Ultrasound and Cell Death of In Vivo Derived and Vitrified Porcine Blastocysts

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ABSTRACT Morphological and molecular signs of injury and cell death and subsequent regeneration following vitrification of porcine blastocysts were evaluated by light (LM) and transmission electron microscopy (TEM) as well as TUNEL/propidium iodide (PI) nuclear staining followed by confocal microscopy (CSM). In vivo derived blastocysts were assigned to one of the following four groups: Controls—(1) fixed immediately after collection (C0h) and (2) after 24 hr culture in vitro (C24h) and vitrified embryos—(3) fixed immediately after vitrification and warming (V0h), and (4) after 24 hr of culture upon warming after vitrification (V24h). Observation by LM and TEM showed that the V0h embryos displayed collapse of the blastocoele cavity (BC) and cell swelling, a general dissection or shrinkage of mitochondria and massive increase in the amount of vesicles, vacuoles, and secondary lysosomes (SLs). Approximately 2/3 of the V24h embryos had recovered, whereas the remaining 1/3 were degenerated. Recovered embryos displayed almost normal blastocyst morphology, except for a widening of the perivitelline space, accumulation of debris and partial dissection of mitochondria, whereas degenerated embryos were disintegrated into a poorly defined mass of cells and debris including cells with abundant degeneration of mitochondria and other organelles. Both recovered and degenerated embryos displayed a persistent abundance of presence of small membrane bounded vesicles, vacuoles, and SLs. Evaluation of TUNEL/PI stained embryos showed only occasional appearance of TUNEL positive nuclei with typical apoptotic morphology in controls (C0h 0.67%, C24h 1.22%) and in the V0h embryos (0.93%). The percentage of apoptotic nuclei in embryos at V24h was significantly higher than in all other groups (2.64%). Vitrified embryos showed significantly increased appearance of DNA fragmented nuclei without typical morphological features of apoptosis (V0h 1.43%, V24h 4.50%) compared with controls (C0h 0.26%, C24h 0.45%). The observed morphological changes and increased DNA fragmentation observed immediately after vitrification and warming probably reflects a direct damaging effect of vitrification. During 24 hr of culture a portion of the embryos was able to regenerate and along with the regenerative process, apoptosis—a possible pathway for elimination of damaged cells—became evident. Mol. Reprod. Dev. 70: 155–165, 2005. © 2005 Wiley-Liss, Inc.

Key Words: embryo; porcine; cryopreservation; electron microscopy; TUNEL assay; apoptosis

INTRODUCTION

The cryopreservation of embryos from many mammalian species has been widely used in reproductive research and in animal breeding (reviewed in Vajta, 2000; Dobrinsky, 2002). The conventional embryo cryopreservation is achieved by a controlled freezing at various developmental stages, from one-cell or two-cells embryos (mouse, human) to morula and blastocyst (cattle, sheep, goat, pig), in the presence of appropriate cryoprotectants before transferring into liquid nitrogen. Despite intensive research efforts, various cryopreservation procedures still cause significant morphological and biochemical alterations which may lead to cell death and loss of embryo viability. The loss of viability associated with freezing can be attributed to the type and concentration of cryoprotectant, freezing protocol, species, genotype, the developmental stage of embryo, and the type of embryo production (reviewed in Rall et al., 2000; Vajta, 2006; Hasler, 2001; Dobrinsky, 2002). Vitrification, i.e., cryopreservation of tissues and cells without ice crystal formation, seems promising for solving some of the problems associated with cryopreservation of embryos.

Compared with other species, pig embryos suffer from severe sensitivity to temperatures below 15°C, limiting their ability to be cryopreserved. This cooling...
intolerance might be due to the high lipid content found in early developing pig embryos prior to the blastocyst stage (Poige et al., 1974; Nagashima et al., 1994).

However, during the past years, new technologies have been developed and porcine embryos have been successfully frozen, thawed, and offspring produced following transfer to recipient gilts (Pujino et al., 1993; Nagashima et al., 1994; Dobrinsky, 1997).

Vitrification has become available as an alternative method also for cryopreservation of porcine embryos (Yoshino et al., 1993). Blastocysts, expanded blastocysts, and hatched blastocysts have been shown to have high survival rates in culture following vitrification. Improvements in procedures have resulted in several litters of offspring born from embryos transferred following vitrification (Kobayashi et al., 1995; Dobrinsky et al., 2000; reviewed in Dobrinsky, 2002).

As a last modification of embryo vitrification, the open pulled straw (OPS) method was developed by Vajta et al. (1998) allowing for increased cooling rates. Using this technique, live offspring have consistently been produced from cryopreserved porcine blastocysts (Berthelot et al., 2000, 2002; Bebo et al., 2002).

