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In vitro survival rate of bovine oocytes following vitrification in glass capillary micropipette (GCM)

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Summary

The purpose of this study was to evaluate the use of glass capillary micropipette (GCM) as a vessel for vitrification of bovine oocytes. Cumulus-oocyte complexes (COCs) were obtained from slaughter-house and washed 5 to 6 times in the washing medium (TCM-199 + 20% FBS) and randomly assigned to treatment and control group. In the first step of vitrification, COCs were exposed to first vitrification solution (VS1) (10% ethylene glycol (EG), 10% DMSO in holding medium (TCM-199 + 10% FBS: HM)) for 1 min at room temperature and then placed in VS2 solution (20% EG, 20% DMSO in HM) for 25 sec and immediately were loaded into the GCM vessel. The filled portion of GCM vessels were placed in liquid nitrogen (LN_2) for 3 to 5 sec and then completely immersed and stored there. The oocytes were thawed by immersing the capillary end of the straw in 1 ml of 0.25 M sucrose in HM and gently expelling the contents. After 1 min the oocytes were transferred into 100 µl of 0.15 M sucrose in HM for another 5 min and then washed with HM twice. For examining the in vitro developmental potential of vitrified-warmed oocytes, the oocytes were placed in 50 µl droplet of maturation medium (TCM-199 + 10% FBS + 10 IU/ml PMSG + 5 IU/ml HCG) covered with paraffin oil in a CO_2 incubator at 38.5ºC for 24 hrs. A high proportion of morphologically normal oocytes (90%) was recovered after vitrification-warming. The percentage of live oocytes after 24 hrs when tested with trypan blue in GCM group was 85.18%, significantly did not differ from control group (90%). The proportion of oocytes which were found to have undergone nuclear maturation did not show statistical difference between the control and GCM group (61.29% vs 40%, respectively). The results of present study demonstrated that vitrification of immature bovine oocytes in the GCM vessels and EG + DMSO solution have high survival rate.

Key words: Vitrification, Bovine, Oocyte, Glass capillary micropipette

Introduction

Cryopreservation of oocytes plays an important role in the preservation and manangement of genetic resources. The first successful cryopreservation of mammalian zygotes and embryos resulting in live births was achieved with mice by Whittingham *et al*., (1972). Based on fertilization of oocytes after thawing and subsequent development, cryopreservation of oocytes has been successfully reported in several mammalian species, including mice (Men *et al*., 1997), cattle (Schellander *et al*., 1994) and humans (Quinn *et al*., 1986). In the past decade, various new methods for ova and embryo cryopreservation have been established (Niemann, 1991; Rall, 1992). Among these methods, vitrification has been widely used and is now regarded as a potential alternative to traditional slow freezing method. Vitrification, glass-like solidification of a solution at a low temperature without ice crystal formation or with the formation of only small extracellular crystals requires higher concentration of cryoprotectants and allows to use an elevated cooling speed. This approach was first applied for oocyte cryopreservation by Critser *et al*., (1986).

Vitrification of cattle oocytes has been performed using a variety of cryoprotectants, including DMSO, acetamide and propylene glycol (Otoi *et al*.,

1995), ethylene glycol and DMSO (Schellander *et al*., 1994) and DMSO, glycerol and 1,2-propanediol (Vajta *et al*., 1997). These cryoprotectants have been used with different concentrations as well as various equilibration times. Based on a study of the literature, ethylene glycol has been the cryoprotectant of choice for vitrification of mammalian oocytes and embryos (Bautista and Kanagawa, 1998). Increasing the speed of cooling and warming may offer two advantages: first it might reduce the concentration of cryoprotectants and decrease subsequent toxicity secondly decrease of chilling injury due to a rapid passage through the dangerous temperature zone (Vajta *et al*., 1998). To gain these goals some techniques have been established: direct immersion in LN_2 (Yang and Leibo, 1999), using an electron microscope grid to provide support (Martino *et al*., 1996) and the open pulled straw (OPS) method (Vajta *et al*., 1997). In the OPS technique, the container is a narrow plastic tube and the approximate volume of 1 µl cryoprotectant solution is loaded into the open end of vessel (Vajta *et al*., 1997). Although, the OPS method is simple to use, the straws will float after immersion in LN_2. To overcome this problem Kong *et al*., (2000) used a glass micropipette vessel for vitrification of mouse blastocysts. To the best of our knowledge no previous attempt has been made to use a glass micropipette vessel for vitrification of bovine ova or embryos therefore, this study was designed to investigate the survival and developmental rate of bovine oocytes following vitrification with ethylene glycol based solution in glass capillary micropipette (GCM).

