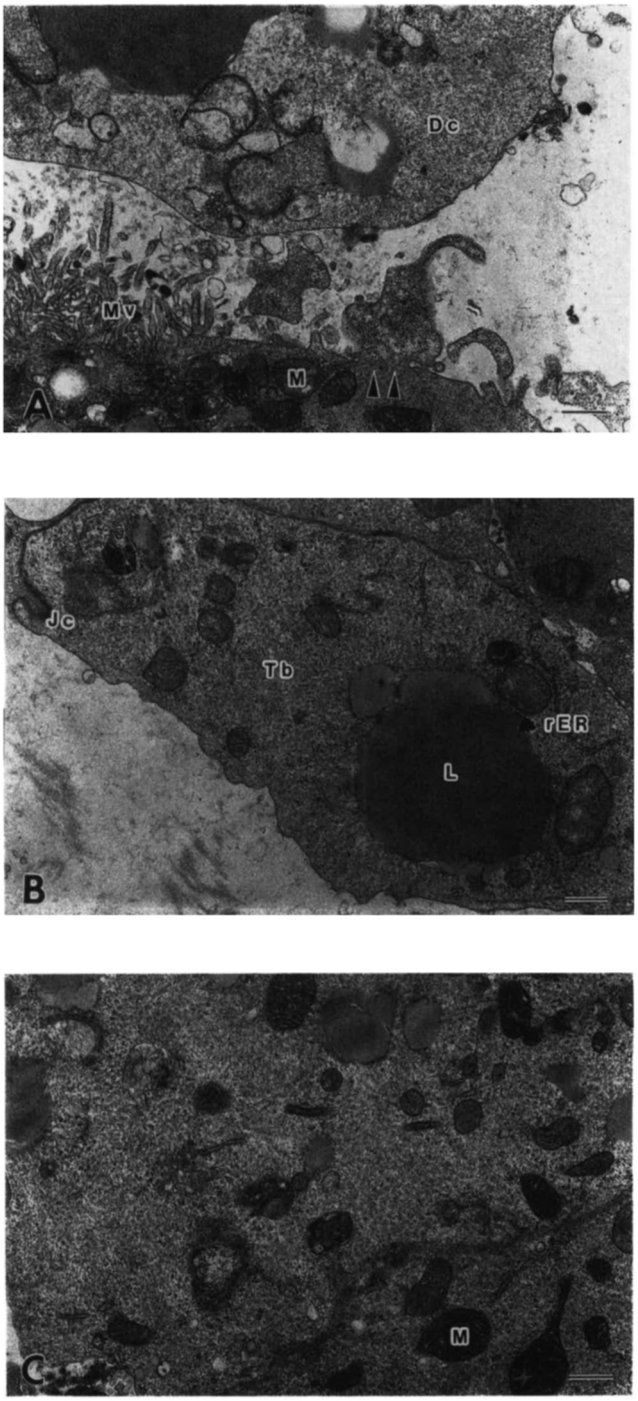

Figure 9 Electron micrographs of post-warming embryos prepared by the two-step procedure. (*A*) Note the damaged plasma membrane (arrowheads) and mitochondria (M) with high electron density. However, microvilli (Mv) remained partially intact. Degenerated cell (Dc) can be seen in the upper part of the micrograph. Scale bar represents 1 μm. (*B*) Note the disappearance of microvilli on the surfaces of the trophoblast cells (Tb). However, rough endoplasmic reticulum (rER) and the junctional complex (Jc) seemed to be intact. A large dark lipid droplet (L) was observed. Scale bar represents 1 μm. (*C*) Note the high electron density in some mitochondria (M) and the disordered inner membrane. Scale bar represents 1 μm.

damage to nuclei, except in the collapsed cells, which had unclear ultrastructure. The outer membrane of mitochondria was intact and the inner membrane was vesicular, and they were more electron-dense than those of fresh embryos (Fig. 8*B*, *C*). Swelling of some parts of the rough endoplasmic reticulum was observed, and it was difficult to detect other cytoplasmic structures with the exception of lipid droplets (Fig. 8*A*, *C*).

In the post-warming embryos treated by the two-step procedure, the ultrastructural features were close to those treated by the one-step procedure, although the extent of damage varied between embryos. However, there was less damage including loss of microvilli, disruption of the plasma membrane leading to loss of cytoplasmic matrix and homogeneity of the cellular matrix (Fig. 9*A*–*C*). Moreover, there were marked differences between these two groups of treated embryos in that parts of mitochondria and rough endoplasmic reticulum were structurally intact (Fig. 9*B*, *C*).