A variety of studies have shown presence of dead cells in mammalian preimplantation embryos (Hardy, 1997), including porcine blastocysts (Long et al., 1998). Most of these cells die by apoptosis, a form of programmed cell death. The function of apoptosis in postnatal life is tissue homeostasis as well as to eliminate cells with abnormal, detrimental, or superfluous potential during preimplantation development. Apoptosis has received an increasing amount of attention also because of its potential role in cellular response to suboptimal developmental conditions and stress (reviewed in Betts and King, 2001).

The TUNEL reaction is the most frequently used method, which enables in situ detection of apoptotic cells by labeling of extensive oligonucleosomal DNA fragmentation generated by endogenous DNase activity during the apoptotic process. However, the specificity of TUNEL assay is relatively low, since nuclei of cells undergoing necrosis are also labeled. Therefore it should be combined with other methods of apoptosis detection, such morphological evaluation of nuclei by DNA dyes (Majno and Joris, 1995; Darzynkiewicz et al., 2001).

So far, TUNEL positive nuclei have not been observed prior to the blastocyst stage in normally developing porcine preimplantation embryos derived in vivo or in vitro (Long et al., 1998; Hao et al., 2003).

Previous experiments showed that embryo survival after in vitro culture following cryopreservation is a valid evaluation of the efficiency of a cryopreservation technique. The purpose of the present study was to characterize the light and electron microscopic changes and to evaluate the incidence of cell death in vitrified in vivo derived porcine blastocysts, immediately after warming and after 24 hr in vitro culture, in order to provide a better understanding of the mechanisms of their injuries and the subsequent regeneration.

**MATERIALS AND METHODS**

Unless otherwise indicated, all chemicals were obtained from Sigma Chemical Co. (St. Louis, MO)

**In Vivo Embryo Production**

Cyclic gilts (Large White hyperprolific, LWh, INRA, Neuzilly, France) around 200 days old were used as embryo donors, and all were synchronised with a progestagen treatment (Regumate, Janssen, France) during 18 days (20 mg/day/gilt) and had an oestrus 5 to 7 days post-treatment in which they were not inseminated.

Non-superovulated gilts (n = 7) received no hormonal treatment (without eCG and hCG) and they were inseminated at their subsequent spontaneous oestrus.

Superovulated gilts (n = 23) were treated with two injections (10 and 16 hr) of 175 μg of Cloprostenol (Planate, Schering-Plough Vétérinaire, France) at Day 13 or 14 or 15 of the oestrous cycle (Day 0 = first day of oestrus induced by Regumate). Superovulation was carried out with an injection of 800 UI eCG (Chronogest PMSG, Intervet, France) administrated 24 hr after the second injection of Cloprostenol and ovulation was induced by administration of 500 IU hCG (Chorulon, Intervet, France), 48 hr after eCG treatment.

Oestrus detection was performed twice a day with a boar (9 and 16 hr). Donors were artificially inseminated (AI) 12 and 24 hr after detection of oestrus. The semen used for AI was collected from adult Pietrain (P) boars and seminal doses (3 x 10⁹ spermatozoa per AI) were prepared at UECIP, INRA. Thus, the embryo genotype was Pietrain x Large White hyperprolific (PxFxLWh).

Donors were slaughtered on Days 5 or 5.5 after first AI. Immediately after slaughter, the genital tract was collected. The embryos were recovered by flushing the uterine horns with saline solution (0.9% NaCl) containing 2% newborn calf serum (NBCS, Bio Whittaker, France) and evaluated under a stereomicroscope at a magnification of x60 and classified as either unfertilised eggs, embryos with abnormal developmental stages, morulae, early blastocysts, blastocysts, expanded blastocysts, and hatched blastocysts.

Blastocysts were then randomly assigned to one of four groups: Controls, which were fixed either (1) immediately after collection (C0h), or (2) after 24 hr culture in vitro (C24h); and vitrification embryos, which were fixed either (3) immediately after vitrification and warming (V0h), or (4) after 24 hr of culture upon warming after vitrification (V24h). In each of the four groups the embryos were processed for LM followed by TEM (C0h, n = 17; 10 superovulated, 7 non-superovulated); C24h, n = 11 (all superovulated); V0h, n = 24 (10 superovulated, 14 non-superovulated); V24h, n = 21 (10 superovulated, 11 non-superovulated) and TUNEL/propidium iodide (PI) staining and confocal laser scanning microscopy (C0h, n = 32; C24h, n = 34; V0h, n = 31; V24h, n = 32 (all superovulated)).

To avoid loss of cells extruded to the perivitelline space, only unhatched blastocysts were subjected to
TUNEL/PI evaluation, and embryos which did not survive 24 hr culture were also excluded.

