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ScienceDirect
Theriogenology 75 (2011) 1211–1220
Theriogenology
www.theriojournal.com

Lipid content and apoptosis of *in vitro*-produced bovine embryos as determinants of susceptibility to vitrification

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Received 21 May 2010; received in revised form 16 November 2010; accepted 16 November 2010

Abstract

The objective was to evaluate supplementation of fetal calf serum (FCS) and phenazine ethosulfate (PES), a metabolic regulator that inhibits fatty acid synthesis, in culture media during *in vitro* production (IVP) of bovine embryos. Taking oocyte fertilization (n = 4,320) as Day 0, four concentrations of FCS (0, 2.5, 5, and 10%) and three periods of exposure to PES (without addition—Control; after 60 h—PES Day 2.5 of embryo culture; and after 96 h—PES Day 4) were evaluated. Increasing FCS concentration in the culture media enhanced lipid accumulation ($P < 0.05$), increased apoptosis in fresh (2.5%: 19.1 ± 1.8 vs 10%: 28.4 ± 2.3, $P < 0.05$; mean ± SEM) and vitrified (2.5%: 42.8 ± 2.7 vs 10%: 69.2 ± 3.4, $P < 0.05$) blastocysts, and reduced blastocoele re-expansion after vitrification (2.5%: 81.6 ± 2.5 vs 10%: 67.3 ± 3.5, $P < 0.05$). The addition of PES in culture media, either from Days 2.5 or 4, reduced lipid accumulation ($P < 0.05$) and increased blastocoele re-expansion after vitrification (Control: 72.0 ± 3.0 vs PES Day 2.5: 79.9 ± 2.8 or PES Day 4: 86.2 ± 2.4, $P < 0.05$). However, just the use of PES from D4 reduced apoptosis in vitrified blastocysts (Control: 52.0 ± 3.0 vs PES Day 4: 39.2 ± 2.4, $P < 0.05$). Independent of FCS withdrawal or PES addition to culture media, the *in vivo* control group had lesser lipid accumulation, a lower apoptosis rate, and greater cryotolerance ($P < 0.05$). The increased lipid content was moderately correlated with apoptosis in vitrified blastocysts ($r = 0.64$, $P = 0.01$). In contrast, the increased apoptosis in fresh blastocysts was strongly correlated with apoptosis in vitrified blastocysts ($r = 0.94$, $P < 0.0001$). Therefore, using only 2.5% FCS and the addition of PES from Day 4, increased the survival of IVP embryos after vitrification. Moreover, embryo quality, represented by the fresh apoptosis rate, was better than lipid content for predicting embryo survival after vitrification.

Keywords: Phenazine ethosulfate; Fetal calf serum; Cryotolerance; Embryo culture; Embryo survival

1. Introduction

Since the birth of the first calf from an *in vitro* produced embryo [1], there have been great advances in IVP systems, with improved culture methods and new knowledge regarding embryo physiology, ultrastructure and morphology [2–5]. Currently, IVP is successfully applied commercially to bovine embryos. Nevertheless, a major obstacle for dissemination of this technology is the great sensitivity of IVP embryos to cryopreservation [3–6]. This reduced cryotolerance is frequently associated with lipid accumulation in the cytoplasm

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doi:10.1016/j.theriogenology.2010.11.033

[6–9]. Characteristics such as very electron-dense cytoplasm, loose blastomeres, buoyant density, slower growth, excessive metabolism of energy substrates and high thermal sensibility [3–10] are indicators that the IVP embryos are more sensitive to cryopreservation, which reduces conception rates relative to *in vivo*-produced embryos [11].

How and why lipid accumulation occurred in the IVP embryo cell's cytoplasm are unknown; however, it is speculated that the accumulation occurred as a result of FCS in the culture media [3–8], or due to abnormalities in energy metabolism [7–9,12].

Serum provides energy substrates, amino acids, vitamins, growth factors and heavy-metal chelators; however, the presence and concentration of these components may vary among batches [6]. Although FCS has useful properties, its use has been associated with large offspring syndrome [13] and several other abnormalities, such as modifications of cell organelles [3], mitochondrial degeneration [14], premature formation of the blastocoele, and modification of gene expression pattern [4,5]. Also, FCS may be a source of pathogenic viruses [8] leading to metabolic deviations, excessive lipid accumulation [3,6,8], and decreased embryo survival after cryopreservation [3,4,6]. Consequently, chemically-defined media (without FCS) were developed [15].

In contrast, lipid accumulation in embryos might also be caused by altered energy metabolism. In pre- and post-compaction IVP embryos, glucose metabolism was characterized by the Crabtree effect [10], which was proposed by Bavister [16] as being caused by an excess of glucose metabolism via glycolysis, with inhibition of oxidative phosphorylation. Lipid accumulation is an effect of excess glycolysis caused by rising cellular concentrations of lipid synthesis precursors [12]. An imbalance in the cellular oxidation-reduction process also occurred, affecting mitochondrial metabolism and impairing metabolism of lipid complexes through β-oxidation [3,7,9,14]. There are two ways to improve embryo survival after cryopreservation: a) modifications in the cryopreservation technique that generally improve results, but are limited; and b) changes in the IVP system that result in an embryo which is more resistant to cryopreservation [17].