Among the post-warming embryos treated by the two-step procedure followed by *in vitro* culture, those judged as viable by showing re-expansion of the blastocoele had well-developed microvilli on the surface of trophoblast cells, and their junctional complex interconnecting with trophoblast cells was further developed to indicate clusters of desmosomes (Fig. 10*A*, *B*). Cytoplasmic projections were also observed. Nuclei had well-developed nucleoli, but excessively high electron density of nuclei was also seen in a few embryonic cells (Fig. 10*C*). A large number of mitochondria with transverse cristae were found throughout cells and their matrix was translucent in appearance. Rough endoplasmic reticulum was abundant, and had assumed conventional morphology (Fig. 10*A*). Other cytoplasmic structures such as ribosomes, lipid droplets and smooth endoplasmic reticulum also appeared normal (Fig. 10*A*, *B*). In the embryos judged as degenerated, some damage remained, including loss of microvilli and desmosomes, indistinct or broken plasma and nuclear membranes and decreased cytoplasmic homogeneity (Fig. 10*D*). Ribosomes were rarely seen. However, some mitochondria were structurally unaltered. Extracellular accumulation of flocculent material was seen in the subzonal space (Fig. 10*D*).

Embryo transfer

Of three recipients, one became pregnant. The fetus and functional corpus luteum were confirmed by ultrasonography at 60 days of pregnancy, and a male calf was born after 261 days of pregnancy.

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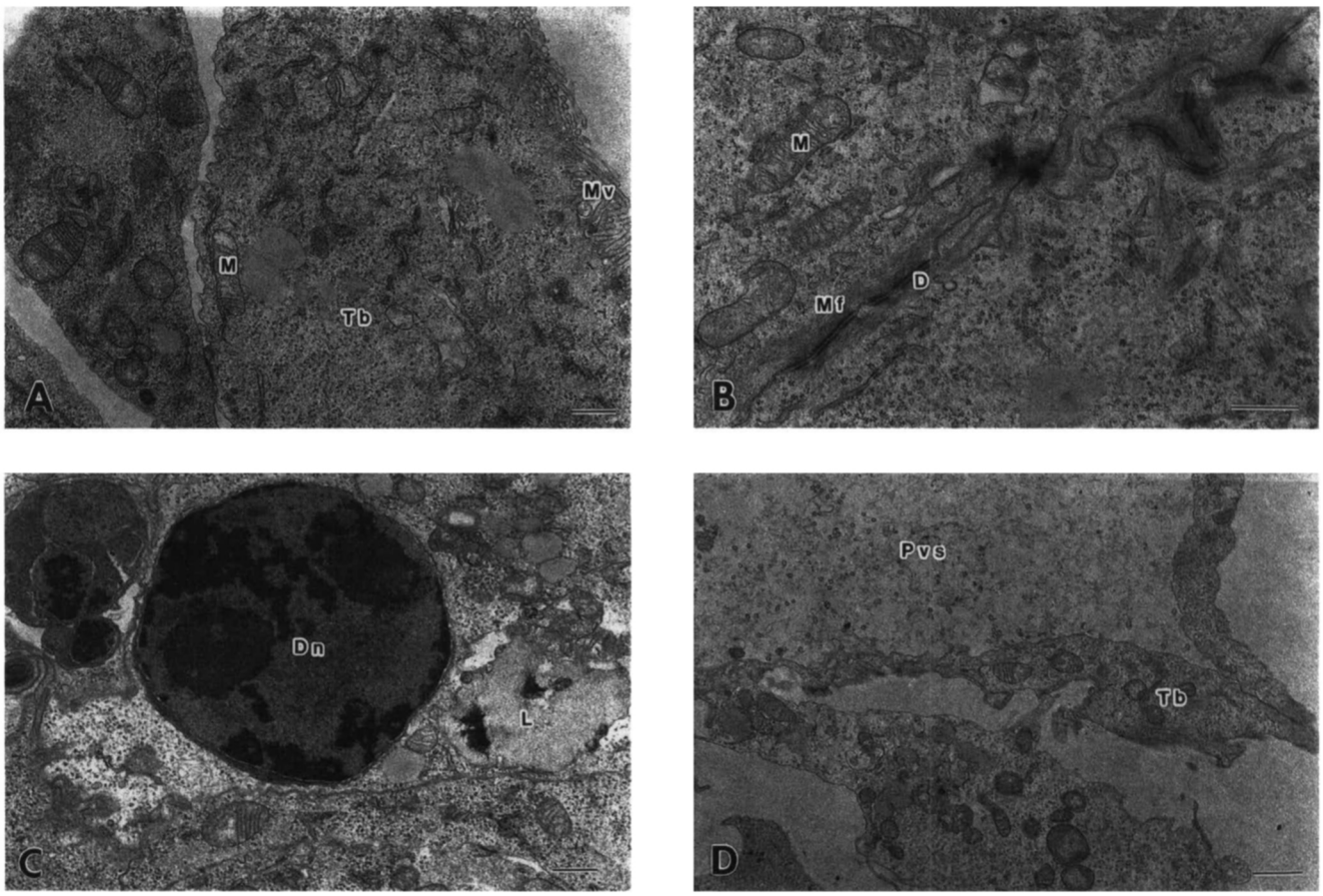

