Dual Labeling of the Cytoskeleton and DNA Strand Breaks in Porcine Embryos Produced In Vivo and In Vitro

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ABSTRACT
In vitro-produced embryos exhibit decreased cell numbers, small inner cell masses and reduced pregnancy rates after transfer. Evaluation of intracellular components of in vitro-produced or -manipulated embryos will lead to improved methodology for embryo production. Whole mount techniques were developed to utilize terminal deoxynucleotidyltransferase 3’ nick end labeling (TUNEL) to detect broken DNA. Subsequent labeling of either tubulin or actin filaments provides further evidence of cytological damage. Porcine embryos produced in vitro or in vivo were evaluated throughout the cleavage and preimplantation stages of development. Early cleavage stages up to the 8-cell stage never contained TUNEL-labeled nuclei. However, TUNEL labeling of in vitro-produced morula revealed some blastomeres with broken DNA. Nearly all in vitro-produced blastocysts displayed some TUNEL positive cells, whereas in vivo-collected embryos at a similar stage displayed few, if any, TUNEL-labeled nuclei. The ratio of TUNEL-labeled DNA to total DNA area of in vitro-derived blastocysts was significantly greater than their in vivo counterparts (P < 0.05). Microtubule and microfilament labeling identified blastomeres of unequal size and shape that were losing cellular integrity. These data suggest that the combination of these labeling techniques may be useful in evaluating cellular damage in embryos produced under in vitro conditions. Mol. Reprod. Dev. 51:59–65, 1998. © 1998 Wiley-Liss, Inc.

Key Words: apoptosis; microtubules; microfilaments; immunocytochemistry; TUNEL

INTRODUCTION
In vitro production of livestock embryos consistently results in embryos with reduced developmental competence following transfer. In vitro-produced embryos from several mammalian species typically exhibit lower cell numbers, altered inner cell mass: trophoderm ratios, blastomeres of irregular size, and cytoplasmic fragmentation (Hardy et al., 1989; van Soom et al., 1996; Funahashi et al., 1997a). Moreover, embryos produced in vitro have increased sensitivity to cryopreservation and manipulation (Hasler et al., 1995). Extended culture to late preimplantation stages is generally related to a further decrease in developmental competence. When compared to in vivo-derived embryos, cultured rabbit (Hegele-Hartung et al., 1988), bovine (Du et al., 1996), and pig (Dobrinsky et al., 1996) embryos exhibit retarded developmental progress. Also, in vitro-produced bovine embryos demonstrate reduced pregnancy rates upon transfer and increased incidence of abnormal offspring, as evidenced by high mortality rate and large calves (Garry et al., 1996; Kruip and den Daas, 1997).

Development in vitro to the morula or blastocyst stage remains a standard experimental endpoint in determining the efficacy of experimental treatments in livestock embryo production systems (Overstrom, 1996). Differential staining of the trophoderm and inner cell mass indicates that the proportion of inner cell mass of cultured bovine embryos is smaller than in vivo counterparts, however, and varies with different culture conditions (Du et al., 1996; van Soom et al., 1996). Evaluation of in vitro produced embryos by standard light microscopy also indicates that blastomeres frequently become dissociated from the embryo proper and blastomere fragmentation is common (Liu and Foote, 1995). Development of techniques to evaluate the nature of these cellular events would be beneficial to understanding the limitations associated with embryo culture systems.

Programmed cell death (PCD) plays an important role in the development and differentiation of higher eukaryotes. Apoptosis is a series of morphological events that is characteristic of some types of PCD. Frequently measured properties of apoptosis include the characteristic intranuclear DNA fragmentation, pronounced nuclear and cytoplasmic condensation, endoplasmic reticulum swelling, and cytoplasmic blebbing. Although the response to many PCD signals are well characterized, the mechanisms for the induction of PCD during embryonic development are still unclear (Ellis et al., 1991).

Programmed cell death, as evidenced by DNA fragmentation, has been reported in early mouse (Brison and Schultz, 1997) and human embryos (Jurisicova et al., 1996). The blastocyst stage of mouse embryos has been reported as the first stage that PCD can be
detected during mammalian cellular differentiation and tissue organization. Jurisicova et al. (1995) suggest that PCD, and subsequently necrosis, occur in earlier stages in response to abnormalities or impaired development of embryonic blastomeres. Possible mediators of PCD include excess spermatozoan enzymes, oxygen free radicals, decreased glutathione, and suboptimal culture conditions. It therefore seems likely that cell death during in vitro production of embryos could be related to inefficient oocyte maturation, fertilization, and culture conditions.

