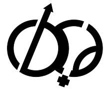

The effect of cryodevice and cryoprotectant concentration on buffalo oocytes vitrified at MII stage

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Abstract

Vitrification is a common method for cryopreservation of gametes and embryos. Although successful oocyte vitrification has been achieved in several animal species, subsequent progress is still limited especially in buffalo. To improve the effectiveness of vitrification of buffalo oocytes, two experiments were conducted. The first experiment evaluated the effect of cryodevices on viability and maturation of vitrified, matured buffalo oocytes. The *in vitro* matured oocytes were divided into two groups, the first was vitrified using conventional French straws, while the other was vitrified using Cryotops. There was a significant reduction in the morphologically normal oocytes after vitrification with both methods. Maturation rates of vitrified thawed buffalo oocytes were significantly higher in Cryotops than straws. The survival rate after vitrification was similar for both straws and Cryotops. The percentages of viable oocytes were significantly lower in straw than in controls. The second experiment evaluated the effect of two concentrations of cryoprotectants on the vitrification of *in vitro* matured buffalo oocytes. Mixtures of DMSO and EG as cryoprotectant (CPA) solutions were prepared in TCM-199 with two concentrations of cryoprotectants. The first concentration was 6 M V2 (3 M E.G + 3 M DMSO), and the second concentration was 7 M V2 (3.5 M DMSO + 3.5 M EG). Each concentration of cryoprotectants was added in two steps, with the first step having half the concentration of the second (and final) concentration. The survival rate after vitrification was similar for both concentration (6 M and 7 M) groups. The maturation rates of vitrified thawed buffalo oocytes were significantly higher in 7 M concentration than in the 6 M group. In conclusion, the survivability and meiotic competence of buffalo oocytes improved with vitrification at higher concentration of cryoprotectants and using cryotops.

Keywords: buffalo, cryoprotectant, Cryotop, maturation, oocyte, vitrification.

Introduction

The primary problem with cooling or cryopreservation of oocytes is the low percentage of oocytes retaining the ability to undergo normal maturation and fertilization (Le Gal, 1996). This may be attributed to the exposure to cryoprotectant and the cooling process. A variety of new techniques and types of holders/devices have been tested with the aim of improving the overall survival of oocytes/embryos post-cryopreservation such as solid surface vitrification (SSV; Somfai *et al*., 2006; Gupta *et al*., 2007), open pulled straw (OPS; Vajta *et al*., 1998; Varga *et al*., 2006), Cryotop (Ogawa *et al*., 2010), electron microscopy grid (Martino *et al*., 1996) and cryoloop (Lane *et al*., 1999). In order to facilitate vitrification with higher cooling rates, it is necessary to minimize the volume of the vitrification solution as much as possible with the use of special carriers (Galeatia *et al*., 2011).

One of the most successful ultra-rapid vitrification techniques is Cryotop vitrification (CTV), which has resulted in excellent survival and developmental rates with human and bovine MII phase oocytes (Kuwayama *et al*., 2005). Muenthaisong *et al*. (2007) demonstrated that *in vitro* matured Swamp buffalo oocytes vitrified by the CTV method retain the capability to cleave and develop into blastocysts following parthenogenetic activation. Moreover, vitrification using Cryotops improved the cleavage rates of both cow and calf oocytes over those obtained using OPS and led to a greater number of blastocysts (Morató *et al*., 2008). During the past few years, CTV has rapidly spread in human medicine, producing impressive results in terms of healthy offspring after oocyte and/or blastocyst cryopreservation (Lucena *et al*., 2006; Cobo *et al*., 2008). Moreover, Cryotops have been successfully used to cryopreserve immature and *in vitro*-matured horse (Bogliolo *et al*., 2006), cow, pig, sheep and buffalo oocytes (Chian *et al*., 2004; Gasparrini *et al*., 2007; Ogawa *et al*., 2010), rabbit zygotes (Hochi *et al*., 2004), cow embryos (Laowtammathron *et al*., 2005) and buffalo embryos (De Rosa *et al*., 2007). The greatest advantage of this method is that an extremely fast cooling rate is achieved

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(-23,000 C°/min) and chilling injury is avoided (Kuwayama *et al*., 2005). The extremely small volume also helps achieve a faster warming rate (-42,000 C°/min), thereby avoiding ice crystal formation during warming. A further benefit of the Cryotop method is that the permeable CPA concentration is reduced to 30%, minimizing potential toxic effects (Kuwayama *et al*., 2005).