Materials and Methods

All chemicals and media were used in this study purchased from Sigma Company (St. Louis, MO, USA) unless otherwise indicated. Fetal bovine serum was purchased from Seromed (Germany) and sucrose from Merck (Darmstadt, Germany). The PMSG (Folligon®) and HCG (Chorulon®) were obtained from Intervet International B. V. Boxmeer (Holland). Disposable petri dishes were purchased from Becton Dickinson Labware (Becton Dickinson and Company Franklin Lakes, NJ. USA).

Collection of oocytes

Bovine ovaries were collected from abattoir and were transported in a thermos flask within 4 to 5 hrs to the laboratory in 0.9% saline at 32 to 37˚C. Ovaries were rinsed in 70% ethanol for 2 sec and then rinsed at least twice with sterile saline. Cumulus-oocyte complexes (COCs) were obtained by aspiration of 2 to 8 mm follicles using an 18G needle attached to a sterile syring. Only oocytes surrounded by compact cumulus cells and homogenous ooplasm were selected (Madison and Fraser, 1992) and randomly assigned to vitrification and control group.

Vitrification of oocytes

The vitrification solution (VS) consisted of VS1 (10% ethylene glycol (EG); 10% DMSO in holding medium, HM (TCM-199 supplemented with 10% FBS)) and VS2 (20% EG, 20% DMSO in HM). Vitrification was done as described by Vajta *et al*., (1998). Briefly, the aspirated oocytes were washed 5 to 6 times in the washing medium (TCM-199 supplemented with 20% FBS), after that these were first incubated in VS1 for 1 min and then were transferred into a 20 µl droplet of VS2 at room temperature (22 to 24˚C). The oocytes were loaded quickly into the GCM straws by using the capillary effect. The time between contact of oocytes with the concentrated cryoprotectant solution and cooling did not exceed 25 sec. The filled portion of the GCM vessels were placed in LN_2 (3 to 5 sec) and then completely immersed and stored there.

Warming and cryoprotectant dilution

Oocytes were thawed by immersing the capillary end of the glass straw in 1 ml of 0.25 M sucrose in HM and gently expelling the contents. After 1 min, the oocytes were transferred into 1 ml of 0.15 M sucrose in HM for another 5 min and then washed with HM twice for 5 min each time. The temperature of the media used for warming was held at approximately 35 to 37˚C.

Culture of oocytes

After thawing, the morphologically

normal oocytes were placed in 50 µl droplets of maturation medium (TCM-199 + 10% FBS + 10 IU/ml PMSG + 5 IU/ml HCG) covered with paraffin oil in a 60 mm petri dish and cultured for 24 hrs in a humidified CO_2 incubator (5% CO_2 in air) at 38.5˚C. In addition, a group of non-vitrified COCs were cultured in maturation medium as control in each in vitro maturation trial.

Assessment of survival and maturation of oocytes

Oocyte normality in vitrified-warmed samples was evaluated by their post-thaw morphological appearance under an inverted microscope (CETI, Belgium). The oocytes with spherical and symmetrical shape and no signs of lyses or degeneration were considered normal, whereas oocytes with ruptured zona pellucida, fragmented cytoplasm or degenerative signs were classified as abnormal. To determine the survival rate of oocytes, the cultured oocytes were stained with trypan blue (0.1%). In this method trypan blue penetrate into the cytoplasm of injured/dead oocytes resulting blue appearance of cytoplasm while live oocytes do not stain (Nasre Sfahani *et al*., 2003). Assessment of in vitro maturation and development status of vitrified-warmed and control oocytes to the second meiotic stage (M II) were made by looking for expansion of cumulus cells and extrusion of the polar body under inverted microscope.

