VITRIFICATION OF CAPRINE EMBRYOS IN MICRODROPS

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ABSTRACT

The aim of this study was to develop a novel and efficacious method for cryopreservation of caprine embryos. In vivo derived embryos at morula and blastocyst stages were vitrified in microdrops by dropping directly into liquid nitrogen (LN2) without using any containers or carriers. Embryos were initially exposed in vitrification solution 1 (VS1) containing 10% ethylene glycol (EG) and 10% dimethylsulfoxide (DMSO) for 45 s, followed by transferring into vitrification solution 2 (VS2) containing either 16.5% EG and 16.5% DMSO or 20.0% EG and 20.0% DMSO for 25 s before vitrification. Microdrops containing embryos were generated from the tip of a pretreated and calibrated glass pipette, followed by dropping into LN2 by flipping the pipette. For locating and manipulating the droplets formed in LN2, we have developed a simple but novel device. The vitrification efficiencies of cryoprotectants in VS2 at different concentrations (16.5% EG, 16.5% DMSO vs 20% EG, 20% DMSO) and different combinations (16.5% EG, 16.5% DMSO vs 12.5% EG, 12.5% DMSO and 8% 1, 3-butanediol, BD) were compared. In addition, the influences of different droplet sizes (1, 2 and 4 μl), developmental stages of embryos (morula, blastocyst and expanded blastocyst), and vitrification methods (microdrop vs open pulled straw, OPS) on vitrification efficiency were determined as well. Embryos vitrified in solution containing 16.5% EG and 16.5% DMSO tended to have higher survival rates in vitro (90.2% vs 83.5%) and in vivo (64.4% vs 52.9%) than those of in 20.0% EG and 20.0% DMSO. Replacing part of EG and DMSO with BD significantly declined the in vitro survival rate of vitrified-thawed embryos (90.3% vs 64.7%, $p<0.05$). Embryos vitrified in 4 μl droplets significantly delayed the initial recovery time after thawing than that of in 1 or 2 μl droplets ($p<0.05$). Although no significant differences on the final embryo survival rate were found irrespective of the drop size in which they were vitrified, embryos vitrified in 2 μl droplet was the most appropriate in terms of the initial time to survive after thawing and the easiness for droplet manipulation.. The survival rates of vitrified-thawed embryos were not influenced by their developmental stages. Also, there was no significant difference on survival rate of embryos vitrified by microdrop and OPS methods was found (81.8% vs 75.0%). These results indicate that directly dropping 2 μl droplet containing 16.5% EG + 16.5% DMSO into LN2 is a promising method for vitrification of caprine embryos.

KEY WORDS: Caprine, Embryo, Microdrop, Vitrification.

INTRODUCTION

Efficient cryopreservation techniques are required to allow the delayed transfer of embryos and to use multiple ovulation and embryo transfer (MOET) schemes more widely to increase the genetic gain resulting from selection. Since the first successes of mammalian embryo cryopreservation in the sixties of last century, two major methodologies have been developed: conventional slow-rate freezing and vitrification. The conventional slow-rate freezing method has been widely and successfully used to cryopreserve embryos in a variety of species including sheep and goat (Cognie *et al*., 2003). However, it suffers from several limitations such as chilling injury, physical damage due to formation of ice crystal, need for elaborate and expensive equipment and tedious freezing protocols. In contrast, vitrification is characterized by its simplified procedures and has proved its efficiency and practicality in the recent years (Liebermann *et al*., 2002; Vajta and Kuwayama, 2006). Currently, vitrification has become a promising and competitive alternative to conventional slow-rate freezing.

