

Vitrification of Bovine Blastocysts Produced *In Vitro* Inflicts Selective Damage to the Inner Cell Mass

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Contents

In contrast to the embryos derived from live animals, the embryos produced *in vitro* undergo increased damage and reduced survival after cryopreservation, particularly when produced with serum. In medium containing serum, retinoic acid increases cell numbers in the inner cell mass and the trophectoderm without altering their relative proportions in the bovine blastocyst. In this work, in medium without serum, we analyzed the contribution of retinoic acid to the development of blastocyst and survival to vitrification, and found a strong cell reduction in the inner mass when compared to the trophectoderm. Day-6 *in vitro*-produced morulae were treated for 24 h with retinoic acid (0.7 and 1.4 μM) and subsequently cultured without additives for a further 24 h period. Day-8 blastocyst production and cell counts in hatched blastocysts were unaffected by retinoic acid. However, Day-7 expanded, vitrified embryos produced with retinoic acid 1.4 μM survived at lower rates than controls when cultured after warming. Vitrification greatly reduced cell numbers in the inner mass ($p < 0.0001$), while cells in the trophectoderm remained unaltered. Differential cell counts analysis in blastocysts should be taken up to replace unspecific determination of total cells to appreciate substantial modifications in their exact terms. The strong reduction we found in the inner cell mass could explain why *in vitro* survival to cryopreservation is sometimes scarcely informative on the viability of the embryo after transfer to recipients.

Introduction

Successful cryopreservation of *in vitro*-produced (IVP) embryos is a major objective in reproductive biotechnology. However, in contrast to embryos derived from live animals, the IVP embryos undergo increased damage and reduced survival after cryopreservation. The quality of the IVP bovine blastocyst has been classically associated to their resistance to cryopreservation (Imai et al. 2002; Lonergan et al. 2003; Rizos et al. 2003) and to the amount and proper allocation of the embryonic cells (Van Soom et al. 1996).

In the blastocyst, two groups of cells can be distinguished. First, the inner cell mass (ICM), which contains two types of cells, the innermost and more abundant ectoderm pluripotent cells, and a cell line facing to the blastocoele cavity, which is the primitive endoderm. Second, the trophectoderm (TE), a spherical shell of epithelial cells surrounding the ICM in a fluid-filled cavity (Wiley 1987; Biggers et al. 1988; Watson 1992). The proper embryo will derive from the ICM, while the placenta is a TE derivative; both the cell populations show contrasting morphology.

The cell numbers are usually reduced in IVP embryos as compared to *in vivo*-derived embryos (De la Fuente and King 1997). The allocation of blastocysts to either

the ICM or TE is crucial to the embryo development, as it is presumed that certain minimal number of ICM cells is required to obtain a pregnancy, and excessive allocation of cells to the TE might lead to pregnancy abnormalities (Van Soom et al. 1996; Leese et al. 1998). Iwasaki et al. (1990) found that IVP embryos had a lower ratio of ICM to TE cells, together with a less compact ICM as compared to both *in vivo*-produced embryos and embryos matured and fertilized *in vivo* but cultured *in vitro*. Recently, we demonstrated that all-trans retinoic acid (ATRA) increases cell numbers in the ICM and TE without alteration of the ICM/TE cell ratio (Rodríguez et al., 2006). These cell counts were made in fresh embryos cultured with serum and not evaluated for cryopreservation survival. However, several reports indicate that the presence of serum in culture both increases the numbers of TE cells (Crosier et al. 2001; Abe et al. 2002; Mucci et al. 2006) and reduces cryo-resistance particularly when embryos are vitrified (Crosier et al. 2001). Vitrification is a cryopreservation procedure, which is simpler than slow freezing, which also results in healthy offspring, although embryos suffer considerable morphological and functional damage when cryopreserved by either freezing or vitrification (Vajta and Nagy 2006). The extent of the cryopreservation injury depends on factors such as the size and shape of the cells, the permeability of the membranes, and the specific quality and sensitiveness of the blastomeres (Vajta and Kuwayama 2006). It is unknown whether specific traits of ICM and TE could lead to differences in survival to cryopreservation. Therefore, whether cryopreservation could alter the distribution of cells between tissues in the blastocyst still remains an open question.

