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Structural Changes Associated with Freezing of Bovine Embryos

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ABSTRACT

The structural changes associated with freezing and thawing were examined in bovine embryos at three developmental stages: Day 5, Day 7, and Day 13 (Day 0 = day of estrus). Embryos collected at Day 5 had 8–16 cells, and contained numerous vesicles and primitive junctional regions between some adjacent blastomeres. After cooling to 4°C, the distribution of organelles within blastomeres as well as the spacial arrangement of blastomeres was disrupted.

Day 7 embryos were at the early blastocyst stage and contained an intact ring of trophoblast cells enclosing a disc of embryonic cells. Adjacent trophoblast cells were attached by a region of junctional complexes which were structurally unaffected by freezing. Damage to blastocysts after freezing included loss of the integrity of the trophoblast plasma membrane, leading to collapse of the blastocoele. When some collapsed blastocysts were cultured for 24 h after thawing, a smaller intact ring of trophoblast cells had reformed around the embryonic cells and debris from cryo-injured cells was excluded from the blastocoele.

Day 13 embryos contained three morphologically distinct cell types: a layer of trophectoderm, a disc of embryonic cells, and a continuous layer of endoderm cells surrounding the blastocoelic cavity. After freezing and thawing, the embryonic cells were structurally intact while the trophectoderm had substantial damage to all cell components.

In conclusion, cryoinjury in bovine embryos may be selective for one cell type within an embryo, and its extent and nature are dependent on developmental stage.

INTRODUCTION

Bovine embryos of the late morula to expanded blastocyst stage of development, collected 6 or 7 days after insemination, can be successfully cryopreserved (Willadsen et al., 1976), but embryos collected at other developmental stages do not survive freezing and thawing. Bovine embryos collected 4 to 5 days after insemination, before compaction of the blastomeres at the morula stage, are sensitive to cooling below 7.5°C and few survive the initial cooling required for freezing (Wilmut et al., 1975; Trounson et al., 1976). Blastocysts collected 10 to 13 days after insemination have hatched from the zona pellucida and can survive exposure to the cryoprotectant dimethylsulphoxide (DMSO) and the initial cooling to subzero temperatures, but cannot recover from deep-freezing to −196°C (Trounson et al., 1978a-c).

Sensitivity of embryos to cooling is even more apparent in the pig where all stages of development fail to survive exposure to temperatures below 15°C (Wilmut, 1972; Polge et al., 1974). In the sheep, Willadsen et al. (1976) reported an increased sensitivity of early cleavage stages (2–16 cell) to cooling. The factors affecting stage-specific sensitivity to cooling are unknown, but changes in cell size, structure, or permeability to cryoprotectants have been suggested (Willadsen, 1977; Trounson, 1977; Mazur, 1977).

Ultrastructural changes accompanying pre-implantation development are well documented for a large number of mammalian species including the mouse (Calarco and Brown, 1969) rat (Dvorak, 1978), rabbit (Hesseldahl, 1971), sheep (Calarco and McLaren, 1976; Wintenberger-Torrès and Fléchon, 1974), and baboon (Panigel et al., 1975). Fine structural analysis of bovine embryos is limited to transmission electron microscopy of follicular

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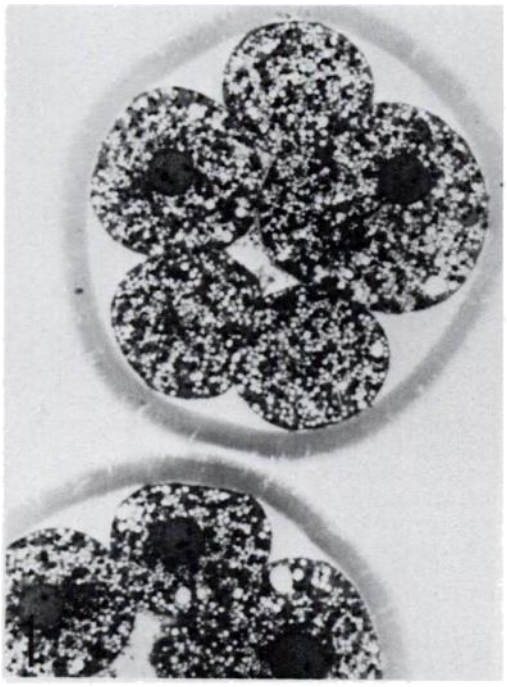
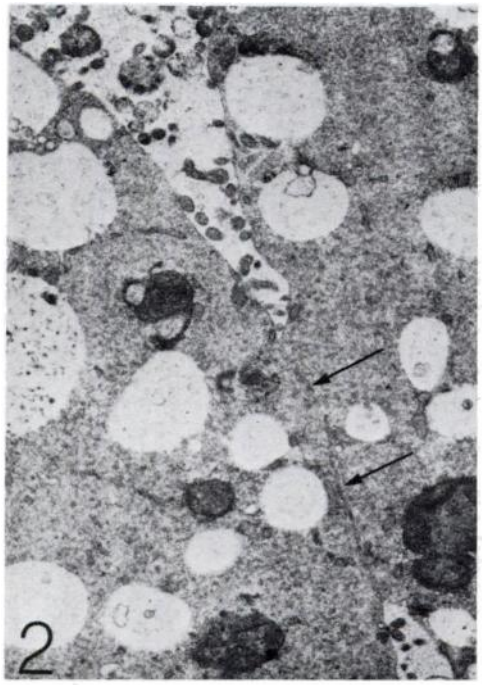




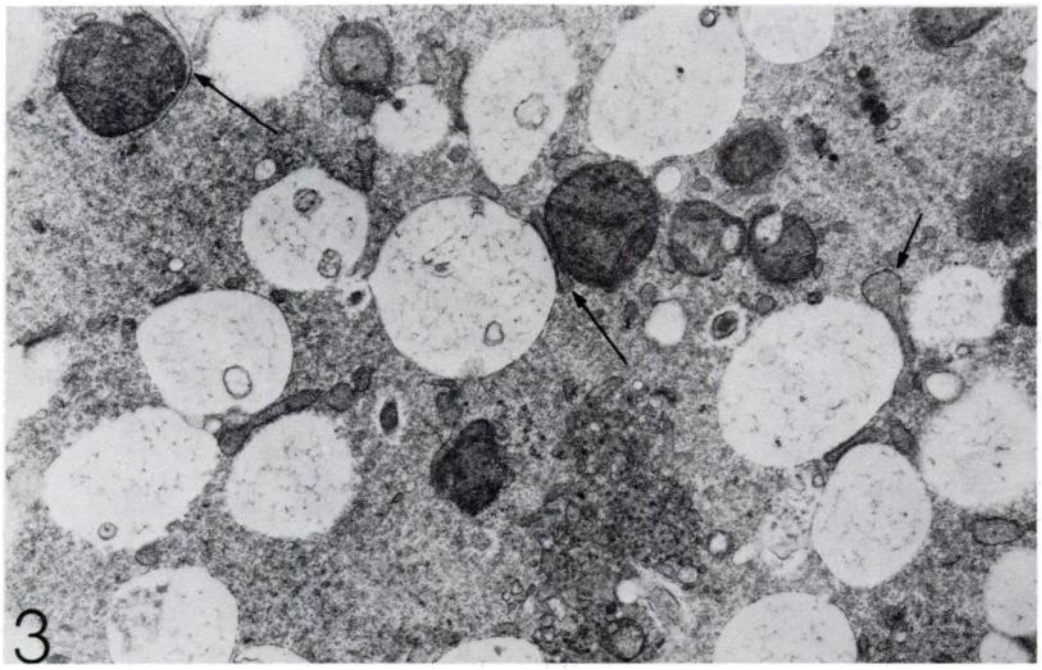


FIG. 1. Light micrograph of a control Day 5 bovine embryo. Note the abundance of vesicles in the cytoplasm. ×540.

FIG. 2. Electron micrograph of the contact region between two blastomeres in a control Day 5 embryo. Note the close apposition of the membranes (arrows). ×6000.

FIG. 3. Representative electron micrograph of a control Day 5 embryo showing numerous vesicles, primitive mitochondria, and smooth endoplasmic reticulum in close association with both vesicles and mitochondria (arrows). ×16,000.

oocytes (Senger and Saacke, 1970; Fleming and Saacke, 1972), embryos fertilized in vivo 2- and 4-cell embryos fertilized and cultured in vitro (Brackett et al., 1980), and scanning electron microscopy of hatched blastocysts (Fléchon and Renard, 1978).