Vitrification and Warming and Embryo Culture

The open pull straw (OPS) method was used for embryo vitrification. The OPS technique allows to reduce simultaneously the volume of medium around the embryos, the internal diameter of the straw, the thickness of the wall of the straw and the direct contact between liquid nitrogen and the embryo-containing medium. The cooling rate by plunging OPS directly into liquid nitrogen is approximately 18,000 °C/min (Vajta et al., 1998).

The basic medium (TCM) used throughout vitrification was TCM199 HEPES medium (Sigma, France) supplemented with 20% newborn calf serum (NBCS, Bio Whittaker, France). Cryoprotectants were dimethylsulfoxide (DMSO, Sigma) and ethylene glycol (EG, Sigma).

At vitrification, two to seven blastocysts were equilibrated in TCM + 7.5% DMSO + 7.5% EG and in TCM + 18% DMSO + 18% EG + 0.4 M sucrose for 3 and 1 min, respectively (Table 1). Media and embryos were maintained at 39°C on a heating plate.

During the last step, blastocysts were gathered in a 2 µl droplet of vitrification medium on the bottom of a petri dish, loaded into the narrow end of the OPS by capillary effect and plunged in liquid nitrogen.

At warming, straws were immediately plunged into TCM containing 0.13 M sucrose. After 1 min, the embryos were transferred into another well with the same medium for 5 min and then onto another 5 min in TCM containing 0.075 M sucrose (Berthelot et al., 2002).

Finally, the V24h embryos were placed into a culture dish containing 100 µl culture medium (HEPES TCM199 + 10% foetal calf serum) into a 5% CO2, 5% O2, and 90% N2 incubator at 39°C during 24 hr.

The C24h embryos were cultured at the same conditions without vitrification and warming procedure.

Light and Electron Microscopy

Embryos were fixed in 3% glutaraldehyde in 0.1 M sodium phosphate buffer (PB), pH 7.2–7.4, for 1 hr, in the laboratory in France. Then they were washed in PB twice for 5 min, packed into straws, and shipped to the laboratory in Copenhagen for further analysis.

**TABLE 1. Outline of Vitrification and Warming Methods** (Berthelot et al., 2002)

<table>
<thead>
<tr>
<th>Vitrification</th>
<th>Warming</th>
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<tr>
<td>TCM alone</td>
<td>1 min</td>
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<tr>
<td>TCM alone</td>
<td>1 min</td>
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<tr>
<td>TCM + 7.5% DMSO + 7.5% EG</td>
<td>3 min</td>
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<tr>
<td>TCM + 18% DMSO + 18% EG + 0.4 M sucrose</td>
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<tr>
<td>Warning</td>
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<tr>
<td>TCM + 0.13 M sucrose</td>
<td>1 min</td>
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<tr>
<td>TCM + 0.13 M sucrose</td>
<td>5 min</td>
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<tr>
<td>TCM + 0.075 M sucrose</td>
<td>5 min</td>
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<tr>
<td>TCM alone</td>
<td>5 min</td>
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TCM: HEPES TCM199 + 20% NBCS.

Subsequently, the embryos were embedded in 4% agar and post-fixed in 1% OsO4 in 0.1 M PB for 1 hr at 4°C. The samples were then stained en bloc with 0.5% uranyl acetate in distilled water, dehydrated by passing them through an ethanol series, embedded in Epon, and serially sectioned into semi-thin sections (2 µm) that were stained with 1% basic toluidine blue for light microscopy (Fig. 1). Selected semithin sections were subsequently re-embedded (Hyttel and Madsen, 1987) and ultra-thin sections (70 nm) were prepared. The ultra-thin sections were collected on copper grids, contrasted with uranyl acetate and lead citrate, and examined on a transmission electron microscope (Philips CM100, Eindhoven, Netherlands; Figs. 2 and 3).

**TUNEL Assay and Confocal Laser Scanning Microscopy**

TUNEL labeling and nuclei staining. Embryos were washed four times in culture media without serum, fixed in 3.7% paraformaldehyde in 0.15 M PBS, pH 7.2–7.4, at room temperature for 1 hr, then stored in 1% paraformaldehyde in 0.15 M PBS at 4°C, packed into straws, and mailed.

Nuclei with degraded DNA were detected by using a cell death-detection technique based on the TUNEL principle using fluorescein-conjugated dUTP with minor modifications (Gjerret et al., 2003).

The embryos were washed three times in PBS with polyvinylpyrrolidone (PBS/PVP; 3 mg/ml) permeabilized for 1 hr in PBS with 0.5% (w/v) Triton X-100, and again washed twice in PBS/PVP.

As positive controls, two to three additional C0h or C24h embryos were pre-incubated in 50 U DNase/ml PBS (RQ; Promega, Biø & Bernstein, Rodovre, Denmark) for 30 min at 37°C and then washed two times in PBS/PVP. Positive controls and all experimental embryos were then incubated in 10 µl of terminal deoxynucleotidyl transferase and 90 µl of fluorescein-conjugated dUTP (TUNEL, In Situ Cell Death Detection Kit, Roche, Eivindværk, Denmark) for 60 min at 37°C in the dark. Negative controls, presented by another two to three additional C0h or C24h embryos, were not incubated with the terminal transferase enzyme.