In this work we investigate: (i) the effects of ATRA added to synthetic oviduct fluid (SOF) medium containing high BSA and no serum on embryo development and survival to cryopreservation; and (ii) whether vitrification exerts a selective effect on survival of ICM and TE.

Materials and Methods

Oocyte recovery

Cumulus-oocyte complexes (COCs) from slaughterhouse ovaries were aspirated from 2- to 8-mm visible follicles. Follicular fluid and COCs were placed in an ovum concentrator (Em-Con; Comextrade, Tarragona, Spain) and rinsed three times in holding medium (HM) consisting of TCM199 (Invitrogen, Barcelona, Spain), 25 mM Hepes and BSA 0.4 g/l, supplemented with 2 IU/ml of heparin.

***In vitro* maturation**

Only oocytes enclosed in a compact cumulus with an evenly granulated cytoplasm were selected for maturation. The COCs were washed three times in HM and twice in maturation medium, which consisted of bicarbonate-buffered TCM199, FSHp (1 µg/ml), LH (5 µg/ml), 17β-estradiol (1 µg/ml) and 10% foetal calf serum. Maturation was performed by culturing approximately 50 COCs in 500 µl of maturation medium in four-well dishes at 39°C in 5% CO₂ in air with high humidity for 22–24 h.

***In vitro* fertilization**

Sperm separation was carried out by the swim-up procedure (Parrish et al. 1986). Briefly, semen from one frozen straw corresponding to a single bull was thawed in a water bath and added to a polystyrene tube containing 1 ml of pre-equilibrated Sperm-TALP. After 1 h of incubation, approximately 700 µl of the upper layer of supernatant containing the motile sperm was removed. The sperms were centrifuged for 7 min at 200 × g and the supernatant aspirated to leave a pellet of approximately 100 µl in volume. The sperm concentration was determined with a haemocytometer. The COCs were washed twice in HM and placed in four-well culture dishes containing pre-equilibrated fertilization medium (Fert-TALP) with heparin (10 µg/ml; Calbiochem, La Jolla, CA, USA). Spermatozoa were then added at a concentration of 2 × 10⁶ cells/ml in 500 µl of medium per well containing 100 COCs for maximum. *In vitro* fertilization (IVF) was accomplished by incubating oocytes and sperm cells together for 18–20 h at 39°C in 5% CO₂ with high humidity.

***In vitro* embryo culture**

The cumulus cells were detached using a vortex, and zygotes cultured in SOF containing amino-acids, citrate and myo-inositol, as per method described by Holm et al. (1999), modified with 20 g/l BSA and no serum (mSOF), a condition reported to enhance survival to vitrification *in vitro* (Díez et al. 2005; Díez et al., unpublished). Droplets of medium were layered under mineral oil and embryos cultured at 39°C in 5% CO₂, 5% O₂ and humidified air. Culture medium was renewed on Day 3. At 139 h post-insemination (Day 6), compacted morulae were treated for 24 h with: (i) ATRA 1.4 µM; (ii) ATRA 0.7 µM; and (iii) ATRA 0 µM, in a volume 400 µl of culture medium without mineral oil overlay. Subsequently, all experimental groups were again cultured in mSOF droplets. Blastocyst development was recorded on Days 7 and 8 at the stages early, middle, expanded or hatched blastocyst.

Vitrification and warming

Days 7 and 8 excellent to good expanded blastocysts were vitrified/warmed by the OPS method described by Vajta et al. (1998) with minor modifications. Briefly, embryos were handled in a basic vitrification medium (BV), consisting of TCM 199-Hepes + 20% FCS. All

procedures were performed in a warm room (30°C) on a heated surface (41°C). Groups of 4–5 blastocysts were exposed to BV with 10% ethylene-glycol (EG) + 10% DMSO (vitrification solution-1; VS1) for 3 min and then moved into a well containing BV with 20% EG + 20% DMSO + 0.5 M sucrose (vitrification solution-2; VS2). After a quick passage of embryos in VS2, a drop of 6 µl VS2 containing the blastocysts was made. Blastocysts were loaded into OPS straws (sOPS; Comextrade) in an approximately 1-mm high cylinder of VS2 containing the embryos, formed by capillary action by touching the narrow end of the straw with the droplet. The straws were plunged in liquid nitrogen (LN). The time spent by the embryos in VS2 (including loading) was 20–25 s.

