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Effect of meiotic stage on viability and developmental competence of bovine oocytes vitrified in TCM-199 and bovine follicular fluid

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Abstract

Cryopreservation techniques for unfertilized oocytes have great potential for cattle breeding; however, the overall success remains low. Oocytes were divided in 8 treatment groups: Immature control, Mature control, Immature oocytes vitrified in Holding Medium (HM), Immature oocytes exposed to cryoprotectors in HM, Immature oocytes vitrified in Follicular Fluid (FF), Immature oocytes exposed to cryoprotectors in FF, Vitrified mature and Exposed mature. Viability and nuclear maturation were analyzed using propidium iodide and Hoescht 33342. Cleavage rate was determined on day 3 and blastocyst formation on day 7 after fertilization. After treatments, all non-vitrified oocytes were viable; however, after vitrification the viability was significantly reduced. Nuclear maturation analysis indicated that vitrification released oocytes from meiotic arrest. However after 22 – 24 hours of *in vitro* maturation no statistical differences were observed between groups ($P > 0.05$). A significantly higher number of zygotes underwent cleavage in the control group compared to the other groups ($P < 0.05$). No statistical differences were observed in numbers of blastocyst between control and exposed oocytes ($P > 0.05$), but blastocyst formation of vitrified oocytes was significantly lower ($P< 0.05$). According to the results obtained, oocytes are sensitive to osmotic and toxic damage during incubation with cryoprotectants and also during vitrification. Although most of the literature reports higher survival and developmental competence for oocytes vitrified after maturation, the present experiment showed no difference between immature and mature oocytes.

Keywords: Bovine, oocytes, vitrification, follicular fluid, mature/immature

Introduction

The establishment of a protocol to cryopreserve mammalian oocytes has several implications for assisted reproduction. Oocyte cryopreservation can preserve genetic material from valuable animals that may die unexpectedly or must be euthanized. Moreover, a successful oocyte banking system would eliminate the need to fertilize or even discard unused oocytes, as is common practice for human oocytes (Coticchio *et al*., 2004).

In Veterinary Medicine, the main goal of this technique is to improve productivity of valuable animals with outstanding genetic material, and to preserve endangered species from extinction (Holt, 1998). Cryopreservation techniques for unfertilized oocytes have great potential for cattle breeding especially when combined with *in vitro* maturation (IVM), *in vitro* fertilization (IVF) and embryo culture. However, the overall success of freezing and thawing procedures remains low (Men *et al*., 2003).

Several attempts have been made to preserve mammalian oocytes by cryopreservation, but results achieved are quite variable between species. The oocyte is a large cell with a high volume-surface ratio, surrounded by a zona pellucida and several layers of granulosa cells, forming the cumulus-oocyte complex (COC). Many specific problems have been described for oocytes at different meiotic stages of maturation, including those associated with the cryopreservation of ovulated oocytes like spindle disorganization (Mandelbaum *et al*., 2004), loss or clumping of microtubules resulting in some scattering of chromosomes (Sathananthan *et al*., 1988), increased polyploidy after fertilization (Al-Hasani *et al*., 1987; Carroll *et al*., 1989; Glenister *et al*., 1987), and a decrease in fertilization (Glenister *et al*., 1987; Wood *et al*., 1992, Mandelbaum *et al*., 2004). Freezing of immature oocytes at the GV stage might circumvent these problems because genetic material is contained within the contours of a nuclear envelope. Nevertheless, very low survival rates have been reported for cryopreserved immature bovine oocytes (Lim *et al*., 1992; Suzuki *et al*., 1996)

Two techniques of cryopreservation are possible options for preservation of oocytes. The traditional method of slow-rate cooling requires an intimate knowledge of membrane permeability to specific cryoprotectants. The results obtained using this method revealed that frozen-thawed oocytes had significantly lower cleavage rates than non-frozen controls (Lim *et al*., 1992, Otoi *et al*., 1993; George *et al*., 1994; Cooper *et al*., 1998). To minimize damage caused by cryopreservation, vitrification protocols were developed. Vitrification is defined as the solidification as cooling occurs in such a way that ice crystals do not form (Vajta, 1997); instead the solution just becomes solid by increasing viscosity. This is achieved by increasing the viscosity of the solution as well as

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increasing the speed of cooling. One method for vitrification has come to the forefront: the Open Pulled Straw method (Vajta *et al.*, 1996).

In face of the difficulties described, the objective of this experiment was to compare the survival and developmental competence of immature and *in vitro* matured bovine oocytes after vitrification in Open Pulled Straws. Bovine follicular fluid obtained from immature follicles was used as a base for vitrification media as to keep immature oocytes in the GV stage. Effects of cryoprotectant solution on oocyte viability and embryo development were also evaluated.

