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Closed system for bovine oocyte vitrification

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Abstract

The aim of our study was to develop a vitrification carrier for bovine oocyte cryopreservation. The carrier was to be cheap enough, elementary in its construction and meet contemporary requirements for a safe closed system. In a closed system, a cell is prevented from direct exposure to liquid nitrogen, thus minimizing the risk of cross-contamination. Furthermore, two questions regarding the proper vitrification technique were resolved: if it is necessary to partially denude the oocytes before the vitrification process or whether intact cumulus oocyte complexes should be frozen; and if it is more advantageous to preheat the vitrification solutions to female body temperature (39 °C) or to keep them at room temperature. Our results show that it is better to partially denude the oocytes prior to vitrification because cryopreserved intact cumulus oocyte complexes often proved dark, non-homogeneous or fragmented cytoplasm after warming, with many of them having visibly widened perivitelline spaces or fractured zonae pellucidae as a result of extensive damage during vitrification. Consequently, intact cumulus oocyte complexes showed significantly lower numbers of cleavage stage embryos on Day 3 compared to partially denuded oocytes (7.4% and 26%, respectively). On the other hand, the survival rate and following development of fertilized oocytes in preheated vitrification solution were equal to results reached at room temperature conditions. In conclusion, results achieved with the newly developed carrier were comparable to previously published studies and therefore they could be recommended for common use.

Cattle, cryopreservation, cumulus cells, preheating

Cryopreservation of farm animal embryos is nowadays a widely used routine technique (Betteridge et al. 2006). However, oocyte cryopreservation produced insufficient results for many years (Chen et al. 2003).

Two basic methods of cryopreservation are commonly used; slow freezing and vitrification. Both of them prevent intracellular ice crystal formation and solution effect during the freezing stage as well as osmotic shock during subsequent warming. To prevent the above mentioned damage of embryos, additional chemicals (cryoprotectants) are applied.

When slow freezing is performed, low concentrations of cryoprotectant chemicals and long freezing time with the assistance of special equipment are essential. To the contrary, vitrification requires high cryoprotectant concentrations and instant refrigeration using liquid nitrogen. During this process, water does not crystallize but transforms into solid state with an amorphous structure and its volume is not expanded (Jain and Paulson 2006). Better results are achieved when vitrification technique is used freezing oocytes (Ambrosini et al. 2006).

Direct transition of water into the amoprhous solid state using vitrification technique is conditioned by instant freezing (Jain and Paulson 2006). The sample is usually deposited in an appropriate carrier and immersed in liquid nitrogen. It still poses a risk of possible bacterial (Bielanski et al. 2003) and viral infections (Bielanski et al. 2000). Mainly in human reproduction, "closed vitrification systems" are implemented as means of prevention in order to keep the carrier hermetically sealed within an additional casing. Based on research data, open vitrification systems have shown results comparable to closed systems (Kuwayama et al. 2005; Larman et al. 2006).

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These safe carriers are commercially produced for human reproduction; however, they are very expensive. The objective of this study was to develop and test a cheap carrier, appropriate enough for closed systems and usable for bovine oocyte vitrification. Further aims included resolving the following questions: what temperature should be maintained while handling cells; and whether it is more advantageous to cryopreserve either intact cumulus oocyte complexes (COC) or partially denuded oocytes.

Materials and Methods

Chemicals were obtained from Sigma-Aldrich (Missouri, USA) unless otherwise stated. The ambient temperature in the laboratory was adjusted to 25 ± 1 °C.

Collection of oocytes and *in vitro* maturation

All procedures were performed following the method described by Hlavicová et al. (2010), with minor modifications only. For maturation, Control Group COCs were cultured for 24 h, COCs from Groups 2 and 3 were cultured for 22 h.

In vitro fertilization

All procedures were performed following the method described by Hlavicová et al. (2010), with minor modifications only. For removing the dead spermatozoa and remaining cumulus cells, a mechanical method of vortexing for 15 s in vortex medium described by Modina et al. (2004) was used (M199 supplemented with 5% calf serum and buffered with 20 mM HEPES).

Carrier

The carrier was obtained from 0.25 ml plastic straw (IMV Technologies). The straw had to be drawn carefully over the flame of alcohol burner in order to lengthen it by additional 20%; then cut with a razor-blade in its narrowest spot. The narrow ends of both halves were then cut obliquely, in order to form a little site at the inner surface of the straw, where oocytes were deposited during the vitrification process. Only by drawing the straw, a sufficiently thin plastic layer is ensured to reduce the influence of its thermal insulation properties on freezing speed during vitrification.

