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Short communications

Reduced competence of immature and mature oocytes vitrified by Cryotop method: assessment by *in vitro* fertilization and parthenogenetic activation in a bovine model

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Summary

This study aimed to evaluate the embryo development competence, the nuclear maturation and the viability of germinal vesicle (GV) and metaphase II (MII) oocytes vitrified by the Cryotop method. Cumulus–oocyte complexes were derived from bovine ovaries and three experiments were conducted. In Experiment 1, GV oocytes were vitrified and underwent *in vitro* maturation (IVM) or not and their nuclear maturation was assessed by orcein staining. In Experiment 2, GV oocytes and MII oocytes were vitrified or not and the viability was assessed by calcein/ethidium homodimer-1 staining. In Experiment 3, MII oocytes matured before or after vitrification were submitted to *in vitro* fertilization (IVF) and parthenogenetic activation (PA) in order to evaluate embryo development. No difference was found for the nuclear maturation rate in the GV group (50%) and the GV control group (67%; $P = 0.23$) and for viability rate (56%; 77%; $P = 0.055$, respectively). However, in the MII group (27%) viability was significantly lower than that of the MII control group (84%; $P < 0.0001$). The cleavage rate by IVF and PA was similar in the GV group and the MII group. In contrast, vitrified MII oocytes showed no capacity for blastocyst development after IVF or PA and vitrified GV oocytes were able to develop to blastocysts only after PA, but not after IVF. In conclusion, oocyte vitrification by the Cryotop method reduced the capacity for embryo development. Vitrification of GV oocytes, however, did not influence the capacity of meiotic nuclear maturation and they exhibited higher viability following vitrification at the MII stage.

Keywords: Bovine oocyte, Embryo Development, Viability, Vitrification

Introduction

Currently, cryopreservation of oocytes has been applied in many species with different goals. Animal models are used due to the limited availability of human oocytes for research purposes to assisted reproduction techniques (ARTs) (Lee *et al.*, 2013), in this matter many aspects of reproductive physiology in bovines are similar to those in humans, including follicle growth, the sizes of the ovaries and oocytes, embryonic genome activation and interaction with the culture medium (Shaw *et al.*, 2000). However gametes cryopreservation also has great importance in animal breeding programmes, storage of female gametes for extended periods and animal reproduction in general (Hochi *et al.*, 1996; Hochi *et al.*, 1998; Mara *et al.*, 2013).

In several mammalian species the ability of cryopreserved oocytes to achieve later embryonic development and succeed to birth is low (Massip, 2003; Sprícigo *et al.*, 2014) regardless of oocyte stage (Versieren *et al.*, 2011). This situation probably arises due to intracellular ice crystal formation and chilling injury caused by the freezing procedure (slow freezing technique), which leads to the destruction

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of intracellular communication between cumulus cells and oocytes (gap junctions) (Hochi *et al.*, 1998) and consequently interferes in oocyte maturation by impairing nutrient supply during IVF (Versieren *et al.*, 2011; Buccione *et al.*, 1990; Chian *et al.*, 1994; Ka *et al.*, 1997). So, to avoid this injury the vitrification technique has been tested and has acquired satisfactory results in human oocytes (Cobo *et al.*, 2010; Rienzi *et al.*, 2010; Herrero *et al.*, 2011; Mukaida & Oka, 2012), which involves cell exposure to high concentrations of cryoprotectants and ultra-rapid cooling (Rall & Fahy, 1985).

On this basis, the retrieval of immature oocytes in the germinal vesicle (GV) stage, their cryopreservation or *in vitro* maturation (IVM) followed by *in vitro* fertilization (IVF) (Cha & Chian, 1998; Tucker *et al.*, 1998; Lin & Hwang, 2006; González *et al.*, 2011; Lee *et al.*, 2013) has been investigated as an option to preserve the genetic diversity in several animal species. In humans it has emerged as an option in cases in which fertility preservation is demanded. The aim of the present study was to evaluate the influence of vitrification protocol on nuclear maturation capacity of immature oocytes, as well as to assess the effect of vitrifying and thawing oocytes at different meiotic nuclear stages in relation to its survival and development capacity after IVF and parthenogenetic activation (PA).

