Ultrastructure and Development of Vitrified/Warmed Bovine Oocytes Matured with 9-*cis* Retinoic Acid

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ABSTRACT

In this work we analyze the effects of vitrification on the ultrastructure and developmental ability of bovine oocytes matured in the presence of 9-*cis*-retinoic acid (RA). Bovine cumulus oocyte complexes (COCs) were matured in a basic medium containing 10% fetal calf serum (FCS), 9-*cis*-RA or polyvinyl alcohol (PVA). Mature oocytes were vitrified using the Open Pulled Straw method with minor modifications. A group of fresh and vitrified/warmed COCs was fixed for ultrastructural analysis, while the remaining oocytes were fertilized *in vitro* and cultured. Vitrification of mature oocytes produced high rates of degeneration regardless of the *in vitro* maturation protocol and low number of cleaved embryos. Development rates up to day 8 were similar between groups undergoing vitrification, which were lower than fresh controls. Morphologic alterations were observed in all groups of vitrified oocytes, although *in vitro* maturation in the presence of 9-*cis*-RA combined with vitrification led to higher ultrastructural damage. More research is needed to explain the low survival rates of the bovine oocyte after vitrification and warming.

INTRODUCTION

ALTHOUGH GAMETES were among the very first cells to be successfully cryopreserved, mammalian oocytes remain one of the most difficult cell types to cryopreserve successfully. The ultrastructure of the cumulus oocyte complexes (COC) is significantly affected by cryopreservation procedures. Several studies focused on cytoplasmic and nuclear alterations after cryopreservation within cumulus cells (CC) and the oocyte,¹⁻⁷ concluded that the reduced developmental competence of cryopreserved oocytes depends on cryodamage inflicted to the oocyte.^{7,8}

In the bovine oocyte, the maturation stage in-

fluences developmental competence after cryopreservation^{1,7-11} with the metaphase II (MII) oocyte being more resistant to cryopreservation than immature oocytes.^{1,7-10} Also, the maturation system is known to condition the oocyte's ability to survive cryopreservation.⁸ Vitamin A (all-*trans*-retinol; ROH) and its metabolites are regulators of cell growth, embryonic morphogenesis, and differentiation in many cell types. In our laboratory, the ROH metabolite, the 9-*cis*-RA stimulates developmental competence of the oocyte during *in vitro* maturation (IVM)^{12,13} and during a period of meiotic inhibition prior to IVM.¹⁴ However, it is unknown whether 9-*cis*-RA during IVM can benefit the viability of the oocyte after cryopreservation. The purpose

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of this work was to analyze the effect of 9-*cis*-RA during IVM on ultrastructure and viability of vitrified/warmed COCs.

MATERIALS AND METHODS

All chemicals were purchased from Sigma (Madrid, Spain) unless otherwise indicated.

Collection of COCs

Ovaries recovered from slaughtered cows were placed in NaCl solution (9 mg/mL) containing antibiotics (penicillin, 100 UI/mL and streptomycin sulfate, 100 μ g/mL) and maintained at 25°–30°C until COCs collection. Visible follicles, 2 to 7 mm in size, were aspirated and COCs were selected and washed as previously reported.¹

The COCs were *in vitro* matured in an IVM medium containing TCM 199 (Invitrogen, Barcelona, Spain) 26 mM HNaCO₃, porcine follicle stimulating hormone (pFSH) (1 μ g/mL), LH (5 μ g/mL), and 17 β -estradiol (1 μ g/mL). Experimental groups were defined adding to IVM medium: (1) 10% fetal calf serum (FCS) (control); (2) 0.3 g/L PVA plus 9-*cis*-RA 5 nM, and (3) 0.3 g/L PVA. Incubations were performed during 22 h in 4-well dishes (Nunc, Biocen, Spain) at 39°C in 5% CO₂ under air and high humidity.

Vitrification and warming

Vitrification and warming of COCs followed the Open-Pulled Straw (OPS) method described by Vieira et al.¹⁵ with minor modifications as recently reported.¹ Vitrification solutions consisted in TCM 199-Hepes plus 20% FCS with 10% EG plus 10% dimethyl sulfoxide (Me₂SO) (vitrification solution 1), and TCM 199-Hepes plus 20% FCS with 20% EG plus 20% (Me₂SO) plus 0.5 M sucrose (vitrification solution 2).