The present studies were undertaken to establish the normal fine structural features of bovine embryos at three developmental stages and to examine the changes in cellular morphology that accompany cooling or freezing and thawing of the stages known to be sensitive to these procedures.

MATERIALS AND METHODS

Animals

Embryos were recovered from superovulated, nonlactating cows inseminated with fresh semen on the afternoon of estrus. Embryos were either collected surgically (Rowson et al., 1969) for Day 5 embryos (Day 0 = day of estrus) or nonsurgically (Brand et al., 1978) on Day 7 or 13. Dulbecco's phosphate-buffered saline (PBS) with 1% fetal calf serum and 25 mg kanamycin sulphate/liter was used to flush embryos from donor animals.

Cooling and Freezing Experiments

Day 5 embryos were placed at 4°C for 30 min and either fixed at 4°C or warmed to 37°C before fixing. Embryos used as controls were kept at 37°C for 30 min before fixing.

Day 7 and Day 13 embryos were equilibrated at room temperature in petri dishes containing 0.25, 0.5, 1.0 and 1.5 M DMSO, prepared in PBS supplemented with fetal calf serum (20 ml serum:80 ml PBS). Equilibration time was 10 min in each dish. The embryos were then transferred to 0.3 ml of 1.5 M DMSO in sterile glass ampules. The ampules were cooled from room temperature (22–28°C) to −6°C at 1.5°C/min, and seeding was induced at −6°C by touching the outside of the ampule with cold forceps. After seeding, embryos were cooled to −20°C at 0.5°C/min, from −20°C to −30°C at 0.3°C/min and from −30°C to −39°C at 0.1°C/min. Embryos were then plunged into liquid nitrogen and stored at −196°C.

Embryos were thawed rapidly by agitating the ampule in a 30°C water bath for ∼10 sec. DMSO was diluted out at room temperature by equilibrating embryos in solutions of 1.5, 1.25, 1.0, 0.75, 0.5, 0.25, and O M DMSO. Equilibration time was 10 min in each solution. Some Day 13 embryos were fixed immediately after thawing and prior to the removal of DMSO. Day 7 embryos were fixed immediately after DMSO removal or after a 24 h culture period. All control Day 7 and Day 13 embryos were fixed immediately after collection.

Embryo Culture

Day 7 embryos were cultured in modified Whitten's media (Hoppe and Pitts, 1973) supplemented with 20% fetal calf serum. The media were placed in tissue culture tubes (Falcon; 12 X 75 mm) and equilibrated in a humidified atmosphere of 5% CO_2:5% oxygen: 90% nitrogen at 37°C for several hours prior to the start of embryo culture. The embryos were transferred to the tubes of medium in a small volume of PBS immediately after thawing and removal of DMSO, and cultured in unsealed tubes in 5% CO_2:5% oxygen:90% nitrogen at 37°C.

Microscopy

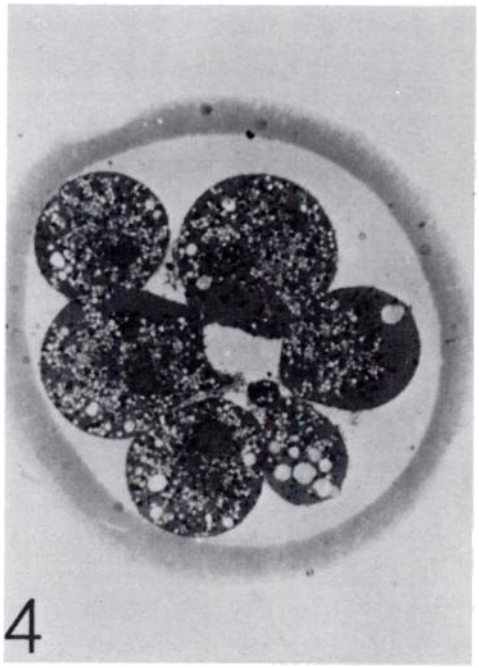
Embryos were fixed in 3% glutaraldehyde in cacodylate buffer, with 1.25 g/liter $CaCl_2$. They were postfixed in 2% osmium tetroxide, dehydrated, and embedded in epon-araldite mixture. Selections for light microscopy were stained with 1% toluidine blue and examined on a Zeiss Photomicroscope III. Thin sections for electron microscopy were stained with uranyl acetate and lead citrate and examined on a Siemens I electron microscope at 80 kv.

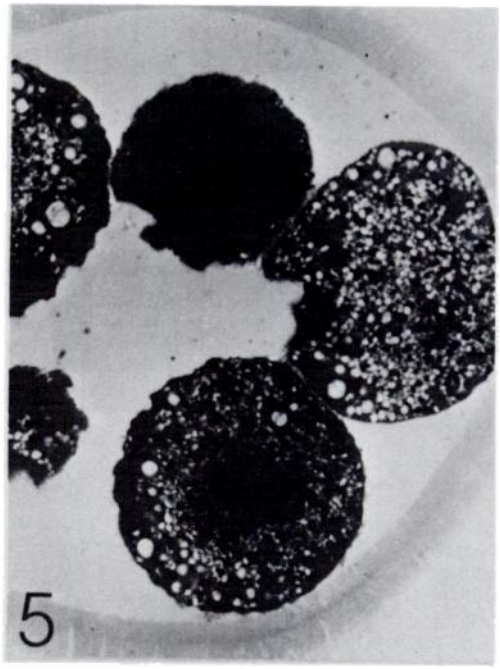
RESULTS

Control Day 5 Embryos

Seven Day 5 embryos were fixed and used to determine normal morphology at this stage. Day 5 embryos consisted of 8- to 16-cells, each containing numerous globular vesicles. When examined by light microscopy, these vesicles were a prominent feature and could be seen evenly distributed in the cytoplasm (Fig. 1). The plasma membrane of the blastomeres contained numerous short microvilli which were very dense in regions between adjacent blastomeres. At the point of contact between blastomeres, the plasma membranes were devoid of microvilli. (Fig. 2). The globular vesicles were 1–10 μm in diameter, and some contained lipid-like material while others contained flocculent and granular material. Some of these vesicles were bound by a unit membrane while others were membrane-free or incompletely membrane-bound (Fig. 3). These vesicles were often closely associated in groups or clusters.

Day 5 embryos contained the unusual "hooded" mitochondria previously reported in bovine occytes (Senger and Saacke, 1970; Fleming and Saacke, 1972). The mitochondria were electron dense and contained a few peripherally arranged cristae (Fig. 3). Smooth endoplasmic reticulum was abundant and closely associated with the mitochondria (Fig. 3). Numerous free ribosomes were present in all blastomeres, but no rough endoplasmic reticulum was detected. The nuclei contained several nucleoli, and pores were frequent along the nuclear membrane.