Therefore, the objective of the present study was to evaluate the effects of the supplementation of culture media with various concentrations of FCS and PES on development of IVP embryos, cytoplasmic lipid accumulation, apoptosis, and cryotolerance.

2. Materials and methods

2.1. Experimental design

A 4 × 3 factorial experimental design was used to test four concentrations of FCS (0, 2.5, 5 and 10%) and three periods of exposure to PES (without addition of PES (Control group), and 0.3 μM of PES beginning either 60 h (PES Day 2.5) or 96 h (PES Day 4) after the start of embryo culture). Overall, 4,320 oocytes were used in 12 replicates (Fig. 1).

2.2. Reagents used

All materials were acquired from Sigma (Sigma-Aldrich Corp., St. Louis, MO, USA), except when specified.

2.3. In vitro maturation (IVM)

Ovaries were recovered from slaughtered Nellore cows (*Bos taurus indicus*). Oocytes were aspirated from follicles 2 to 8 mm in diameter; only oocytes with three or more compact layers of cumulus cells and homogeneous cytoplasm were used. Selected oocytes (n = 4 320) were maturated *in vitro* through incubation at 38.5 °C in 5% CO_2 with saturated humidity for 24 h. Drops of 90 μL of TCM 199 with Earle salts and L-glutamine (Gibco®, Invitrogen Co., Grand Island, NY, USA) supplemented with 5 mg/mL BSA (fatty acid free, Sigma A-8806), 0.2 mM sodium pyruvate, 5 mg/mL LH (Lutropin-V®, Bioniche Co., Belleville, ON, Canada), 1 mg/mL FSH (Folltropin®, Bioniche Co.), 100 μg/mL of streptomycin sulfate, and 100 IU/mL of penicillin containing 30 oocytes each, were placed in petri dishes and covered with mineral oil.

2.4. In vitro fertilization (IVF)

At the end of the maturation period, groups of 30 oocytes were transferred to 90 μL drops of fertilization media covered with mineral oil. Oocytes were subjected to IVF with frozen semen from a single sample of a Nellore (*Bos taurus indicus*) bull with proven fertility. Sperm were selected by the Percoll method [18], and the concentration was adjusted to 2×10^6 sperm/mL. Fertilization occurred in HTF (Irvine Scientific Co., Santa Ana, CA, USA) supplemented with 5 mg/mL BSA (fatty acid free, Sigma A-8806), 0.2 mM pyruvate, 30 μg/mL heparin, 18 μM penicilamine, 10 μM hypotaurine, 1.8 μM epinephrine, 100 μg/mL streptomycin sulfate, and 100 IU/mL penicillin. Oocytes and sperm were incubated under the same conditions as IVM for approximately 18 h. The day of fertilization was defined as Day 0.