Statistical analysis

Data were analysed by chi-squared test. Differences were considered to be statistically significant at $p<0.05$.

Results

A high proportion of morphologically normal oocytes (90%) was recovered after vitrification-warming using GCM (Table 1).

As showed in Table 2, a high proportion of live oocytes resulted from both groups after 24 hrs in culture (85.18 for GCM vs 90% for control group). There was no significant difference in the percentage of live oocytes between the GCM and control group (P>0.05).

The in vitro maturation and development rate of vitrified-warmed oocytes is showed in Table 3. Although the proportion of oocytes which were found to have undergone nuclear maturation did not differ between the control and GCM group (P>0.05), the rate of extrusion of polar body tended to be higher in control than that for GCM group (61.29 vs 40%, respectively).

Table 1: Morphological status of bovine oocytes following vitrification-warming in GCM

Experimental group	No. of oocytes (%)		
	Total treated	Mn	Md
GCM	30	27(90)	3(10)
Control	40	40(100)	0.00

Mn: morphologically normal. Md: morphologically damaged. Each group of experiments were replicated 4 times

Table 2: Effect of GCM method on post-thaw survival of vitrified bovine oocytes after 24 hrs culture

Experimental group	Replicated	No. of oocytes (%)	
		Total vitrified	Survival rate
GCM	4	27	23 (85.18)
Control	4	40	36 (90)

No significant difference was observed between groups (P>0.05)

Table 3: Post-thaw maturation rate (development to M II oocytes) of vitrified bovine oocytes in GCM followed by 24 hrs culture

Experimental group	Replicated	No. of oocytes (%)	
		Total vitrified	Maturation rates
GCM	3	25	10 (40)
Control	3	31	19 (61.29)

No significant difference was observed between groups (P>0.05)

Discussion

The present investigation demonstrates that vitrification of bovine oocytes in the GCM vessels using EG + DMSO solution is a simple and efficient method. Most of the vitrified bovine oocytes (90%) had morphologically normal appearance after thawing and these oocytes following in vitro culture showed high rate of maturation to M II phase.

Various type of cryoprotectants (e. g. glycerol, propylene glycol (PG), DMSO, polyethylene glycerol and 1,2-propanediol) have been used in different combinations for the vitrification of mammalian oocytes and

embryos (Palasz and Mapletoft, 1996). Recently, EG has gained importance as an effective cryoprotectant for the vitrification of mouse (Miyake *et al*., 1993) and cattle oocytes (Saha *et al*., 1996), since it offers advantages over other cryoprotectants in terms of higher permeation into oocytes or embryos for vitrification and faster removal during dilution, as its molecular weight is lower than that of glycerol and PG. Previously, DMSO was thought to be more toxic to embryos than glycerol and PG and was generally used in combination with acetamide, which was believed to neutralize its toxicity (Hamano *et al*., 1992; Tada *et al*., 1993). However, later studies using 2-cell mouse embryos failed to find any benefit of using acetamide (Tada *et al*., 1993) and in many subsequent studies DMSO alone has been found to be an effective cryoprotectant for vitrification of mouse and hamster oocytes (Wood *et al*., 1993). Therefore, we have used a combination of EG and DMSO. The concentration of EG and DMSO used in the present study was based on those used for the successful vitrification of bovine ova and embryos (Ishimori *et al*., 1993; Vajta *et al*., 1998).

Various types of morphological abnormalities may be observed after vitrification-warming like cracking of zona pellucida, leakage of cellular contents, change in shape, vacuolization and etc. In the present study the percentage of morphologically damaged oocytes was 10% (Table 1) and mostly cracking of zona pellucida was observed. This is in agreement with previous studies for buffalo oocytes vitrified in EG and DMSO (Dhali *et al*., 2000) and bovine oocytes vitrified in EG, ficoll and sucrose (Hochi *et al*., 1998). In order to study physical damage during vitrification, mouse blastocysts were subjected to 10 cycles of rapid vitrification and warming. As a result, 75% of the embryos were found to have an injured zona pellucida. This physical injury is thought to be caused by non-uniform volume change of the solution during phase change (Kasai, 1996).