The objective of the current experiment was to observe and quantitate DNA strand breaks in the nuclei of porcine embryonic blastomeres concurrently with localization of cytoskeletal components. Utilization of both techniques, in combination, would provide additional information about both the nuclear and cytoplasmic events in embryonic blastomeres. In vivo- and in vitro-produced embryos at similar developmental stages were compared using terminal transferase 3’ end labeling to identify broken DNA of embryos in situ and fluorescent localization of either microtubules or microfilaments. Our results indicate that blastomere fragmentation and DNA damage, in the form of broken strands, was prevalent in cultured embryos. Cytoskeletal labeling provided additional information about cell size and cytoplasmic organization. These techniques could be utilized to evaluate modifications to embryo production systems, as well as evaluation of the detrimental effects of embryo manipulations.

**MATERIALS AND METHODS**

**In Vitro Maturation**

Methods for in vitro maturation of porcine oocytes in our laboratory have been previously described (Long et al., 1998). Ovaries from pre- and peripuberal gilts were collected at an abattoir and transported to the laboratory in 0.9% saline at 25°C within 6 hr post mortem. Cumulus oocyte complexes (COC) were aspirated from 2–8 mm follicles using an 18-gauge short bevel needle attached to a 50 ml conical tube in line to a vacuum pump set to a flow rate of 50 ml of H2O per minute. Cumulus oocyte complexes were allowed to settle and follicular fluid was removed; COC were then washed twice with Hepes-PVA (Bavister, 1989) and held until addition of sperm.

Following the final wash, oocytes with multiple layers of intact cumulus cells were selected for maturation. The selected COC were washed twice more in Hepes-PVA and washed three times in maturation medium prior to culture. Maturation medium (MAT) consisted of NCSU 37 (Petters and Wells, 1993), without BSA, supplemented with 5 mg/ml insulin, 10.0% porcine follicular fluid, 50 µM b-mercaptoethanol, 0.6 mM cysteine, 10 ng/ml EGF, 10 U/ml PMSG, 10 U/ml hCG, and 1.0 mM db-cAMP (Abeydeera et al., 1997; Funahashi et al., 1997b). All chemicals for media were purchased from Sigma (St. Louis, MO), excluding PMSG and hCG, which were from Intervet, Inc. (Millsboro, DE). Fifty to sixty COC were cultured per well containing 500 µl of MAT in 4-well Nunc plates (Nunc, Roskilde, Denmark) for 22 hr. Oocytes with an expanded cumulus cell complex were washed in fresh MAT without PMSG, hCG, and db-cAMP, and cultured an additional 22 hr prior to preparation for fertilization.

**Sperm Preparation**

Semen from the sperm-rich fraction of the ejaculate was collected from mature boars of proven fertility and transported immediately to the laboratory in a warmed thermos. Antibiotic/antimitotic solution (Gibco) was added at 10 µl/ml, the semen was placed in a room-temperature water bath, and held in a 18°C cooler for storage until washing. Semen was generally held 3 to 5 hr. One milliliter semen was diluted in 9 ml 0.9% saline containing 100 mg/L BSA (Sigma catalog number A-7906, St. Louis, MO) and 100mg/L kanamycin sulfate (saline-BSA), and washed 3 times in 10 ml of saline-BSA by centrifugation at 900g for 5 min. The sperm were resuspended in 3 ml mTLP-FERT (Yoshida et al., 1993) and the sperm concentration and motility were determined using a haemocytometer and visual observation by a single technician.

**In Vitro Fertilization and Culture**

Oocytes cultured a total of 44 hr in maturation medium were stripped of cumulus by addition of 500 µl 0.1% hyaluronidase in MAT and gentle aspiration with a small-bore pipet. Oocytes were washed 3 times in FERT (mTLP-FERT with 2 mM caffeine sodium benzoate) and moved to fertilization drops. Drops were prepared by placing 2–3 µl of medium in a 35 mm petri dish (Falcon) covering with light mineral oil (Fisher) then adding 90 µl of additional medium to each starter drop. Oocytes were added in a small amount of medium and held until addition of sperm.