Warming was conducted by immersing the pulled end of the straw directly in 1.2 ml of 0.25 M sucrose in BV. They were kept for 5 min, and then transferred into a 0.15 M sucrose medium in BV for another 5 min, and subsequently transferred and washed twice in BV. After warming, blastocysts were rinsed twice in the culture medium and then cultured in drops of 25 µl of B2 medium supplemented with 5% FCS, seeded with Vero cells and overlaid with mineral oil. Embryo survival was evaluated for re-expansion at 24 h and hatching at 24, 48 and 72 h.

Differential cell counts

Embryonic cells were counted in the ICM and the TE of hatched blastocysts both fresh (Days 7 and 8) and after vitrification, warming and *in vitro* culture, at the day of the full re-expansion or hatching occurrence. Embryos were fixed and stained as per method reported by Thouas et al. (2001). Blastocysts were incubated in 500 µl BSA-free TCM199 Hepes (Invitrogen, Barcelona, Spain) with 1% Triton X-100 and 100 µg/ml propidium iodide for 30 s. Samples were then fixed in 500 µl ethanol with 25 µg/ml bisbenzimidazole (Hoechst 33342) and stored overnight at 4°C. These fixed and stained blastocysts were transferred directly to a glycerol droplet on a glass microscope slide. Cell counts were made using digital images obtained with an inverted microscope equipped with a UV excitation filter at 330–385 nm and a barrier filter at 420 nm. TE cells were identified by their red fluorescence; ICM cells appeared blue.

Statistical analysis

The data were analyzed in two steps. First, factors showing significant influence were identified by categorical data modelling (CATMOD) using SAS Version 8.2 software (1999; SAS Institute Inc., Cary, NC, USA). The factors found to have a significant effect on the dependent variables were treatment and replicate and blastocyst stage at cryopreservation but not at which the cell counts were performed. Second, these significant factors were used to produce a linear model using the general linear models procedure (GLM; SAS software). The GLM was used to estimate the least square means (LSM) for each fixed effect having a significant *F* value. Duncan's multiple-range test was used to compare the

raw means calculated for the main effects. Development data were transformed to frequency percentages, while blastocyst cell counts were expressed as absolute values.

Results

A total of 917 morulae were used for blastocyst development, accounting for 38% of 2413 cultured oocytes. Embryo development can be seen in Table 1, showing no significant differences in blastocyst rates between the two ATRA-treated groups and ATRA 0 μM .

Upon vitrification and post-warming culture (Table 2), Day-7 embryos produced with ATRA 0.7 μM and controls re-expanded earlier at higher rates, while survival of embryos produced with ATRA 1.4 μM did not vary according to the day at which they were vitrified. Hatching rates were higher in Day-7 embryos cultured without additives, although these differences were non-significant concerning Day-7 embryos cultured with ATRA 0.7 μM .

Cell counts (Table 3) of embryos surviving vitrification were not affected by the stage at which counting was made, so data of fully expanded and hatched embryos are presented pooled. Under the stereomicroscope we appreciated that ICMs in blastocysts re-expanding in culture after warming were mostly unapparent. Once the cells of blastocysts were counted before and after vitrification, the ICM showed a striking reduction in embryos that survived vitrification as compared to non-cryopreserved blastocysts. In contrast, the TE remained unaffected after vitrification, while total cells and ICM/total cell ratio significantly reflected the detrimental effects occurred in the ICM. Among the 27 embryos counted after vitrification and warming, 18 (66%) showed lesser than 10 cells in the ICM, including nine ones that showed no cells at all (33%). Only three ICMs contained 20 cells or more, and just one approached the cell average (33 cells) proper of embryos counted before vitrification. In contrast, in fresh embryos only two ICMs showed no cells (4%), and seven ICMs contained lesser than 10 cells (15%).