After warming, COCs were cultured for 2 h to accomplish the maturation period before being submitted to *in vitro* fertilization (IVF) and culture.

In vitro fertilization

Sperm separation was carried out using a swim-up procedure similar to that reported by

Parrish et al. (16) and IVF was performed as is usually done in our laboratory.¹

Embryo culture

Presumptive zygotes were gently passed through a small gauge pipette to eliminate CC and sperm, washed three times in HM, and twice in culture medium.

Before culture, morphologic appearance of the ova was evaluated under a stereomicroscope. Only presumptive zygotes with normal morphology were cultured. Embryo culture was performed in modified synthetic oviduct fluid (SOF) containing amino acids, citrate, and myoinositol.¹⁷ The FCS (5%) was added at 42 h post-fertilization (post-FIV). Incubations were carried out at 39°C, 5% CO₂, 5% O₂, and 90% N₂. Culture media were renewed at 66 h (day 3) and 138 h (day 6) post-FIV, and embryonic development was recorded on days 3, 6, 7, and 8.

Experimental design

The experiment analyzed the ability of the bovine oocytes to develop after vitrification/warming and the oocyte ultrastructure after maturation in a defined medium containing 9-*cis*-RA. We used as a control a maturation medium containing FCS, which was replaced by an experimental group with 9-*cis*-RA plus PVA. A group with PVA alone served to control the effect of merely replacing serum. Thus, the basic maturation medium consisted in TCM 199 26 mM HNaCO₃, pFSH (1 μ g/mL), luteinizing hormone (LH) (5 μ g/mL), and 17 β -estradiol (1 μ g/mL) to which we added: (1) 10% FCS (FCS group); (2) 0.3 g/L PVA plus 9-*cis*-RA 5 nM (RA group), and (3) 0.3 g/L PVA (PVA group). Oocytes from each experimental group were processed fresh (i.e., unvitrified) as controls.

Electron microscopy

Vitrified/warmed oocytes and their corresponding fresh controls were fixed for ultrastructural analysis as previously described.¹ We analyzed the degree of expansion in CC, ultrastructure of both CC and the oocyte focusing on organelle status, gap junctions (GJ) between CC and the oocyte, integrity of the plasmatic membrane and mitochondria, loca-

TABLE 1. CULTURED EMBRYOS AND EMBRYO DEVELOPMENT RATES AFTER VITRIFICATION/WARMING OF BOVINE OOCYTES SUBMITTED TO THREE DIFFERENT *IN VITRO* MATURATION PROTOCOLS

Group	N	R	Day 1 % Cultured	Day 3 % Cleaved	Day 3 % 5–8 cells	Day 6 % Mor+B1	Day 7 % Blastocysts
FCS	378	9	68.2 ± 7.5	39.4 ± 6.1 ^a	15.8 ± 4.7 ^a	5.1 ± 4.4 ^a	0 ^a
RA	181	6	73.5 ± 13.9	40.3 ± 7.8 ^a	20.7 ± 6.0 ^a	1.7 ± 5.6 ^a	0 ^a
PVA	210	7	78.5 ± 10.1	42.9 ± 7.2 ^a	21.4 ± 5.5 ^a	4.5 ± 5.2 ^a	0 ^a
c-FCS	214	9		81.1 ± 6.2 ^b	69.7 ± 4.8 ^b	44.8 ± 4.5 ^b	36.8 ± 3.4 ^b
c-RA	161	7		82.7 ± 7.3 ^b	55.1 ± 5.6 ^b	28.1 ± 5.2 ^c	16.1 ± 4.0 ^c
c-PVA	154	7		78.3 ± 7.3 ^b	60.0 ± 5.6 ^b	30.8 ± 5.2 ^c	17.4 ± 4.04 ^c

N, number of oocytes recovered after warming; R, replicates; values are least square mean (LSM) ± standard error (SE); groups named with “c” indicate fresh control group of oocytes; different superscripts differ significantly (^{a,b,c} $p < 0.05$). Development data refer to cultured oocytes.

FCS, fetal calf serum; RA, retinoic acid; PVA, polyvinyl alcohol.

tion of cortical granules (CG), smooth endoplasmic reticulum (SER), and lipid droplets as well as cytoplasmic vesicles.