Materials and methods

All the chemicals used in this study were purchased from Sigma-Aldrich (Oakville, ON, Canada), unless otherwise indicated. This study was performed in three experiments: Experiment 1, GV oocytes were vitrified or not and underwent IVM and the nuclear maturation was assessed by orcein staining. Experiment 2, GV oocytes and MII oocytes were vitrified or not and their viability were assessed by calcein/ethidium homodimer-1 staining. Experiment 3, MII oocytes matured before or after vitrification were submitted to IVF and PA in order to evaluate embryo development.

Bovine ovaries collection

Bovine ovaries were collected from random stages of the oestrous cycle in a slaughterhouse and transported to the laboratory in saline solution (0.9% NaCl) containing gentamycin (50 μg/ml) at 35–37°C.

Selection of cumulus–oocyte complexes (COCs)

COCs were aspirated with a syringe and needle from 2–8 mm antral follicles washed in holding medium (HM) consisting of HEPES-buffered TCM-199 and only COCs with compact, with no more than three layers of cumulus cells and an oocyte with homogeneous cytoplasm (immature oocytes), were used in the experiments. For each replicate of the experiments these COCs were randomly assigned to the three study groups: control group (immature oocytes submitted to IVM), GV group (immature frozen oocytes followed by post thawed IVM), and MII group (fresh IVM followed by oocyte vitrification).

In vitro maturation (IVM)

The basic maturation medium was TCM-199 supplemented with 0.2 M sodium pyruvate, 0.5 μg/ml follicle-stimulating hormone (FSH), 5 μg/ml luteinizing hormone (LH), 1 μg/ml estradiol, 100 IU/ml penicillin, and 0.1 mg/ml streptomycin. Maturation was accomplished in 35 mm Petri dishes covered with 3 ml of M8410 mineral oil at 38.5° C, in an atmosphere with 5% CO_2 in air for a period of 22 h. COCs were cultured in a volume of 70 μl culture medium.

Oocyte vitrification and warming

All of the vitrification solution was used at room temperature (25–27°C), only the thawing solution (TS) step was used at 37°C. HM supplemented with 20% synthetic serum substitute (SSS) was used for handling oocytes during vitrification and warming. For vitrification, the oocytes were washed (three times) in an equilibrium solution containing 7.5% (v/v) ethylene glycol (EG) and 7.5% (v/v) dimethylsulfoxide (DMSO) in HM for a total of 9 min. Then, the oocytes were transferred to vitrification solution containing 15% EG, 15% DMSO, and 0.5 M sucrose for 1 min. Under a stereomicroscope (Nikon-SMZ 650), the oocytes were loaded onto the film strip of a Cryotop device (three oocytes/device) and were immediately submerged into liquid nitrogen for storage. Warming was performed by immersing directly the Cryotop device in 1 ml of TS containing 1 M sucrose (1 min). After that, the oocytes were transferred to HM with 0.5 M sucrose (3 min) and washed twice in original HM for 5 min. The thawed oocytes were then incubated in TCM-199 medium at 38.5°C, in 5% CO_2 and a 95% humidified air atmosphere for 2 h. Those oocytes vitrified at GV stage were then submitted to IVM for 22 h.

Assessment of meiotic stage of bovine oocytes by orcein staining

After 22 h of IVM, before or after vitrification/warming, the COCs were denuded in a 0.03% hyaluronidase solution by repeated pipetting. The nuclear status of the oocytes was assessed microscopically