Materials and Methods

Preparation of bovine oocytes: Oocytes were recovered from 2- to 8-mm follicles into Dulbecco's phosphate-buffered saline by aspiration using a 6-ml syringe with an attached 18 gauge needle. Ovaries were transported in sterile saline at 25 to 30°C to the laboratory within 3 h of slaughter. During selection the oocytes were maintained in fresh filtrated bovine follicular fluid. Oocytes were evaluated at 25x magnification, and only oocytes having intact, compact cumulus cells and evenly granulated cytoplasm were used.

In vitro maturation (IVM): Oocytes for IVM were matured in 4-well dishes containing 400µl of media and 20 – 25 oocytes in each well at 38.5° C in 5% CO_2 in air for 24 h. Maturation media was composed by TCM-199 with Earle's salts (Sigma, St. Louis, MO) + 10% of FCS, 1 µg/mL estradiol 17ß (E_2 – Sigma, St. Louis, MO), 50µg/ml hCG (Profasi®, Serono, Brazil, 5.000IU), 5 µg/mL FSH (Folltropin - V®, Vetrepharm, Canada), and gentamicin (25 µg/mL).

Vitrification and exposure to cryoprotectants: Two vitrifications solutions were used. In the first one, the basic medium was Hepes-TCM 199 (Sigma, St. Louis, MO) + 10% FCS (holding media - HM), and in the second one, bovine FF obtained from immature follicles was added to HM (1:1).

The COC selected for vitrification were equilibrated at 39o C in 10% ethylene-glycol (EG) + 10% DMSO in HM or in FF:HM for 30 – 45 seconds, and transferred to the vitrification solution with 20% EG + 20% DMSO + 0,5M sucrose in HM of in FF:HM for 25 sec. During the last step oocytes were aspirated into open pulled straws (OPS) according to the technique described by Vajta *et al.* (1997) and plunged in liquid N_2.

The oocytes selected for exposure to cryoprotectants were washed in the same solutions, for the same times as describe previously. However, the oocytes were not vitrified and after 25 sec in 20% EG + 20% DMSO = 0.5M sucrose, the oocytes were transferred to the warming solution.

Warming: For warming and re-hydration of oocytes, the thinner extremity of the OPS was embedded in HM solution + 2.5M sucrose at 39°C for groups IHM and GMV, and in solution of FF + 2.5M sucrose for group IFF. The oocytes release from OPS and the oocytes exposed to cryoprotectants remained in this solution for 5 min. Oocytes were them transferred to a 0.15M sucrose solution for an extra 5 min and finally to HM or FF.

Experimental groups: Selected oocytes were utilized in two different experiments.

Experiment 1

The aim of this experiment was to verify the viability and nuclear maturation of immature and *in vitro* matured oocytes after exposure to cryoprotectants and after vitrification.

Oocytes were divided in 8 different treatment groups.

- Group IC (Immature control) – oocytes analyzed just after collection.
- Group MC (Mature control) – oocytes analyzed after 24 h of *in vitro* maturation.
- Group IHM (Immature oocytes vitrified in HM) – oocytes were vitrified in Hepes-TCM 199 (Sigma, St. Louis, USA) + 10% FCS (HM) + 20% ethylene-glycol, 20% DMSO and 0.5M Sucrose just after collection.
- Group IHMexp (Immature oocytes exposed to cryoprotectants in HM) – just after collection, oocytes were exposed to cryoprotectants in HM but not vitrified.
- Group IFF (Immature oocytes vitrified in FF) - oocytes were vitrified in filtrated bovine follicular fluid (FF) with HM (1:1) + 20% Ethylene-glycol, 20% DMSO and 0.5M Sucrose just after collection.
- Group IFFexp (Immature oocytes exposed to cryoprotectants in FF) – just after collection, oocytes were exposed to cryoprotectants in FF:HM but not vitrified.
- Group VM (vitrified mature) – oocytes vitrified in HM after *in vitro* maturation;
- Group VMexp (Exposed Mature) – Oocytes exposed to cryoprotectants in HM after *in vitro* maturation.

Summary of groups - Experiment 1

	control	exposed		vitrified	
immature	IC	IHMexp	IFFexp	IHM	IFF
mature	MC	VMexp		VM	

Experiment 2

The aim of this experiment was to compare the cleavage rates and blastocyst formation after exposure to cryoprotectants or vitrification of immature and *in vitro* matured bovine oocytes. The same groups described to Experiment 1 were used, but group IC (immature control) was omitted.