Sperm were diluted to $4 \times 10^4$ motile sperm in 2 ml of FERT. Diluted sperm were introduced in a volume of 5 µl to the 95 µl drops of FERT containing 50 oocytes. Final sperm concentration was $2 \times 10^4$ motile sperm per ml corresponding to approximately 40 motile sperm per oocyte. Sperm and oocytes were coincubated at 38.7°C for 6 hr in a humidified atmosphere of 5.0% CO2 and air. Presumptive zygotes were removed and washed twice in embryo culture medium to remove excess sperm. Zygotes were cultured in groups of 30–50 in 4-well Nunc plates in 500 µl of NCSU 23 (Petters and Wells, 1993). Cleavage-stage embryos and blastocysts were removed from culture at appropriate time points from 30–144 hr post insemination (hpi) and prepared for TUNEL labeling. In total, 237 embryos (55, 4–8 cell; 17, 8–16 cell; 39, morula and early blastocyst; and 126, expanded and hatched blastocyst) with at least two replicates at each stage, are represented in the analysis. During the course of these experiments, the in vitro embryo production system averaged 44.9 ± 16.1% cleavage rate and 21.8 ± 7.5% development to blastocyst from total fertilized oocytes cultured.
DNA AND CYTOSKELETAL LABELING OF EMBRYOS

In Vivo Embryo Collection

Naturally cycling crossbred gilts at least 6 months of age and 100 kg were used as embryo donors in these experiments. Gilts were bred, reproductive tracts collected, and embryos flushed as previously described (Dobrinsky et al., 1996). Embryos were immediately moved to fixative for further processing. A total of 274 embryos distributed across embryonic stages from 4-cell to hatched blastocyst were used (79, 4–8 cell; 13, 8–16 cell; 36, morula and early blastocysts; 146, expanded and hatched blastocysts).

Fixation and Permeabilization

Zona intact and hatched pig embryos were fixed in 2.0% paraformaldehyde in PBS (pH 7.0) for 30 min at 38°C. After washing in PBS, embryos were held no more than two weeks at 4°C in PBS until TUNEL and cytoskeletal labeling. Embryos were permeabilized for 6 hr in PBS with 1% Triton X-100, washed briefly in PBS-Tw (PBS with 0.1% Tween 20) and then moved to blocking solution (PBS with 2.0% BSA, 2.0% normal goat serum, 2.0% nonfat dry milk, and 0.15 M glycine) for 30 min, followed by additional PBS-Tw washes.

Terminal Transferase Assay (TUNEL)

Methods for TUNEL labeling embryos were modified from previously reported techniques (Gorczyca et al., 1994). After a PBS-Tw wash, embryos were rinsed in Hank’s Balanced Salt Solution with 0.1% Triton (HBSS-Tr) 3 times for 5 min. Incorporation of biotinylated deoxyuridine triphosphate (b-dUTP; Boehringer Mannheim, Indianapolis, IN) to damaged DNA in situ was accomplished by incubating embryos in 50 µl of TUNEL buffer (200 mM sodium cacodylate, 25 mM Tris-HCl, 2.5 mM cobalt chloride and 0.025% BSA) containing 0.5 nmoles b-dUTP and 5 units of calf thymus terminal transferase enzyme (Boehringer Mannheim, Indianapolis, IN) at 37°C for 1 hr. The reaction was stopped by moving embryos to 4× SSC (0.6 M sodium chloride and 0.6 M sodium citrate) and 0.1% Triton X-100 for 15 min. Embryos were rinsed in HBSS-Tr with 0.5% BSA and incubated in the same solution with a 1:1000 dilution of streptavidin conjugated to FITC or Cy3 (depending on the secondary label) for 30 min at 25°C.

Negative control embryos were treated identically except that the terminal transferase enzyme was not added to the b-dUTP incorporation buffer. Positive controls were generated by incubating fixed and permeabilized embryos in 10 units per ml DNAase I in PBS for 30 mins at 37°C. Following TUNEL label, all embryos were washed in HBSS-Tr with 0.5% BSA and prepared for either microtubule or microfilament labeling.

Immunolabeling of Embryos

The embryos were incubated at 37°C for 2 hr or overnight at 4°C in a 1:1 ratio of α and β tubulin monoclonal antibody (Sigma, St. Louis, MO) diluted 1:100 in PBS-Tw. Embryos were washed for a minimum of 1.5 hr in PBS-Tw and moved to secondary label with affinity-purified goat antimouse IgG conjugated to FITC (Sigma, St. Louis, MO) diluted 1:200 in PBS-Tw at 37°C for 2 hr. Following incubation, embryos were washed a minimum of 2 hr in PBS-Tw. Microfilament label was applied by incubating the embryos at 37°C for 45 min in PBS-Tw with 10 units per ml phalloidin-Texas Red X (Molecular Probes, Eugene, OR). All embryos were mounted on glass slides in PBS with 20% glycerol, 100 mg/ml DABCO, and 2.0 µg/ml Hoechst 33342 (Sigma, St. Louis, MO). Coverslips were sealed to slides with nail polish and stored in the dark at 4°C prior to evaluation.