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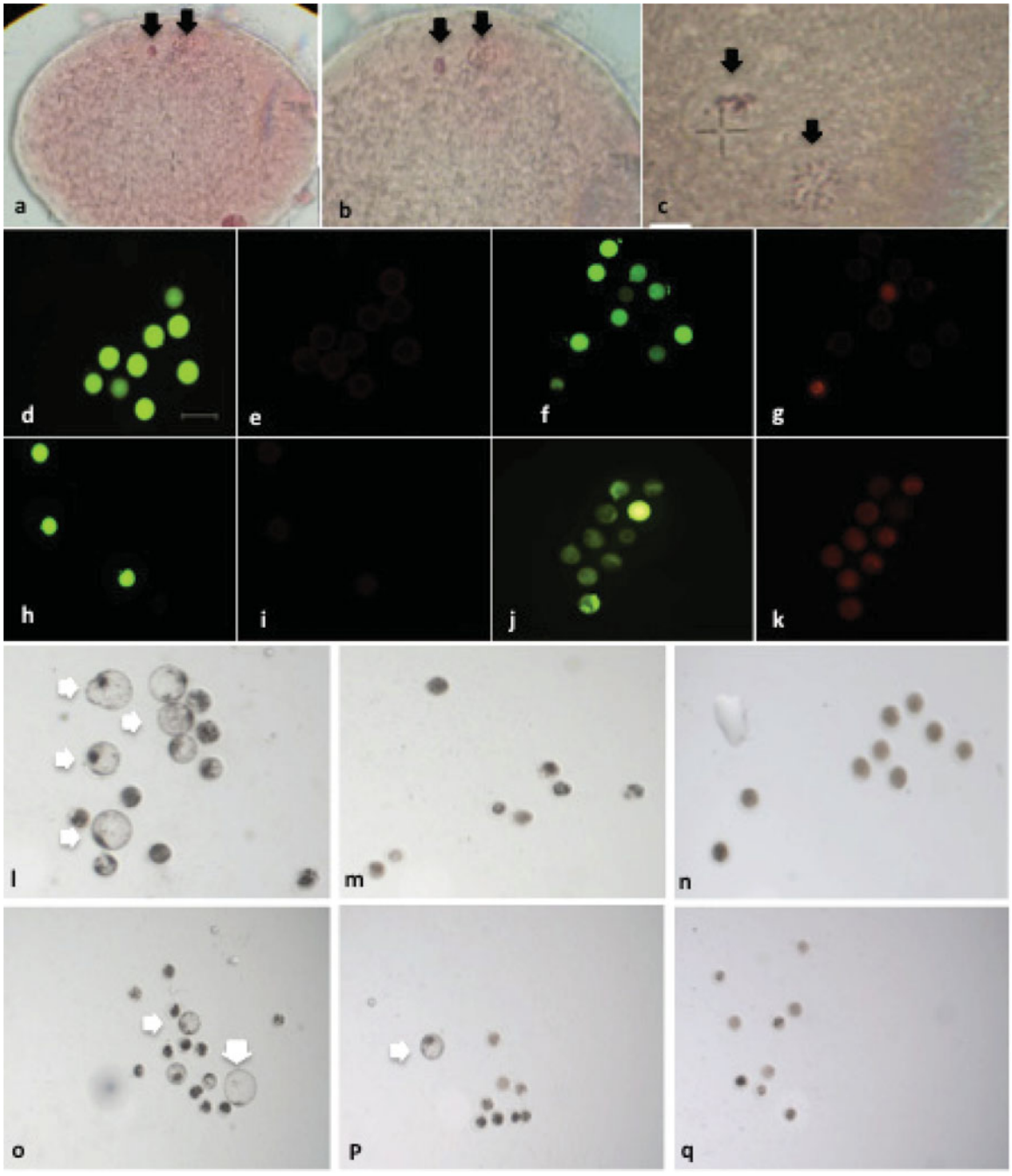

Figure 1 (*a–c*) Representative images of nuclear maturation (metaphase II stage) obtained from immature oocytes vitrified and submitted to *in vitro* maturation (IVM), in which black arrows indicate two groups of unequally spread chromosomes, the polar body set clustered together and the haploid set of the oocyte spread out – MII stage (*a* ×10 magnification; *b*, ×40 magnification; and *c*, ×100 magnification). Images of oocytes under viability assessment by dead/live technique staining (×40 magnification) where calcein/green fluorescence indicates viable oocytes and dead oocytes/red fluorescence. (*d*, *e*) Oocytes from the GV control group; (*f*, *g*) oocytes from GV group; (*h*, *i*) oocytes from MII control group; and (*j*, *k*) oocytes from the MII group. Representative images of embryo development to blastocyst stage in day 8 by *in vitro* fertilization (IVF) of oocytes from (*l*) control group; (*m*) GV group; and (*n*) MII group; and by parthenogenetic activation (PA) of oocytes from (*o*) the control group, (*p*) the GV group and (*q*) the MII group. Scale bar = 50 μm.