To prevent cells from direct exposure to liquid nitrogen, the carrier was afterwards inserted into extra 0.5 ml straw (Minitübe). A metal weight, obtained from steel wire (Graupner, 2 mm diameter) cut into 0.5 cm pieces, was placed inside the 0.5 ml straw from the cotton plug end. This end was thereafter sealed up with burner heated tweezers.

Vitrification and warming

Vitrification and warming of COCs followed the method described by Vajta et al. (1998) with several modifications. One of them concerned replacing DMSO, dangerous for its staff toxicity, by less hazardous PROH (Stachecki et al. 2008). Oocytes were firstly washed and equilibrated in a holding medium (HM) consisting of TCM199-HEPES supplemented with 20% foetal calf serum (FCS) for 5 min. After equilibration, two oocytes were always incubated for 30 s (or 45 s in Group 3 in Experiment 2) in first vitrification solution, composed of 10% ethylene glycol (EG) + 10% propylene glycol (PROH) dissolved in HM, and subsequently transferred into second vitrification solution, composed of 20% EG + 20% PROH + 0.5 M sucrose dissolved in HM and deposited with a minimum amount of second vitrification solution onto the carrier. This carrier was later inserted into the outer straw previously fixed in a polystyrene box filled with liquid nitrogen. The outer straw had to be attached vertically to the wall of the box, in order to keep the open end about 1 cm above the nitrogen level. Immediately after the carrier insertion, the open end of the straw had to be sealed up with burner heated tweezers. The time span of 30 s (or 45 s within Group 3 in Experiment 2) between the oocyte exposure to second vitrification solution and the carrier insertion into the outer straw should not be exceeded.

Warming was performed by placing the end of the carrier, where oocytes had been previously deposited, directly into first warming solution composed of HM supplemented with 0.25 M sucrose for 5 min. Oocytes were thereafter transferred into second warming solution, composed of HM containing 0.15 M sucrose for another 5 min. After two subsequent washes in HM for 5 min each, oocytes were transferred back into maturation 4-well plates (NUNC), matured for an additional 2 h and fertilized.

Vitrification and warming solutions were pipetted into 4-well plates at 500 µl each (only the first warming solution at 1 ml). Solutions were preheated on a 39 °C hot plate for 15–20 min (except for vitrification solutions within the third group in Experiment 2, equilibrated to an ambient temperature) before use.

Experimental design

Experiment 1

Oocytes were separated randomly into three groups. Group 1 (Control) was matured *in vitro* for 24 h; then fertilized. Groups 2 and 3 were matured for 22 h and vitrified. Within Group 2, intact COC were vitrified, whereas within Group 3, COC were vortexed for 15 s before the insertion into HM, wherefore only two to four layers of cumulus cells remained on the surface.

Experiment 2

Oocytes were separated randomly into three groups. First group (control) was matured *in vitro* for 24 h; then fertilized. Groups 2 and 3 were partially denuded 22 h after the onset of maturation by vortexing for 15 s, wherefore only two to four layers of cumulus cells remained on the surface, and vitrified. Within Group 2, vitrification solutions were preheated on a 39 °C hot plate for 15–20 min and oocytes from Group 2 were then placed into the first and second vitrification solution for 30 s. Within Group 3, vitrification solutions were equilibrated to an ambient temperature and oocytes were placed into the first and second vitrification solution for 45 s.

Within Group 3, relevant time interval was lengthened due to the fact that membrane permeability is lower at room temperature than at 39 °C (Mazur 1970; Ambrosini et al. 2006). In order to reach comparable penetration of cryoprotectants into the cell at lower temperatures it was necessary to prolong exposure time. Aforementioned 45 s match the reference of exposure intervals previously stated in Chian et al. (2009), who used similar vitrification solutions at room temperature.

Statistical analysis

The obtained data were analyzed by the $\chi 2$ test (Fisher's exact test, $P < 0.001$ and $P < 0.02$).

Results

Experiment 1

Vitrified intact COCs often proved dark, non-homogeneous or fragmented cytoplasm after warming and numerous COCs had visibly widened perivitelline spaces or fractured zonae pellucidae. Significantly lower cleavage rate on Day 3 (Table 1) was induced by oocyte degeneration on Day 1 after warming.

