Improvement of vitrification of *in vitro* produced buffalo embryos with special reference to sex ratio following vitrification

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Summary

Cryopreservation and sexing of embryos are integrated into commercial embryo transfer technologies. To improve the effectiveness of vitrification of *in vitro* produced buffalo embryos, two experiments were conducted. The first evaluated the effect of exposure time (2 and 3 min) and developmental stage (morula and blastocysts) on the viability and development of vitrified buffalo embryos. Morphologically normal embryos and survival rates (re-expansion) significantly increased when vitrified morulae were exposed for 2 min compared to 3 min ($P<0.001$). On the other hand, morphologically normal and survival rates of blastocysts significantly increased when exposed for 3 min compared to 2 min ($P<0.001$). However, there were no significant differences between the two developmental stages (morulae and blastocystes) in the percentages of morphologically normal embryos and re-expansion rates after a 24 h culture. The second experiment aimed to evaluate the effect of viability on the sex ratio of buffalo embryos after vitrification and whether male and female embryos survived vitrification differently. A total number of 61 blastocysts were vitrified for 3 min with the same cryoprotectant as experiment 1. Higher percentages of males were recorded for live as compared to dead embryos; however, this difference was not significant. In conclusion, the post-thaw survival and development of *in vitro* produced morulae and blastocysts were found to be affected by exposure time rather than developmental stage. Survivability had no significant effect on the sex ratio of vitrified blastocysts; nevertheless, the number of surviving males was higher than dead male embryos.

Key words: Buffalo embryo, Cryoprotectant, Exposure time, Sexing, Vitrification

Introduction

Approaches such as slow freezing and vitrification have been employed to cryopreserve the embryos of humans and animals (Saragusty and Arav, 2011; Arav, 2014). Vitrification protocols are particularly attractive cryopreservation strategies for embryos as they are inexpensive, fast, and simple, but most of them are embryo-toxic as the solutions contain high cryoprotectant concentrations (Kuleshova and Lopata, 2002). Considerable effort is therefore still being directed toward improving vitrification protocols for embryos (Mazur *et al*., 2008). *In vitro* and *in vivo* survival rates of vitrified embryos are reasonable in buffaloes (Hufana-Duran *et al*., 2004; Manjunatha *et al*., 2008; Manjunatha *et al*., 2009). Nevertheless, intrinsic biological problems such as high chilling sensitivity and high embryo lipid content have impeded progress in this species (Gasparrini, 2002). Fundamental research is thus needed to improve the results, mainly with *in vitro* produced embryos.

Exposure time is a very important parameter when selecting cryoprotectants. The main strategy used to avoid cryoprotectant toxicity is to shorten exposure time. Optimal exposure time for successful vitrification must prevent toxic injury and intracellular ice formation (Kasai, 1996). Despite many advances in the field of embryo cryopreservation, there is still no consensus on the optimal developmental stage for embryo cryopreservation, especially in buffalo. Little research has been conducted on the effect of development stage on buffalo and bovine embryo post vitrification survival (De Rosa *et al*., 2007; Manjunatha *et al*., 2009).

Faster-developing blastocysts in *in-vitro* culture systems are generally considered more viable, and more capable of surviving cryopreservation or embryo transfer than those that develop more slowly (Dinnyés *et al*., 1999). However, there is evidence that female embryos might take longer than male embryos to reach the developmental transition stage, form a blastocoel and develop from an early to an expanded blastocyst (Gutierrez-Adan *et al*., 2001). Several other studies indicated that *in vitro*-produced bovine blastocysts were predominantly male (Xu *et al*., 1992; Lonergan *et al*., 1999); nonetheless, their findings were contrary to Larson *et al*. (2001) who claimed that *in vitro*-produced male bovine embryos did not have inherently faster growth rates than females. Darwish *et al*. (2009) reported the sex ratio of *in vitro* produced buffalo embryos to be nearly 1:1. Their study included all embryos produced *in*

vitro from fertilization up to the 7th day regardless of their developmental stages.

