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Effect of Equilibration Temperature on *In vitro* Viability and Subsequent Embryo Development of Vitrified-Warmed Immature Bovine Oocytes

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Abstract: Problem statement: Vitrification is replacing conventional slow freezing to cryopreserve gametes and embryos especially for *in vitro* production of embryo in domestic animal species. However, the results are still not satisfactory. The aim of this experiment was to study the effect of different equilibration temperatures on *in vitro* viability of immature bovine oocytes after vitrification. **Approach:** Oocytes were obtained from slaughterhouse ovaries. Only grade one oocytes were used. Oocytes were equilibrated in three different temperatures: 32, 37, or 41°C. Immature oocytes were equilibrated in VS1 (7.5 Ethylene Glycol (EG) + 7.5% DMSO) for 10-12 min and then exposed to VS2 (15% EG + 15%DMSO + 0.5M sucrose) for one min. Thereafter oocytes were loaded on hand-made Cryotop and directly plunged into liquid nitrogen. After warming, oocytes were examined for viability, maturation, cleavage and blastocyst production. **Results:** Oocytes that were equilibrated at 37°C had significantly higher ($p<0.05$) viability than 41°C, but there were no significant difference between 37 and 41 with 32°C. Maturation rate in 37°C group was significantly higher compared with other groups. The highest percentage of degenerated and germinal vesicle stage oocytes were obtained from 41°C than 32 and 37°C. Cleavage rate of 37°C group (38.77%) was greater than other groups (30.84 and 28.95% for 32 and 41°C, respectively). The highest blastocyst rate was also produced when oocytes equilibrated at 37°C (6.45%). **Conclusion:** In conclusion, these results indicated that immature bovine oocytes can be equilibrated successfully at 37°C while higher or lower temperature can significantly decrease their subsequent viability and development.

Key words: Vitrification, equilibration temperature, bovine, immature oocytes

INTRODUCTION

Cryopreservation of reproductive cells has been initiated decades ago and currently its importance are becoming more notable. Preservation of fertility in patients suffer from cancer, conservation of endangered animal species, improvement of genetic breeding and assisted reproductive biotechnologies are all the recognized aspects that could be applicable by using established cryopreservation techniques (Fuller and Paynter, 2004; Rall, 1992). The hazardous part of cryopreservation systems which ultimately can be the cause of cell death is the risk of ice crystal formation inside the cells (Liebermann *et al*., 2002). This possibility of harm vanishes by using vitrification (Kasai, 1996; Rall, 1992; Vajta and Kuwayama, 2006) that employs high concentrations of cryoprotectants with ultra rapid cooling rates (Smorag and Gajda, 1994).

Cryoprotectants potentially could be the source of toxicity which also increases by rising the temperatures. According to the literature, researchers are utilizing room (Chian *et al*., 2005; Kuwayama *et al*., 2005), biological (Vajta *et al*., 1998; Yoon *et al*., 2003) or higher than biological temperatures (Albarracin *et al*., 2005; Morat *et al*., 2008) for handling of the gametes and embryos at the time of vitrification. On the other hand, permeability and sensitivity of gametes and

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embryos are different with relation to the species and developmental stages. Previous studies have shown that MII bovine oocytes have greater permeability to cryoprotectants and water than Germinal Vesicle (GV) oocytes (Agca *et al*., 1998). However, there have been little fundamental studies which compare handling temperatures specified for vitrification of immature bovine species. The aim of the present study is to determine the effects of three different equilibration temperatures on *in vitro* viability and subsequent embryo developments of immature bovine oocytes after vitrification.

MATERIALS AND METHODS

Reagents: Unless otherwise indicated, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Oocyte collection: Ovaries were collected from local abattoirs and transported to the laboratory within 3 h at 34-36°C in phosphate-buffered saline (PBS; P-4417) containing penicillin-streptomycin (100,000 IU of penicillin and 100 mg of streptomycin per liter). Cumulus Oocyte Complexes (COCs) were obtained by slicing method in tissue culture medium 199 (Medium 199, 12340; Gibco) containing 25 mM HEPES, Earl's salts, L-glutamine and 2 mg mL^{-1} sodium bicarbonate modified by the addition of 4 mg mL^{-1} bovine serum albumin (BSA, fraction V, A-3311) and gentamycin 50 μg mL^{-1} (G-1264).