Cryoprotectants are compounds used to achieve cellular dehydration and avoid intracellular ice crystal formation upon freezing (Fahy, 1986). Cryoprotectants influence the ability of buffalo oocytes to survive cryopreservation (Wani *et al*., 2004a, b). Dimethyl sulfoxide (DMSO), ethylene glycol (EG), 1,2-propanediol (PROH), propylene glycol and glycerol have been used in different combinations for vitrification of mammalian oocytes and embryos (Palasz and Mapletoft, 1996). Several studies demonstrated that EG would be the ideal cryoprotectant (Shaw *et al*., 1997), because it penetrates membranes faster than glycerol (Cha *et al*., 2000) and is less toxic than other permeable cryoprotectants (Martino *et al*., 1996; Cha *et al*., 2000; Dinnyes *et al*., 2000). The damaging effects of solutes can be lessened by using a mixture of two or more cryoprotectants or by stepwise equilibration (two or more steps) in solutions of intermediate concentrations at room temperature or after cooling to 5°C. At present, the combined use of two types of cryoprotectants is the standard. It was reported that cryoprotectant mixtures have some advantages over solutions containing only one permeable cryoprotectant (Vajta *et al*., 1998; Chian *et al*., 2004; Mahmoud *et al*., 2010). The aim of the present study was to improve the vitrification of buffalo oocytes by using different devices and cryoprotectant concentrations.

Materials and Methods

Chemicals

Chemicals for *in vitro* maturation including fetal calf serum and tissue culture medium (TCM 199) were obtained from Gibico BRL (Grand Island, New York, USA). Cysteamine, ethylene glycol (EG) and dimethyl sulfoxide (DMSO) were obtained from Sigma Chemical Company.

Oocyte recovery and selection

Buffalo ovaries were collected from abattoir within 2 h of slaughter. The ovaries were transported to the laboratory in physiological saline (0.9% NaCl) containing antibiotics (100 µg/ml streptomycin sulfate and 100 IU/ml penicillin) maintained at 30°C. Ovaries were washed three times in phosphate-buffered saline (PBS). Oocytes were aspirated from 2 to 5 mm follicles with a 20-gauge needle attached to a 5-ml syringe containing PBS with 3% bovine serum albumin (BSA), fraction V and antibiotics (100 µg/ml streptomycin sulfate and 100 IU/ml penicillin). Oocytes were searched using a stereo zoom microscope. The oocytes with intact layers of cumulus cells and homogenous cytoplasm were selected for the study (Warriach and Chohan, 2004).

In vitro maturation (IVM) of oocytes

Oocyte maturation was carried out as described by Mahmoud (2001). Briefly, the recovered oocytes were cultured in groups of 10 to 20 in 100 µl droplets of maturation medium (TCM-199 supplemented with 10% fetal calf serum, 50 µM cysteamine and 50 µg/ml gentamycin sulfate. The droplets were covered with mineral oil and pre-incubated for a minimum of 2 h in a humidified 5% CO_2 atmosphere at 38.5°C. The oocytes were placed into the droplets and incubated for 22 h in a humidified 5% CO_2 atmosphere at 38.5°C.

Vitrification and warming by straw method

Oocytes were exposed to two-step addition of cryoprotectants (Mahmoud *et al*., 2010). Briefly, oocytes were exposed to VS1 (1.5 M EG + 1.5 M DMSO) for 45 sec. and VS2 (3 M EG + 3 M DMSO) for 25 sec. The holding medium was TCM 199 containing 2.5 mM HEPES + 20% fetal calf serum. Oocytes (n = 5-10) were immediately loaded in 0.25 ml straws in the middle column of VS2 and separated by air bubbles, sealed with straw plugs, pre-cooled by exposure to liquid nitrogen (LN2) vapor for at least 60 sec and dipped vertically in LN2 and stored for two months. Straws transferred rapidly in less than 5 sec in air to prevent zona fracture (Subramaniam *et al*., 1990); and then to a water bath at 35-37°C for 20 sec for warming; the expelled oocytes were equilibrated for 5 min in 0.5 M galactose solution (Horvath and Seidel, 2006) in TCM-199 for one-step dilution to remove the cryoprotectants. Oocytes were washed 4-5 times in a fresh washing medium and cultured in IVM medium for 24 h.