Statistical analysis

Data from embryo development were analyzed in two steps. First, because these values can be represented by contingency tables, those factors significantly affecting the variables were identified by Categorical Data Modeling (CATMOD) using the SAS model (version 8.2, SAS Institute, Cary, NC). CATMOD extends analysis from continuous data to categorical data, fitting linear models to functions of response frequencies. Treatment and replicates were found to have significant influence on dependent variables. Second, those factors identified to be significant were used to fit a linear model using the General Linear Models (GLM; SAS). The GLM procedure was used to estimate least square means (LSM) for each fixed effect having a significant F value. Duncan's multiple-range test was carried out on raw means calculated for the main effects, and data are referred to frequency percentages of oocytes.

RESULTS

Morphologic appearance of COCs immediately after warming was similar to fresh COCs in all groups; consequently, all recovered oocytes after warming were submitted to IVF. Those zygotes derived from vitrified/warmed oocytes appeared as much more fragile struc-

tures than fresh controls, and removal of CC after fertilization was usually associated with fragmented cytoplasm, indistinct oolema, increased perivitelline space, or damaged zona pellucida.

Vitrification produced high rates of embryos showing degenerated appearance after IVF, as showed by the percentages of zygotes selected to be cultured on day 1 (Table 1). However, no differences between maturation treatments were detected at this stage. Zygotes derived from fresh oocytes developed up to day 7 at higher rates ($p < 0.05$) than those derived from vitrified/warmed oocytes. Oocytes matured fresh with FCS showed higher 5- to 8-cell, day 6 morulae plus blastocysts and day 7 blasto-

FIG. 1. Fresh complex oocyte cumulus after maturation in the presence of fetal calf serum (FCS). Cumulus cells appear expanded (200×).

FIG. 2. Mitochondria of a vitrified/warmed oocyte from the PVA group. Crestae and the external double membrane (arrows) are observed although the matrix shows signs of vacuolization (stars) (44,000 $\times$).

cysts rates than their counterparts matured with PVA and RA. No blastocysts were obtained in any treatment from vitrified/warmed oocytes.

Ultrastructural study

Vitrification/warming of oocytes matured both with FCS and PVA yielded similar alter-

FIG. 3. Fresh complex oocyte cumulus matured with 9-*cis*-retinoic acid (RA). Cumulus cells present nuclei showing lysis (arrow) and apoptosis (stars) phenomena (400 $\times$).

ations. The CC remained expanded around oocytes after vitrification (Fig. 1), but these cells contained a large number of cytoplasmic vacuoles that appeared to damage the plasma membrane. Necrotic nuclei were observed within these cells. Moreover, most cumulus cells projections appeared to be damaged and interrupted. In the oocyte the perivitelline space was smaller, and cytoplasmic vesicles migrated peripherally. These vesicles became confluent and often disrupted the oolema. The numbers of CG in the ooplasm decreased and we observed an abnormal CG premature release into the perivitelline space. Mitochondria exhibited extensive vacuolization and scarce cristae, although the mitochondrial double membrane was mostly conserved (Fig. 2).

FIG. 4. Residual bodies (arrows) from a vitrified/warmed oocyte matured with 9-*cis*-retinoic acid (RA) (32,000 $\times$).

Similar to oocytes matured with FCS and PVA, vitrified/warmed oocytes matured with 9-*cis*-RA showed cumulus cells expansion, although a lower incidence of necrotic nuclei (Fig. 3). Mitochondria appeared swollen, with degeneration of the internal structures, but keeping the integrity of the double membrane. The most interesting finding in this group of oocytes was the high numbers of residual bodies present in the cytoplasm of both cumulus cells and oocytes. This structure appeared as electron-dense with an evident membrane (Fig.

4). Oocytes vitrified and warmed showed lipid vesicles darker than fresh controls.

DISCUSSION

In this work we analyzed the ability of 9-*cis*-RA to influence the development and the ultrastructural appearance of the vitrified oocyte matured with 9-*cis*-RA. Within all experimental groups, vitrification of oocytes increased degeneration after IVF, and embryonic development rates from days 3 to 7 were lower than fresh controls. We did not obtain blastocysts from vitrified/warmed oocytes.