Discussion

Short (24 h) culture of morulae with ATRA 0.7 μM increased ICM and TE cell numbers in the presence of serum (Rodríguez et al., 2006). However, in the present report addition of ATRA to a medium with high BSA concentration, a condition reported to enhance survival to vitrification *in vitro* (Diez et al. 2005; Díez et al.,

Table 2. Percentages of expanded blastocysts produced in synthetic oviduct fluid medium with 20 mg/ml BSA containing all-trans retinoic acid (ATRA) 1.7 μM , ATRA 0.7 μM or no additives, vitrified at days 7 or 8 and warmed, that re-expanded and hatched during a 72 h co-culture in medium B2 + vero cells

Group	Day	n	% Re-expanded		% Hatched		
			24 h		24 h	48 h	72 h
ATRA 1.4 μM	7	51	16.2 $\pm$ 10.0 ^{bc}		3.2 $\pm$ 8.2 ^b	6.4 $\pm$ 8.3 ^b	8.3 $\pm$ 8.2 ^b
ATRA 1.4 μM	8	23	32.7 $\pm$ 10.0 ^{bc}		7.6 $\pm$ 8.1 ^b	7.2 $\pm$ 8.3 ^b	6.0 $\pm$ 8.2 ^b
ATRA 0.7 μM	7	41	38.1 $\pm$ 10.0 ^{ab}		4.0 $\pm$ 8.2 ^b	10.3 $\pm$ 8.3	15.1 $\pm$ 8.2
ATRA 0.7 μM	8	21	20.9 $\pm$ 9.7 ^c		4.2 $\pm$ 7.9 ^b	4.1 $\pm$ 8.0 ^b	4.0 $\pm$ 7.9 ^b
ATRA 0 μM	7	37	50.4 $\pm$ 10.0 ^a		31.2 $\pm$ 8.2 ^a	31.4 $\pm$ 8.3 ^a	35.0 $\pm$ 8.2 ^a
ATRA 0 μM	8	17	18.4 $\pm$ 9.7 ^c		0.8 $\pm$ 7.9 ^b	0.8 $\pm$ 8.0 ^b	0.7 $\pm$ 7.9 ^b

Data are LSM $\pm$ SE (four replicates).

n, blastocysts cultured.

Different superscripts express significant differences: ^{a,b,c} $p < 0.05$.

unpublished), did not improve embryo quality. The effects of ATRA were non-existent during embryonic development and cell counts. However, as seen with blastocysts vitrified on Day 7, ATRA reduced embryo survival to vitrification in a dose-dependent manner. These effects would be not related to activation of apoptotic pathways, as 24 h of ATRA 0.7 μM has previously been shown not to increase apoptosis (Rodríguez et al., 2006). However, 48 h of the same concentration increased the expression of pro-apoptotic genes such as p53, p66 and Bax (submitted). The consequences of ATRA overexposure are comparable to high dosage, the latter reported by Huang et al. (2003) in mice embryos exposed for 24 h to 10 μM ATRA. These consisted of antiproliferative and proapoptotic effects mainly targeted to the ICM (Huang et al. 2005). In our work neither development nor proliferation (cell counts) were inhibited by ATRA 0.7 or 1.4 μM , so these concentrations cannot be considered as overdose. Therefore, it could be expected from the above that the effects of ATRA on vitrification survival were unrelated to apoptosis. Alternatively, Na⁺ and water transport, and in turn permeability (see below), in the TE could be altered, because Na/K-ATPase, subunit $\alpha 1$ gene expression experiences changes in response to ATRA 0.7 μM for 24 h (Rodríguez et al., 2006).