Almost all vitrification strategies are based on two main factors: concentration of cryoprotectants and cooling-warming rates. Extensive research in the past 20 years has resulted in new approaches and created an acceptable compromise by decreasing cryoprotectant toxicity and increasing cooling rates. To date, the concentration and combination of cryoprotectants, and the addition and removal protocols have been almost fully exploited (Vajta and Kuwayama, 2006). Considerable efforts have been invested to develop alternative methods to increase cooling and warming rate to gain real benefit over conventional methods. Several vitrification methods attempting to increase the cooling rate have been described for embryos from different species. Most of these methods used a variety of carriers or containers to hold embryo-containing solutions while they were plunging into LN_2. The carrier tools which have been developed to minimize the volume and to submerge the embryos quickly into LN_2 included electron microscopic grids (Martino *et al*., 1996), open pulled straw (OPS) (Vajta *et al*., 1997), cryoloop (Lane *et al*., 1999), cryotop (Kuwayama and Kato, 2000) and nylon mesh (Matsumoto *et al*., 2001). All those carrier tools caused vapor formation around the samples while they were submerged into LN_2, thus hamper the cooling rate of embryos. An alternative way to avoid vapor formation around the sample was the solid surface vitrification (SSV) (Dinnyes *et al*., 2000; Atabay *et al*., 2004). However, drawbacks of this method included the difficulty to control the drop size exactly and uniformly, and the temperature on the metal surface could be as low as -150℃ which might relatively decrease the cooling rate of the sample (Dinnyes *et al*., 2004). Theoretically, dropping the minimum volume, as small as 1 to 2 μl of embryo-containing solution directly into the LN_2 without the aid of any containers or carriers can minimize the vapor formation and maximize heat exchange between sample and LN_2, thus to maximize the cooling rate (Riha *et al*., 1991). Unfortunately, this method has not been reported so far. The technical difficulties such as control of a uniform microdrop size as well as the delay before the drop floating on the surface of LN_2 sank were the limitations of this method (Papis *et al*., 2000). Furthermore, studies on vitrification of caprine embryos are much less abundant (Baril *et al*., 2001; Begin *et al*., 2003; Branca *et al*., 2000; El-Gayar and Holtz, 2001; Traldi, 2000; Traldi *et al*., 1999) when compared to that of bovine and ovine species. Development a novel simplified technique to improve the vitrification efficiency of caprine embryos is in need. The main objective of the present study was to develop a method to vitrify caprine embryos by dropping microdrops of minimum volume directly into LN_2 to maximize the cooling rate. The vitrification efficiency of different concentrations and combinations of cryoprotectants,

droplet sizes, developmental stages of embryos and the efficiency of OPS method were compared as well.

MATERIALS AND METHODS

Superovulation and Estrus Synchronization

Multiparous Nubian and Boer crosses with the age ranging from 2 to 5 years old were used as embryo donors and recipients. Synchronization of estrus in donor and recipient does was induced by progestagen treatment with a vaginal releasing device containing 366 mg progesterone (Controlled Internal Drug Release, CIDR®, EAZI-breed, Rydalmere, Australia) inserted for 11 days. Two days before CIDR removal, each of the recipients were given 500 IU eCG (Sera-Gona, China Chemicals, Taipei, Taiwan) and 125 μg cloprostenol (Estrumate®, Schering-Plough, Baulkham, Australia) by intramuscular injection.

Superovulation of donor does were induced with a total dose of 16 mg pFSH (Sigma F-2293, Saint-Louis, USA) in 6 decreasing doses, administered at 12 h intervals, starting 60 h before CIDR removal. Two doses of pLH (Sigma L-5269, Saint-Louis, USA), 1.7 units of each, were administered at the same time with the fifth and sixth doses of pFSH. Following CIDR removal, does exhibiting behavioral estrus were mated to bucks two to three times 12 h apart upon its duration of estrus.

Embryo Collection

Embryos were collected surgically 7 days after the onset of estrus. General anesthesia of donor doe was induced by an injection (i. m.) of 0.11 mg/kg of xylazine (Rompun®, Bayer, Leverkusen, Germany) and 5.5 mg/kg (i. m.) of ketamine hydrochloride (Soon-Soon®, Yung-Shin Co., Taipei, Taiwan) 10 minutes later. Followed, the genital tract was exteriorized through a mid-ventral laparotomy. Ovulatory response was assessed by counting the number of corpora lutea, and embryos was collected by flushing uterine horn with 30 ml Dubelco's phosphate buffer solution (DPBS, Gibco, 21300-025, Grand Island, USA) supplemented with 2% bovine serum albumin (BSA) through a flushing needle connected a 10 ml syringe. Recovered embryos were graded by their morphological appearance using the criteria similar to that reported by Lindner and Wright (1983). Embryos of excellent to good quality collected on the same day were pooled and allocated to the different treatment groups within each experiment.