Morphologic appearance of COCs immediately after warming was normal (i.e., comparable to fresh, unvitrified COCs), allowing selection of all COCs for IVF. However, signs of degeneration (fragmented cytoplasm, indistinct oolema, increased perivitelline space, or damaged zona pellucida) were observed at the onset of culture. In addition, zygotes from vitrified and warmed oocytes appeared much more fragile, and removal of CC was usually associated to damage to the zona pellucida and plasma membrane.

Men et al.⁸ reported that composition of IVM medium influences cryosurvival. These authors replaced FSH for LH and found improved cryoresistance of the MII bovine oocyte. In our experiments, maturation media contained gonadotropins FSH and LH, added either with FCS, PVA, or 9-*cis*-RA, and no differences among maturation protocols were found in terms of survival after warming. Our degeneration percentages were lower, while cleavage rates were higher than those obtained by other authors,^{9,18,19} with the development up to the morula and blastocyst stage being compromised. The low correlation we found between cleavage and subsequent embryonic development has been cited elsewhere.^{9,15} In the present work we did not obtain blastocysts from vitrified/warmed oocytes, but development rates up to day 6 were comparable to our previous work.¹ Many other works reported controversial results after vitrification and warming of bovine oocytes,^{7,9,15,18–21} with observed differences being probably based on maturation media, cryoprotectants, and *in vitro*

culture system. Live calves have been cited to arise from vitrified and warmed immature oocytes.¹⁵

The altered morphology observed in vitrified/warmed COCs matured both with FCS and PVA, agrees with the findings of Fuku et al.,^{3,4} who suggested that exposure to cryoprotectants together with low temperatures may contribute to migration and fusion of vesicles in the oolema, causing major damage at the level of the plasma membrane. In our work, most gap junctions, which communicate intercellularly with CC and oocytes during the IVM process, appeared as damaged and interrupted. Mitochondria exhibited extensive vacuolization, swelling, and disappearance of most crestae. Within the oocyte, we observed premature release of GC into the perivitelline space. The perivitelline space was reduced. The crestae of the mitochondria were electron-dense, as a sign of morphologic alteration in the organelle, but the double membrane was conserved in most cases. Vesicles within the SER were more numerous than in fresh oocytes, and they appeared isolated from the mitochondria. Organization and metabolic activity of mitochondria are necessary features of cytoplasmic maturation and meiotic resumption.²² In bovine oocytes the major relocation of mitochondria occurs during IVM and it is influenced by hormones and energy substrates in the maturation medium.²⁴ The mitochondrial swelling observed in our vitrified/warmed oocytes can be responsible for an impaired development of these oocytes. In accordance with other authors,^{1–4,7,24} ultrastructural findings in our work seriously compromised embryo development. On the other hand, vitrified/warmed COCs matured with 9-*cis*-RA showed CC with an expansion degree comparable to those in FCS and PVA, whereas alterations in GJ appeared to be reduced, with mitochondria showing fewer degenerative signs. Interestingly, we found a high, unusual concentration of residual bodies in both CC and the oocyte. Residual bodies derive from lysosomes, which are membrane-bounded cytoplasmic vesicles enclosing 50 or more essentially hydrolytic enzymes. The boundary lysosomal membrane selectively admits substrates and protects the host cell from digestion by enclosed enzymes.²⁵

The origin of many of these residual bodies cannot be determined from their ultrastructure, but their number is notable in metabolically active cells. Skeletal cells treated with vitamin A in an organ culture show increased activity of releasing lysosomal enzymes and higher population of residual bodies.²⁶ On the other hand, the administration of cyclosporine A to rats increases the occurrence of numerous anomalous spermatids and residual bodies in the epididymal ducts.²⁷ To the best of our knowledge, no data about the presence of residual bodies in COCs have been provided. It should be hypothesized that the presence of RA, together with the cryodamage derived from the vitrification protocol produced this alteration, because it is not observed in the fresh oocyte treated with 9-*cis*-RA or in oocytes vitrified in the absence of 9-*cis*-RA.

We conclude that cryopreservation causes bovine oocytes to undergo degeneration during subsequent culture. In agreement with Men and coworkers^{7,8} the reduced developmental competence of our vitrified and warmed oocytes depends on cryodamage inflicted to the oocytes. Upon characterization of the mechanisms explaining degeneration within cryopreserved oocytes, improvements in oocyte cryopreservation could be possible by the addition of inhibitors in the cryopreservation solution and/or cell culture media.

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