Table 1. Effect of partial denudation on the development of vitrified-warmed bovine oocytes

Group	No. oocytes	Day 3	Day 6	Day 8	
		Cleaved (%)	Morulae (%)	Blastocysts (%)	Expansion (%)
Control	230	171 (74.3)^{a}	77 (33.5)^{a}	44 (19.1)^{a}	30 (13)^{a}
Partially denuded	196^{N}	51 (26)^{b}	10 (5.1)^{b}	2 (1)^{b}	2 (1)^{b}
Intact COCs	189^{N}	14 (7.4)^{c}	4 (2.1)^{b}	1 (0.5)^{b}	0

N - number of oocytes recovered after warming; different letters differ significantly (a, b, c: $P < 0.001$)

Experiment 2

There was no difference in development of fertilized oocytes, which were handled in preheated vitrification solution or in vitrification solution equilibrated to ambient temperature (Table 2). Based on the amount of cleavage stage embryos on Day 3, there was no difference in survival and fertilization ability.

Table 2. Effect of vitrification solutions preheating on the development of vitrified-warmed bovine oocytes

Group	No. oocytes	Day 3	Day 6	Day 8	
		Cleaved (%)	Morulae (%)	Blastocysts (%)	Expansion (%)
Control	121	83 (68.6)^{a}	38 (31,4)^{a}	19 (15,7)^{a}	11 (9.1)^{x}
39 °C	105^{N}	27 (25.7)^{b}	6 (5.7)^{b}	2 (1.9)^{b}	1 (1)^{y}
25 ± 1°C	104^{N}	21 (20.2)^{b}	5 (4.8)^{b}	1 (1)^{b}	0

N - number of oocytes recovered after warming; different letters differ significantly (a, b, c: $P < 0.001$, x, y: $P < 0.02$)

Discussion

Better results are achieved when vitrification technique is used for freezing oocytes compared to slow freezing (Ambrosini et al. 2006). This is most likely caused by

oocyte chilling injury, significant damage following exposure to low but not freezing temperatures, a phenomenon that accompanies the slow-freezing method (Arav et al. 1996). This damage concerns in particular cell membranes. In the lipid phase transition, some membrane parts become solidified, separated ("lipid phase separation") leading to ultimate disruption of membrane function (Quinn 1985). Furthermore, chilling injury affects the cytoskeleton, especially the spindle microtubules in MII oocytes, causing them to disassemble. During the warming phase the spindle re-forms, often incorrectly. This leads to consequent aneuploidy resulting in embryo loss (Chen et al. 2003). Chilling injury, devastating for the spindle, is noticeable mostly at temperatures around 0 °C (Martino et al. 1996). Even the cooling of the oocyte to 25 °C leads to abnormal spindle formations (Aman and Parks 1994).

Nevertheless, as Vajta et al. (2009) write, the survival of vitrified oocyte is determined by a number of factors and the effect on the overall spindle condition should not be overestimated when compiling the protocol. This fact is also supported by results from MII stage oocyte cryopreservation quite comparable to GV stage (in which the spindle is not yet assembled) oocyte cryopreservation results (Fuku et al. 1992; Kubota et al. 1998; Zhou et al. 2010). Our data confirmed that because the survival rate and following development of fertilized oocytes in preheated vitrification solution were equal to results reached at room temperature conditions.

Apart from the vitrification solution temperature value, we had to solve another question: if it is more advantageous to partially denude the oocytes before the vitrification process, or whether intact cumulus oocyte complexes should be frozen. Cumulus cell in cattle are often considered essential for maturation (Im et al. 1996) and fertilization (Chian et al. 2009). It is possible to freeze completely denuded oocytes but it requiers a unique composition of manipulation medium leading to a lower blastocyst rate (Modina et al. 2004). A complete cumulus may obstruct penetration of cryoprotectants into oocytes (Hyttel et al. 2000) and affects lipid phase transition during the chilling procedures, modifying the dynamic response of cytoskeleton and plasma membrane (Bogliolo et al. 2007). This corresponds with our results. Based on the morphological condition of oocyte cytoplasm and cumulus cells after warming, the significantly lower amount of cleavage stage embryos on Day 3 from vitrified-warmed intact cumulus oocyte complexes was a clear consequence of more frequent damage during vitrification and the resultant decrease in survival and fertilization ability.

The achievements of vitrification with our newly developed carrier are comparable to results achieved by following research teams using similar vitrification protocol and open carriers (Luna et al. 2001; Diez et al. 2002; Vieira et al. 2002; Modina et al. 2004; Albarracín et al. 2005; Morató et al. 2008a, 2008b, 2008c; and Hou et al. 2009). Hence, we can declare this new carrier a safe variant for bovine oocyte vitrification, which is inexpensive to manufacture from affordable components.

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