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(×1000 magnification), after fixation (methanol:acetic acid 3:1) and orcein staining (2%). Oocytes were classified as follows: (i) immature: oocytes with a germinal vesicle visible (GV stage); or (ii) mature: metaphase II oocytes with two groups of unequally spread chromosomes, the polar body set clustered together and the haploid set of the oocyte spread out (MII stage) (Süss *et al.*, 1988) (Fig. 1*a–c*).

Viability assessment

Denuded oocytes were assessed by calcein AM/ethidium homodimer-1 (CaAM/EthD-1) staining, based on the simultaneous determination of live cells using calcein (AM) for the detection of intracellular esterase activity and ethidium homodimer-1 (EthD-1) to label the nucleus acids of non-viable cells with plasma membrane disruption (Cortvrindt & Smitz., 2001; Martinez-Madrid *et al.*, 2004; Sanfilippo *et al.*, 2011; Merdassi *et al.*, 2011). Fresh and thawed oocytes were submerged in DPBS containing 2 μM AM and 5 μM EthD-1 (Molecular Probes, Invitrogen-Life Technologies, Germany) for 15 min, washed in DPBS and mounted on glass slides for examination under a fluorescence microscope (AxioScope A1, Zeiss, Germany). The labelled oocytes were classified for fluorescent staining as follows: (i) as live oocytes (viable) when the cytoplasm was stained positively with AM (green fluorescence); or (ii) as dead oocytes (unviable) when chromatin was labelled with EthD-1 (red fluorescence) (Fig. 1*d–k*).

In vitro fertilization and parthenogenetic activation

COCs submitted to IVM, before or after vitrification/warming, were selected for either IVF or PA. For IVF the COCs were washed in HM supplemented with 50 μg/ml gentamycin, 0.2 M sodium pyruvate and 5% FCS. Semen for all replicates was collected from a single male and stored at liquid nitrogen (LN_2). Sperm were obtained by a swim-up method (Parrish *et al.*, 1988), sperm were washed with Sperm-TALP HEPES medium for 1 h at 38.5°C. Following this step, the supernatant sperm were then centrifuged at 500 *g* for 10 min and motile sperm were selected. The fertilization medium was FERT-TALP (Gordon, 1994), supplemented with 20 μg/ml heparin and 6 mg/ml bovine albumin essential fatty-acid free (BSA; Sigma A-7030). Sperm concentration was approximately 2 × 10^6 spermatozoa/ml and the sperm were co-incubated with 20 COCs for 18 h in a humid atmosphere containing 5% CO_2 at 38.5°C. For PA, COCs were denuded by incubation with 0.03% hyaluronidase solution for 5 min and by repeated pipetting. Then, the oocytes at metaphase II with the presence of a first polar body were activated using 10 μM calcium ionophore for 5 min followed by 2 mM 6-DMAP for 3 h at 38.5°C, in the presence of 5% CO_2 in air and high humidity. Activated oocytes were subsequently cultured in embryo medium (CR2aa medium) (Rosenkrans & First, 1994) under mineral oil at 38.5°C.

Assessment of embryonic development *in vitro*

After IVF or PA, the presumptive zygotes were mechanically denuded (partially – up to two or three layers of cumulus cells) and cultured in 50 μl drops of embryo medium (CR2aa medium) (Rosenkrans & First, 1994) supplemented with 5% FCS and 1 mg/ml BSA under mineral oil, in a moist atmosphere containing 5% CO_2 at 38.5°C. The embryo medium was replaced by fresh medium every 48 h. Embryo cleavage rate was recorded on day 3 after insemination (day 0 = day of insemination) and was calculated as the number of cleaved embryos (2- to 8-cell stage) divided by the number of COCs placed in culture. Further, embryo development was assessed on day 8, when blastocyst development rate was calculated by the number of blastocyst stage embryos divided by the number of COCs placed in culture. In addition, the proportion of oocytes that developed to the hatched blastocyst stage was recorded 240 h after insemination or activation. For the *in vitro* embryo production experiment, five replications of IVF and seven of PA were performed in different experiments for IVF or PA.