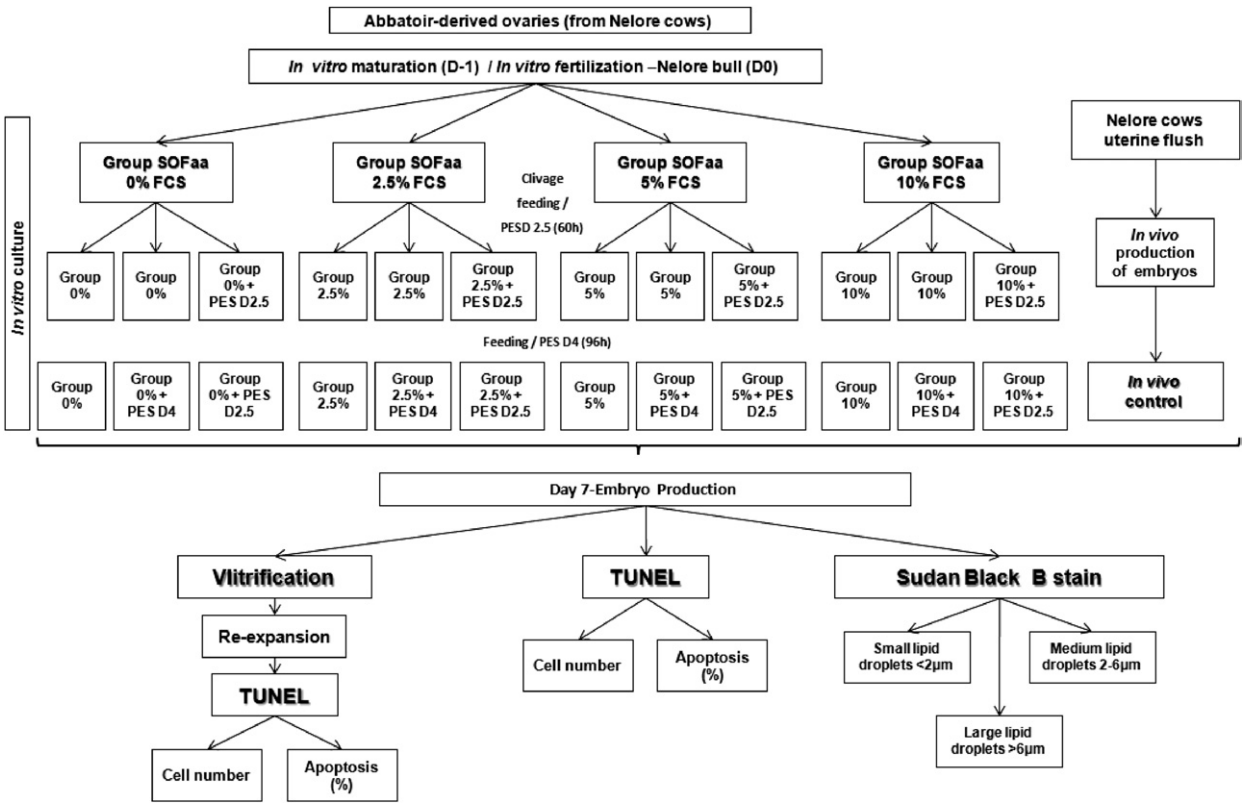

Fig. 1. Experimental design.

2.5. In vitro culture (IVC)

Eighteen hours post-IVF, presumptive zygotes were denuded by repeated pipetting and transferred to culture plates in drops of 90 μL of culture media (30 structures per drop), covered with mineral oil, and cultured in an atmosphere of 5% CO_2, 5% O_2 and 90% N_2. The culture medium used was SOFaa [19] with 2.7 mM myo-inosytol, 0.2 mM pyruvate, either 0, 2.5, 5 or 10% of FCS, 5 mg/mL BSA (fatty acid free, Sigma A-8806), 100 μg/mL streptomycin sulfate, and 100 IU/mL of penicillin. After 60 h of culture (Day 2.5), cleavage was checked, and structures that were not cleaved were discarded. Embryos were transferred to new drops of 90 μL containing the respective culture media (0, 2.5, 5 or 10% of FCS). At this point, 0.3 μM of PES (Sigma-P4544) were added in only the PES Day 2.5 groups (for each concentration of FCS; Fig. 1). The culture conditions were the same as described above. On Day 4, 96 h after the onset of embryo culture, one more change of the culture media was done, conserving the respective media used before for each group, while starting another treatment with the addition of 0.3 μM PES to make group PES Day 4 (Fig. 1). Embryos remained in these conditions until Day 7, at which time blastocyst production was evaluated, and then they were vitrified or lipid content and apoptosis were assessed.

2.6. In vivo production of embryos

For the *in vivo* control group, embryos were collected from nine adult, non-lactating Nellore (*Bos taurus indicus*) cows with good body condition scores. The superstimulation protocol was based on that used by Nogueira and Barros [20]. On Day 0 (without regard to the stage of the estrous cycle), cows were given an auricular implant containing 3 mg of norgestomet (Crestar®, Intervet/Schering-Plough Animal Health Co., Cruzeiro, SP, Brazil) and 2 mg of estradiol benzoate im (Estrogin®, Farmavet Co., São Paulo, SP, Brazil). Starting on Day 4, cows received 200 mg of FSHp (Folltropin-V, Bioniche Co.) im twice a day, in eight decreasing doses (40, 30, 20 and 10%), and on Day 6, two doses of 150 μg of sodium cloprostenol im (Ciosin®, Intervet/ Schering-Plough Animal Health Co.) were given, 12 h apart. The implant was removed 36 h after the first application of $PGF_{2\alpha}$, and then 12.5 mg of LH (Lutropin-V®,

Bioniche Co.) were given im 48 h after the first dose of $PGF_{2\alpha}$. Cows were artificially inseminated (12 and 24 h after LH) with frozen semen of proven fertility from the same Nellore bull used for IVF in the *in vitro* experimental groups. Embryo recovery (n = 45) was done through uterine flushing with a phosphate-buffered saline solution (PBS, Nutricell Co., Campinas, SP, Brazil) the morning of Day 16, 7.5 d after the first AI.