Vitrification of Embryos

Unless in the experiment to compare the effect of development stages (morula, blastocyst and expanded blastocyst) on efficiency of vitrification, all embryos were vitrified at expanded blastocyst stages. Therefore, collected embryos at morulae and blastocysts were briefly cultured in 50-μl drops of M-199 (Gibco, 12340-030, Grand Island, USA) supplemented with 5% fetal bovine serum (FBS, Gibco, 10270-106, Grand Island, USA) under paraffin oil at 38.5 ℃ in a controlled atmosphere.

Holding medium for vitrification was prepared by supplementing M-199 with 20% FBS (v/v). Vitrification solution 1 (VS1) contained 10% EG, 10% DMSO and 10% FBS in M-199, while vitrification solution 2 (VS2) contained 16.5% EG, 16.5% DMSO, 10% FBS and 0.5 M

sucrose in M-199. In another experiment we also compared the efficiency of VS2 containing different combinations of cryoprotectants (16.5% EG, 16.5% DMSO vs 12.5% EG, 12.5% DMSO and 8% 1, 3-butanediol, BD) with the total concentration remained unchanged. Different concentrations of cryoprotectants in VS2 (16.5% EG, 16.5% DMSO vs 20% EG, 20% DMSO) and different volume sizes of droplet for vitrification (1, 2, 4 μl) were compared as well.

Two to four embryos were initially placed in the holding medium for 5 min, followed by VS1 for 45 s. Subsequently the embryos were transferred into 3 sequential drops of VS2 for 25 s. During equilibration in VS2, the exact volume of solution containing embryos were aspirated into a fine pretreated and calibrated glass pipette connected to a silastic tubing and manipulated by mouth. Immediately after aspiration of embryo-containing VS2 upon the calibration of glass pipette, a microdrop containing 3 embryos was generated at the tip of pipette by mouth manipulation, followed by dropping the droplet onto LN_2 by flipping the pipette above LN_2. Microdrop floating on the surface of LN_2 was immediately pressed into LN_2 by a pre-cooled metal mesh. We have developed a novel device that could be used to facilitate locating and handling the solidified microdrops in LN_2. The vitrified droplets at the bottom of the device were then moved into a 1 ml cryovial with LN_2-cooled forceps for long-term storage (2-3 wk).

Open pulled straws (OPS) were purchased from Szig Ta (Denmark). Treatments of embryos before OPS vitrification were the same as described above except the embryos were loaded into the straw by capillary action and then plunged the straw vertically into LN_2 immediately.

Thawing and Culture of Vitrified Embryos

Microdrops containing embryos were withdrawn from cryovial and poured back into the described novel device that was pre-filled with LN_2. The vitrified droplets were then picked up with LN_2-cooled forceps and plunged into a 38.5℃ M-199 containing 0.5 M sucrose and 20% FBS for 30 s, followed by serial dilutions in 0.25 M sucrose solution for 2 min and 0.15 M sucrose solution for 5 min to remove cryoprotectants. Embryos vitrified in OPS were thawed by placing the narrow end of straw into 38.5℃ M-199 and the removal of cryoprotectants was conducted by three-step dilution as described above.

After removal of the cryoprotectants, embryos were transferred into M-199 containing 5% FBS and were incubated at 38.5℃ in a humidified atmosphere of 5% CO_2 in air for 24 h. Survivability of embryos was assessed at 2, 4, 8, 12 and 24 h after culture. Well rehydrated embryos with intact zona pellucida and blastomere, having further development to hatching stage before the end of culture, were assessed as survival from vitrification.

Embryo Transfer

Vitrified-thawed embryos were transferred surgically into uterine horns ipsilateral to the corpus luteum on day 7 after estrus of the recipients using a glass capillary connected to a 1 ml syringe.

Pregnancy diagnosis was performed by transrectal ultrasonography 60 d after embryo transfer. Number of transferred embryos survived to term was recorded.

Statistics

Data of in vivo and in vitro survival rates of embryos, and conception rate of recipients were analyzed by the chi-square test (SAS, 1987).

RESULTS

Effects of the concentration and combination of cryoprotectants on the in vitro and/or in vivo survival rate of vitrified-thawed embryos

A total of 124 embryos were vitrified to compare the efficiency of different concentration of cryoprotectants in vitrification solution. As shown in Table 1 and 2, embryos vitrified in 2 μl droplet containing 16.5% EG and 16.5% DMSO yielded as high as 90.2% in vitro survival rate within 24 h culture after thawing and 64.4% transferred embryos survived to term, though there were no significant differences were found when compared to that of 20% EG and 20% DMSO (83.5% and 52.9%). Therefore, vitrification solution containing 16.5% EG and 16.5% DMSO was used in the rest of experiments in present study.