Vitrification and warming by the Cryotop method

The cryotop procedure was described by Kuwayama and Kato (2000). Briefly, oocytes were exposed to VS1 (1.5 M EG + 1.5 M DMSO) for 45 sec and then transferred to VS2 (3 M EG + 3 M DMSO) for 25 sec; 3 oocytes were placed on a sheet of Cryotop (Kitazato Supply, Tokyo, Japan) in a small volume of the vitrification solution (<1 µl). The Cryotop was plunged into liquid nitrogen. After storage in liquid nitrogen for two months, oocytes were thawed by removing the protective cap from the Cryotop under liquid nitrogen and the polypropylene strip of Cryotop was immersed directly into TCM-199 following a stepwise dilution with 0.5 M galactose solutions. Finally, oocytes were washed, evaluated, and cultured in TCM-199 for another 2 h to complete the maturation period.

Morphological evaluation of vitrified, warmed oocytes

The vitrified, warmed oocytes were examined under an inverted phase contrast microscope (Olympus, Tokyo, Japan).The criteria used for assessing morphology were as follows: morphologically normal oocytes, with spherical and symmetrical shape with no sign of lysis, membrane damage, swelling, vacuolization, degeneration or leakage of the cellular content; morphologically abnormal oocytes with a ruptured zona pellucida or ruptured vitelline membrane or having fragmented cytoplasm with signs of degeneration.

Evaluation of viability with trypan blue

Viability was determined after thawing and two hours after incubation. Oocytes were stained with 0.4% trypan blue for 1 min and viability was determined by examination under inverted phase contrast microscope. Uptake of dye by COCs indicated non-viable (blue color) and exclusion of dye by COCs was considered as viable.

Evaluation of nuclear maturation of vitrified-warmed oocytes

At the end of the culture period, chromosome slides were prepared according to the procedure described by Tarkowski (1966). Briefly, cumulus cells were removed mechanically by vortexing. Each oocyte was transferred to a 1% hypotonic sodium citrate solution for 10 min and then placed on a microscope slide with a minimal amount of hypotonic solution. Three drops of fixative (methanol: acetic acid, 3: 1) were dropped onto the oocytes. Subsequently, the fixed oocytes were stained with 1% orcein. The state of nuclear maturation was determined as described earlier by Mahmoud (2004). Oocytes which reached telophase I or metaphase II stages were considered matured. Post thaw maturation rate (matured oocyte ⁄ survived oocyte) was calculated.

Experimental design

Experiment 1: The effect of cryodevice on viability and maturation of vitrified matured buffalo oocytes. The *in vitro* matured oocytes were vitrified in a mixture of 3 M DMSO and 3 M EG and oocytes were divided into two groups: the first was vitrified using conventional French straws, while the other was vitrified using Cryotops.

Experiment 2: The effect of two concentrations of cryoprotectants on the vitrification of *in vitro* matured buffalo oocytes. Mixtures of DMSO and EG as CPA solutions were prepared in TCM-199. The first concentration was 6 M (V2: 3 M E.G + 3 M DMSO) and the second concentration was 7 M (V2: 3.5 M E.G + 3.5 M DMSO). Each concentration of cryoprotectants was added in two steps, with the first step concentration being half of the second (and final) concentration.

Statistical analysis

Data were subjected to ANOVA using SPSS for Windows version 13.0, statistical software. Comparison of means was carried out by Duncan's Multiple Range Test.

Results

Experiment 1: Effect of using cryodevices on the vitrification of buffalo oocytes

Table 1 illustrates the morphological evaluations immediately after warming of vitrified buffalo oocytes using two cryodevices, straws and Cryotops. The vitrification solution was a mixture of 3 M DMSO + 3 M EG. There were no significant differences between the two cryodevice groups in the percentages of morphologically normal and abnormal oocytes.

Table 1. Morphological evaluation immediately after thawing of vitrified buffalo oocytes using straws and Cryotop cryodevices (Mean S ± E).