2.7. TUNEL (terminal deoxinucleotil transferase uracil nick end labeling)

Samples of fresh and vitrified blastocysts (n = 60–134, except for the *in vivo* control group of n = 15) were submitted to the TUNEL reaction (*In Situ* Cell Death Detection Kit with Fluorescein, Roche®, Mannheim, BW, Germany), according to the technique adapted by Paula-Lopes and Hansen [21]. In summary, embryos were fixed and permeabilized. A positive control was performed by treating samples with 1 IU/μL of DNase (Promega Co., Madison, WI, USA). All samples were incubated in micro-drops of the TUNEL Kit containing 10% of the enzymatic solution (deoxinucleotidil terminal transferase enzyme) with 90% of the marking solution (2′-deoxyuridine 5′-triphosphate-dUTP+fluorescein isothiocyanate-conjugated-FITC) for 1 h in a humid chamber at 37 °C in the dark. The negative control was incubated in micro-drops containing only marking solution. After washing, control and experimental samples stained with Hoechst 33342 were mounted in glycerol on histological slides and observed under a fluorescence microscope. Nuclei with green fluorescence (FITC) were considered TUNEL positive (fragmented DNA). Hoechst 33342 stained all healthy and apoptotic cells, based on the presence of blue fluorescence. To count apoptotic and total number of cells, Image J 1.41 software (Wayne Rasband National Institutes of Health, Bethesda, MD, USA) was used.

2.8. Lipid quantification

A sample of blastocysts (n = 45–60, except for the *in vivo* control group of n = 15) was randomly selected during the experimental replications and stained with Sudan Black B (Sigma S-0395), adapted from [3]. These embryos were previously fixed in 10% formaldehyde in PBS, pH 7.4, for 2 h at room temperature. After fixation, they were washed in distilled water containing 0.05% PVA and then transferred to drops of 50% ethanol. After 2 min, embryos were stained in drops of 1% Sudan Black B (w/v) in 70% ethanol for 1–2 min, then they were washed 3× with 50% ethanol, 5 min each, followed by a 5 min wash in 0.05% PVA in distilled water. Prepared embryos were mounted in 10 μL glycerol on cover slips and examined under a light microscope at 600× magnification. To estimate the relative amount of lipid droplets in the cytoplasm in each embryo, a grid with five squares of 1,600 μm^2 (40 × 40 μm) each was designed with AutoCAD 2000 Software (Autodesk, Inc., San Francisco, CA, USA); each square was subdivided by alternating continuous and dotted lines 5 μm apart (Fig. 2). Lipid droplets were classified as small, medium, and large (<2, 2–6 μm, and >6 μm, respectively). The number of droplets per category in the 1,600 μm^2 square were counted, and the average number of droplets from five squares for each embryo were calculated. Data regarding lipid accumulation are presented as number of lipid droplets per 1,000 μm^2. For the relationship between total lipid droplet value and apoptosis in fresh and vitrified blastocysts, small and medium droplets were converted to an equivalent number of large droplets; taking into account an average size of 1, 4 and 8 μm for a small, medium and large lipid droplets, respectively. Since the comparison was done on the basis of volume, one large droplet was equivalent to eight medium and 512 small droplets, respectively.

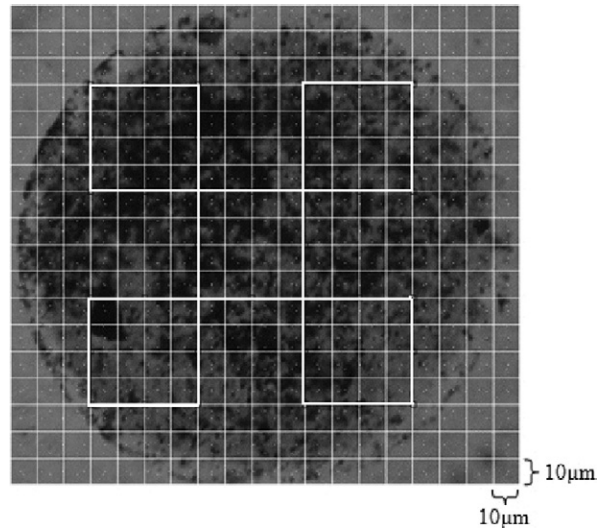

Fig. 2. Grid for the lipid quantification of embryos stained with Sudan Black B.

2.9. Vitrification

A two-step technique was used for vitrification of blastocysts (n = 2,647), as described by Campos-Chillòn et al [22]. Blastocysts (grades 1 and 2) were washed in holding solution (HS; Vigro®, Bioniche Co.) and transferred to 500 μL vitrification solution VS1 (5

M of ethylene glycol in HS) for 3 min. Then, embryos were put in a 10 μL drop of vitrification solution VS2 (7 M ethylene glycol in HS + 0.5 M galactose + 18 % (w/v) of Ficoll 70) for 45 s. Immediately afterwards, a 0.25 mL straw was mounted containing 1 cm galactose solution (GS) (1 M of galactose in HS) + 0.5 cm air + 7 cm GS + 0.5 cm air + the drop of SV2 with the embryos + 0.5 cm air + GS until the straw was full. Straws remained for 1 min in N_2 liquid vapor before being immersed in the liquid. Vitrification was done in groups of five to 10 blastocysts per straw, except for the *in vivo* control group in which three blastocysts were vitrified per straw.