In vitro fertilization: To perform the *in vitro* fertilization sperm were selected using the swim up procedure and the concentration adjusted to 1 x 10^7 sperm cells/ml.

After maturation, oocytes were transferred to 30µl drops of Fert-TALP covered with mineral oil. To each drop containing 10 oocytes was added a 10µl volume of the prepared sperm (a total of 100.000 sperm). Sperm were co-incubated with oocytes for 18 h at 38.5° C in 5% CO_2 in air.

After fertilization presumptive zygotes were washed in culture media to remove the remaining granulosa cells and spermatozoa. The same dish used during maturation was prepared for *in vitro* culture. The culture medium was composed of Human Tubal fluid (HTF®, Irvane, New Zeeland) + 10% FCS, 0,01% myo-inositol (Sigma, St. Louis, MO) and 75µg/ml gentamycin.

Cleavage rate was determined on day 3, and blastocyst formation on day 7 after fertilization.

Viability and nuclear maturation analysis: Oocytes were stripped in Hepes-TCM199 + 10% FCS and 300IU hyaluronidase using a fine-bore glass pipette. Denuded oocytes were incubated in DBPS + 1% BSA + 125µg/ml propidium iodide during 10 min and transferred to droplets of a 90% glycerol in DPBS + 100µg/ml Hoescht 33342 on histological slides. A cover slip was deposit over the droplet, and the oocytes gently squeezed. After 5 min incubation, the slides were examined on a fluorescence inverted microscope using a blue filter (535 – 617 nm). The oocytes heavily stained red were considered damaged. Nuclear maturation was determined by examination of chromatin stained in blue with Hoescht 33342 using a UV filter (350 – 461 nm).

Statistical analysis: Three replicates were completed for the viability test and 5 for IVF and IVC. Goodmann test was used to compare treatment differences in percentages of oocytes according to nuclear maturation. To compare differences in cleavage and blastocyst formation rates, the Kruskal Wallis non-parametric test was used.

Results

Experiment 1

Almost all non-vitrified oocytes (IC, MC, IHMexp, IFFexp and VMexp) were viable (Table 1). However, after vitrification (GIV, GIFFV and GMV) the viability of oocytes was significantly reduced (Table 1).

Nuclei of the majority of oocytes were in the germinal vesicle stage (GV) just after collection (IC), as well as in the groups of immature oocytes exposed to cryoprotectants (IHMexp and IFFexp) (Table 2). However, when nuclear maturation was analyzed in vitrified immature oocytes, greater germinal vesicle breakdown (GVBD) was observed than with IC (Table 2).

No oocytes in GV or GVBD were observed after 22 – 24 h of in vitro maturation (CM, GMV and GMexp). Numbers of oocytes that reached metaphase II are shown in Table 3. No statistical differences were observed between groups (P > 0.05).

Table 1. Cell viability in experimental groups

Classification	IC	MC	IHM	IHMexp	IFF	IFFexp	VM	VMexp
	(n)	(n)	(n)	(n)	(n)	(n)	(n)	(n)
Viable	97%[a]	100%[a]	57%[b]	97%[a]	72%[b]	100%[a]	51%[b]	100%[a]
	(38)	(30)	(35)	(33)	(43)	(35)	(37)	(35)
Damaged	3%	0%	43%	3%	28%	0%	49%	0%

[a,b]percentages with different superscripts differ (P < 0.05) ANOVA.

IC = Immature control

MC = Mature control

IHM = Immature oocytes vitrified in holding medium

IHMexp = Immature oocytes exposed to cryoprotectants in holding medium

IFF = Immature oocytes vitrified in follicular fluid + holding medium (1:1)

IFFexp = Immature oocytes exposed to cryoprotectants in follicular fluid + holding medium

VM = Vitrified mature

VMexp = Exposed mature.

Table 2. Nuclear maturation of bovine immature oocytes

Classification	IC (n=38)	IHM (n=35)	IHMexp (n=33)	IFF (n=43)	IFFexp (n=35)
GV	84%[a]	49%[b]	85%[a]	61%[b]	97%[a]
GVBD	0%[a]	34%[b]	15%[ab]	30%[b]	3%[a]
Unidentified	16%	17%	0%	9%	0%

[a,b]percentages without common superscript differ (P < 0.05).
GV – Germinal Vesicle,
GVBD – Germinal vesicle break down
IC = Immature control
IHM = Immature oocytes vitrified in holding medium
IHMexp = Immature oocytes exposed to cryoprotectants in holding medium
IFF = Immature oocytes vitrified in follicular fluid + holding medium (1:1)
IFFexp = Immature oocytes exposed to cryoprotectants in follicular fluid + holding medium

Table 3. Nuclear maturation of bovine oocytes *in vitro* matured after vitrification (GMV) or exposure to cryoprotectants (GMexp).