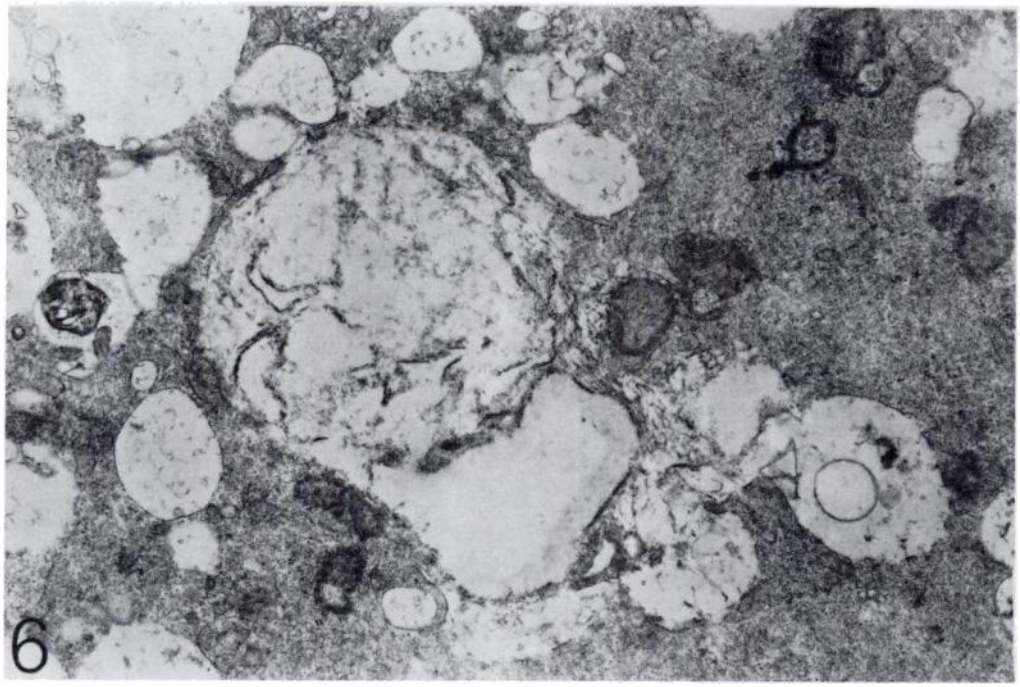
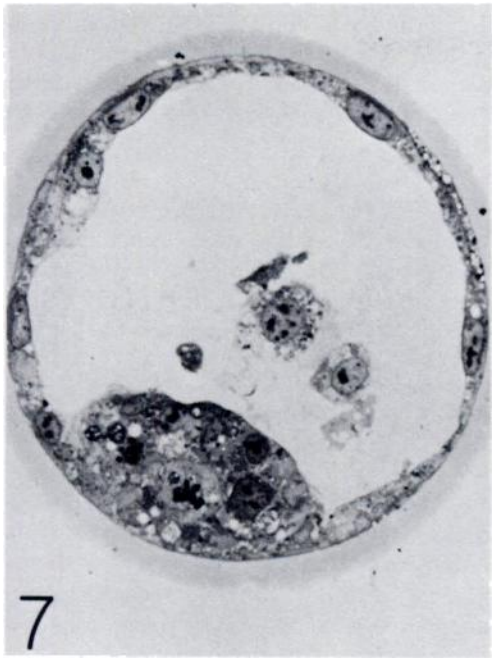
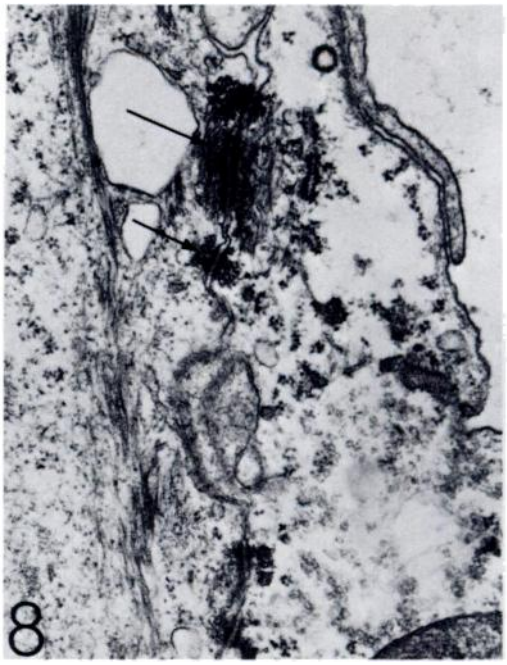


FIG. 4. Light micrograph showing a Day 5 embryo after cooling to 4°C. Note the regions of contact are retained but blastomere shape is distorted. ×540.

FIG. 5. Light micrograph of a cooled Day 5 embryo showing uneven distribution of organelles and irregularities in blastomere shape. ×675.

FIG. 6. Electron micrograph of a cooled Day 5 embryo. Note the large inclusion showing lack of homogeneity and disturbances in the adjacent cytoplasm. ×32,000.





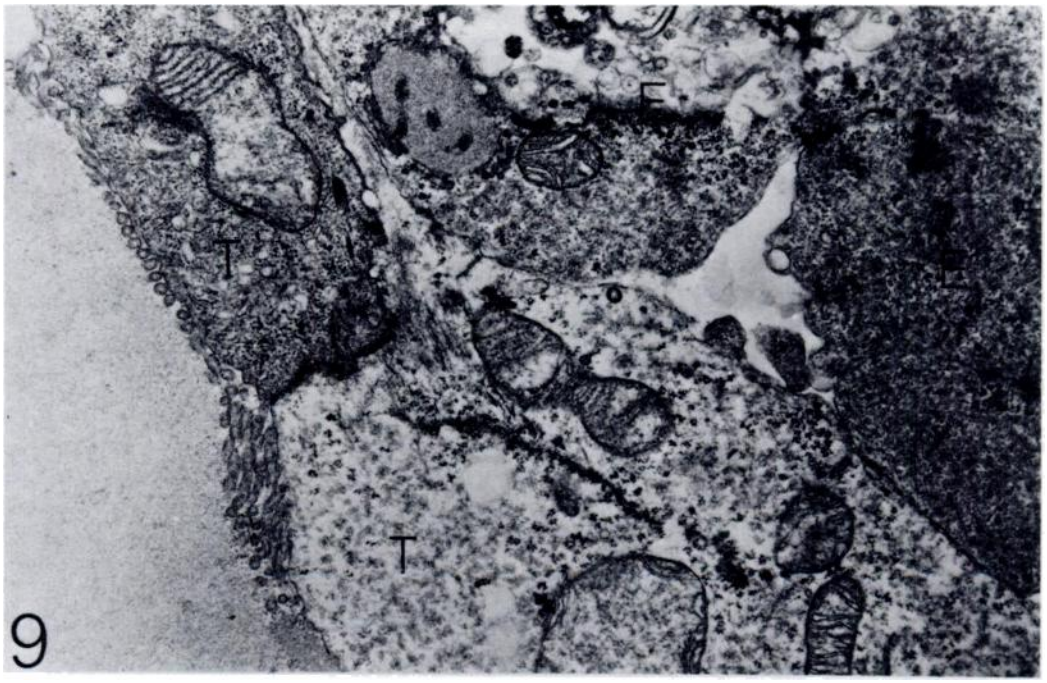


FIG. 7. Light micrograph of a Day 7 blastocyst. A disc of embryonic cells is enclosed within a ring of trophoblast cells. X540.

FIG. 8. Electron micrograph of the junction region between two trophoblast cells in a Day 7 blastocyst. Note the desmosome complexes (arrows) and numerous fine filaments. X32,000.

FIG. 9. Electron micrograph of a Day 7 blastocyst showing two trophoblast cells (T) and two embryonic cells (E). Note the light and dark trophoblast cells wtih elongated mitochondria, abundant ribosome clusters, and rough endoplasmic reticulum. X12,000.

Day 5 Embryos After Cooling

After embryos were cooled to 4°C, the distribution of organelles within the blastomeres as well as the spacial arrangement of blastomeres was disrupted (Figs. 4, 5). Regions where contact between adjacent blastomeres had occurred were still intact after cooling but blastomere shape was distorted. Organelles were no longer evenly distributed in the cytoplasm, but they appeared structurally unaltered by cooling. Many of the globular vesicles were distorted after cooling, and some fusion of adjacent vesicles could be seen (Fig. 6).

Control Day 7 Embryos

Four Day 7 embryos were fixed immediately after collection and used to determine normal morphology. The Day 7 bovine embryo is a blastocyst with an intact ring of trophoblast cells surrounding a disc of embryonic cells (Fig. 7). The vesicles seen in abundance in Day 5 embryos were no longer present in the blastocyst and only a few lipid inclusions were detected in the trophoblast cells. Adjacent trophoblast cells formed junction regions characterized by a tight junction along the external surface followed by numerous desmosomes. Microfilaments could be seen running parallel to the plasma membrane in the junction region (Fig. 8). Microvilli were present on the outer surface of the trophoblast cells while the lateral surface showed cytoplasmic projections which often interdigitated. Some trophoblast cells contained numerous free ribosomes and polysomes giving them a darker appearance (Fig. 9). The mitochondria in both trophoblast and embryonic cells had assumed a more conventional appearance. They were lighter, spherical, or oval with several transverse cristae (Fig. 9); no "hooded" mitochondria were seen. Smooth and rough endoplasmic reticulum was abundant, particularly in association with mitochondria.

The embryonic cells were cuboidal with large prominent nuclei. Several cells in the embryonic mass contained large lysosome-like complexes. An increased number of free ribosomes were present in the embryonic cells giving them a darker appearance than the adjacent trophoblast (Fig. 9). Rough endoplasmic reticulum, annulate lamellar structures, and a few lipid droplets were also present in the embryonic cells.

Adjacent cells in the embryonic disc were closely apposed and formed desmosome junctions with one another (Fig. 9). The embryonic disc was attached to the polar trophoblast by junctional complexes. The cavity of the blastocyst contained fine granular and flocculent material.