2.10. Warming and subsequent culture of embryos

Vitrification straws were withdrawn from the liquid N_2 and kept for 10 s in air, followed by 15 s in water at 35–37 °C. Straws were slightly agitated to mix the columns of vitrification media. After 4 min, embryos were washed and cultured in drops of SOFaa supplemented with 10% FCS, 2.7 mM myo-inositol, 0.2 mM pyruvate, 5 mg/mL BSA (fatty acid free—Sigma A-8806), 100 μg/mL streptomycin sulfate, and 100 IU/mL penicillin covered with mineral oil, in saturated humidity and an atmosphere of 5% CO_2, 5% O_2, 90% N_2 for 12 h to allow embryo re-expansion. Thereafter, embryos were evaluated to consider their re-expansion rates and submitted to the TUNEL reaction for determination of apoptosis in vitrified blastocysts. Embryo survival after vitrification was defined as re-expansion of the blastocoele associated with a reduced apoptosis rate.

2.11. Statistical analysis

Data were analyzed with ANOVA, using the general linear model (GLM) procedure with the SAS statistical software package (SAS Inst. Inc., Cary, NC, USA). Sources of variation in the model included FCS concentration (0, 2.5, 5 and 10%), PES period exposure (Control, PES Day 2.5 and PES Day 4) and first order interactions; all factors were considered fixed effects. The arcsine transformation was applied to percentage data. If the ANOVA was significant, means were separated by a Tukey's test. Data are reported as untransformed least-squares means. In the absence of significant interactions, only main effect means are presented. Pearson's correlations were calculated (CORR procedure in SAS) between the total lipid droplets value and apoptosis in fresh and vitrified blastocysts, and between apoptosis in fresh and vitrified blastocysts.

3. Results

3.1. Embryo production

The use of various concentrations of FCS (0, 2.5, 5 and 10%) did not affect cleavage rate. However, the addition of FCS increased blastocyst production ($P < 0.05$). The use of PES from Day 2.5 and Day 4 did not affect cleavage rate. Furthermore, blastocyst yield was not affected by the addition of PES from Day 4. Nevertheless, the addition of PES from Day 2.5 reduced blastocyst production ($P < 0.05$).

3.2. Apoptosis

The cell number per blastocyst was not affected by the concentration of FCS. The apoptosis rate in blastocysts produced with 2.5% FCS was similar to the group without FCS. However, higher serum concentrations (5 and 10%) augmented the apoptosis rate in blastocysts ($P < 0.05$, Table 1). The addition of PES from Day 2.5 or Day 4 did not affect cell number or apoptosis rates. The lowest apoptosis rate occurred in blastocysts from the *in vivo* control group ($P < 0.05$, Table 1).

3.3. Lipid accumulation

Cytoplasmic lipid accumulation augmented with increased FCS concentration in the culture media ($P < 0.05$, Fig. 3). Additionally, the increase of total lipid droplet value was strongly correlated ($r = 0.76$; $P = 0.0028$) with the increase of apoptosis in fresh blastocysts. The addition of PES to the media, on both Day 2.5 and Day 4, reduced the content of medium and large lipid droplets. Nevertheless, only the addition of PES on Day 2.5 reduced the number of small droplets and promoted an even more pronounced reduction of large droplets ($P < 0.05$, Fig. 4). Despite the decrease in the lipid droplet number achieved by the reduction in FCS supplementation and by the use of PES, the *in vivo* control group had the lowest lipid content ($P < 0.05$).

3.4. Cryotolerance

Supplementation of media with 2.5% FCS did not reduce embryo cryotolerance when compared to the group cultivated in FCS-free media. However, higher FCS concentrations (5 and 10%) decreased embryo survival after vitrification ($P < 0.05$, Table 2), due to impaired blastocoele re-expansion ($P < 0.05$) and increased percentage of cell death in vitrified blastocysts ($P < 0.05$). Also, an increased FCS concentration reduced the total number of cells after vitrification ($P <$

Table 1
Effects of fetal calf serum (FCS) and phenazine ethosulfate (PES) on cleavage and blastocyst production rate, total cell number and apoptosis in fresh bovine blastocysts (mean ± SEM).

Responses	Embryo production				Embryo quality	
	Oocyte (No.)	Cleavage (%)**	Blastocyst/oocyte (%)**	Blastocyst/cleaved (%)**	Cell number (No)***	Apoptosis (%)***
FCS						
0%	1080	86.7 ± 1.7	30.5 ± 2.5^{a}	35.2 ± 3.0^{a}	131.6 ± 4.1	13.8 ± 1.2^{a}
2.5%	1080	82.3 ± 1.6	41.8 ± 2.4^{b}	50.8 ± 2.9^{b}	136.0 ± 5.9	19.1 ± 1.8^{a}
5%	1080	86.3 ± 1.4	40.5 ± 2.6^{b}	46.9 ± 3.1^{b}	141.0 ± 8.2	20.7 ± 1.9^{ab}
10%	1080	87.0 ± 1.5	47.2 ± 2.8^{b}	54.3 ± 3.3^{b}	148.8 ± 6.5	28.4 ± 2.3^{b}
In vivo control*	—	—	—	—	127.9 ± 6.5	6.3 ± 1.1^{cA}
PES						
Control	1440	87.0 ± 1.3	42.0 ± 2.8^{A}	48.3 ± 3.3^{A}	136.6 ± 5.5	20.7 ± 2.0^{B}
PES Day 2.5	1440	85.4 ± 1.5	35.0 ± 2.3^{B}	41.0 ± 2.8^{B}	138.2 ± 6.2	22.8 ± 1.5^{B}
PES Day 4	1440	84.4 ± 1.3	43.0 ± 2.0^{A}	51.0 ± 2.5^{A}	144.8 ± 4.7	18.9 ± 2.1^{B}