The high survival rate of vitrified-warmed oocytes observed in the present study (85.18%) compares favorably with those of buffalo oocytes vitrified in 4.5 M EG and 3.4 M DMSO (Dhali *et al*., 2000) or of bovine oocytes vitrified in a mixture of DMSO, acetamide and PG (Hamano *et al*., 1992) or in mouse oocytes vitrified in either 6.0 M DMSO (Wood *et al*., 1993) or in a mixture of DMSO, PG, acetamide and polyethylene glycol (Shaw *et al*., 1991). This is higher than the survival rates of 36 to 39% for mouse oocytes vitrified in EG, ficoll and sucrose (Miyake *et al*., 1993). To increase the survival rates of cryopreserved ova and embryos, efforts are focused either to vary the cryopreservation technology (Mahmoud Zadeh *et al*., 1995; Martino *et al*., 1996; Vajta *et al*., 1997) or to improve in vitro conditions (Massip *et al*., 1993; Shamsuddin *et al*., 1994). When embryos are cryopreserved by vitrification, ice crystal formation is prevented by the use of high concentration of cryoprotectants and high cooling and warming rates. These may lead to osmotic and toxic effects (Vajta *et al*., 1998). To overcome these problems, Vajta *et al*., (1997) reported a new technology called the OPS method. In the OPS straw technology the carrier is a narrow plastic tube with an inner diameter of approximately 0.8 mm and the approximate volume of 1 µl cryoprotectant solution is loaded into the open end of straw (Vajta *et al*., 1997). The cooling rate in this method reaches approximately 20000˚C/min. In the GCM method the carrier is a narrow glass tube (outer/inner diameter: 1.5/1.1 mm) which has heat conductivity higher than the OPS plastic vessels and also, density of a GCM container is higher than an OPS straw therefore, the GCM straw will remain immersed in LN_2.

The rate of extrusion of the polar body after treatment with gonadotropins in our culture system for the GCM group (40%) was comparable to that for the control group (61.29%, $P>0.05$), showed in Table 3. The maturation rate of the oocytes vitrified in the present study are in agreement with that reported by Wani *et al*., (2004) and higher than reported by Dhali *et al*., (2000). The maturation rates of the immature bovine oocytes (3.77-30.91%) vitrified in a mixture of 40% EG + 0.5 M sucrose with or without 5% polyvinylpyrrolidone in OPS or 0.25 ml straws as reported by Yang *et al*., (2000) are lower than that of the maturation rate

obtained in the present study. Saxena and Maurya (1999) have reported 73% maturation rates of buffalo oocytes vitrified in 4 M EG and Hurtt *et al*., (2000) have reported 60% maturation rates of bovine oocytes vitrified in 2.5 M EG + 18% ficoll + 0.5 M sucrose, which are higher than the maturation rate observed in the present study. The lower maturation rate rather than survival rate in our study may be because of the vitrification of immature oocytes, since the developmental ability of oocytes frozen at germinal vesicle has been reported to be much lower than that of in vivo or in vitro matured oocytes in mice (Schroeder *et al*., 1990), cattle (Hurtt *et al*., 2000) and horse (Hurtt *et al*., 2000). This reduction could also be due to a possible multifactorial cause, including toxic effects of cryoprotectants, ultrastructural damage to the oocytes and deleterious effects on chromosomes and other cytoplasmic structures. Since permeating cryoprotectants interact intracellularly and affect microfilament and microtubule dynamics, exposure to vitrification solutions cause rapid cellular and nuclear dehydration and virtually complete depolymerization of cytoplasmic microtubules and microfilaments, the failure of which to repolymerize during rehydration may alter their cellular distribution, leading to compromised embryo quality (Dobrinsky, 1996).

The present study demonstrated that vitrification of immature bovine oocytes in GCM vessels using 20% EG + 20% DMSO solution has high survival rate.