Oocytes	MC (n=30)	VM (n=37)	VMexp (n=35)
MI	33%	24%	43%
MII	67%	70%	49%
Unidentified	0%	6%	8%

MI – Metaphase I
MII – Metaphase II
MC = Mature control
VM = Vitrified mature
VMexp = Exposed mature.

Experiment 2

Cumulus expansion was similar in all groups of oocytes vitrified just after collection (P > 0.05).

Cleavage rate were higher for the control (MC) than for any other group (Table 4). The group of oocytes vitrified after in vitro maturation (VM) showed a lower cleavage rate than the groups of non-vitrified oocytes (Table 4). Concerning blastocyst formation rates, there were no statistical differences in number of blastocysts between groups of oocytes exposed to cryoprotectants and the control group (Table 4). However, the mean blastocyst formation in groups of vitrified oocytes was significantly lower than the control group (Table 4).

Table 4. Mean cleavage and blastocyst formation rates per oocyte for experimental groups.

Group	CLEAVAGE (N)	BLASTOCYST FORMATION (N)
MC	68%[a] (338)	34%[a] (338)
IHM	19%[bc] (193)	5%[b] (193)
IHMexp	35%[bc] (208)	13%[ab] (208)
IFF	13%[bc] (226)	4[b] (226)
IFFexp	43[b] (227)	17[ab] (227)
VM	6[c] (213)	4[b] (213)
VMexp	42[b] (121)	21[a] (121)

[a,b,c]Mean without common superscript differ (P < 0.05).
MC = Mature control
IHM = Immature oocytes vitrified in holding medium
IHMexp = Immature oocytes exposed to cryoprotectants in holding medium
IFF = Immature oocytes vitrified in follicular fluid + holding medium (1:1)
IFFexp = Immature oocytes exposed to cryoprotectants in follicular fluid + holding medium
VM = Vitrified mature
VMexp = Exposed mature.

Discussion

The first step in any cryopreservation protocol is exposure of the cells to cryoprotectant. This procedure can result in extreme fluctuations in volume, causing cell damage or making the cell more susceptible to damage during subsequent cooling. Martino *et al.* (1996) suggested that osmotic stress produced by cryoprotectants has deleterious effects on survival of mature bovine oocytes after cryopreservation. However, in this experiment incubation of oocytes with cryoprotectans had no detrimental effect on oocyte viability either in immature or in *in vitro* matured oocytes.

According to Fahy (1986) no cryopreservation technique allows 100% of cellular survival after thawing. During cryopreservation, oocytes experience severe adverse physiological conditions and these result in oocytes with various cytological injuries (Men *et al*, 2003). The results obtained in this experiment showed that after vitrification lower percentages of viable oocytes were observed. Meiotic stages of oocytes also influence the ability of oocytes to survive cryopreservation. According to Ford *et al*. (2000), in rat oocytes permeability of the plasmalemma decreases during maturation in vivo or in vitro. However, according to Agca *et al.* (1998) bovine mature oocytes were more permeable to EG and DMSO than immature ones. In fact, the permeability characteristics of the plasmalemma are one of the questions on which research on oocyte cryopreservation will have to focus on in the future. Moreover, one of the major concerns caused by oocyte freezing is possible effects on the cytoskeletal structures. The meiotic spindle, present in oocytes that resume meiosis, is a particularly sensitive structure, showing only partial ability to restore its original organization following cooling and thawing (Pickering *et al*., 1990). These data indicate that the results of oocyte cryopreservation would be lower after maturation. However, the literature shows that the germinal vesicle (GV) stage is more sensitive to cryopreservation than any other nuclear stages (Parks and Ruffing, 1992; Otoi *et al*., 1995; Parks *et al*., 1997) for reasons yet unknown. The results obtained in this study disagree with most of the data found in the literature, since no statistical differences were observed in the viability of oocytes vitrified just after collection (immature) and after *in vitro* maturation.