After TUNEL reaction, all embryos were washed three times in the Triton X-100 in PBS, once in PBS/PVP and stored at 4°C over night in the dark. Then, the embryos were washed once in Tris-buffer (40 mM Tris, 10 mM NaCl, and 6 mM MgCl2, pH 8.0) and incubated with 0.2 mg/ml of RNase A in Tris-buffer for 1 hr at 37°C in the dark. The nuclear material was counterstained with 7.5 µg/ml of PI in the Tris-buffer for 45 min at 37°C in the dark. Then, the embryos were transferred through a gradient of Vecta-Shield (Vector Laboratories, Burlingame, CA) at 50, 75, and 100% (w/v) in PBS in the dark, with each step lasting for 30 min; in the 100% Vecta-Shield, 0.07 mg/ml of PI was added.

Then, the embryos were mounted on a slide with a hole enforcement in 4 µl of the 100% Vecta-Shield PI solution and covered with a coverslip. Slides were stored at 4°C.
for up to 7 days before confocal laser scanning microscopy was performed.

**Confocal microscopy and image processing.** The embryos were subjected to confocal laser-scanning microscopy on a Leica TCS4D microscope (Leica Laser Technik GmbH, Heidelberg, Germany) fitted with 25/40x PL Fluotar 0.75 oil objectives. An argon/krypton laser was used for excitation at 488 and 568 nm, and two-channel scanning was performed with a double-dichroic DD488/568 beam splitter and a band-pass BP530 barrier and a long-pass OG590 barrier filter for detection of TUNEL reaction and PI, respectively. A complete Z series of 20 to 25 optical sections at 3 to 4 µm intervals was acquired from each embryo using Leica Scanware software (Leica Laser Technik), and image stacks were reconstructed on a Silicon Graphics computer equipped with an Imaris image-analysis software package (Bitplane, Zurich, Switzerland).

**Quantitative analysis of TUNEL labeling and cell death.** Digitally recombined, composite images were analyzed using the Imaris software. All 20 to 25 optical sections were divided using a standard grid over each layer to count all nuclei as a measure of the total cell number of the embryo. Allocation of nuclei to inner cell mass (ICM) and trophoblast (TB) was based on their position in the reconstructed images. The nuclei belonging to the polar TB were counted together with the ICM nuclei, leading to an overestimation of the ICM (Gjerrit et al., 2003). The nuclei located in the perivitelline space (PvS) were counted together with TB nuclei whereas the nuclei in blastocoel cavity (BC) were counted together with ICM nuclei.

According to their morphology, nuclei were classified as normal (oval with uniform PI staining), condensed (dense PI staining, smaller than normal nuclei), or fragmented (disintegrated into apoptotic bodies). Mitotic configurations were classified as normal nuclei (Fabian et al., 2004). The combination of morphological classification of nuclei with TUNEL labeling was used for their following exact categorization into four groups: normal T−M− nuclei (TUNEL negative and normal morphology), T−M+ nuclei (TUNEL positive and condensed or fragmented morphology), T+M+ nuclei (TUNEL negative but condensed or fragmented morphology) and T+M− nuclei (TUNEL positive but normal morphology) (Fig. 4).

A dead cell index (DCI) was calculated as the percentage of nuclei with fragmented DNA (T+M+, T+M−) relative to the total number of cells, and an Apoptotic cell index (ACI) was calculated as the percentage of nuclei with both fragmented DNA and apoptotic morphology (T+M−) relative to the total number of cells. Additionally, the percentage of nuclei showing DNA fragmentation but no morphological features of apoptosis (T+M−) were counted.

**Statistical Analysis**

The results are expressed as mean values ± SD. Statistical analysis of total cell numbers were done using Student’s t-test. The Chi-square test was used
to detect differences in the profiles of nuclei. Values $P < 0.05$ were considered as significant.

**RESULTS**

**Light and Electron Microscopy**

LM and TEM observation showed no morphological differences between embryos recovered from superovulated or nonsuperovulated gilts. Hence, in the following the two groups of embryos are pooled.

**Morphology of control embryos (C0h).** Evaluated C0h embryos were either early (nonexpanded, $n = 10$) or expanded (n = 7) blastocysts, all displaying a spherical shape, well-defined TB with flattened cells, and an ICM markedly protruding into the BC and a narrow or even lacking perivitelline space. In some areas among the ICM cells, intercellular spaces of the size of an average embryonic cell were formed (Fig. 1a).