Cryodevice	Total number of oocytes	Number of normal oocytes (percentage in parenthesis)	Number of abnormal oocytes (percentage in parenthesis)
Straw	110	97 (87.7 ± 1.77^a)	13 (12.3 ± 1.77^a)
Cryotop	95	86 (90.9 ± 2.37^a)	9 (9.1 ± 2.73^a)
Control (unvitrified)	120	118 (98.5 ± 0.70^b)	2 (1.5 ± 0.70^b)

[a,b]values within a column with different lower case letters differ ($P < 0.01$).

Post-thaw survivability 2 h after thawing of control and vitrified oocytes after trypan blue staining and microscopic examination is shown in Table 2. The survival rate after vitrification was similar for both straws and Cryotops. The percentages of viable oocytes were significantly lower in both straws ($P < 0.05$) and Cryotops ($P < 0.001$) than in the control. Also, the percentages of dead oocytes were significantly higher ($P < 0.05$) in straws and Cryotops ($P < 0.001$) than in the control. Table 3 shows maturation rates of vitrified thawed buffalo oocytes using two cryodevices, straws and Cryotops. The maturation

rates were higher (P < 0.05) in Cryotops (70.3 ± 2.47) than straws (62.4 ± 2.53). The percentages of matured oocytes were significantly lower in both straws (P < 0.001) and Cryotops (P < 0.05) than in the control.

Table 2. Survivability evaluation 2 h after thawing and staining with trypan blue using straws and Cryotops (Mean S ± E).

Cryodevice	Total number of oocytes	Number of viable oocytes (percentage in parenthesis)	Number of dead oocytes (percentage in parenthesis)
Straw	101	73 (72.8 ± 2.24[a])	28 (27.0 ± 2.00[a])
Cryotop	84	67 (80.2 ± 3.09[ab])	17 (19.8 ± 3.09[a])
Control (unvitrified)	102	91 (89.0 ± 2.28[b])	11 (11.0 ± 2.28[b])

[a,b]values within a column with different lower case letters differ (P < 0.05 to P < 0.001).

Table 3. Maturation rates of vitrified thawed buffalo oocytes using straw and cryotop cryodevices (Mean S ± E).

Cryodevice	Total number oocytes	Number of matured oocytes (percentage in parenthesis)
Straw	90	56 (62.4 ± 2.53)[a]
Cryotop	71	51 (70.3 ± 2.47)[b]
Control (unvitrified)	83	66 (79.8 ± 1.79)[c]

[a] *vs*. [b] and [b] *vs*. [c] (P < 0.05), [a] *vs*. [c] (P < 0.001).

Experiment 2: Effect of two concentrations of cryoprotectants on vitrified buffalo oocytes

The *in vitro*-matured buffalo oocytes were vitrified in a mixture of two cryoprotectants DMSO and EG at either 3 M DMSO + 3 M EG or 3.5 M DMSO + 3.5 M EG. Table 4 illustrates the morphological evaluation immediately after warming of vitrified buffalo oocytes. There were no differences (P > 0.05) in oocyte morphology between the two concentrations. The percentages of morphologically normal oocytes were significantly lower in both 6 M (P < 0.01) and 7 M (P < 0.05) groups compared to the control. Post-thaw survivability of oocytes two hours after thawing of control and vitrified groups following trypan blue staining and microscopic examination is shown in Table 5. The survival rate after vitrification was similar for both concentration (6 M and 7 M) groups. The percentages of viable oocytes were significantly higher (P < 0.001) in control than in the two vitrified groups. The percentages of dead oocytes were also higher (P < 0.01) in the two vitrified groups than in the control. Table 6 shows the maturation rates of vitrified, thawed buffalo oocytes with two concentrations of cryoprotectants (6 M and 7 M). The overall maturation rate averaged 61.8 ± 0.99% for oocytes vitrified with 6 M and 69.6 ± 2.11% for oocytes vitrified in 7 M cryoprotectant. The maturation rates were higher (P < 0.01) in the 7 M group than in the 6 M cryoprotectant.

Table 4. Morphological evaluation immediately after thawing of vitrified buffalo oocytes with two concentrations of cryoprotectants (Mean S ± E).