Statistical analysis

Data regarding oocyte nuclear maturation, oocyte viability and embryonic development (cleavage and blastocyst production) were analyzed by the chi-squared (Q^2) test. The hatching rate was evaluated by Fisher's exact test. The results are shown as percentages, and the level of significance was set at $P < 0.05$.

Results

Assessment of meiotic stage of bovine oocytes

In total, 114 oocytes from the control group and 152 oocytes from GV group were destined to meiotic stage analysis. For this assessment orcein staining was applied. As the oocyte position during fixation is crucial for meiotic stage identification – the meiotic spindle shall be visible and the chromosomes aligned in the metaphasic plate – an expressive number of oocytes were considered not analysable [GV control group = 30 (26%) and GV group = 70 (46%)]. Among the analysable oocytes, 90% ($n = 76$) of the immature oocytes cryopreserved before IVM (GV group) presented meiotic nuclear maturation after IVM and 92.7% ($n = 76$) of the immature oocytes not submitted to

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Table 1 Oocyte viability rate of immature and mature oocytes cryopreserved and matured *in vitro*

Treatment groups	*N* oocytes	Viable oocytes *n* (%)	Non-viable oocytes *n* (%)
GV control group	48	37 (77)^{a,b}	11 (23)
GV group	34	19 (56)^{a,c}	15 (44)
MII control group	38	32 (84)^{b}	6 (16)
MII group	41	11 (27)^{c}	33 (75)

Treatment groups: GV control group (immature oocytes subsequent to IVM without cryopreservation); GV group (immature oocytes vitrified and then submitted to IVM); MII control group (oocytes matured *in vitro* and then vitrified); MII group (immature oocytes subsequent to IVM without cryopreservation). Values with the same superscripts letters are significantly different ($P < 0.05$).

cryopreservation (GV control group) were considered matured (MII) ($P = 0.78$; Experiment 1).

Viability assessment

Immature oocytes vitrified before IVM showed similar viability rate between the GV group 56% (19/34) and the GV control group 77% (37/48) ($P = 0.055$). However, the MII group showed lower viability 27% (11/41) in relation to the MII control group 84% (32/38) ($P < 0.0001$). Thus, higher viability rate was observed in immature oocytes (GV group) in comparison to *in vitro*-matured oocytes (MII group) ($P = 0.017$) (Table 1; Experiment 2).

Assessment of embryonic development *in vitro* by IVF

When analyzing the capacity of oocytes for embryonic development *in vitro* by IVF, no difference was found in cleavage rate between oocytes from GV group 28% (11/39) and MII group 26% (12/46) ($P = 1.0$). However, cleavage rates in both groups were significantly lower than those observed in immature oocytes submitted to IVM without vitrification (MII control group) 80% (169/211) ($P < 0.001$). The embryo development in MII control group (fresh) in relation to blastocyst formation was 26% (54/211) and hatching 41% (22/54) while in both vitrified groups (GV group and MII group) no blastocyst formation or hatching was observed (Table 2).

Assessment of embryonic development *in vitro* by PA

To examine the influence of the fertilization capacity of the spermatozoa, PA was performed. The cleavage rates were significantly lower in both vitrified groups, GV group 28% (14/50) and MII group 22% (14/63) ($P = 0.59$). However, cleavage rates were statistical different in the MII control group 58% (118/205) in relation to the GV group ($P < 0.002$) and MII group ($P < 0.001$). Development to the blastocyst stage was significantly lower in the GV group 2% (1/50) while in the MII control group the cleavage rate was 24% (50/205) ($P < 0.0001$). However, in MII group there was no blastocyst formation ($P < 0.001$). No significant difference was founded between the vitrified groups (GV group and MII group) in relation to blastocyst formation ($P = 0.65$; Experiment 3) (Table 3). In this way, the hatching rates in GV group were 100%, which represents one hatched blastocyst in 50 cultured embryos (Table 3) while in the MII control group the hatching rate was 18% (9/50) ($P = 0.04$). In MII group there was no hatching after PA ($P = 0.003$) (Table 3 and Fig. 1*l*–*q*).