Figure 10 Electron micrograph of a post-warming embryo prepared by the two step procedure followed by 18 h *in vitro* culture. (*A*) Note the well-developed microvilli (Mv) on the surface of trophoblast cell (Tb). Normal-shaped mitochondria (M) with transverse cristae were observed. Scale bar represents 1 μm. (*B*) Note the clusters of desmosomes (D) between trophoblast cells in which microfilaments (Mf) were apposed. Mitochondria (M) appeared to be normal. Scale bar represents 1 μm. (*C*) Note the degenerated nuclei (Dn) with extremely high electron density. Unidentifiable structures of cellular components were observed close to the degenerated nucleus. An irregularly shaped lipid droplet (L) was also seen. Scale bar represents 1 μm. (*D*) Note the absence of microvilli on the surface of the trophoblast cells (Tb). Powder-like debris was seen in the perivitelline space (Pvs). Scale bar represents 2 μm.

Discussion

Our ultrastructural observations of *in vitro*-produced bovine blastocysts before and after cryopreservation by vitrification indicate that the extent of injury depends on the procedure used for equilibration in the vitrification solution. Embryos equilibrated in a single step (1 min in VS on ice) exhibited more deleterious changes at both the light and electron microscope level than embryos equilibrated in two steps (10% ethylene glycol for 5 min at room temperature and then 1 min in VS on ice). Our assessments of post-warming survival indicate that embryos vitrified using the two-step equilibration exhibited a high rate of *in vitro* development and yielded a normal bull calf after embryo transfer.

Rall (1987) was the first to suggest that stepwise equilibration in the vitrification solution is an important strategy for obtaining high survival of mammalian embryos. Osmotic injury during equilibration has been suggested to be lethal (Massip *et al.*, 1989) and osmotic injury caused by water outflow might be avoided by increasing the number of equilibration steps, resulting in higher survival rates (Kuwayama *et al.*, 1992). Kuwayama *et al.* (1994) reported that complete vitrification was accomplished when embryos were equilibrated by the two-step method. However, small vesicles and distinct intramembrane particle aggregation were frequently observed in the plasma membranes of embryos. They also suggested that increasing the number of equilibration steps is useful not only for achieving the amorphous state in both intra- and extracellular solution, but also for minimising ultrastructural damage to the plasma membrane.

In this study, although we used different cryo-

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protectants and procedures, the cause of low embryonic survival in the post-warming embryos treated by the one-step procedure was suggestive of damage to the membrane functions of the embryonic cells, as we observed disruption of the plasma membrane, changes in the mitochondrial cristae and matrix, and swelling of rough endoplasmic reticulum. Mohr & Trounson (1981) reported that the plasma membranes of bovine frozen-thawed morulae and early blastocysts sustained the most damage, and in the hatched embryos most damage was observed in the trophectoderm cells, undifferentiated embryonic cells appearing to survive. The epithelium of the trophoblast cells was characterised by well-developed microvilli projecting to the perivitelline space and junctional complexes. These structures have been considered to contribute to formation and expansion of the blastocyst by active fluid transport and maintenance of pregnancy by secretion of factors (Linares & Plöen, 1981; Ohboshi *et al.*, 1995). The marked decrease in microvilli indicates difficulties in further embryonic development. Junctional complexes between cells seemed to be resistant to cryoinjury. Within the same cell, intact junctional regions were observed with disruption of the plasma membrane in other regions. This resistance of junctional regions could be due to the structural support given by microfilament bundles or to altered chemical composition of the membrane itself in these regions (Mohr & Trounson, 1981). However, frozen-thawed collapsed embryos with trophoblast cell damage would form another layer of trophoblast cells and a new blastocoele while in culture (Wilson *et al.*, 1987).