Cryoprotectant concentration	Total number of oocytes	Number of normal oocytes (percentage in parenthesis)	Number of abnormal oocytes (percentage in parenthesis)
6M (3 DMSO + 3 EG)	113	96 (85.4 ± 3.21[a])	17 (4.6 ± 3.21[a])
7M (3.5 DMSO+3.5 EG)	111	98 (88.5 ± 2.34[a])	13 (11.1 ± 2.34[a])
Control (unvitrified)	116	114 (98.9 ± 2.34[b])	2 (1.8 ± 0.95[b])

[a,b]values within a column with different lower case letters differ (P < 0.05 to P < 0.01).

Table 5. Survivability evaluation 2 h after thawing and staining with trypan blue using two concentrations of cryoprotectants (Mean S ± E).

Cryoprotectant concentrations	Total number of oocytes	Number of viable oocytes (percentage in parenthesis)	Number of dead oocytes (percentage in parenthesis)
6M (3 DMSO + 3 EG)	88	65 (73.6 ± 2.21[a])	23 (26.4 ± 2.21[a])
7M (3.5 DMSO + 3.5 EG)	89	68 (75.4 ± 2.06[a])	21 (24.6 ± 2.06[a])
Control (unvitrified)	101	89 (87.5± 1.57[b])	12 (12.5 ± 1.57[b])

[a,b]values within a column with different lower case letters differ (P < 0.001 to P < 0.01).

Table 6. Maturation rates of vitrified thawed buffalo oocytes using two concentrations of cryoprotectants (Mean S ± E).

Cryoprotectant concentrations	Total number of oocytes	Number of matured oocytes (percentage in parenthesis)
6M (3 DMSO+3 EG)	77	48 (61.8 ± 0.99)[a]
7M (3.5 DMSO+3.5 EG)	80	55 (69.6 ± 2.11)[b]
Control (unvitrified)	84	66 (78.6 ± 0.69)[c]

[a,b,c]values within a column with different lower case letters differ (P < 0.01-P < 0.001).

Discussion

In the present study the rate of morphologically intact oocytes following vitrification/warming was high, ranging from 87.7% in straws to 90.8% in Cryotops. This high rate of cryosurvival can be attributed to the extreme reduction of the volume of vitrified solution that is achievable with the Cryotop (less than 0.1 µl), resulting in a dramatic increase of cooling/warming rates (Kuwayama *et al*., 2005; Kuwayama, 2007). It was also demonstrated that by using small volumes and open systems of vitrification, the incidence of cracking, leading to zona pellucida damage and lysis of the plasma membrane can be reduced (Vajta, 1997). In our study the survival rate of *in vitro* matured, vitrified, warmed buffalo oocytes ranged from 72.7 - 80.2% for straws and Cryotops respectively, which is higher than that obtained by Muenthaisong *et al*. (2007), but still lower than those reported for buffalo (Attanasio *et al*., 2010) and those recorded in cattle (Kuwayama *et al*., 2005) oocytes. The lower survival rate reported by these authors may be the consequence of several factors including the one-step dilution procedure employed after warming, as well as the ultra-rapid temperature changes during vitrification and warming, which may have caused extensive damage to membranes and structures in the ooplasm. In the present study, Cryotops and 0.25-ml straws as cryodevices for packaging buffalo COCs for vitrification were compared. From the data, we deduced a higher nuclear maturation in COCs vitrified on Cryotop than in 0.25-ml straws. This can be attributed to extremely fast cooling and warming rates achieved using the cryotop (Kuwayama *et al*., 2007). Cryotop allows COCs to be loaded with minimal VS2 by aspirating the maximum VS2 surrounding COCs. This helped in achieving an ultra-rapid cooling rate, thereby avoiding chilling injury. Additionally, the extremely small volume allowed oocytes to undergo a rapid warming rate which helped to avoid devitrification. In 0.25-ml straws, COCs were loaded with a larger volume of VS2 than Cryotops, which reduced the cooling rate. Our results are consistent with those previously reported for vitrification of bovine (Morató *et al*., 2008) and porcine oocytes (Liu *et al*., 2008). In our study the maturation rate of vitrified warmed buffalo oocytes ranged from 62.3 - 70.3% for straws and Cryotops respectively, which is higher than the 47.5% maturation rate described previously by Mahmoud *et al*. (2010) for vitrification of buffalo oocytes by straws and OPS. This result is in accordance with Prentice (2010) who stated that the nuclear maturation rate was higher in oocytes vitrified on Cryotops than those vitrified in straws, but still lower than that of Morató *et al*. (2008), who reported a 76.9% maturation rate. In conventional semen and embryo cryopreservation methods, the French straw is a popular cryodevice (packaging system) as it is inexpensive, and cells and tissues remain sterile. However, vitrification of bovine oocytes in 0.25-ml straws causes a delay in heat loss from the solutions, possibly leading to devitrification, i.e. intracellular recrystallization during warming (Morató *et al*., 2008). Considerable progress has been made in increasing cooling and warming rates by developing alternative packaging systems (Succu *et al*., 2007). Cryodevices such as OPS (Vajta *et al*., 1998) and Cryotops (Kuwayama, 2007) have drastically improved the cooling rate by reducing the surface to volume ratio and thus exposing the minuscule vitrification drop directly to LN2 (Liebermann *et al*., 2002).