$^{a–c}$ Within a column, means without a common superscript differ ($P < 0.05$).
^{A,B} Within a column, means without a common superscript differ ($P < 0.05$).
* n = 15, ** n = 12, *** n = 60–80.

0.05, Table 2). In contrast, the addition of PES from Days 2.5 or 4 increased the blastocoele re-expansion rate ($P < 0.05$) and preserved cell numbers in vitrified blastocysts. Nevertheless, reduced apoptosis in vitrified blastocysts occurred only with the addition of PES from Day 4, which increased cryotolerance of IVP embryos ($P < 0.05$, Table 2).

Independent of the concentration of FCS in the media or the use of PES, the *in vivo* control group had the greatest survival after vitrification; i.e., this group had the highest blastocoele re-expansion rate, highest cell number of blastocysts after vitrification and the lowest apoptosis rate in vitrified blastocysts ($P < 0.05$).

Whereas the total lipid droplet value had a moderate correlation with the increased apoptosis in vitrified blastocysts ($r = 0.64$; $P = 0.01$), increased apoptosis in fresh blastocysts was very strongly correlated with increased apoptosis in vitrified blastocysts ($r = 0.94$; $P < 0.0001$).

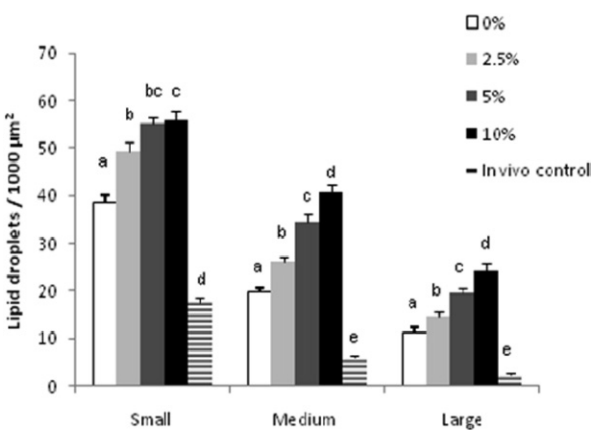

Fig. 3. Effect of various concentrations of fetal calf serum (0, 2.5, 5, and 10%) on number of small, medium, and large (<2, 2–6, and >6 µm, respectively) cytoplasmic lipid droplets in bovine blastocysts (mean ± SEM).

$^{a–e}$Refers to the difference ($P < 0.05$) between groups in the categories of small, medium and large droplets.

N = 45 – Groups 0, 2.5, 5, 10%. N = 15 – *In vivo* control group.

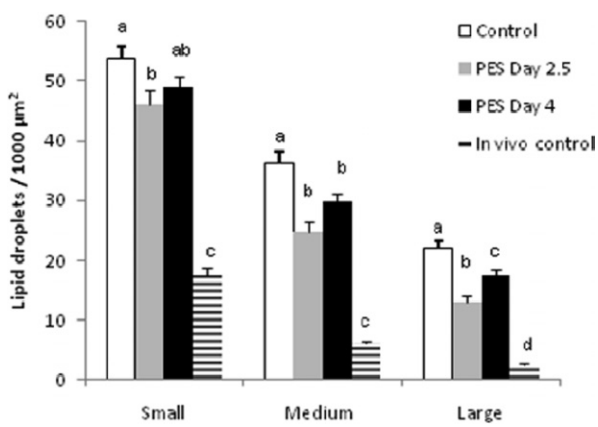

Fig. 4. Effect of Control, PES Day 2.5 and PES Day 4 groups on number of small, medium, and large (<2, 2–6, and >6 µm, respectively) cytoplasmatic lipid droplets in bovine embryos (mean ± SEM).

$^{a–d}$Refers to difference ($P < 0.05$) between groups in the categories of small, medium and large droplets.

N = 60 - Control, PES Day 2.5 and PES Day 4 groups. N = 15 – *In vivo* control group.

Table 2
Effects of fetal calf serum (FCS) and phenazine ethosulfate (PES) on blastocoele re-expansion, cell number, and apoptosis in vitrified bovine blastocysts (mean ± SEM).