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References

1- Bautista, JA and Kanagawa, H (1998). Current status of vitrification of embryos and oocytes in domestic animals: ethylene glycol as the emerging cryoprotectant of choice. Jpn. J. Vet. Res., 45: 183-191.

2- Critser, JK; Arneson, BW; Aaker, DW and Ball, GD (1986). Cryopreservation of hamster oocytes: effects of vitrification or freezing on human sperm penetration of zona-free hamster oocytes. Fertil. Steril., 46: 277-284.

3- Dhali, A; Manik, RS; Das, SK; Singla, SK and Palta, P (2000). Vitrification of buffalo (*Bubalus bubalis*) oocytes. Theriogenology. 53: 1295-1303.

4- Dobrinsky, JR (1996). Cellular approach to cryopreservation of embryos. Theriogenology. 45: 17-26.

5- Hamano, S; Koikeda, A; Kuwayana, M and Nagai, T (1992). Full term development of in vitro matured, vitrified and fertilized bovine oocytes. Theriogenology. 38: 1085-1090.

6- Hochi, S; Ito, K; Hirabayashi, M; Veda, M; Kimura, K and Hanada, A (1998). Effect of nuclear stages during IVM on the survival of vitrified-warmed bovine oocytes. Theriogenology. 49: 787-796.

7- Hurtt, AE; Squires, EL and Seidel, GEJr (2000). Vitrification of immature and mature equine and bovine oocytes in an ethylene glycol, ficoll and sucrose solution using open pulled straw. Theriogenology. 54: 119-128.

8- Ishimori, H; Saeki, K; Inai, M; Nagao, Y; Itasaka, J; Miki, Y; Seike, N and Kainuma, H (1993). Vitrification of bovine embryos in a mixture of ethylene glycol and dimethyl sulfoxide. Theriogenology. 40: 427-433.

9- Kasai, M (1996). Simple and efficient methods for vitrification of mammalian embryos. Anim. Reprod. Sci., 42: 67-75.

10- Kong, IK; Lee, SI; Cho, SG; Cho, SK and Park, CS (2000). Comparison of open pulled straw (OPS) vs glass micropipette (GMP) vitrification in mouse blastocysts. Theriogenology. 53: 1817-1826.

11- Madison, V and Fraser, LR (1992). Selection of immature bovine oocytes for developmental potential in vitro. Anim. Reprod. Sci., 27: 1-11.

12- Mahmoud Zadeh, AR; Van Soon, A; Bols, P; Yesbaert, MT and De Kruif, A (1995). Optimization of a simple vitrification procedure for bovine embryos produced in vitro: effect of developmental stage, two step addition on embryonic survival. J. Reprod. Fertil., 103: 33-39.

13- Martino, A; Songsasen, N and Leibo, SP (1996). Development into blastocysts of bovine oocytes cryopreserved by ultra-rapid cooling. Biol. Reprod., 54: 1059-1069.

14- Massip, A; Mermillod, P; Wils, C and Desy, F (1993). Effects of dilution procedure and culture conditions after thawing on survival