Cryopreservation treatments in both horse (Rieger *et al.*, 1991) and cattle (Rieger *et al.*, 1993) embryos increased glutamine metabolism, possibly as a result of disturbances in mitochondrial function (Rieger *et al.*, 1995). Ultrastructural changes in mitochondria, and blebbing of the inner membrane and electron-dense matrix, were also observed in this study. Wilson *et al.* (1987) showed that cryopreservation or glycerol treatment results in changes to the mitochondria of horse embryos including thickening of the cristae, coalescence of the inner membrane and blebbing of the outer membrane, suggesting that mitochondrial damage would result in cell death. The endoplasmic reticulum also changes structurally, as observed by swelling of its cavity. Since rough endoplasmic reticulum and ribosomes are organelles associated with protein synthesis, damage to these organelles might seriously affect the subsequent viability of the embryos.

Damage to post-warming embryos treated by the two-step procedure was also detected, albeit to a lesser extent than in those treated by the one-step procedure. This reduction in damage was due to the increase in the number of equilibration steps, which prevents osmotic injury as mentioned above. However, resumption of normal development would require time, causing a slow and gradual restoration of normal metabolic and synthetic activity of the post-warming embryo (Wilson *et al.*, 1987) and would be dependent on the culture environment (Massip *et al.*, 1993; Mahmoudzadeh *et al.*, 1995) As not all the post-warming embryos treated by the two-step procedure survived (approximately 60% data not shown), even reduced damage would be detrimental in some embryos. Moreover, pregnancy rate after embryo transfer was low, as described previously (Ohboshi *et al.*, 1997*a*), although the surviving embryos *in vitro* showed structurally normal development and organelles. Massip *et al.* (1986, 1995), Tachikawa *et al.* (1993) and Wurth *et al.* (1994) suggested that there is not a high correlation between *in vitro* survival and *in vivo* development. Ali & Shelton (1993) concluded that a significant number of blastomeres of sheep embryos were irreversibly damaged during one-step vitrification with the result that subsequent viability was reduced *in vivo* but not *in vitro*. The survival of blastocysts was unaffected by the cell numbers in embryos before cryopreservation (Ohboshi *et al.*, 1997*a*), although subsequent development may be dependent on the number of living cells in post-warming embryos.

Cryopreservation of embryos produced *in vitro* by either vitrification or conventional freezing reduced their ability to establish pregnancy when a single embryo was transferred into each recipient (Wurth *et al.*, 1994). Tachikawa *et al.* (1993) reported that high pregnancy rate and live calves were obtained after transfer of two vitrified-warmed embryos into each recipient. Hernandez-Ledezma *et al.* (1993) showed that hatched blastocysts of good or excellent quality produced significantly more trophoblast protein than those of fair quality, and the amount of such protein might be a useful objective indicator of embryo quality prior to embryo transfer. In fact, dead cells were also observed in the inner cell mass, and a proportion of the trophoblast cells were denatured by the vitrification protocol used in this study, these degraded cells being extruded from the tight junction during *in vitro* culture. Iwasaki *et al.* (1994) showed that the total cell number of frozen-thawed embryos was lower than that of untreated embryos and the one-step freezing technique resulted in a higher proportion of dead cells in frozen-thawed embryos compared with the three-step technique. As there is a deficiency in production of luteotrophic factors due to the decrease in number of living cells in the post-warming embryos (Hernandez-Ledezma *et al.*, 1993) co-transfer of these embryos with trophoblastic vesicles might improve transfer success (Heyman *et al.*,

1987). Moreover, addition of antifreeze glycoproteins to vitrification solution may improve viability by protecting cell membranes from damage caused by low temperatures (Rubinsky *et al.*, 1992). Further investigations of changes at the molecular level that may not be discernible by ultrastructural examination will be required for evaluation of embryonic viability.

The vitrification solution used in this study successfully cryopreserved bovine *in vitro*-produced blastocysts by vitrification. Damage to embryos after cryopreservation by vitrification included cellular membranous structures, i.e. decreases in numbers of microvilli, loss of integrity of the plasma membrane, mitochondrial changes and swelling of the rough endoplasmic reticulum. Marked ultrastructural modifications in embryos can be induced by additional equilibration steps, which might prevent osmotic shock. These modifications may protect against lethal changes in cellular membranous structures, resulting in higher survival rate.

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