The result of replacing part of EG and DMSO with BD, as presented in Table 3, significantly decreased the in vitro survival rate of vitrified-thawed embryos (90.3% vs 64.7%; $P < 0.05$).

Table 1. The in vitro survival rate of caprine embryos vitrified in 2 μl droplets of different concentrations of cryoprotectants

Concentration of Cryoprotectants	No. of embryos vitrified	No. of embryos survived in vitro	Survival rate of embryos in vitro (%)
33% (16.5% EG＋16.5% DMSO)	51	46	90.2
40% (20.0% EG＋20.0% DMSO)	73	61	83.5

Table 2. The conception rate of recipients and the kidding rate of transferred embryos vitrified in 2μl droplets of different concentrations of cryoprotectants

Concentration of Cryoprotectants	No. of embryos transferred	No. of recipient	No. of doe conceived	Conception rate (%)	Embryos survived to term (%)
33% (16.5% EG＋16.5% DMSO)	14	7	6	85.7	9(64.4)
40% (20.0% EG＋20.0% DMSO)	17	9	7	77.8	9(52.9)

Table 3. The in vitro survival rate of caprine embryos vitrified in 2 μl of droplet with different combinations of cryoprotectants

Combination of cryoprotectants	No. of embryos vitrified	No. of embryos survivied	Survival rate of embryos (%)
16.5% EG＋16.5% DMSO	31	28	90.3[a]
12.5% EG＋12.5% DMSO＋8% BD	34	22	64.7[b]

Values with different superscripts in the same column are significantly different ($P < 0.05$)

Effect of the droplet volume on the in vitro survival rate of vitrified-thawed embryos

The results of embryos vitrified in the droplet of different volumes were summarized in Table 4. There were no significant differences observed among 1, 2 and 4 μl droplets in terms of the final in vitro survival rate of vitrified-thawed embryos. Whereas, as shown in Table 5, the embryos vitrified in 4 μl droplet significantly delayed the initial recovery time by 4 h after thawing and culture when compared to those in the 1 and 2 μl group (40.0% vs 70.8% and 69.2%; $P < 0.05$).

Table 4. The in vitro survival rate of caprine embryos vitrified in different volumes of droplet containing 16.5% EG＋16.5% DMSO

Volume of Droplet (μl)	No. of embryos vitrified	No. of embryos survived	Survival rate of embryos (%)
1	32	24	75.0
2	30	26	86.7
4	36	30	83.3

Table 5. The effect of droplet volume on recovery rate of vitrified embryos after thawing and culture.

Volume of Droplet (μl)	No. of embryos thawed and survived	No. of embryos (%) recovered after thawing and cultured for: 2hrs	4hrs	12hrs	24 hrs
1	24	17(70.8)[a]	20(83.3)	22(91.6)	24(100)
2	26	18(69.2)[a, b]	22(84.6)[a]	25(96.1)	26(100)
4	30	12(40.0)[c]	18(60.0)[b]	25(83.3)	30(100)

Values with different superscripts in the same column are significantly different ($P < 0.05$)

Vitrification efficiency of the embryos vitirified at different developmental stages

The results of the in vitro survival rates of embryos vitrified at morula, blastocyst or expanded blastocyst were shown in Table 6. There were no significant differences on final survival rate of vitrified-thawed embryos during 24 hr culture irrespective of the developmental stages. However, embryos vitrified at blastocyst or expanded blastocyst stages tended to have higher in vitro survival rate.

Table 6. The in vitro survival rate of caprine embryos vitrified at different developmental stages in 2 μl droplet containing 16.5% EG＋16.5% DMSO

Developmental stages	No. of embryos vitrified	No. of embryos survived	Survival rate of embryos (%)
Morula	20	16	80.0
Blastocyst	18	16	88.8
Expanded blastocyst	11	10	90.9

Effect of the different vitrification methods on the in vitro survival rate of embryos

There was no significant difference on survival rate of embryosvitrified by microdrop and OPS methods was found (Table 7). However, as presented in table 8, higher percentage of embryos vitrified in microdrops tended to recover earlier by 2 hrs after culture (81.4% vs 63.6%).