With respect to sexing vitrified embryos, it was reported in bovine that male embryos developed faster than females (Tominaga, 2004) and that blastocysts that survived vitrification and hatched 48 h after warming were male (Nedambale *et al*., 2004). A small number of experiments have been conducted on vitrified sexed embryos in bovine (Akiyama *et al*., 2010); nevertheless, to the authors' knowledge no studies have compared the survivability of male and female buffalo embryos following cryopreservation.

The present study was carried out to examine the effect of exposure time and developmental stage on the viability and development of vitrified buffalo embryos and to determine whether male and female embryos survive vitrification differently.

Materials and Methods

Chemicals

Chemicals for *in vitro* maturation including fetal calf serum (10106-151) and tissue culture medium (TCM 199, 31100-027) were obtained from Gibco BRL (Grand Island, New York, USA). Cysteamine, M-6500, heparin, H-5515, caffeine, C-4144, ethylene glycol (EG, E9129) and dimethyl sulfoxide (DMSO, D 2650) were obtained from Sigma Chemical Company.

Oocyte recovery and selection

Buffalo ovaries were collected from an 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. The ovaries were then washed three times in phosphate-buffered saline (PBS). Oocytes were aspirated from 2 to 5 mm follicles using an 18-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 of oocytes

Oocyte maturation was carried out as described by Mahmoud (2001) with some modifications. Briefly, the recovered oocytes were cultured in groups of 10 to 20 in 50 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 paraffin oil (M-4810 Sigma) and pre-incubated for a minimum of 2 h in a humidified 5% CO_2 atmosphere at 38.5°C. The oocytes were then placed into the droplets and incubated for 24 h in a humidified 5% CO_2 atmosphere at 38.5°C.

In vitro fertilization and culture

The procedure was performed as described by Niwa and Ohgoda (1988). Straws of frozen buffalo semen were thawed in a water bath at 35-37°C for 30s. The spermatozoa were washed twice by centrifugation (800 g for 10 min) in BO medium (Brackett and Oliphant, 1975) without BSA containing 10 µg /ml heparin and 2.5 mM caffeine. The sperm pellets were diluted with BO medium containing 20 mg/ml BSA to adjust the concentration of spermatozoa to 12.5×10^6 sperm/ml. After removing cumulus cells, matured oocytes were washed three times in BO medium containing 10 mg/ml BSA and introduced into 50 µL sperm suspension droplets (about 5-10 oocytes/droplet) under paraffin oil (M-4810 Sigma). The spermatozoa and oocytes were co-cultured for 5 h under the same culture conditions, 5% CO_2, 38.5°C and 95% humidity. Groups of 10-20 oocytes were again cultured for 6 and 7 days with previously prepared co-culture 100 µL droplets consisting of TCM-199 + 10% calf serum at 5% CO_2, 38.5°C and 95% humidity.

Experimental design

Experiment 1

The purpose of the first experiment was to find the effect of exposure time (2 and 3 min) of the first vitrification solution and the stage of development (morula and blastocysts) on the viability and development of vitrified buffalo embryos. To this end, Morula and blastocysts were vitrified for 2 and 3 min, respectively.

Experiment 2

The second experiment aimed to measure the effect of survivability after vitrification on the sex ratio of buffalo embryos and whether male and female embryos survived vitrification differently. Blastocysts were vitrified for 3 min and sexed after being vitrified/ warmed. The experiment was carried out on 61 vitrified embryos (29 live and 32 dead).

Embryo vitrification and warming

All vitrification solutions (VS) were prepared in TCM 199 containing 2.5 mM HEPES + 20% fetal calf serum. Embryos (morula and early blastocysts) were vitrified in 0.25 ml straws following a two step addition of cryoprotectant. Two embryos were placed in VS1: 1.75 M EG + 1.75 M DMSO (step one) for 2 or 3 min. For the second step, they were placed in 30 µL of 7 M of VS2: 3.5 M EG + 3.5 M DMSO for 45 s. Immediately after this, 0.25 ml straws were placed in liquid nitrogen vapor in a goblet for 1 min and then plunged into liquid nitrogen for a week. Straws were then held in air for 10 s to warm, placed in water at 37°C for 30 s, and flicked four to six times to mix columns. After warming, embryos were washed in 0.5 M galactose for 4 min at 20-22°C. Finally, the embryos were washed in TCM plus 5% FCS five times and cultured at 38.5°C, 5% CO_2 with the previously mentioned culture media for another 24 h period. Fresh or non-vitrified embryos were used as

controls.