of frozen bovine blastocysts produced in vitro. J. Reprod. Fertil., 97: 65-69.
15- Men, H; Chen, W; Shang, E; Yang, S and Zou, R (1997). Cryopreservation of Kunming mouse oocytes using slow cooling, ultra-rapid cooling and vitrification protocols. Theriogenology. 47: 1423-1431.
16- Miyake, T; Kasai, M; Zhu, SF; Sakurai, T and Machida, T (1993). Vitrification of mouse oocytes and embryos at various stages of development in an ethylene glycol-based solution by a simple method. Theriogenology. 40: 121-134.
17- Nasre Sfahani, MH; Behdadi Pour, Z; Baharvand, H; Eimani, H; Ghanei, MS; Darabi, MR and Hajipour, MR (2003). Effect of vitrification and slow freezing on development of bovine cumulus oocytes complex. In: 10th Middle East Fertility Society Annual Scientific Meeting. P: 63 (abs).
18- Niemann, H (1991). Cryopreservation of ova and embryos from livestock: current status and research needs. Theriogenology. 35: 109-124.
19- Otoi, T; Yamamoto, K and Suzuki, T (1995). In vitro fertilization and development of immature and mature bovine oocytes cryopreserved with ethylene glycol and sucrose. Cryobiology. 32: 455-460.
20- Palasz, AT and Mapletoft, RJ (1996). Cryopreservation of mammalian embryos and oocytes: recent advances. Biotech. Adv., 14: 127-149.
21- Quinn, P; Kerin, J; Stone, B and Wilson, L (1986). Successful cryopreservation of human oocytes. In: 42nd Annual Meeting of American Fertility Society and 18th Annual Meeting of Canadian Fertility and Andrology Society. P: 72 (abs).
22- Rall, WF (1992). Cryopreservation of oocytes and embryos: methods and applications. Anim. Reprod. Sci., 28: 237-245.
23- Saha, S; Boediono, A; Sumantri, E; Murakami, M; Kikkawa, Y and Suzuki, T (1996). Vitrification of bovine in vitro matured and pronuclear oocytes with different vitrification solutions. Theriogenology. 45: 179 (abs).
24- Saxena, MK and Maurya, SN (1999). One step freezing of immature buffalo oocytes. Indian. J. Anim. Reprod., 20: 3.
25- Schellander, K; Peli, J; Schmoll, F and Brem, G (1994). Effects of cryoprotectants and carbohydrates on freezing of matured and unmatured bovine oocytes. Theriogenology. 23: 909-915.
26- Schroeder, AC; Champlin, AK; Mobraatin, LE and Eppig, JJ (1990). Developmental capacity of mouse oocytes cryopreserved before and after maturation in vitro. J. Reprod. Fertil., 89: 43-50.
27- Shamsuddin, M; Larsson, B; Gustafsson, H and Rodriguez-Martinez, H (1994). A serum free culture system for development of bovine one-cell to blastocyst stage with improved viability. Theriogenology. 41: 1033-1043.
28- Shaw, PW; Fuller, BJ; Bernard, A and Shaw, RW (1991). Vitrification of mouse oocytes: improved rates of survival, fertilization and development. Mol. Reprod. Dev., 29: 373-378.
29- Tada, N; Sato, M; Amann, E and Ogawa, S (1993). A simple and rapid method for cryopreservation of mouse 2-cell embryos by vitrification: beneficial effect of sucrose and raffinose on their cryosurvival rate. Theriogenology. 40: 333-344.
30- Vajta, G; Booth, PJ; Holm, P; Greve, T and Calleson, H (1997). Successful vitrification of early stage bovine in vitro produced embryos with the open pulled straw (OPS) method. Cryo-letters. 18: 191-195.
31- Vajta, G; Holm, P; Kuwayama, M; Booth, PJ; Jacobsen, H; Greve, T and Calleson, H (1998). Open pulled straw (OPS) vitrification: a new way to reduce cryoinjuries of bovine ova and embryos. Mol. Reprod. Dev., 51: 53-58.
32- Yang, BS and Leibo, SP (1999). Viability of in vitro-derived bovine zygotes cryopreserved in microdrops. Theriogenology. 51: 178.
33- Yang, BC; Yang, BS; Seong, HH; Im, GS and Park, SJ (2000). Effect of vitrification methods and polyvinylpyrrolidone supplementation on the viability of immature bovine oocytes. Theriogenology. 53: 25-65.
34- Wani, NA; Mista, AK and Maurya, SN (2004). Maturation rates of vitrified-thawed immature buffalo (*Bubalus bubalis*) oocytes: effect of different types of cryoprotectants. Anim. Reprod. Sci., 84: 327-335.
35- Whittingham, DG; Leibo, SP and Mazur, P (1972). Survival of mouse embryos frozen to -196 and -269ºC. Science. 178: 411-414.
36- Wood, MJ; Barros, C; Candy, CJ; Carroll, J; Melendez, J and Whittingham, DG (1993). High rates of survival and fertilization of mouse and hamster oocytes after vitrification in dimethyl sulfoxide. Biol. Reprod., 49: 489-495.