At the ultrastructural level, the cytoplasm of both TB and ICM cells contained cytoplasmic organelles with normal appearance and an abundant number of lipid droplets (LD) (Fig. 2a,c). Majority of mitochondria displayed an oval or pleomorphic shape and low number of cristae leaving major spaces with mitochondrial matrix (Fig 2c). TB cells were closely connected by tight junctions and desmosomes, and microvilli protruded into the PrS but were absent on the basal cell membranes facing the BC (Fig 2b).

**Morphology of vitrified embryos (V0h).** In all V0h embryos ($n = 24$), collapse of the BC and cell swelling were evident by LM. The BC was either missing ($n = 16$; Fig. 1b) or small and eccentrically positioned ($n = 8$; Fig. 1b) and ICM and TB cells were hardly distinguishable. The PrS was widened and contained prominent masses of cellular debris, and intercellular
Fig. 3. Morphological features associated with cell death in cultured porcine blastocysts. a: Light micrograph of a 24-h re-expanded blastocyst showing thin zona pellucida (ZP), single layer of T assays (TC) and inner cell mass (ICM) protruding into blastocoele cavity (BC). Note cells extruded to BC (arrowhead) and PV/S (arrowhead) (×880). b: Electron micrograph showing a detail of PV/S extruded cell inside the zona pellucida (ZP) with dense cytoplasmic matrix and some degenerated mitochondria (M) (×33,400). c: Electron micrograph of BC extruded cell with typical oval shape showing blebbing of the plasma membrane (×64,000). d: Electron micrograph of dead cell showing typical features of apoptosis: shrinkage, higher density of the cytoplasmic matrix, crowding of organelles, chromatin margination and condensation, degeneration of mitochondria (M), and increased presence of vacuoles (V) and SL (×10,000). e: Electron micrograph of lysed cell with low density of the cytoplasmic matrix, degenerated mitochondria (M), and damaged nuclear envelope (N). f, f': Electron micrographs of normal ICM cell with phagocytosed material (arrowhead). Dotted micrograph showing the remnants of organelles (arrowheads) and a condensed nucleus (N) (f: ×3,000, f': ×19,000).
VITRIFIED PORCINE BLASTOCYSTS

spaces between both TB and ICM cells were also widened.

At the ultrastructural level, extensive injuries were seen including a massive increase in the amount of vesicles, vacuoles, and secondary lysosomes (SLs) (Fig. 2d). A part of the mitochondria appeared distended, resulting in electrolucent areas in the mitochondrial matrix, whereas others were shrunken and displayed increased electron density. In many cases, the smooth endoplasmic reticulum (SER) was swollen. Tight junctions and desmosomes were also less developed than in the C0h group, however, microvilli appeared intact.

Morphology of cultured control embryos (C24h). In the C24h group, expanded (n = 6), hatching or hatched (n = 5) blastocysts were evaluated by LM and they displayed a well-defined TB and ICM, expanded BC and a narrow or even lacking Pvs (Fig. 1c). Almost no intercellular spaces were observed between TB and ICM cells and in some blastocysts a hypoblast layer was developing.

At the ultrastructural level, only the size and number of lipid drops were decreased, and occurrence of SLs was slightly increased when compared with C0h embryos. Further, mitochondria seemed to have reached a higher step in development characterized by prolongation and increased abundance of cristae.

Morphology of vitrified and cultured embryos (V24h). Approximately 2/3 of the V24h embryos, processed for LM observation, had recovered (re-expanded or even hatched, n = 14; Fig. 1d), whereas the remaining 1/3 were degenerated (n = 7; Fig. 1d').

Recovered embryos displayed almost normal blastocyst morphology, very similar to C24h embryos, including clearly distinguishable ICM and TB cells with an abundant population of microvilli. However, the Pvs was widened and still contained substantial amounts of cellular debris.

At the ultrastructural level, an increased electron-lucidity of the ICM cytoplasmic matrix, partial distension of mitochondria, limited reduction or dilatation of the SER and persistent presence of small vesicles, vacuoles, and SLs were observed. Nevertheless, the majority of organelles were regenerated and the intercellular contacts between TB cells were totally restored. The number and size of lipid drops were the same as in C24h blastocysts (Fig. 2c).

Degenerated embryos were disintegrated into a poorly defined mass of cellular debris and cells displaying either decreased or increased electron-density of the cytoplasm and with abundant degeneration of mitochondria and other organelles. They also displayed a persistent abundance of small vesicles, vacuoles, and SLs.

LM and TEM assessment of cell death. In both C0h and C24h embryos, the presence of dead cells in the ICM and TB was occasional. Dead cells were predominantly located in the ICM, and such cells displayed typical morphological features of apoptosis—shrinkage,
higher density of the cytoplasmic matrix, crowding of organelles, nuclear condensation, degeneration of mitochondria, and increased presence of vacuoles and primary and SLs (Fig. 3d). Occurrence of phagocytosed cells in the C0h group was very low, but appeared slightly increased in the C24h group (Fig. 3f, f').