Microscopy and Image Processing

Immunolabeled embryos were observed using a laser scanning confocal microscope system (LSM 410, Carl Zeiss Inc., Thornwood, NY). Fluorochromes were excited using appropriate combinations of excitation and barrier filters and an Argon/Krypton laser for FITC (488 nm) and Texas Red X (568 nm) conjugates, or UV laser for excitation of Hoechst (354 nm). Images were obtained using a 40× water immersion objective (1.2 NA) with either 1.5× or 2.0× zoom and projected onscreen. Optical sections of each fluorochrome were obtained individually, pseudocolored, and digitally recombined into composite images using LSM software (Carl Zeiss Inc.). Images were saved to optical disks and printed using a dye-sublimation printer (Codonics NP-1600 Photographic Network Printer; Codonics, Inc., Middleburg Heights, OH).

Quantitative Analysis of TUNEL Labeling

The ratio of TUNEL labeled fluorescence was quantitatively assessed by comparison to total Hoechst staining of nuclei. Digitally recombined composite images were analyzed using Kontron Elektronik Imaging System (KS 300 v.2.1, Kontron Elektronik, Germany). Total area of TUNEL- and Hoechst-labeled DNA was measured for a random sample of in vitro- and in vivo-derived blastocysts (n = 62 and 24, respectively) and values exported to a database for calculation of proportion of TUNEL-labeled area. Data were analyzed by using SAS, General Linear Models procedures (v. 6.12; SAS Institute Inc., Cary, NC). Least Squares Means and standard errors for proportion of TUNEL-labeled area of in vivo and in vitro embryos were calculated from individual embryos.

RESULTS

Morphological Comparisons

Controls. Representative images of negative and positive controls are presented in Fig. 1a and Fig. 1b, respectively. No TUNEL label was observed in negative control embryos (Fig. 1a; incubated without terminal transferase enzyme), indicating no nonspecific incorporation of b-dUTP or binding of the streptavidin. Alternatively, the nuclei of DNase I treated embryos (Fig. 1b) were heavily labeled. The label was evenly distributed across the entire interphase nuclei. No significant autofluorescence or crossover from other fluorochrome...
channels was observed under the established parameters of the confocal microscope.

**Four- to eight-cell.** Representative images of in vivo and in vitro 4-cell embryos are compared in Fig. 1c and d. No differences were noted in the pattern of Hoechst staining and no TUNEL labeling was observed in any 4- to 8-cell embryos in either group. This indicates that DNA damage is not detectable in early stages of embryonic development. Microtubule labeling patterns are also similar, showing a distinctive perinuclear localization pattern, typical of interphase cells. These microtubules radiate outward and extend throughout the cytoplasm in both in vitro and in vivo embryos. Although not shown, cleavage-stage embryos, both in vivo and in vitro, were labeled with phalloidin-Texas Red X. The label was predominately at the cell-to-cell junctions, but also in a diffuse pattern across the cytoplasm. The microfilament label became more intense at the blastomere borders as development proceeded.

**Morula and early blastocyst.** Figure 1e and 1f represent morula produced in vivo and in vitro, respectively. No TUNEL label was present in the in vivo derived embryos (Fig. 1e), whereas DNA damage was more prevalent in the in vitro produced embryos (Fig. 1f). Microtubules still maintain the perinuclear staining pattern; cells of the in vivo embryos generally displayed a more distinct label, however, and contain defined bundles of microtubules at the cell borders. **Expanded and hatched blastocyst.** Figure 2a and 2b represent typical patterns of TUNEL and microtubule labeling for in vivo and in vitro blastocyst-stage embryos, respectively. In vivo-derived embryos in advanced stages (Fig. 2a) exhibited only a few TUNEL-labeled nuclei in 70.8% of scanned and analyzed embryos. In vitro embryos again revealed the perinuclear labeling pattern of microtubules, which radiated from the nuclear periphery and bundled at the cell borders. In vitro-produced blastocysts (Fig. 2b) exhibited TUNEL-labeled nuclei in 90.3% (56/62) of embryos scanned and analyzed. The labeled nuclei were predominately condensed and often fragmented. In vitro embryos displayed prominent microtubule label but not in the same organized pattern of the in vivo counterparts. Microtubules of in vitro-cultured embryos were generally bundled or clumped in appearance.

Microfilament labeling by phallolidin Texas Red X (here pseudocolored green) indicated distinct labeling of the cell borders of in vivo produced embryos (Fig. 2c). This pattern was also present in embryos cultured in vitro, but displays the uneven cell shape typical of in vitro produced embryos (Fig. 2d).