Vitrification and warming: The vitrification protocol was adapted from Kuwayama *et al*. (2005) with minor modifications. Briefly, oocytes were washed twice in Holding Solution (HS, Hepes-buffered TCM medium supplemented with 20% Fetal Calf Serum, FCS) and kept there for about 15 min. Group of four COCs were incubated in the first Vitrification Solution (VS1; 7.5% DMSO and 7.5% EG in WS) for 10-12 min and then transferred to the second Vitrification Solution (VS2; 15% DMSO, 15% EG and 0.5 M sucrose in WS) for a further 60 sec. Instantly oocytes were loaded on a hand-made cryostrip and submerged into Liquid Nitrogen (LN_2) for storage. The time of exposure from VS2 to LN_2 was less than 90s. Vitrified samples were maintained in Liquid Nitrogen (LN_2) for at least 10 days.

Immediately after removing Cryotop from LN_2, thin strip of Cryotop was submerged in 3-ml pre-warmed (39°C) HS plus 1 M sucrose (T1) and smoothly tried to detach oocytes from Cryotop device. Immature oocytes were left in T1 for 1 min and then transferred to HS plus 0.5 and 0.15 M sucrose solution for 3 and 5 min, respectively. Finally, the immature oocytes were washed twice in HM for 5 min each and processed for *in vitro* maturation.

***In Vitro* Maturation (IVM):** Immature oocytes were washed two times in medium 199 supplemented with 4 mg mL^{-1} BSA and washed again in maturation solution containing Hepes-buffered medium 199 supplemented with 10% fetal calf serum, 0.2 mM sodium pyruvate (P-5280), 50 μL mL^{-1} Gentamycin (G-1264) and 1 μg mL^{-1} oestradiol-17β (E-8875). Approximately 20-30 oocytes were incubated in 400 μL of maturation solution under mineral oil in 4-well plates for 22-24 h at 38.5°C under 5% CO_2 atmosphere with high humidity. The vitrified separated oocytes also underwent the same maturation process with control group after warming.

Nuclear maturation stage: For the purpose of maturation determination, 24 h after maturation, oocytes were denuded using 0.1% (w/v) hyaluronidase (Type 1-S) in Hepes-buffered medium 199 by vortexing. The cumulus-free oocytes were fixed in aceto-ethanol mixture (1:3, v/v) at 5°C for 24 h. Once fixed, oocytes in groups of 5-10 were mounted on slides. Cover-slip with 4 spots of silicon glue at the corners was gently pressed down until it touched and secured the oocytes. Afterward, aceto-lacmoid stain was passed under the cover-slip and remained there for 5 min. Subsequently, a decolorizing solution (aceto-glycerol) was passed through to remove the stain residuals. Stained oocytes were examined under light microscope (400x) for nuclear stages determination.

***In Vitro* Fertilization (IVF):** The fertilization method was previously described by Parrish *et al*. (1988) with some modifications. Briefly, matured oocytes were washed three times in tyrode's albumin lactate pyruvate buffered with HEPES Talp-Hepes (Parrish *et al*., 1988) and twice in fertilization medium. Viable spermatozoa were obtained by centrifugation of frozen–thawed spermatozoa on Bovipure (Nidacon International AB, Gothenburg, Sweden) discontinuous density gradient (2 ml of top-layer over 2 ml of bottom-layer) for 20 min at 300×g at room temperature. Viable spermatozoa collected at the bottom of the tube, were washed with 5 mL of sperm-TALP (Parrish *et al*., 1988) supplemented by 6 mg mL^{-1} BSA (fraction V, A-3311) and centrifuged for 10 min at 300×g. After centrifugation 150 μL of the pellet was selected. Spermatozoa were counted using a hemacytometer and diluted with IVF-Talp to give the final concentration of 1×10^6 spermatozoa mL^{-1}. IVM-oocytes were transferred

in groups of up to 7 into 50 μL of fertilization solution supplemented with 10 μg mL^{-1} heparin-sodium salt (Sigma, H-3393). Spermatozoa suspension was added to each fertilization droplet. Oocytes and sperms were incubated for 18 h at 38.5°C in 5% CO_2 in humidified air.