Day 7 Embryos After Freezing

Four Day 7 embryos were fixed immediately after thawing. All of these embryos showed some damage after freezing and thawing although the extent of damage varied between embryos. Cryoinjury in these embryos included large vacuolated regions in cells, dark staining organelle-free blebs from cells, indistinct or broken plasma and nuclear membranes, and a decrease in cytoplasmic homogeneity (Fig. 10). In two blastocysts damage to several trophoblast cells was sufficient to cause collapse of the blastocoele cavity (Fig. 11). This type of damage was not confined to the trophoblast cells. Junction regions between adjacent cells were very resistant to freezing injury (Fig. 13). A large proportion of cell components including rough endoplasmic reticulum, microfilaments, and mitochondrial and nuclear membranes were structurally unaltered by freezing and thawing.

Frozen-Thawed Day 7 Embryos After 24-h Culture

Five thawed Day 7 embryos that collapsed after freezing were placed in culture for 24 h. Three of these embryos had reformed blastocoele cavities at the end of the culture period, and these were fixed for microscopy. Normal junctional regions had reformed between the trophoblast cells, and cellular debris from cryoinjured cells was excluded from the blastocoele (Fig. 12). The blastocoele cavity was smaller than that of a normal Day 7 embryo. The frozen-thawed and cultured embryos were retarded in development when compared with an unfrozen control. Four unfrozen Day 7 blastocysts were cultured for 24 h and found to be expanded and hatching or hatched from the zona pellucida after the culture period.

Control Day 13 Embryos

Six freshly collected Day 13 embryos were processed to determine normal morphology. Size and shape varied greatly between these embryos which were spherical or oval and from 0.5 to 2 mm in diameter. The embryos

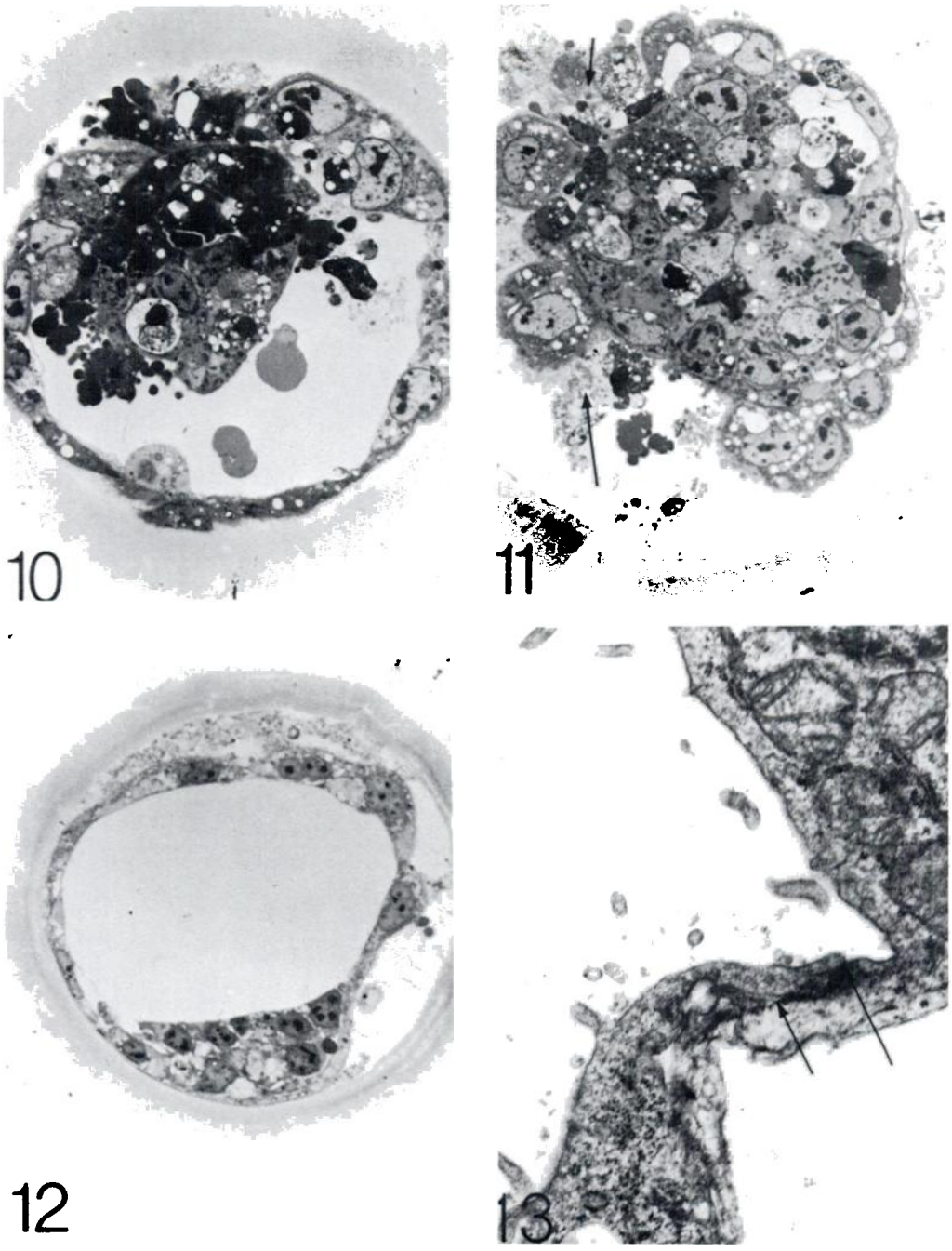
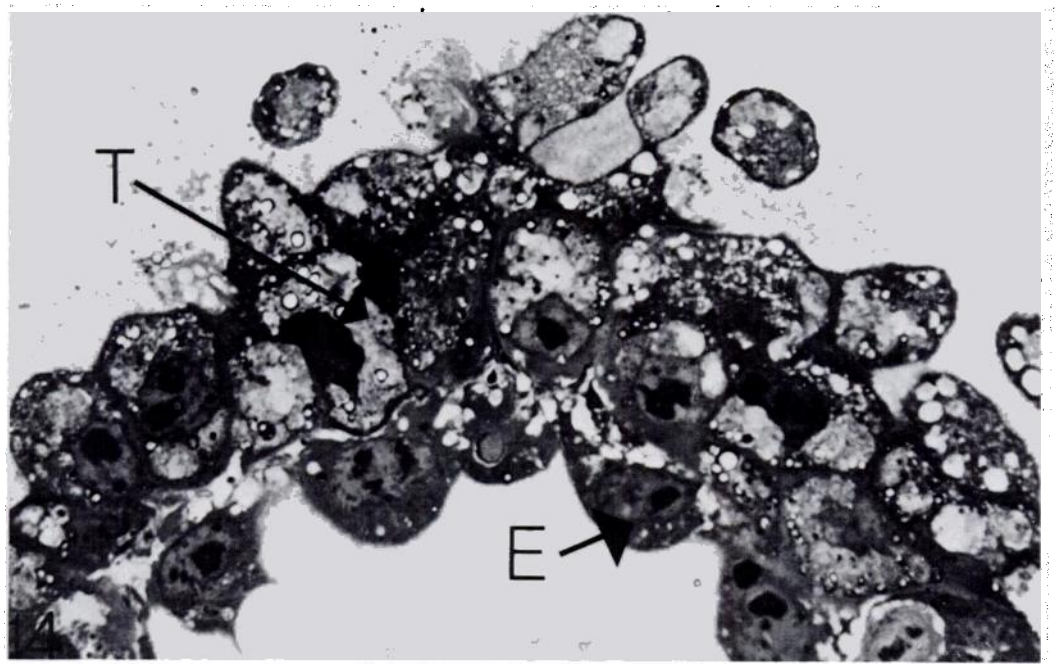


FIG. 10. Light micrograph of a frozen-thawed Day 7 blastocyst. The blastocoele cavity is intact but blebs of cytoplasm and vacuoles indicate cryoinjury. X 540.

FIG. 11. Light micrograph of a frozen-thawed Day 7 blastocyst. The blastocoele cavity has collapsed due to damage to several trophoblast cells (arrows), and the zona pellucida is broken. X675.

FIG. 12. Light micrograph of a frozen Day 7 blastocyst that collapsed after thawing and was cultured for 24 h. Note that a smaller blastocoele had reformed and the cellular debris from cryoinjury was excluded from the cavity. X 540.

FIG. 13. Electron micrograph depicting two adjacent trophoblast cells of a collapsed Day 7 blastocyst after freezing and thawing. Note that the trophoblast cells are still attached by an apical junction region (arrows). X12,000.