A few extruded cells were noticed in the perivitelline space or in the BC (approximately one in almost each embryo). Extruded cells ranged from being rather normal to apoptotic. The shape of extruded cells in PS was oval or flat (Fig. 3b) and in BC round or pleomorphic (Fig. 3c). Occasionally, cell blebbing or fragmentation into apoptotic bodies was observed and the microvilli were reduced on some cells extruded into PS. Cytoplasmic matrix of the cells showed normal or higher density and the nuclear morphology ranged from normal to condensed or fragmented. Highly damaged cells showed mitochondria degeneration and increased presence of vesicles, vacuoles and SLs.

All V0h and V24h embryos displayed increased occurrence of dead and phagocytosed cells in the ICM and TB as well as increased occurrence of extruded cells—approximately one (V0h) and two or more (V24h) into either PS or BC in each embryo. Dead cells located in ICM or TE showed either shrinkage or swelling with increased or decreased density of cytoplasmic matrix respectively. Nuclei of such cells were often condensed or fragmented or in decay. Incidence of phagocytosis was considerably higher in the V24h group.

Extruded cells ranged from being rather normal to apoptotic, with similar morphology as observed in the control groups, or lysed. Lysed cells showed lower density of cytoplasmic matrix, disorganized nuclear morphology, extensive degeneration of mitochondria, and highly increased presence of vesicles and vacuoles of all sizes and of primary and SLs (Fig. 3e).

**TUNEL Assay and Confocal Microscopy**

**Numbers of nuclei.** The total numbers of nuclei per embryo in the noncultured blastocysts were not significantly different: C0h: 97.84 ± 33.99 vs. V0h: 96.74 ± 25.57, P > 0.05; Table 2).

The C24h group of regenerated embryos reached higher total numbers of nuclei than the C24h group (C24h: 117.74 ± 44.34 vs. V24h: 143.25 ± 29.79, P > 0.01).

**Cell death incidence.** Embryos subjected to preincubation in DNase (positive controls) displayed TUNEL reaction in all nuclei, whereas when terminal transferase was omitted (negative controls), no labeling of any nuclei was observed.

The DCI in the V0h group was higher than in the C0h group (2.36 vs. 0.93%, P < 0.001). However, there was no significant difference in the ACI between the two groups (V0h: 0.93% vs. C0h: 0.67%, P > 0.05). Thus, the increase in DCI was caused mainly by the presence of T+ M- nuclei.

In the C0h group, the percentage of TUNEL positive cells and the percentage of apoptotic cells were similar in both TB + PS (TB including cells extruded into PS) and ICM + BC (ICM including cells extruded into BC) (DCI: 0.59 vs. 0.35, P > 0.05; ACI: 0.35 vs. 0.22, P > 0.05). It was not possible to calculate the proportion of dead cells between TB + PS and ICM + BC in the V0h group because of collapsed blastocysts morphology, not allowing precise TB/ICM differentiation.

The dead cell indexes (DCI) showed that the DNA damage was increased in both C24h and V24h groups when compared with 0h groups (C24h: 1.67% vs. C0h, P < 0.01; V24h: 6.94% vs. V0h, P < 0.001). However, a detailed analysis showed that in the C24h group, only the incidence of apoptotic nuclei (ACI) was increased during 24 h culture (C24h: 1.22% vs. C0h, P < 0.05), not the proportion of T+ M- nuclei (C24h: 0.45% vs. C0h, P > 0.05). On the contrary, the ACI in V24h embryos (2.64%) and the percentage of T+ M- nuclei in V24h embryos (4.30%) were both significantly higher than in V0h embryos. Moreover, in the V24h group, all parameters (including DCI, ACI and % of T+ M- nuclei) reached significantly higher values than in the C24h group (P < 0.001). Thus, vitrification apparently accelerated the incidences of all markers of cell death even further than IVF alone.

In both C24h and V24h groups, the Apoptotic cell index (ACI) was higher in ICM and blastocoel area (ICM + BC) than in TB and perivitelline space area (TB + PS) (C24h: 0.83 vs. 0.46%, P < 0.05; V24h: 1.89 vs. 0.79%, P < 0.001; Table 3), whereas the percentage

| Table 2. Total Numbers of Nuclei and Cell Death Indexes of Control (C0h, C24h) and Vitrified (V0h, V24h) Porcine Blastocysts Evaluated by TUNEL Labeling and Propidium Iodide Staining |
|---------------------------------------|-------|-------|-------|-------|
| No. of evaluated blastocysts | 32    | 31    | 34    | 32    |
| Number of nuclei * | 97.84 ± 33.99 | 96.74 ± 25.57 | 117.74 ± 44.34 | 143.25 ± 29.79 |
| DCI (% of T+ M±, T+ M- nuc.) | 0.63% | 2.36% | 1.67% | 6.94% |
| ACI (% of T+ M± nuc.) | 0.67% | 0.83% | 1.29% | 2.64% |
| % of T+ M- nuclei | 0.26% | 1.43% | 0.45% | 4.30% |
| Blastocysts with T+ or M± nuc. | 56.25% | 90.32% | 73.53% | 100.00% |

C0h, controls fixed immediately after collection; C24h, controls fixed after 24 h culture in vitro; V0h, vitrified embryos fixed immediately after vitrification and warming; V24h, vitrified embryos fixed after 24 h of culture upon warming after vitrification.