Regarding the effect of cryoprotectant concentration on the vitrification of mature buffalo oocytes, in this study no significant differences were recorded between 6 M and 7 M concentrations in the percentage of morphologically normal oocytes and survivability after thawing using trypan blue. The survival rate of this study is lower than that of Dhali *et al*. (2000), who reported 88% survival rate for buffalo oocytes, but higher than the 36% survival rate for mouse oocytes vitrified in EG, ficoll-70 and sucrose (Miyake *et al*., 1993). In our work, EG and DMSO were used for the cryopreservation of oocytes with the straw method. Due to their low molecular weight, these compounds can easily permeate through cell membranes rapidly (25-30 sec) to achieve concentration equilibrium across the cell membrane. Moreover, EG, because of its high permeability and low cytotoxicity, has been found to be convenient for vitrification of bovine and equine oocytes (Hurtt *et al*., 2000; Cetin and Bastan, 2006). The present study indicates that the nuclear maturation rate was higher for oocytes vitrified in 7 M of EG + DMSO than 6 M, but the two groups were lower than the control unvitrified group. This reduction could also result from possible multifactorial causes, including toxic effects of cryoprotectants, ultrastructural damage to the oocytes and deleterious effects on chromosomes and other cytoplasmic structures (Johnson and Pickering, 1987; Aman and Parks, 1994; Almeida and Bolton, 1995; Liu *et al*., 2003). Moreover, the cryoprotectants, despite their protective effect during

cryopreservation, may impose concentration, time, and temperature-dependent toxicity (Fahy *et al*., 1990). The greater lipid content present in the buffalo oocytes may also be one of the factors responsible for decreasing maturation rates, since it has been reported that high lipid content in oocytes makes them more sensitive to chilling injury (Ledda *et al*., 2001). Our maturation rate with EG + DMSO using either 6 M or 7 M was higher than that of Mahmoud *et al*. (2010) in buffaloes, which was 41.5 and 40.3%, maturation rates were obtained in buffalo oocytes vitrified by 7 M of DMSO (Wani *et al*., 2004b) and 31.5% for 3-min equilibration for buffalo oocytes vitrified in 4.5 M EG and 3.4 M DMSO (Dhali *et al*., 2000), but this is lower than the 73.1% maturation rate obtained by Hou *et al*. (2005) for bovine oocytes vitrified in 20% EG, 20% DMSO, ficoll, sucrose and FBS. A common practice to reduce the toxicity of the cryoprotectant, but not its effectiveness, is to place the cells first in a lower concentration cryoprotectants solution to partially load the cells before transferring them into a full strength cryoprotectant. In this study we tried to reduce the higher concentration toxicity of cryoprotectant by combining cyoprotectants and two step exposures to oocytes. The combination of EG and DMSO was reported to be the mixture of choice (Vajta *et al*., 1999). In contrast Yadav *et al*. (2008) had found no benefit of combining DMSO with EG. Also, Albarracýn *et al*. (2005) reported that using either 20% EG or 20% DMSO gave similar results.

In conclusion, the maturation rates were higher in oocytes vitrified by Cryotops than straws, thus, Cryotop is one of the most efficient methods for vitrification of *in vitro*-matured buffalo oocytes. Cryoprotectant mixtures of EG and DMSO at 7 M concentration result in a higher maturation rate ($P < 0.05$) than the 6 M concentration for the vitrification of matured buffalo oocytes.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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