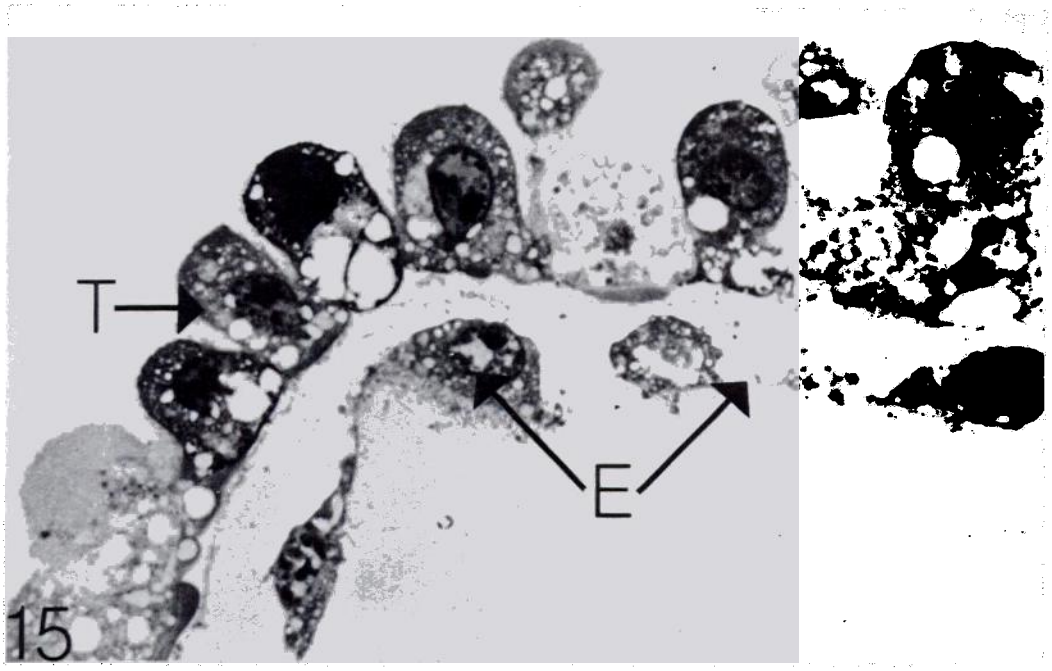


FIG. 14. Light micrograph showing the trophectoderm (T) and endoderm (E) of a Day 13 embryo. Note the large number of inclusions in the trophectoderm and the close apposition of the endoderm to trophectoderm. ×675.

FIG. 15. Light micrograph showing the trophectoderm (T) and endoderm (E) of a frozen-thawed Day 13 embryo. There are many vacuoles in the trophectoderm. The endoderm is discontinuous and torn free from the trophectoderm. ×675.

contained a layer of cuboidal trophectoderm cells one or two cells thick and a disc of embryonic cells. A ring of endoderm cells completely encircled the blastocoele cavity (Fig. 14).

Trophectoderm Cells

These cells contained numerous inclusions: small lipid-like inclusions, small membrane-bound vesicles containing granular material, and large lysosome-like structures often occupying most of the cytoplasm (Fig. 16). The cuboidal trophectoderm cells were very closely apposed and joined by numerous desmosomal junctions. A large number of filament bundles were found running parallel to the plasma membrane along these junction regions and ramifying into the cytoplasm (Fig. 17a). Microtubules were found throughout the trophectoderm cell cytoplasm, and spherical electron-translucent mitochondria with many transverse cristae were present. Other organelles included many free ribosomes and smooth and rough endoplasmic reticulum. The outer surface of the trophectoderm had many long tubular microvilli and showed signs of pinocytotic activity while the inner surface adjacent to the endoderm was devoid of microvilli.

Endoderm

Endodermal cells had a large number of free ribosomes giving them a very electron-dense appearance. They contained a few lipid droplets but no lysosome-like bodies or other inclusions found in the trophectoderm (Fig. 14). Mitochondria were cylindrical with well developed transverse cristae. Microfilament bundles and rough and smooth endoplasmic reticulum were abundant (Fig. 17b). Flocculent, extracellular accumulations could be seen between trophectoderm and endoderm indicating the formation of basement membranes. The sheet of endoderm was exceedingly thin in parts and adjacent endoderm cells were joined by apical junctional complexes.

Embryonic Disc

The embryonic disc was wedged in the trophectoderm and separated from the blastocoelic cavity by a layer of endoderm cells. The embryonic cells were structurally very different from the neighboring trophectoderm or endoderm. They were cuboidal cells with prominent nuclei and an electron-translucent cytoplasm containing few organelles (Fig. 18). These included free ribosomes, rough and smooth endoplasmic reticulum, and spherical or elongated mitochondria. The embryonic cells were attached to one another by junction regions.

Day 13 Embryo After Freezing to −196°C

Five Day 13 embryos were fixed for electron microscopy immediately after thawing but before removal of DMSO. This was found to be necessary as a high proportion of embryos disintegrated during removal of the cryoprotectant. The trophectoderm cells were consistently and severely structurally damaged after freezing. This damage included disruption of the plasma membrane leading to loss of cytoplasmic matrix, mitochondrial membrane damage, and loss of matrix homogeneity and large vacuoles within cells (Fig. 19). The embryonic cells, by contrast, appeared structurally intact, the integrity of the plasma membrane was maintained, and only slight swelling of some mitochondria could be detected. The close apposition of the embryonic cells was well preserved during the freezing and thawing (Fig. 20). A few endoderm cells appeared structurally intact after the freezing with some swelling of mitochondria and small vacuoles, while other endoderm cells suffered severe damage with loss of membrane integrity and cytoplasmic contents (Fig. 19). The continuity of the endodermal sheet was lost, and the endoderm layer was no longer closely apposed to the trophectoderm and appeared as broken sheets within the blastocoele cavity (Fig. 15).

DISCUSSION

The 8–16-cell bovine embryos appeared morphologically similar to embryos of other mammalian species at comparable stages of development. Primitive junctional complexes had commenced forming between some blastomeres of the 8-cell bovine embryos, and similar junctions have been reported at the 8-cell stage in the mouse (Ducibella, 1977), rat (Schlafke and Enders, 1967), sheep (Calarco and McLaren, 1976), and pig (Norberg, 1973).

Vesicular inclusions have been reported in preimplantation embryos of a large number of mammalian species and were a prominent feature of the Day 5 bovine embryos. Inclusions, which are present from the 1-cell