***In Vitro* Culture (IVC):** Eighteen hours after IVF, oocytes were freed of cumulus cells by high speed vortexing and washed twice in Talp-Hepes and culture medium. For each well, 10-15 oocytes were transferred to 400 μL^{-1} of Synthetic Oviductal Fluid (SOFacci), (Holm *et al*., 1999) supplemented with 5% adult bovine serum (B-9433) and 1 μg mL^{-1} gentamycin under mineral oil. Every two days after transfer to culture medium, 200 μL of culture medium were replenished with 200 μL freshly incubated SOF medium. Cleavage and blastocyst rates were recorded at 48 h, 7 and 9 days post insemination, respectively.

Statistical analysis: All experiments were repeated six times. Significant differences among treatments were revealed by non parametric one-way analysis of variance followed by Duncan's multiple range test for mean comparisons ($p<0.05$) using SAS software ver. 9.1.

RESULTS

After morphological assessment of denuded oocytes for presence of polar body, equilibration at 37°C resulted in higher percentage of oocytes with extruded polar body and lower percentage of degenerated oocytes ($p<0.05$) while in group 41°C, degeneration of oocytes happened in higher percentage than other groups ($p<0.05$; Table 1). Out of 40 stained oocytes in group 37°C, 23 (57%) were seen in M-II stage of maturation which were significantly higher compared with 32 and 41°C. However, it can be seen from the data in Table 2 that the oocytes of 41°C group reported significantly more GV-stage oocytes than the other two groups which show that they failed to mature after 24 h IVM.

As shown in Table 3, the *in vitro* viability rate of vitrified-warmed immature bovine oocytes were 76.32±2.18%, 87.95±1.66% and 70.73±2.30% for oocytes that equilibrated at 32, 37 and 41°C, respectively. Although viability rate was not significantly differ between 32°C and other groups but oocytes equilibrated at 37°C had significantly higher viability than 41°C. The rate of cleaved oocytes were significantly greater in the 37°C group (38.77±0.76%) compared to 32 and 41°C (30.84±0.58% and 28.95±1.20%, respectively), but there was no difference between 32 and 41°C.

Table 1: Polar body rate after maturation in equilibrated groups

	32°C[a] (n = 43)	37°C[a] (n = 47)	41°C[b] (n = 42)
Polar body +	9 (21%)[b]	17 (36%)[a]	6 (14%)[b]
Polar body -	24 (55%)	23 (50%)	23 (55%)
Degenerated	10 (24%)[a]	7 (14%)[b]	13 (31%)[a]

[a], [b], [c]: Values in the same row with different superscripts differ significantly ($p<0.05$)

Table 2: Nuclear maturation of vitrified immature oocytes after staining

Nuclear stage	32°C (n = 33)	37°C (n = 40)	41°C (n = 29)
GV	10 (29.84%)[b]	8 (19.82%)[b]	16 (55.55%)[a]
GVBD-MI	7 (21.27%)[a]	5 (12.82%)[ab]	2 (7.14%)[b]
MII	14 (42.54%)[b]	23 (57.57%)[a]	9 (30.95%)[c]
Unclassified	2 (6.34%)	4 (9.77%)	2 (6.34%)

[a], [b], [c]: Values in the same row with different superscripts differ significantly ($p<0.05$)

Table 3: *In vitro* viability of vitrified-warmed immature bovine oocytes equilibrated at different temperatures