NS, not significant, P > 0.05.

*Means ± S.D.; DCI, dead cell index; ACI, apoptotic cell index; T+/-, TUNEL positive/negative nuclei; M+/-, nuclei with/without apoptotic morphology.

**Significant, P < 0.05; Student's t-test for the number of nuclei, Chi-square test for the percentage of damaged nuclei.**
of T + M - nuclei was higher in TB and perivitelline space area (TB + Pvs) than in ICM and blastocoele area (ICM + BC) (C24h: 0.55 vs. 0.07%, P < 0.01; V24h: 3.49 vs. 0.81%, P < 0.01). In C24h embryos, TUNEL-positive nuclei (DCI) were distributed evenly (TB + Pvs 0.77% vs. ICM - BC 0.90%, P > 0.05), but in V24h embryos, the high percentage of T + M - nuclei located in perivitelline space increased Dead cell index in TB + Pvs (4.28%) compared with Dead cell index in ICM + BC (2.66%, P < 0.001).

### DISCUSSION

The ultrastructural features observed in nonvitrified in vivo derived porcine blastocysts were essentially consistent with those reported by Hyttel and Niemann (1999).

Immediately fixed in vivo derived porcine blastocysts occasionally contained cells displaying both ultrastructural characteristics of apoptosis as well as biochemical features such as DNA degradation detected by TUNEL. The observations of such cells are comparable to observations made in other species like cattle (Gjerret et al., 2003; Knijn et al., 2003) and mice (Brisson and Schultz, 1998; Kamajo et al., 2002), but the Dead cell index and Apoptotic cell index observed in porcine blastocysts appeared to be somewhat lower than the average percentages of apoptotic cells presented in bovine and murine in vivo derived embryos. Indexes correlated with previous reports of Long et al. (1998) in pg. Random samples of in vivo derived porcine blastocysts in their experiment show comparable small area of TUNEL labeling from total area of Hoechst-stained DNA (1.2 ± 2.1%).

Dead cells in porcine embryos were evenly distributed in both ICM and TB as previously reported only in human in vivo and in vivo produced embryos (reviewed in Hardy, 1999). Increased incidence of cell death in ICM, as described in cattle (Gjerret et al., 2003; Knijn et al., 2003) or mouse and rat (reviewed in Hardy, 1997; Pampfer and Donnay, 1999; Brison, 2000) in vivo derived blastocysts, was not observed.

Vitrification of porcine blastocysts in our experiment caused direct osmotic injuries characterized mainly by the collapse of BC, cell swelling, and partial subcellular damage. These alterations were similar to features observed in in vivo derived and vitrified bovine (Fair et al., 2001) and ovine (Cocero et al., 2002) Day 7 blastocysts. Bovine embryos show a blastocyst collapse, accumulation of debris in Pvs and swelling of mitochondria. However, the damage of microvilli was more evident in bovine blastocysts compared to our observations. In addition to similar mitochondria alteration, ovine embryos show increase of vacuoles and vesicles with various type of content, but they differed from vitrified porcine (present data) and bovine (Fair et al., 2001) blastocysts by higher desmosomal stability in TE. Minor discrepancies were probably caused by the use of different cryopreservation protocol, but could also be due to species specificity. However, bovine in vitro produced Day 7 blastocysts vitrified by similar vitrification method evaluated by Vajta et al. (1997) showed almost identical morphology as our porcine embryos. Vajta et al. assumed that the disintegration of functional specializations between adjacent TB cells lead to collapse of the blastocoele. The mitochondria in general as well as other organelles and cells of such embryos react by either distension or shrinkage, which in the case of whole cells (at least a proportion of them) results in degeneration. Some of these degenerated cells are extruded (Vajta et al., 1997). Our experiments also proved the damage of degenerated cells on the biochemical level. The percentage of nuclei showing DNA fragmentation without typical morphological features of apoptosis in V0h embryos was significantly increased compared with the COh group. The observed ultrastructural and biochemical effects probably resulted from the vitrification process, but they may also to some degree have resulted from the exposure to the vitrification media and cryoprotectants.