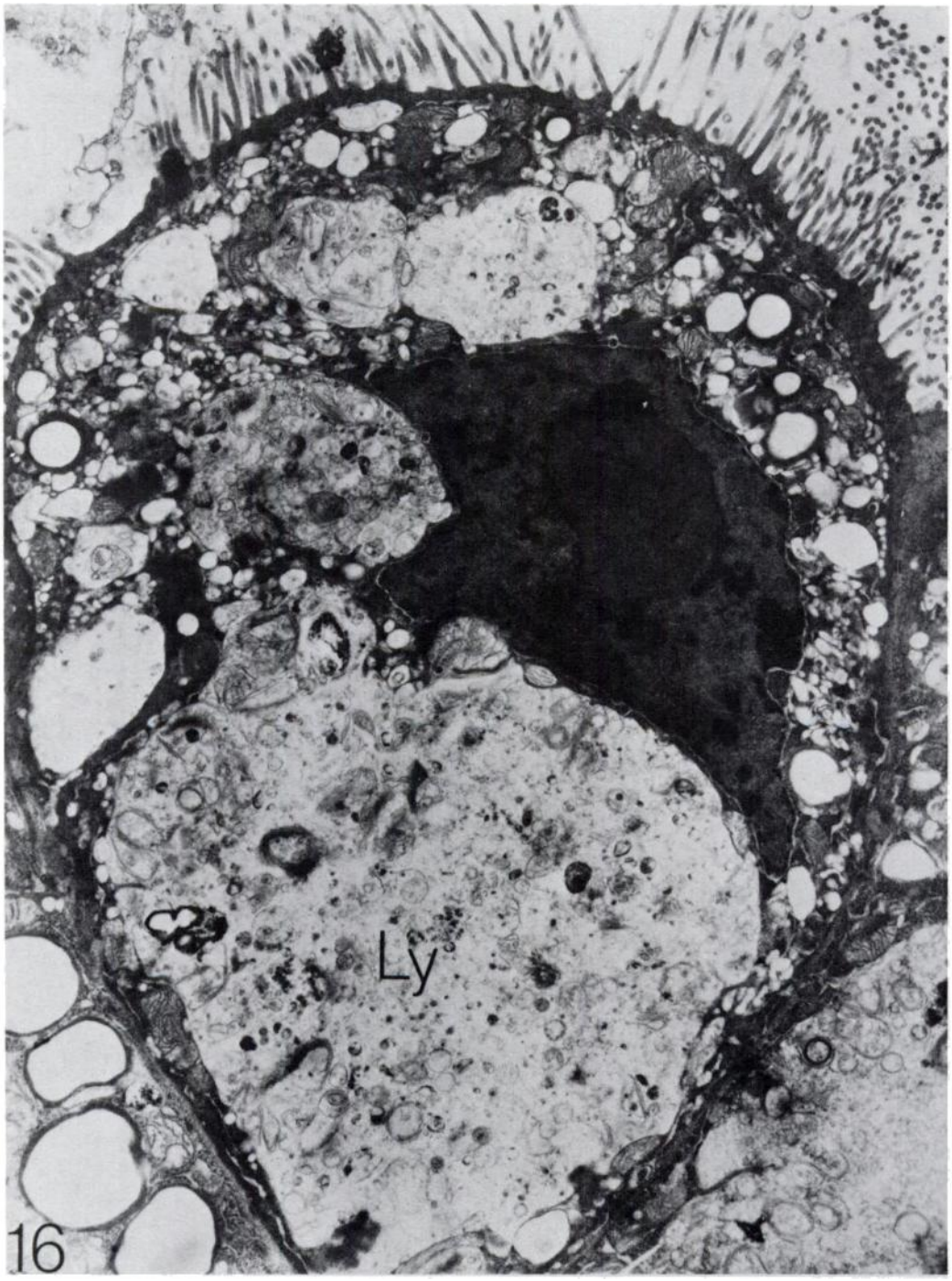


FIG. 16. Electron micrograph showing a typical trophectoderm cell in a control Day 13 embryo. Note the numerous long microvilli, large lysosome-like complexes (Ly), and other vesicular inclusions. ×12,000.

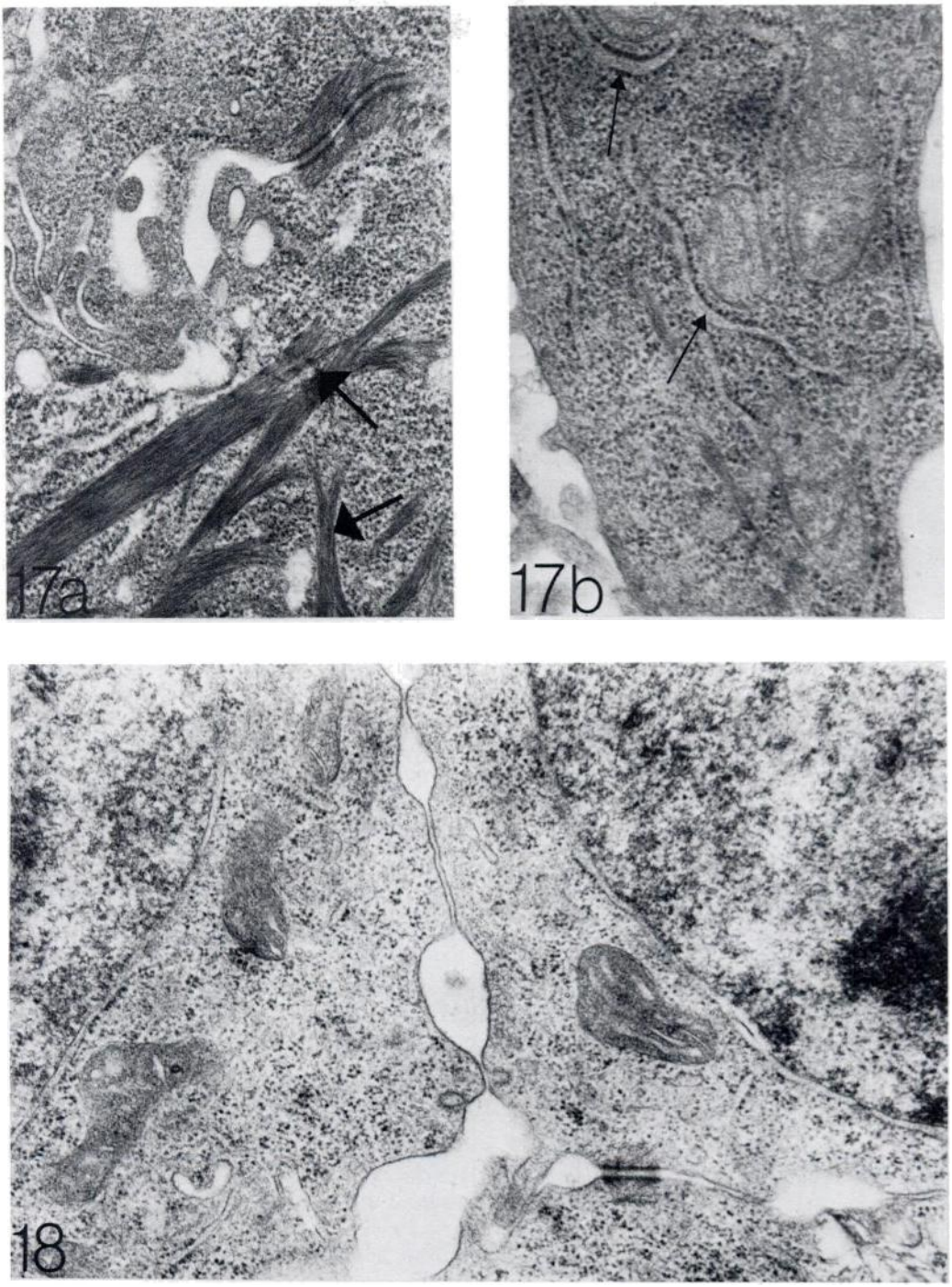


FIG 17 a) Electron micrograph of two adjacent trophectoderm cells in a control Day 13 embryo. Note the numerous microfilament bundles (arrows). X 20,000.

b) Electron micrograph showing the abundant rough endoplasmic reticulum (arrows) in an endoderm cell of a control Day 13 embryo. X 25,000.

FIG. 18. Electron micrograph showing three cells in the embryonic disc of a control Day 13 embryo. Ribosomes, rough endoplasmic reticulum, and mitochondria are the predominant organelles. X 30,000.

stage and decline in number with development, may be a form of stored material that is utilized during cleavage (Enders, 1971). In the mouse, rat, and hamster these inclusions have a linear form and have been variously referred to as plaques, fibrous elements, and cytoplasmic lamellae (Weakley, 1968). The ova and early embryos of the cow, sheep, and pig contain large numbers of spherical inclusions variously referred to as yolk, globules, lipid droplets, and cytoplasmic vesicles. In the pig these globules persist throughout cleavage, are closely associated with mitochondria, and may contribute lipid for oxidation by mitochondrial oxidases (Norberg, 1973). In the sheep, vesicles very similar to those seen in the 8–16-cell bovine embryos are abundant from the 1-cell stage to blastulation (Calarco and McLaren, 1976). No apparent decrease in vesicle number could be detected during cleavage, which would support the theory of yolk utilization, and no vesicles were present in cells after cavitation (Calarco and McLaren, 1976). In the mouse, vesicles of $\sim$1 μm diameter accumulate during the first two cleavages (Calarco and Brown, 1969), Subsequent to the 4-cell stage, these vesicles accumulate along the cell boundaries and can be found in large numbers by the morula stage. At the time of cavitation the vesicles form clusters along the cell borders, and the clefts that form between blastomeres are considered a result of secretory-like activity of these vesicles (Wiley and Eglitis, 1980). At the blastocyst stage, most vesicles have disappeared. The contents of some vesicles observed in the 8–16-cell bovine embryos were very similar to the PAS-positive granulated vesicles in bovine oocytes and were presumed to be glycogen (Fleming and Saacke, 1972). Whether these vesicles in bovine embryos represent storage material for use during early cleavage or play a role in cavitation is unclear.