Table 7. The in vitro survival rate of caprine embryos vitrified in microdrops or open pulled straws

Vitrification method	No. of embryo vitrified	No. of embryo survived	Survival rate of embryos (%)
Microdrops	33	27	81.8
OPS	44	33	75.0

Table 8. The effect of different vitrification methods on recovery rate of vitrified-thawed embryos after thawing.

Vitrification Methods	No. of embryos survived	No. of embryos (%) recovered after thawing and cultured for:			
		2hrs	4hrs	12hrs	24 hrs
Micro drops	27	22(81.4)	22(81.4)	24(88.8)	27(100)
OPS*	33	21(63.6)	26(78.7)	30(90.9)	33(100)

Values with different superscripts in the same column are significantly different ($P < 0.001$)

* Means the embryos were vitrified by using open pulled straw method.

DISCUSSION

To our knowledge, this is the first report dealing with vitrification of animal embryos in microvolume, as small as 1 to 2 μl droplets by direct contact with LN_2. Cooling rate has been one of the crucial factors for the success of embryo or oocyte vitrification (Vajta and Kuwayama, 2006). In the present study, we attributed the relative high in vitro and in vivo survival rate of vitrified embryos to the maximum cooling and warming rate achieved by the direct contact of microdrops with LN_2 and a warm diluting solution. This ultrarapid freezing process allow no pressure changes occur during freezing, avoiding fracture damage (Vajta *et al*., 1997) and implies minimal de- and re-hydration of cells, reducing the degree of strain placed on the cell membrane. Container or carrier free vitrification method favors rapid heat exchange between the embryo-containing drop and LN_2, thus preventing chilling injury.

In the present study, the use of very small amount of vitrification solution (2 μl) to reduce the risk of drop cracking occurred instantaneously contacting with LN_2 might partly contribute to the high survival rate of vitrified-thawed embryos. Cracking, which leads to rupture of the zona pellucida or plasma membrane lysis, is generally caused by extreme temperature gradients between the outer layer and the core of the vitrified drop with the volume larger than 2 μl (Begin *et al*., 2003; Dinnyes *et al*., 2004). According to our experience, dropping down a 1 or 2 μl drop smoothly from an un-pretreated fine glass pipette's tip is somewhat technically difficult. Misumi *et al*. (2003) used a fine-tipped Pasteur pipette to form 1 μl drop and immersed the drop together with the pipette tip into LN_2 for solidification, followed by cutting off 1 cm of the tip with droplet for cryopreservation. Papis *et al*. (2000) use 6 μl droplet to vitrified bovine oocytes and suffered from an inconsistency of results due to technical problem. In present study, we confirmed the 4 μl droplet suffered from a cracking problem during cooling as described in previous reports. The results in Table 5 showed that embryos vitrified in 4 μl droplet delayed their recovery in vitro after thawing. Though Dinnyes *et al*. (2004) reported to drop 1 to 2 μl droplet onto the LN_2 pre-cooled metal surface for vitrification of bovine oocytes, this approach is challenging since the drop size could not be controlled precisely and some ova could stick to the pipette or be lost due to random drop dispersion caused by the flipping movement. In the present study, we pre-treated and calibrated the glass pipette to facilitate the formation of droplet of exact small volume smoothly and to let the microdrops dropping down feasibly from the pipette's tip. No case of embryos sticking to the pipette was found. We did not find extra advantage from vitrification of 1 μl droplet over that of 2 μl droplet in terms of embryo survival rate. Moreover, 1 μl droplet was less easy to get rid of the pipette's tip while applying flipping movement due to its lighter gravity. Furthermore, 1 μl droplet was much difficult for recovery and manipulation in LN_2. Upon the results of the present study, we suggest the droplet size of 2 μl is appropriate for microdrop vitrification.

Plunging a warm object onto LN_2 usually creates an isolating layer of nitrogen vapor around the object and might hamper the cooling rate of embryos. Although the droplets used in this study were much smaller in size when compared to those embryo-holding containers or carriers used in other studies, the resulting vaporization of LN_2 while plunging onto LN_2 remained more or less unavoidable. Floating of microdrops on LN_2 following plunging might

limit the cooling rate. For circumventing this problem, we designed a handled metal screen, pre-cooled by LN_2, to press the floating droplets submerging into LN_2 immediately after dropping onto LN_2.