Responses	Cryotolerance			
	Vitrified embryos (No.)	Re-expansion rate (%)	Cell number (No.)**	Apoptosis (%)**
FCS				
0%	233	90.5 ± 2.7^{a}	95.1 ± 4.5^{ab}	37.3 ± 2.6^{a}
2.5%	346	81.6 ± 2.5^{b}	97.6 ± 4.0^{ab}	42.8 ± 2.7^{ab}
5%	332	78.0 ± 2.8^{bc}	83.4 ± 3.9^{ab}	51.7 ± 3.6^{b}
10%	405	67.3 ± 3.5^{c}	81.1 ± 5.3^{a}	69.2 ± 3.4^{c}
In vivo control*	15	93.3 ± 6.7^{aA}	111.3 ± 7.1^{bA}	15.7 ± 2.0^{dA}
PES				
Control	474	72.0 ± 3.0^{B}	90.2 ± 3.6^{AB}	52.0 ± 3.0^{B}
PES Day 2.5	362	79.9 ± 2.8^{C}	83.0 ± 4.6^{B}	61.3 ± 3.7^{B}
PES Day 4	480	86.2 ± 2.4^{AC}	94.7 ± 3.8^{AB}	39.2 ± 2.4^{C}

[a–d] Within a column, means without a common superscript differ ($P < 0.05$).
[A–C] Within a column, means without a common superscript differ ($P < 0.05$).
* n = 15, ** n = 73–134.

4. Discussion

Reducting FCS to 2.5% in the culture media and adding PES from Day 4 decreased the lipid content and facilitated embryo survival after vitrification without modifying the developmental pattern and quality. However, embryo lipid content did not seem to be the major determinant of cryotolerance. In contrast, greater apoptosis in fresh blastocysts was very strongly correlated with increasing apoptosis in vitrified blastocysts, emphasizing the importance of embryo quality over lipid content as a determinant of survival after cryopreservation.

Fetal calf serum is widely used in the majority of culture media for cells or embryos. However, in embryos, FCS in the culture media may inhibit early cleavage divisions, although later it seemed to accelerate embryo development until the blastocyst stage [4,5]. In the present experiment, FCS did not affect cleavage, but instead favored blastocyst production. Moreover, the addition of FCS impaired embryo quality due to higher fresh apoptosis rates (Table 1). Some authors [10,23] reported that serum supplementation increased embryonic cell number and consequently quality, whereas others reported a negative effect [24,25]. Based on the conflicting results in the literature regarding the effect of FCS on embryo culture, we inferred that the effects of FCS on embryo development, production, and quality were complex.

Phenazine ethosulfate is a metabolic regulator that inhibits fatty acid synthesis and favors the pentose-phosphate pathway, since it oxidizes NADPH to $NADP^{+}$ [7–9,26]. The addition of this metabolic regulator in culture media from Day 2.5 had a deleterious effect and impaired embryo development. However, the addition of PES from Day 4 did not affect embryo development and apoptosis in fresh blastocysts when compared to the control group. The addition of 0.9 μM PES to the culture media starting on Day 2.5 was toxic, but smaller doses (0.1 and 0.3 μM) had no effect on blastocyst production rate, inner cellular mass (ICM) and cell number as compared to the control group [9]. Independent of the FCS concentration or PES addition, the *in vivo* control group had the lowest average apoptosis in the present study, which demonstrated, as postulated by Gjorret et al [27], that the embryos produced *in vivo* have lower apoptosis rates than IVP embryos (5.4 and 8.9%, respectively).

The reason for the increased lipid deposits in IVP embryos is unknown. In the present experiment, increasing FCS concentration in the embryo culture media directly affected the amount of lipid in the cell's cytoplasm. Similarly, lipid accumulation was greater when 2.5% [28], 5% [3] or 10% [7] FCS were added to the media. Currently, there are many speculative explanations for the increased lipid contents in embryos cultured in FCS, such as: a) the lipoproteins from the serum are absorbed by the embryonic cells [29]; b) the embryo is induced to perform neo-synthesis of triglycerides due to the presence of serum [30]; and c) the serum changes the function of β-oxidation in the mitochondria [3,14]. In this context, an imbalance in the oxidation-reduction state affecting mitochondrial metabolism could impair the metabolism of lipid complexes through β-oxidation, leading to an increase in lipid accumulation [3,7,9,14].

In the present experiment, the addition of PES to the culture media efficiently reduced the lipid increase in

IVP embryos, which was consistent with previous reports [7,26]. Nevertheless, the *in vivo* control group still had the lowest lipid accumulation. The addition of PES to the media from Day 2.5 on promoted a more pronounced reduction in the lipid content than when the addition was performed from Day 4. Apparently, the duration of the embryo exposure to PES regulated the amount of lipid accumulated. However, a probable greater lipid content observed in Day 4 embryos, compared to Day 2.5 embryos, before exposure to this compound, must also be considered. There were more medium and large lipid droplets in the embryo from 2 to 8 cells to the morule stage, when the most lipid deposits occurred in serum-supplemented media [3]. This occurred concurrent with the period of exposure to PES from Day 4.