The changes in mitochondrial morphology accompanying cleavage of bovine embryos are similar to the patterns displayed during mitochondrial development in most mammalian embryos. Small, electron-dense, "hooded" mitochondria in the bovine oocyte (Senger and Saacke, 1970) and the 2-, 4- and 8-cell stage (Brackett et al., 1980) develop to form the long, slender, electron-translucent mitochondria with transverse cristae seen in the Day 7 bovine blastocyst. Similar mitochondrial development occurs in the sheep (Calarco and McLaren, 1976) and the mouse (Stern et al., 1971). The intimate relationship between mitochondria and smooth endoplasmic reticulum reported here in the 8–16-cell bovine embryo has also been reported in bovine oocytes (Fleming and Saacke, 1972) and in the 8-cell sheep embryo (Calarco and McLaren, 1976). The function of this close association is unknown, but may involve the exchange of metabolic intermediates between mitochondria and endoplasmic reticulum.

Bovine blastocysts contain apical junctional complexes between adjacent trophoblast cells characteristic of blastocysts of a large number of mammalian species (Enders, 1971). The morphology of the cow Day 7 blastocyst was very similar to that reported for the sheep Day 8 blastocyst (Winterberger-Torrès and Fléchon, 1974). Unusually large numbers of find filaments were found in association with desmosomes and extending along the junction region between bovine trophoblast cells. Similar filaments have been reported in the armadillo blastocyst (Enders, 1971), the sheep blastocyst (Winterberger-Torrès and Fléchon, 1974), and the pig blastocyst (Hall et al, 1965). These microfilaments are thought to have a structural role in the cell, imparting shape, rigidity, and support to the cell body (Hall et al, 1965).

The columnar trophoblast of the Day 13 bovine embryo contained abundant microvilli on the outer surface, numerous cytoplasmic microfilaments, microtubules, and large lysosome-like structures, and closely resembled the trophoblast of the Day 14 sheep embryo (Winterberger-Torrès and Fléchon, 1974). Tonofibrils and microtubules are also abundant in the Day 14 sheep trophoblast. Evidence of pinocytosis could be seen along the outer surface of the cow Day 13 trophoblast and has also been reported in the sheep Day 14 trophoblast (Winterberger-Torrès and Fléchon, 1974). The role of the large lysosome-like complexes is unknown; however, the disappearance of some of these inclusions by Day 16 in the sheep is consistent with their being a form of storage material. The prominent microvilli seen on the cow Day 13 and sheep Day 14 embryos may provide an increased surface area for nutrition of the rapidly expanding embryo.

Maternal recognition of pregnancy, in the cow, occurs around Day 16 when a signal of embryonic origin must directly or indirectly prolong the life-span and function of the corpus luteum. In the sheep this signal is thought to be a protein secreted by the trophoblastic tissue of

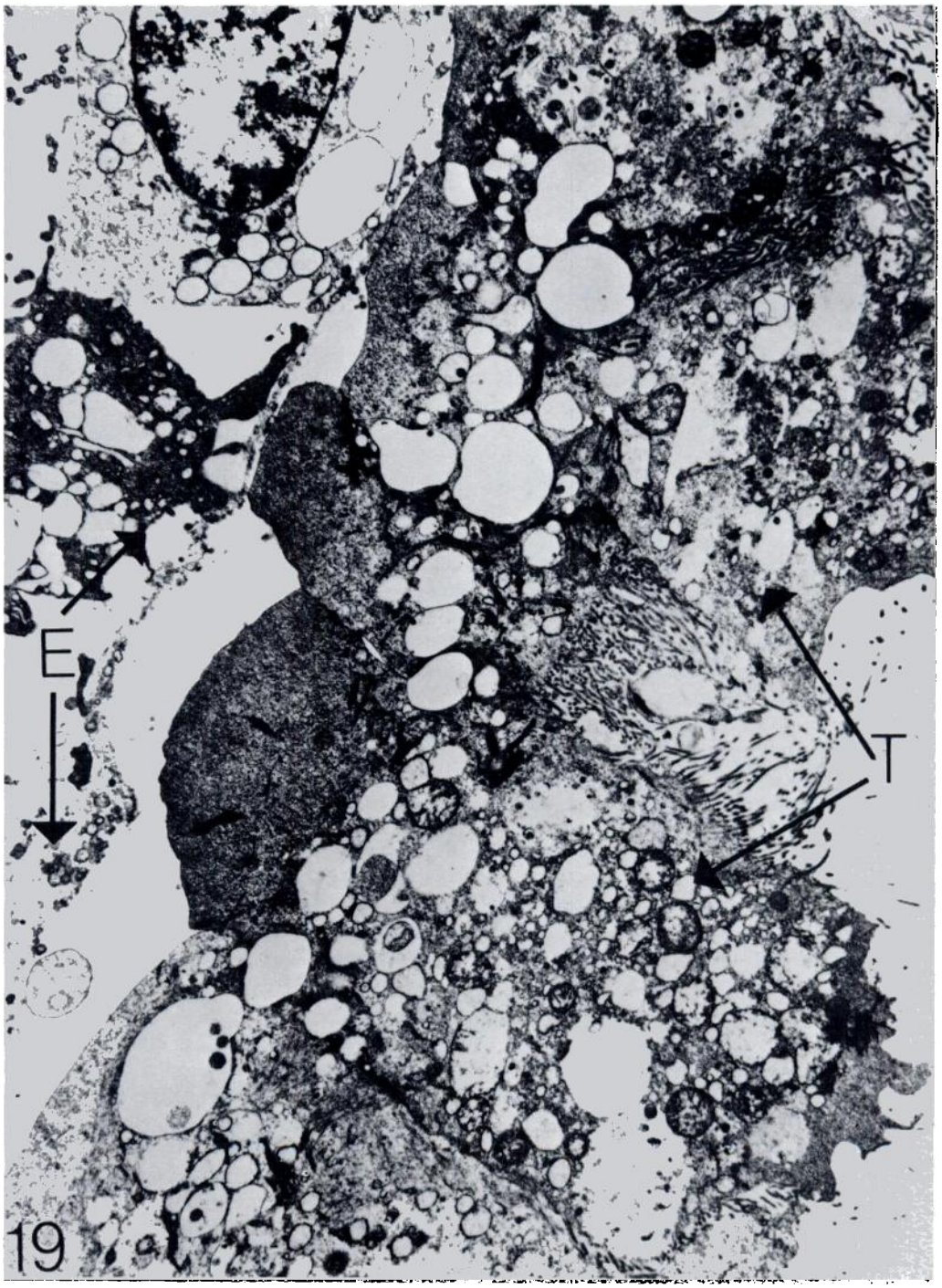


FIG. 19. Electron micrograph showing the trophectoderm (T) and endoderm (E) of a frozen-thawed Day 13 embryo. Note the swelling of trophectoderm mitochondria, numerous vacuoles, and loss of cytoplasmic matrix. The endoderm layer has severe damage to plasma membranes with a loss of cell contents. ×6000.

the Day 12–13 blastocyst (Heap, 1979). Ultrastructural examination of the Day 14 sheep trophoblast revealed several factors consistent with protein synthetic capacity but evidence of secretory capacity was not observed (Winterberger-Torrès and Fléchon, 1974). Similarly, the Day 13 bovine embryos contained organelles associated with protein synthesis such as abundant rough endoplasmic reticulum and ribosome clusters; however, the Golgi apparatus was poorly developed and little evidence of secretory activity could be found.

The survival of 8- and 16-cell bovine embryos is severely reduced after cooling below 10°C (Wilmut, 1975). Porcine embryos do not continue to develop when cooled below 15°C (Wilmut, 1972). The sensitivity to cooling in mammalian embryos is associated with the presence of a large number of vesicular inclusions within the embryonic cells. Bovine embryos have a large number of these inclusions in the early cleavage stages, and the absence of these vesicles at the blastocyst stage is coincident with a loss of sensitivity to cooling (Trounson et al., 1976). Porcine embryos contain a large number of globular inclusions throughout preimplantation development (Norberg, 1973) and cannot be cooled below 15°C at any stage of development. Preimplantation mouse embryos do not have a large number of vesicular inclusions and can be successfully frozen at all stages (Whittingham et al, 1972). The critical temperature for porcine embryos is around 15°C; this is also the temperature at which there is an anomalous increase in lateral diffusion within membranes being cooled (Petit and Edidin, 1974). This increase in diffusion is attributed to changes in the lipid order caused by lipid-lipid or lipid-protein interactions (Edidin and Petit, 1977). These lipid-phase changes would occur in plasma membranes, internal membranes such as mitochondria or endoplasmic reticulum, and also in lipids located within inclusions. While the phase changes themselves are reversible, they may result in irreversible interactions between proteins or other molecules normally kept apart. When porcine embryos were held at 15°C, structural changes were observed within lipid droplets and they also appeared to coalesce to form larger droplets (Willadsen, 1977). When 8–16-cell bovine embryos were cooled to 4°C, some coalescing vesicles and loss of homogeneity within vesicles was observed. The most conspicuous feature of these cooled embryos was a loss of cytoplasmic organization leading to a massive disruption of the subcellular localization of organelles. The control Day 5 embryos contained an abundance of smooth endoplasmic reticulum intimately associated with vesicles and mitochondria, and ramifying throughout the cytoplasm. This smooth endoplasmic reticulum would be subjected to lipid phase-changes during cooling, and while this organelle appeared structurally intact after cooling, the result of these phase changes may not be detectable by transmission electron microscopy.