**Quantitation of TUNEL-Labeled DNA**

Due to low or absent TUNEL labeling of cleavage-stage embryos, only a random sample of blastocysts were evaluated for comparing the total area of TUNEL labeling between in vitro- and in vivo-derived embryos. Blastocysts produced in vitro exhibited TUNEL label over 7.3 ± 1.3% (least squares mean ± SEM) of the total area of Hoechst-stained DNA. This level of TUNEL labeling is significantly different than the level found in embryos derived in vivo of 1.2 ± 2.1% (P < 0.05).

**DISCUSSION**

The interpretation of the observed labeling patterns suggests that DNA strand breaks are a serious side effect of in vitro culture of porcine embryos. The increase in occurrence of labeled nuclei over the course of development in vitro suggests the conditions, although favorable for cleavage and cavitation, may still lack the necessary factors to prevent cellular death of these blastomeres.

The conditions for TUNEL labeling of fixed and permeabilized embryos were effective as demonstrated by the complete label of all cells in the DNase I treated positive control. Terminal transferase labeled blastomere nuclei exhibited a wide variety of morphology, from condensed, fragmented chromatin to diffuse, even label over the entire nuclei. These are typical patterns of advanced and early stages of intranucleosomal DNA fragmentation associated with apoptotic death. The TUNEL labeling of broken DNA has been used extensively in many cell types to detect final stages of cell death (Hale et al., 1996). Caution should be taken in interpretation of these results, however, because no attempt was made to distinguish the mode of DNA damage whether by apoptosis or necrosis (Charriaut-Marlangue and Ben-Ari, 1995). It is conceivable that both apoptotic and necrotic cell death could be responsible for the TUNEL patterns observed.

These results confirm previous reports of a significant increase in the proportion of TUNEL-labeled blastomeres in cultured embryos of other species. Mouse and human cultured blastocysts display DNA fragmentation as assessed by TUNEL label (Jurisicova et al.,
In the mouse, the results of Brison and Shultz (1997) suggest that embryos cultured singly had cell numbers that were not different than in vivo-derived embryos. However, the percentage of dead cells labeled with TUNEL increased from 1.95% for in vivo embryos to 6.17% for singly cultured embryos. Culturing the mouse embryos in groups of 30 per 25 µl drop reportedly decreased the percentage of dead cells by 50%. In our study, embryos were also cultured in groups, perhaps attenuating the effect of culture conditions on proportion of fragmenting nuclei. Alternatively, since significantly greater TUNEL labeling was observed in cultured embryos, groups of 30-50 embryos in 500 µl of medium may have diluted reported survival factors diminishing the beneficial effect (Brison and Schultz, 1997).

The TUNEL label in expanded and hatched blastocysts produced in vivo indicates that PCD of a few blastomeres may be a mechanism of cellular reorganization as the embryo begins to remodel and form the primary germ layers. Weil et al. (1996) have suggested that all cells constitutively express all the proteins required to undergo apoptotic cell death in the absence of survival factors. Also, blastomeres of cleaving embryos were reported to be less sensitive to apoptotic death than more advanced developmental stages. Weil et al. (1996) have suggested that all cells constitutively express all the proteins required to undergo apoptotic cell death in the absence of survival factors.
DNA undergoes cleavage during extended in vitro port previous results in the mouse and human that from limited experimental materials. These data sup-
mammalian embryos, providing increased information cytoplasmic components can be labeled concurrently in indicate that multiple cytoskeletal and perhaps other dard light microscopy. Furthermore, these procedures opmental state of embryos not obtainable with stan-
cytoplasmic components, provides insight into the devel-
meres of both the trophectoderm and ICM. In both in vivo and in vitro embryos, and the size decreased with advanced development.

Overall, TUNEL labeling was observed in blasto-
meres of both the trophectoderm and ICM. In both groups of embryos, the TUNEL-labeled nuclei present in the trophectoderm of embryos often appeared to be extruded cells that no longer contributed to the embryo proper. Microtubule and microfilament labeling would also suggest that the labeled cells had become disen-
gaged from the remaining embryo. Together this infor-
mation suggests that the dying cells are perhaps being actively removed from the embryo.

In summary, this study provides evidence for the decreased viability of porcine embryos in culture. Identi-
fication of broken DNA by TUNEL labeling in blasto-
meres nuclei, in addition to fluorescent labeling of cytoskeletal components, provides insight into the de-
velopmental state of embryos not obtainable with stan-
ard light microscopy. Furthermore, these procedures indicate that multiple cytoskeletal and perhaps other cytoplasmic components can be labeled concurrently in mammalian embryos, providing increased information from limited experimental materials. These data sup-
port previous results in the mouse and human that DNA undergoes degradation during extended in vitro culture, perhaps by apoptosis.

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