Treatment	No. of oocytes	Viability (%)	Oocytes Cleaved (%)	≥8 cell embryos (%)	Blastocyst (%)
32°C	96	76.32±2.18[ab]	30.84±0.58[b]	13.05±4.21[b]	3.65±1.18[ab]
37°C	104	87.95±1.66[a]	38.77±0.76[a]	21.13±2.82[a]	6.45±0.54[a]
41°C	94	70.73±2.30[b]	28.95±1.20[b]	10.71±3.44[b]	1.94±1.24[b]

[a], [b], [c]: Values in the same column with different superscripts differ significantly ($p<0.05$)

The rate of embryos that developed to 8-cell or beyond were significantly higher ($p<0.05$) in 37°C group (21.13±2.82%) compared with other groups (13.05±4.21%, 10.71±3.44% for 32 and 41°C, respectively). However, the blastocyst rate for 37°C (6.45±0.54%) only significantly differed from 41°C (1.94±1.24%). There were no significant differences in term of blastocyst rate between 32°C group (3.65±1.18%) and other groups.

DISCUSSION

For vitrification process and prevention of intracellular ice formation, high concentrations of permeating cryoprotectants (CPAs, around 6-8 M) are usually used. These concentrations could be relatively toxic to oocytes and embryos base on CPAs mixture, time and temperature of exposure (Kasai, 1997; 2002). There is a parallel relationship between biochemical toxicity, exposure time and temperature. Enhancing the passage of permeating cryoprotectants by increasing the temperature can be the cause of biochemical toxicity (Chen and Yang, 2009).

The current study found that equilibration of immature bovine oocytes at 32 and 37°C were superior compared to 41°C. As exposure temperatures

employed by researchers are in different categories such as room (25-27°C; (Chian *et al*., 2005; Kuwayama *et al*., 2005)), biological (37-39°C; (Vajta *et al*., 1998; Yoon *et al*., 2003)) and sometimes above the biological temperatures (41°C; (Albarracin *et al*., 2005; Morat *et al*., 2008)). By increasing the temperature, the possibility of biochemical toxicity will also increase. Thus, short equilibration for high temperature such as 2-3 min at 37°C and 10-15 min at room temperature are sufficient for equilibration. On the other hand, it should be kept in mind that oocytes from various species and also developmental stages differ in physiology and respond to the procedures differently (Kasai, 1997). For instance, mice oocytes can tolerate low temperatures easier than species like bovine and porcine oocytes, as a result of existing lipids in their ooplasm (Zhou and Li, 2009). It has been proven that oocytes containing high lipids are more susceptible to cold damages (Critser and Men, 2007; Zhou and Li, 2009). Also, oocytes at different stages of development are different in permeability and sensitivity to cryoprotectants (Papis *et al*., 2000; Magnusson *et al*., 2008). Germinal vesicle oocytes surrounded by compact cumulus cells react differently than metaphase-II oocytes originated from the same species. By using strategy invented by Kuwayama *et al*. (2005) which lowering the concentration of cryoprotectant mixture and direct contact are employed together, the problem of biochemical toxicity decreases to some extent. As shown in Table 1, 2 and 3, immature oocytes equilibrated at 32 and 37°C had higher maturation, 8-cell embryos and blastocysts. These results can be due to interactions between optimum equilibration temperature, cryoprotectant concentration and high cooling and warming rates. It has been shown that matured bovine oocytes equilibrated in relatively low concentration of cryoprotectants for 10 min followed by vitrification using high cooling rate had higher blastocyst rate than shorter equilibration time (Checura and Seidel, 2007). The authors mentioned that they have handled the oocytes at room with 37°C temperature. However, the possibility of precise control of temperature is almost expectable by using of stage warmer plus controlling the room temperature. Another possibility for this response from this study is due to the presence of cumulus cells which act as a barrier to slow down the permeation of cryoprotectants into the oocytes compared with mature oocytes. The previous studies have shown that MII oocytes have greater permeability to cryoprotectants and water than GV oocytes (Agca *et al*., 1998).

CONCLUSION

In conclusion, it could conceivably be hypothesized that optimum temperature for equilibration of bovine oocytes in low concentration of cryoprotectant mixtures is almost around biological temperature (32-37°C).

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