The reduced effects of ATRA on development and cell counts can be due to its molecular binding by BSA, as this protein has been described to interact with retinoids (Klaassen et al. 1999). Retinoid binding prolongs the half-life of the compound in culture and makes a slower release to the cells, reducing the effective ATRA

Table 1. *In vitro* development of bovine morulae in synthetic oviduct fluid medium with 20 mg/ml BSA containing all-trans retinoic acid (ATRA) 1.7 μM , ATRA 0.7 μM or no additives

Treatment	n	% Day-7 blastocysts			% Day-8 blastocysts		
		Early	Blastocysts	Expanded	Blastocysts	Expanded	Hatched
ATRA 1.4 μM	294	53.1 $\pm$ 3.2	33.9 $\pm$ 3.5	18.7 $\pm$ 2.7	48.6 $\pm$ 3.6	38.0 $\pm$ 3.0	7.0 $\pm$ 2.6
ATRA 0.7 μM	320	56.7 $\pm$ 3.0	36.1 $\pm$ 3.2	19.7 $\pm$ 2.5	49.9 $\pm$ 3.4	37.5 $\pm$ 2.8	9.7 $\pm$ 2.2
ATRA 0 μM	303	53.8 $\pm$ 3.0	37.3 $\pm$ 3.2	18.5 $\pm$ 2.5	49.3 $\pm$ 3.4	37.4 $\pm$ 2.8	8.5 $\pm$ 2.2

Data are LSM $\pm$ SE from percentages of morulae (n) that were blastocysts at the indicated stages (10 replicates).

No significant differences ($p > 0.10$).

Table 3. Differential cell counts before and after vitrification, warming and co-culture in medium B2 + vero cells of bovine blastocysts produced in synthetic oviduct fluid medium with 20 mg/ml BSA containing all-trans retinoic acid (ATRA) 1.7 μ M, ATRA 0.7 μ M or no additives

Group	Time ^c	n	ICM	TE	Total cells	ICM/TE (%)
ATRA 1.4 μ M	Before	15	28.3 $\pm$ 3.1 ^z	133.9 $\pm$ 10.0	160.0 $\pm$ 9.8 ^a	16.9 $\pm$ 2.7 ^x
ATRA 1.4 μ M	After	9	8.6 $\pm$ 4.1 ^{β}	122.1 $\pm$ 11.9	130.0 $\pm$ 12.2 ^b	6.1 $\pm$ 3.2 ^y
ATRA 0.7 μ M	Before	17	27.7 $\pm$ 3.5 ^z	141.7 $\pm$ 8.8	165.3 $\pm$ 8.8 ^a	17.2 $\pm$ 2.3 ^x
ATRA 0.7 μ M	After	9	2.2 $\pm$ 4.1 ^{β}	121.3 $\pm$ 11.5	123.2 $\pm$ 11.7 ^b	2.0 $\pm$ 3.0 ^y
ATRA 0 μ M	Before	14	31.3 $\pm$ 3.1 ^z	129.7 $\pm$ 9.0	161.2 $\pm$ 9.2 ^a	20.6 $\pm$ 2.4 ^x
ATRA 0 μ M	After	6	7.0 $\pm$ 5.1 ^{β}	125.3 $\pm$ 14.8	131.0 $\pm$ 15.1 ^b	4.3 $\pm$ 3.9 ^y
Cumulative	Before	46	29.2 $\pm$ 1.9 [*]	135.4 $\pm$ 5.5	162.5 $\pm$ 5.5 [*]	18.3 $\pm$ 1.5 [*]
	After	24	5.9 $\pm$ 2.6 ⁱ	121.6 $\pm$ 7.5	127.2 $\pm$ 7.6 ⁱ	4.2 $\pm$ 2.0 ⁱ

Data are LSM $\pm$ SE.

ICM, inner cell mass; TE, trophectoderm; n, blastocysts counted.

Different superscripts express significant differences: ^{*} $p < 0.0001$; ^{z, β} $p < 0.002$; ^{x,y} $p = 0.01$; ^{a,b} $p < 0.05$.

^cTime at which cell counts were made before (seven replicates) and after (four replicates) vitrification and warming.

concentrations. Therefore, we doubled the original ATRA concentration 0.7 μ M (Rodríguez et al., 2006), including a group with ATRA 1.4 μ M in our experiments, so far without positive results. Current work in our laboratory confirms the above reported binding effect, as 3 g/l BSA in the same conditions and culture medium led to increased blastocyst development and cell counts (unpublished).