The specific mechanism through which lipid accumulation in IVP embryo influenced cryotolerance is unknown; however, peroxidation of the lipids might account for this decrease [17]. Therefore, elevation of the lipid content in the embryos might increase the production of free radicals and stimulate embryo death [8].

Apoptosis is a highly conserved form of cell death with important roles in embryo development and organism homeostasis; it acts as a mechanism of cell quality control by removing damaged, impaired, nonfunctional, or even excessive cells [21,31]. However, a disproportionate incidence of this kind of cell death is associated with the reduction of embryo viability [24].

The majority of apoptosis in fresh blastocysts of this study occurred in the ICM, when compared to the trophoblast, as described in murine [32] and bovine [24] embryos, affecting ~10% of the ICM cells, but only 4% of trophoblast cells. The greater incidence of apoptosis in ICM might be explained by removal of damaged or excessive cells, which are not needed or competent for the embryo's development. In this case, apoptosis exerts a rigorous control over the quality of the ICM, which is the cell lineage from which the fetus originates [24].

Events that exert any kind of stress on the embryo are potential causes of apoptosis, e.g., thermal stress [21,33], oxidative stress [34], unfavorable conditions in culture media composition [35,36], and cryopreservation. In the present work, the stress generated by cryopreservation corresponded to an increase of 20.3 to 40.8% in apoptosis rate when fresh and vitrified blastocysts were compared. Moreover, that there was a random distribution of apoptosis incidence in both ICM and trophoblast cells in vitrified blastocysts, corroborated the reduction in quality.

A low concentration (2.5%) of FCS did not affect cryotolerance. Furthermore, PES treatment from Day 4 increased survival of vitrified blastocysts. Nevertheless, addition of PES from Day 2.5 did not alter apoptosis in vitrified blastocysts, consistent with a previous report [28]. Perhaps exposure to PES from Day 2.5 altered the equilibrium of the oxidation-reduction state by acting as an NADPH oxidase and promoted the increase of ROS due to NADPH oxidation [37], since this coenzyme plays a great role in reduction of intracellular glutathione and is an important antioxidant for the embryo [18]. Furthermore, greater sensitivity of cryopreserved embryos to ROS [34] might potentiate this effect, thereby contributing to the higher incidence of apoptosis with this treatment. An alternative explanation might be related to the great reduction of the lipid content induced by PES in Day 2.5 embryos, which might have compromised synthesis of cell membranes and induced the apoptosis cascade.

Barceló-Fimbres and Seidel [8] reported that the addition of PES to the culture media from Day 2.5 had a beneficial effect on embryo survival after conventional freezing and vitrification (with 92, 85 and 60 % survival of embryos exposed to PES, control, and 10% FCS; all significantly different from each other), which contrasted with the results obtained in the present experiments in relation to the PES group Day 2.5.

Excessive lipid accumulation was suggested as the main cause for reduced cryotolerance of IVP embryos [3,4,6,8]. However, in the present study, there was only a moderate correlation between lipid content and increased apoptosis in vitrified blastocysts. In contrast, there was a very strong correlation between increased apoptosis in fresh and vitrified blastocysts, emphasizing the great importance of embryo quality for post-thaw viability. Nevertheless, vitrification must also be considered as a potential cause of physical, chemical and thermal damage, which could have increased apoptosis in blastocysts.

The existing data regarding relationships between lipid content and embryo survival after cryopreservation were controversial. Whereas lipid content was greatest in the morule stage [3], Rodrigues et al [38] observed that those embryos had the greatest conception rate after thawing and transfer to recipient cows. Although taurine embryos (*Bos taurus taurus*) produced *in vivo* presented greater lipid accumulations in the cytoplasm than did zebuine embryos (*Bos taurus indicus*), the former were more resistant to vitrification [39].

Both the current results and the literature call into question the actual influence of embryo lipid content on cryotolerance. Perhaps lipid accumulation might be a consequence of a series of depreciating events on IVP

embryo quality, for example, the addition of FCS to the culture media; the imbalance of the oxidation-reduction metabolism and the increase in ROS, could lead to the increased sensitivity of IVP embryo to cryopreservation. In conclusion, the reduction of the concentration of FCS in the culture media to 2.5% and the addition of the metabolic regulator PES starting on Day 4 improved the survival of vitrified blastocysts without affecting embryo development and quality. Moreover, embryo quality, represented by the apoptosis in fresh blastocysts, was a more reliable predictor than lipid content for embryo survival after vitrification.

Acknowledgments

We thank the Fundação de Amparo à Pesquisa do Estado de São Paulo (São Paulo Research Foundation - FAPESP), grants 07/57766-4 and 08/51378-5; and professor Dr. Roberto Sartori Filho and researcher Dr. Rui Machado for their helpful suggestions in this work.

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