Discussion

Different from human oocytes that showed high survival rates and embryo development after vitrification in comparison to slow freezing (Katayama *et al.*, 2003; Kuwayama *et al.*, 2005; Kuwayama, 2007), bovine oocytes have repeatedly been tested for vitrification with poor results. In this short study, we demonstrated that bovine oocyte vitrification by the Cryotop method significantly reduced oocyte viability and developmental competence despite the nuclear maturation stage at the time of cryopreservation. However, immature oocytes (GV stage) were shown to be more resistant to the cryopreservation process than mature oocytes due to the ability to mature and its viability by Dead-Live technique was not compromised. The immature oocytes, after vitrification and IVM, were able to progress from the GV stage to the MII stage. Similar data were obtained by Sripunya *et al.* (2014), in a study in which surviving bovine oocytes vitrified by Cryotop method were lately subjected to IVM for 24 h and showed a 58% rate of MII, while oocytes *in vitro*-matured without vitrification showed an 81% MII rate (Sripunya *et al.*, 2014). It should be noted that several are the mechanisms involved in the correct progression of meiosis; among them the activities of the cytoplasmic proteins of the maturation promoting factor (MPF) complex and of the proteins of the mitogen-activated protein kinase (MAPK) family, which have been demonstrated to be preserved after IVM and the cryopreservation process (Kano *et al.*, 2000; Lefebvre *et al.*, 2002).

The integrity of the plasma membrane is an important cryobiological property that affects oocyte viability after vitrification because it influences

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Table 2 Embryo development after *in vitro* fertilization of immature and mature cryopreserved oocytes

Treatment groups	*N* oocytes	Embryo development		
		% cleavage 72 hpi (*n*)	% blastocysts 168–192 hpi (*n*)	% hatching 224 hpi (*n*)
Control group	211	80 (169)^{a,b}	26 (54)^{c,d}	41 (22)
GV group	39	28 (11)^{a}	0 (0)^{c}	0 (0)
MII group	46	26 (12)^{b}	0 (0)^{d}	0 (0)

hpi, hours post insemination. ^{a,b,c,d}Values with the same superscripts letters are significantly different ($P < 0.05$).

Table 3 Embryo development after parthenogenetic activation of immature and mature cryopreserved oocytes

Treatment groups	*N* oocytes	Embryo development		
		% cleavage 72 hpi (*n*)	% blastocysts 168–192 hpi (*n*)	% hatching 224 hpi (*n*)
Control group	205	58 (118)^{a,b}	26 (50)^{c,d}	18 (9)^{e,f}
GV group	50	28 (14)^{a}	2 (1)^{c}	100 (1)^{f}
MII group	63	22 (14)^{b}	0 (0)^{d}	0 (0)

hpi, hours post insemination. $^{a–f}$Values with the same superscripts letters are significantly different ($P < 0.05$).

membrane permeability to water and to cryoprotective agents (CPAs), which if unbalanced can lead to excessive osmotic edema and induce rupture and lysis of the membrane and consequent oocyte death (Kuwayama, 2007). The vitrification of immature oocytes, followed by IVM, did not damage the cellular esterase metabolism of the oocytes (which were able to metabolize calcein) or the integrity of the plasma membrane (as inferred by the non-penetration of ethidium bromide), once their viability was similar to the control group oocytes. In contrast, oocyte viability was impaired in mature oocytes, reducing from 77% in the control group to 27% in the vitrified mature group; this change may indicate that oocyte vitrification in the MII stage might have impaired, at least in part, the oocyte metabolism and promoted disruption of the plasma membrane. Opposite results were demonstrated by Chian *et al.* (2004). Based on the integrity of the oocyte membrane and the zona pellucida, they showed high survival rates (91.8%) of bovine oocytes submitted to IVM and subsequently to vitrification and thawing (Chian *et al.*, 2004). In the same way, Jin *et al.* (2011) showed that the plasma membranes of bovine oocytes were highly permeable, facilitating CPA movement during freezing and thawing and preventing in part the damage caused by the formation of intracellular ice, by CPA toxicity and by excessive osmotic edema (Jin *et al.*, 2011).