After freezing and thawing of bovine blastocysts, a number of features characteristic of cellular damage were observed. The extent of this damage varied between embryos and also between cells within an embryo. All junction regions between cells seemed surprisingly resistant to freezing injury. An intact junction region could be seen whereas in another region of the same cell the plasma membrane was discontinuous. This resistance of junction regions to freezing damage could be due to the structural support given by microfilament bundles or to altered chemical composition of the membrane itself in those regions. Some cattle blastocysts that collapse during freezing and thawing can reexpand after culture at 37°C and develop to live young after transfer (Bilton and Moore, 1979). The ability of trophoblast cells to reform junctions with one another is not a transitory feature in bovine embryos and is still present at Day 13 (Hare et al., 1976).

Freezing injury in the Day 13 embryo was related to cell type rather than location within the embryo. The undifferentiated embryonic cells survived freezing and thawing very well and appeared structurally intact while the neighboring and highly differentiated trophectoderm was severely damaged. The trophectoderm cells at this stage have a high density of organelles in a well developed cytoskeleton. This structure would allow little flexibility to withstand the shrinkage of cells that takes place during dehydration accompanying slow freezing. The large lysosome-like structures, a prominent feature in the trophectoderm, may be sensitive to freezing. There is some evidence that lysosomes may be one of the primary targets of cryoinjury in cells frozen in the presence of DMSO (Persidsky, 1971). Exposure to DMSO itself, at the concentrations and equilibration times required to achieve cryoprotection, is associated with a number of structural changes in cells (Malinin, 1973). These include swelling

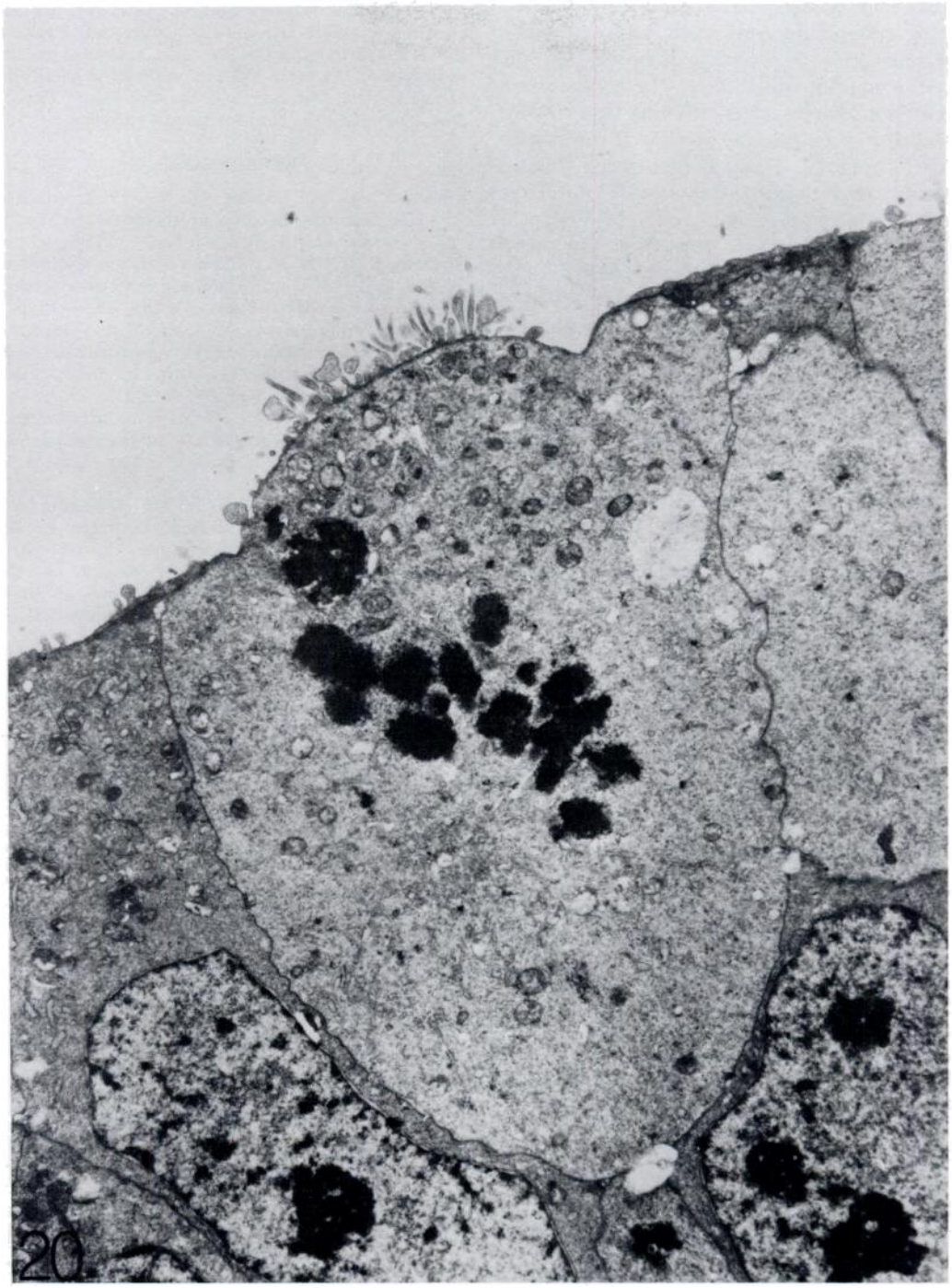


FIG. 20. Electron micrograph showing a portion of the embryonic disc of a frozen-thawed Day 13 embryo. Note the plasma membrane integrity, cytoplasmic homogeneity, absence of vacuoles, and preservation of the close apposition of the cells. ×6000.

of mitochondria with damage to their membranes and cristae, dilation and degranulation of the endoplasmic reticulum, and damage to the nuclear and plasma membranes. In the experiments in which Day 7 embryos were fixed immediately after thawing and DMSO removal, it would not be possible to distinguish subtle structural changes that were due to freezing from those due to DMSO. The large vacuoles and complete destruction of some cells due to intracellular ice formation can, however, be readily distinguished as cryoinjury. The Day 13 embryos were fixed in the presence of 1.5 M DMSO but the excellent structural preservation of the embryonic disc argues against any nonspecific deleterious effect of DMSO on cell structure. An increased sensitivity of the trophectoderm to DMSO-induced damage is unlikely as Day 13 embryos can continue development after exposure to DMSO and cooling to subzero temperatures (Trounson et al., 1978c).

These ultrastructural findings are consistent with the marked reduction in survival when later stage (Day 8–Day 13) bovine blastocysts are frozen and thawed. Transfer of these more advanced stages after freezing and thawing does not result in fetal development (Trounson et al., 1978b,c). The successful cryopreservation of Day 13 embryos is most desirable, because due to their size they are more easily recovered and their sex determination by chromosome examination is more readily achieved.

Using the present procedures for cryopreservation, it is not possible to freeze and transport these advanced bovine embryos, and the development of new methods is necessary to enable both the trophoblast and embryonic cells to survive. The freezing rate giving maximal survival of different mammalian cell types can differ by several orders of magnitude (Leibo, 1977). When more than one cell type is present, each may have its own optimum freezing and thawing rate and these need not overlap. The precise nature of factors determining the optimun freeze-thaw protocol for each cell is not known but may include cell size, permeabilty to water, ability to withstand shrinkage, and sensitivity to solution effects. In the Day 13 bovine embryo, or in an organ, where a number of different cell types are present, it may be a substantial problem to reach a freeze-thaw protocol that would allow adequate survival of all cell types.

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