Vitrification seemed to exert a detrimental effect on the ICM, while TE cells survived cryopreservation in numbers comparable to embryos counted before vitrification. This effect is not consistent with an accumulation of lipids in the TE higher than ICM (Abe et al. 2002), as cytoplasmic lipid contents appear strongly related with survival to cryopreservation (Leibo et al. 1995; Diez et al. 2001; Ushijima et al. 1999). The high nucleus to cytoplasm ratio in the ectoderm cells within the ICM determines a low volume for storing lipids. However, besides a specific sensitivity, it is unknown other differential traits between the ICM and TE could make the former more susceptible to cryopreservation. Chromosomal abnormality, typical in cells dividing rapidly, is more abundant in the TE than in the ICM (Viuff et al. 2002), which could be due to the intense proliferative activity of the TE. Interestingly, embryos that develop in serum-containing media have a higher proportion of TE cells (Crosier et al. 2001). A smaller size and a regular spherical shape in the ectoderm cells within the ICM are in principle favourable conditions to cryopreservation. In contrast, TE cells show morphology of a polarized epithelium with specific activity and tight cell-to-cell communication. Therefore, it is likely that cryoprotectants may encounter more difficulties to diffuse to the ICM, as the TE acts as a selective entry and exit system to a variety of molecules ultimately required by the cells of the ICM (Brison et al. 1993; Hewitson and Leese 1993). Permeability between the bovine TE and ICM cells can also be different, as in the mouse blastocyst aquaporin subtypes and Na/K-ATPase vary in their trophectodermal localization and distribution (see a review by Huang et al. 2006). Aquaporins are channels that permit water and low molecular weight cryoprotectants movements across the membranes. In addition, Na/K-ATPase, located in the basolateral membrane of the trophectoderm cells, was demonstrated to play a role in water movement across the epithelium in the process of cavitation (Betts et al. 1998; Watson and Barcroft 2001). Overall, the above mor-

phological and functional differences could explain that ICM and TE have specific sensitivity and therefore differ in their survival to cryopreservation.

We and others reported that ICM has a higher apoptotic rate than the TE in mammalian blastocysts (Rodríguez et al., 2006; Gjørret et al. 2003; Davidson et al. 2004). This suggests that apoptosis occurs naturally for eliminating the non-committed ICM cells, and that the ICM is submitted to a more stringent control than the TE, consistent with the higher impact that programming errors in the ICM can have. As the apoptotic cascade can be activated in bovine embryos in response to vitrification (Park et al. 2006), the presence of more active apoptotic pathways in the ICM could determine death and disappearance of these cells in vitrified embryos. In the present work, an apoptosis analysis concerning specific cryopreservation damage in the ICM was discarded because of the low number of cells present in the ICMs. Our procedure could be an extreme example of damage to ICM, as several works reported on vitrified – warmed embryos that were able to produce pregnancies (see Xu et al. 2006 and a review by Vajta and Nagy 2006). Therefore, it must be assumed that embryos vitrified that led to pregnancies to term did contain enough cells in their ICM.

Reductions in total cell counts after embryo cryopreservation have been reported (Kaidi et al. 1999, 2001; Mucci et al. 2006). However, to our knowledge, our work is the first to analyze the effects of cryopreservation on blastocyst cell distribution and describing the specific damage of vitrification to the ICM. In high BSA concentrations, ATRA shows limited effects on embryo development and ICM and TE cell counts, although survival to cryopreservation is affected in a dose-responsive manner. Our work emphasizes the need to make differential cell counts in the blastocyst instead of unspecific determination of total cells. Otherwise, structural changes thought to be relevant to the embryonic viability could be not appreciated in their exact terms. The TE in surviving blastocysts would contain enough cells to hatch (i.e. similar to non-cryopreserved embryos), but the ICM would not have a minimum cell number to support embryonic and foetal viability. The loss of cells in the ICM might also be related to the reduced survival rates obtained after warming. The strong cell reduction found in the ICM could explain why, sometimes, *in vitro* tests of survival

to cryopreservation are scarcely informative of the embryonic viability after transfer to recipients.

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