Several studies have been conducted in order to better vitrify bovine oocytes at different meiotic stages, however the optimal meiotic stage is still not clear. Regardless of the protocol, the devices and cryoprotectants agents adopted the vitrification process impairs the capacity of embryo formation (rates lower than 5%) from bovine oocytes (Otoi *et al.*, 1997; Shaw *et al.*, 2000; Men *et al.*, 2002; Chian *et al.*, 2004; Zhou *et al.*, 2010; Prentice *et al.*, 2011; Sripunya *et al.*, 2014; Sprícigo *et al.*, 2014)). Prentice *et al.* (2011) obtained lower cleavage rates (93% versus 42%) and blastocyst formation rates (31% versus 0.4%) in oocytes vitrified by the Cryotop method when compared with non-vitrified bovine oocytes (Prentice *et al.*, 2011), similar results were also described by Sripunya *et al.* (2014) and by Arcarons *et al.* (2015).

These poor results in reproductive outcomes, especially in the cleavage rates, may be, at least partially, a result of the zona pellucida hardening that occurs after oocytes cryopreservation (Carroll *et al.*, 1990; Gook *et al.*, 1994) With this concern, in the present study, PA was performed in an attempt to overcome the influence of the fertilization capacity of the spermatozoa. However, despite PA of the vitrified GV and MII oocytes, there was no improvement in cleavage rates or blastocyst formation. Thus, these data suggest that, in addition to ZP impairment, there must have been further damage that bypassed this structural event and severely compromised the reproductive capacity of the oocytes. As an example, if inadequate cytoplasm maturation occurs, processes such as protein synthesis (Sirard *et al.*, 1998), molecular modifications (Kubelka *et al.*,

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2000), migration and reorganization of organelles (Stojkovic *et al.*, 2001) will be impaired. According to Meirelles *et al.* (2004) transcript and protein stock in the oocyte cytoplasm is extremely important for ensuring ideal cytoplasm maturation and initial embryonic development (Meirelles *et al.*, 2004). Thus, the lower cleavage rates in the groups submitted to cryopreservation compared with controls indicated that the first stages of embryonic development were partially impaired by vitrification, although the stage of nuclear maturation among cryopreserved oocytes did not influence this impairment. It should be noted that no specific evaluation was performed in the present study regarding this topic. After these first stages of embryonic development (eight cells in cattle), maternal–zygotic transition (MZT) occurs, with activation of the embryonic genome and synthesis of new proteins (Meirelles *et al.*, 2004). In this phase we detected a marked interference of cryopreservation in MZT, and we did not obtain blastocysts from cryopreserved oocytes submitted to IVF, regardless of the meiotic stage.

Other studies have corroborated our study and showed that although vitrification might not compromise the capacity for nuclear maturation of oocytes in GV stage, the cryopreservation process itself impairs embryonic development (Aman & Parks., 1994; McWilliams *et al.*, 1995; Rojas *et al.*, 2004). Furthermore, in theory, oocytes in the MII stage would be more susceptible to meiotic anomalies and consequently to poor embryonic development because of the distribution of the chromosomes throughout the metaphase plate and the presence of a meiotic spindle, which are targets of the deleterious effects of the vitrification process (Brunet *et al.*, 2003). In accordance, studies with vitrified mature bovine oocytes has observed that vitrification modifies the cytoskeletal components as alterations in the meiotic spindle and consequently an increase in chromosomal abnormality occurs that compromises the developmental capacity of *in vitro*-matured bovine oocytes (Bogliolo *et al.*, 2007; Morató *et al.*, 2008). Besides this theoretic impact of cryopreservation on oocytes genetics, there have already been many newborn babies born from human vitrified oocyte with no reports of increased fetal malformation or neonatal complications, and this technique may be applied for human mature oocytes cryopreservation, no longer an experimental practice (Practice Committees of American Society for Reproductive Medicine, 2013). However, few data are available concerning immature oocytes, which may be considered for specific clinical situations in fertility preservation in the routine of assisted reproduction clinics.

In conclusion, we cannot define the best or more resistant meiotic stage for cryopreservation, as this procedure has a great impact on the capacity for embryonic development of vitrified bovine oocytes despite the nuclear meiotic stage. Thus, better protocols specifically designed for bovine oocytes must be developed, and only then the influence of the meiotic stage on cryotolerance may be tested.

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