Although oocytes collected from a given batch of slaughtered cattle ovaries constitute a heterogeneous follicular population, most of the oocytes were arrested at the GV stage. When nuclear maturation was analyzed in immature vitrified oocytes, a higher number of GVBD was observed compared to non-vitrified oocytes. These data indicate that somehow, the vitrification process released the oocytes from meiotic arrest. It has been demonstrated that follicular fluid collected from small follicles inhibits meiotic resumption (Ayoub and Hunter, 1993; Choi *et al*., 1998). So in order to allow them to acquire developmental competence at the same time, after thawing, oocytes were vitrified in FF. However the results showed that the addition of FF to the vitrification media did not prevent vitrified oocytes from resuming meiosis. In fact, previous results obtained in the same laboratory showed that the addition of FF to vitrification media had no influence on nuclear maturation rates (Siqueira *et al*., 2002)

After i*n vitro* maturation no oocytes in GV or GVBD were observed, and there were no statistical differences between groups concerning nuclear maturation. Although the highest blastocyst rate in the literature was obtained from cryopreserved MII oocytes (Vajta *et al*., 1998), consistent results are still difficult to achieve. One of the possible causes is the disorganization of spindle, with the consequent dispersion of chromosomes in MII (Aman and Parks, 1994; Mandelbaum *et al*., 2004). In this experiment the fine structure of the spindle was not analyzed, but the percentage of oocytes with undetected chromatin after vitrification was not different between groups of MII oocytes.

The ability of an oocyte to be fertilized and develop normally requires both nuclear and cytoplasmic maturation. Although in experiment I, no statistical differences were observed in oocyte viability between controls and oocytes exposed to cryoprotectants, in experiment II, the cleavage rates obtained in the control group (68%) were significantly higher than those observed in groups of oocytes exposed to cryoprotectants (36, 39 and 46% for IHMexp, FFexp and MVexp respectively). This may be related to a toxic effect of the cryoprotectants. Arnold *et al* (1983) determined that cryoprotectants may have a direct action on cryoinjuries by altering membrane polarity, resulting in permeability changes. In an experiment of Men *et al*. (2003), more than 90% of oocytes were viable just after warming. Nevertheless, the detrimental effect of cryopreservation was expressed gradually during in vitro culture. However, in this experiment no differences on cleavage rates were observed between oocytes exposed to cryoprotectants before and after *in vitro* maturation.

The cleavage rates obtained after fertilization of vitrified oocytes were significantly lower than in the control group. These results are in agreement with Fukui *et al* (1995), who showed that cooling leads to low fertilization rates due to premature release of cortical granules and zona pellucida hardening.

According to the literature, blastocyst formation is lower when immature are oocytes vitrified compared with mature oocytes. Distribution of organelles in immature oocytes is more sensitive to cold shock, resulting in impairment of protein synthesis necessary for fertilization and embryo development (Parks and Ruffing, 1992). However the results obtained in the present experiment did not show statistical

differences in blastocyst formation rates between oocytes vitrified before and after *in vitro* maturation.

Vajta *et al.* (1998) reported very high rates of development of vitrified oocytes to blastocyst (up to 25%). Many reasons could explain such differences between their results and ours: origin of biological material (dairy vs beef cows), reduced vs unreduced size of the cumulus and culture system (SOF vs HTF). For *in vitro* matured oocytes, the manner in which the oocytes are matured may also affect the oocyte's response to cryopreservation. It has been demonstrated that oocytes must undergo not only nuclear maturation, but also cytoplasmic maturation to become developmentally competent. Cytoplasmic maturation is much more difficult to achieve than nuclear maturation in an *in vitro* system, and inadequate cytoplasmic maturation could seriously compromise the ability of oocytes to continue development (Leibfred-Rutledge *et al*., 1989). In this experiment blastocyst formation in the control group was 34%. Although this rate is in accordance to the results obtained in several laboratories, it could be improved by modifications in the culture system, such as culture in a semi-defined sequential system. This procedure may results in better blastocyst formation after cryopreservation.

Apparently the detrimental effect of the vitrification procedure is due to cumulative effects of exposure to vitrification solution (as cleavage rates for exposed oocytes were lower than those for controls) and to vitrification per se (as blastocyst rates for vitrified oocytes were significantly lower than those for exposed oocytes at the same meiotic stage). The effect of the exposure to vitrification solution appears at the time of first cleavage, and the detrimental effect of the vitrification procedure became more evident with the time length of culture.

According to the results obtained in this study, oocytes are sensitive to osmotic and/or toxic damage during incubation with cryoprotectants and also during vitrification. The addition of FF to the media did not prevent oocytes from resuming meiosis and was not efficient in protecting oocytes during vitrification. Although most publications report a higher survival rate and developmental competence for oocytes vitrified after maturation, in this experiment no difference was observed between groups of immature and mature oocytes concerning oocyte viability, cleavage and blastocyst formation rates.

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