After 24 hr culture, 2/3 of observed vitrified embryos showed normal regenerated morphology. Survival of in vivo derived and vitrified (OPS) porcine blastocysts after 24 or 48 hr culture had been documented in previous studies of Berthelot et al. (2000) and ranged from approximately 30 to 70%, depending on vitrification protocol, cryoprotectant dilution media, and genotypes.

In our experiment, the cell numbers of both control (C24h) and regenerated vitrified blastocysts (V24h) were increased after 24 hr culture. Paradoxically, regenerated vitrified embryos reached higher total

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<th>C24h</th>
<th>V24h</th>
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<tr>
<td></td>
<td>TB + Pvs</td>
<td>ICM + BC</td>
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<tr>
<td>DCI (% of T + M -, T + M - nuc.)</td>
<td>0.77%</td>
<td>0.90%*</td>
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<tr>
<td>ACI (% of T + M + nuc.)</td>
<td>0.40%</td>
<td>0.83%*</td>
</tr>
<tr>
<td>% of T + M - nuclei</td>
<td>0.37%*</td>
<td>0.07%</td>
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DCI, dead cell index; ACI, apoptotic cell index; T+/-, TUNEL positive/negative nuclei; M+/-, nuclei with/without condensed or fragmented morphology. NS, not significant, P > 0.05. *Significant, P < 0.05, Chi-square test.
number of nuclei than cultured control group embryos. We can hypothesize that the need for replacement of high amount of extruded and dead cells forced vitrified blastocysts to increase their mitotic activity.

As shown in previous studies, the morphology of bovine in vitro produced and vitrified embryos after 24 hr culture was also normalized, except for similarly persistent increased amount of cellular debris in PVS (Vajta et al., 1997). In both porcine and bovine embryos, junctional contacts between TB cells were restored, a blastocoele was formed, and mitochondrial morphology was to some degree normalized. Still, degenerated and extruded cells were present.

The effect of culture conditions on the incidence of cell death has been investigated in several species. Both bovine and mouse in vitro produced blastocysts show higher occurrence of TUNEL-positive nuclei than in vivo derived blastocysts (Brison and Schultz, 1997; Byrne et al., 1999; Gjerrit et al., 2003). Further, Hao et al. (2003) also reported that later stages of in vitro produced porcine blastocyst development (Day 6) usually show higher incidence of DNA fragmentation compared with earlier stages (Day 5).

A similar increase in the incidence of TUNEL-labeled nuclei could be observed after 24 hr culture in the current study. However, a more thorough analysis by combination of TUNEL reaction and morphology evaluation revealed that the incidence of nuclei with apoptotic morphology was slightly increased in ICM + BC and T + M - nuclei were predominantly located in TB + PVS.

Compared with cultured controls, cultured vitrified embryos showed highly significant increases in all parameters—dead cell index, apoptotic cell index, and the percentage of T + M - nuclei. The majority of the cells displaying DNA degradation with or without nuclear condensation or fragmentation were probably damaged during vitrification. We hypothesize that all of them underwent the same apoptotic process, but were fixed at different stages of it.

Observations made by Kressel and Groscurth (1994) are in favor of such a hypothesis. Thus, they report that apoptosis is an asynchronous event in a cell population. This could be due to asynchrony of cell cycles. As shown in previous studies (Baran et al., 2003; Fabian et al., 2004), a part of apoptotic blastomeres in cultured embryos terminally appear to undergo necrosis. Liu et al. (1999) similarly hypothesized that embryonic cells exposed to an apoptotic inducer (diamine) initiate the apoptotic pathway, but not all of them can complete the terminal sequences of the apoptotic process in vitro conditions. In somatic cells this leads to secondary necrosis. Accordingly, we suggest that T + M - blastomeres in the C24h and the V24h groups in the present study are likely to be primary apoptotic cells undergoing secondary necrosis.

The difference observed in distribution of apoptotic and secondary necrotic cells may be caused by the different environment in ICM and TB. Extrusion of damaged cells to PVS may be easier in the TB layer, and the subsequent loss of contact with neighboring cells possibly leads to lack of phagocytosis and later to secondary necrosis. In contrast, dead ICM cells usually display typical morphological features of apoptosis and are adequately phagocytosed.

CONCLUSIONS

Freshly collected porcine blastocysts contained a very low proportion of dead cells displaying both ultrastructural and biochemical evidence of apoptosis, but 24 hr of subsequent embryo culture significantly increased the incidence of such cells.

Immediately after vitrification and warming porcine embryos displayed collapse of BC, severe subcellular damages and increased presence of nuclei with degraded DNA but without typical apoptotic morphology. This possibly reflected the direct damaging effect of vitrification. However, following 24 hr of culture a major portion of vitrified embryos was able to regenerate. Along with the regenerative process, apoptosis—a possible pathway for elimination of damaged cells, became much more widespread.

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REFERENCES