Like most ultrarapid vitrification procedures, the present employed method depends on direct contact of the embryo containing solution with the liquid nitrogen for cooling and on subsequent storage without hermetical isolation. Although the possibility of cross-contamination through LN_2 in embryology is very low (Vajta and Kuwayama, 2006), it cannot be neglected. To solve this problem, cooling can be performed in factory derived, and/or filtered or UV-sterilized LN_2 followed by storage of droplets in a sealable container.

Concentration of cryoprotectants in solution is one of important factors to affect vitrification efficiency. Considerable efforts were invested to decrease the toxicity of cryoprotectants by applying less toxic and more permeable chemicals, lowering total concentration of chemicals and using two or more cryoprotectants to decrease the specific toxic effect of each. Vitrification solution containing 20% EG + 20% DMSO has been used in vitrifying ovine (Dattena *et al*., 2004; Isachenko *et al*., 2003; Papadopoulos *et al*., 2002) and caprine embryos (El-Gayar and Holtz, 2001) and attained good results. In the present study, the vitrification efficiency of 16.5% EG + 16.5% DMSO was firstly tested and compared with that of 20%EG + 20% DMSO in caprine species. Although no significant difference on the survival rate of vitrified-thawed embryos between treatments was found, embryos vitrified in solution containing 16.5% EG + 16.5% DMSO tended to survive better in vitro and in vivo than that of 20% EG + 20% DMSO counterparts (Table 1, 3). This might be attributed to the improvement of cooling rate in present vitrification method since one characteristic of vitrification is that as the cooling rate chosen increased, the cryoprotectant concentration can be lowered and vice versa (Vajta and Kuwayama, 2006). In this study, substituting part of EG and DMSO for BD in vitrification solution significantly lowered the survival rate of embryos. This is not consistent with the results reported by Pugh *et al*. (2000). The BD has been proved to has excellent glass-forming characteristics and vitrify at low molar concentrations compared to EG (Ali and Shelton, 1993). It is possible that the addition of a third highly permeable cryoprotectant such as butanediol, further affects the permeability of the other cryoprotectants (Pugh *et al*., 2000).

Although no statistical difference on post thaw survival rate of embryos vitrified at different developmental stages was found, it was likely that embryos vitrified at blastocyst or expanded blastocyst stages survived better than that at morula stage (Table 6). Blastocyst and expanded blastocyst have been proved to survive better than morula from vitrification damage in bovine (Leibo *et al*., 1996), ovine (Martinez and Matkovic, 1998; Naitana *et al*., 1996) and caprine (Li *et al*., 1990) embryos. The reason for this difference has been attributed to that the cellular membranes of embryos become more resistant to osmotic and toxic stress after the formation of the blastocoelic cavity. The diversification of cell types and in particular the increase of Na/K ATPase activity which occurs during blastocoelic formation in trophoblastic cells may determine more active transport mechanisms of cryoprotectants (Naitana *et al*., 1996). Other aspects that can have an influence on survival rate may be explained by differences in blastomere size. The cells of compacted morulae are slightly larger than the cells of blastocysts, and this may render them more sensitive to the osmotic stress induced by the removal of the permeated cryoprotectants (Tachikawa *et al*., 1993). In this study, the high survival rate of morulae might be attributed to the improvement of cooling rate.

The OPS method has been used to vitrify in vivo derived caprine embryos and yielded

64% embryos surviving to term (El-Gayar and Holtz, 2001). In this study, we obtained 75% post thaw in vitro survival rate of embryos which was not different from that of vitrified by microdrop method (81.8%). However, no straws are needed in our microdrop method so that the risk of cracking of OPS straw tip during handling and storage can be avoided.

In conclusion, the present results demonstrated that vitrification of caprine embryos in 2 μl drop containing 16.5% EG + 16.5% DMSO followed by dropping into LN_2 without the aid of containers or carriers resulted in high in vitro and in vivo survival rates. This method was characterized by its simplicity and high efficiency for cryopreservation of advanced stages of caprine embryos. Further experiments are needed to determine the efficiency of the present method on vitrification of caprine oocytes and early embryos.

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