Survival assay

Embryos were evaluated morphologically after thawing and the viability of morulae and blastocysts were assessed by *in vitro* culture for 24 h. Embryos with a clearly visible inner cell mass that had developed to more advanced stages together with morulae that developed into blastocysts and re-expanded blastocysts were defined as surviving.

Extraction of DNA from embryos

A total of 61 *in vitro* produced-vitrified buffalo embryos at blastocyst stage were used. Embryos were taken individually in a minimum amount of culture medium, washed twice by TE buffer (10 mM Tris, 1 mM EDTA) and kept at -20°C until used for DNA extraction according to Darwish *et al*. (2013). Briefly, 20 µL of Chelex-100 (10%) were added to each embryo which was then boiled in a water bath for 10 min. A final centrifugation step was followed to pellet the cell debris and the supernatant was transferred to a clean tube, stored at -20°C to be used as a source for template DNA.

Primers

The primers used in the PCR reaction are shown in Table 1. Two pairs of primers were used; a male-specific primer (BuRYNI 1 and 2) which targeted male-specific sequences in the buffalo DNA and amplified the 164 bp DNA fragment in males, and a gender-neutral primer which targeted bovine satellite sequences common to both males and females (Sat I and II) and amplified the 216 bp DNA fragment in both male and female buffaloes.

Multiplex PCR assay

Multiplex PCR assay was carried out according to Darwish *et al*. (2009) on the DNA extracted from embryos. PCR was performed in 20 µL reaction volumes containing 5 µL template DNA, 20 pmol of each primer and 1 X PCR master mix (Dream Taq Green PCR Master Mix, Fermentas Life Science). Amplification cycles were carried out in a Nexus gradient Master cycler (Eppendorf, Germany). Reaction conditions were 95°C for 3 min as initial denaturation, followed by 35 cycles of 94°C for 45 sec, 58°C for 1 min and 72°C for 1 min. A final extension step followed at 72°C for 10 min. Positive male and female DNA isolated from adult buffalo whole blood and negative control (no template) were included in each PCR to ensure no cross contamination or amplification failure due to the presence of inhibitors. PCR products were analyzed by electrophoresis in 2% agarose gel in a 0.5 X TBE buffer containing ethidium bromide and visualized under a UV transilluminator. To assure that amplification products were of the expected size, a 100 bp DNA ladder was run simultaneously as a marker.

Statistical analysis

Data were subjected to an ANOVA using SPSS for windows version 13.0. Means comparisons were carried out by LSD. Differences were considered to be significant at $P<0.05$. Chi-square tests (χ^2) were also run to analyze the differences between the percentages of male and female live and dead embryos after vitrification, between the overall male to female sex ratio and the expected 1:1 sex ratio.

Results

Experiment 1

Morphologically normal embryos and their survival rates (re-expansion) significantly increased when vitrified morulae were exposed to 2 min compared to 3 min ($P<0.001$). As Table 2 shows, these rates increased significantly with blastocysts when exposed to 3 min compared to 2 min ($P<0.001$). As the table also indicates, vitrification significantly reduced survival rates of both morula and blastocyst at 2 and 3 min groups compared to non-vitrified control groups ($P<0.001$).

There were no significant differences between the two developmental stages (morulae and blastocysts) in

Table 1: Oligonucleotide primers used in the PCR

Repeat sequence	Primer name	Primer sequence (5´-3´)	PCR product size (bp)	Reference
Satellite	Sat I	TGGAAGCAAAGAACCCCGCT	216 bp	Pucienniczak and Skowronski (1982)
	Sat II	TCGTGAGAAACCGCACACTG		
BuRYNI	BuRYN 1	CGTGGTGGGTGACCCCACAGCCCC	164 bp	Appa Rao and Totey (1999)
	BuRYN 2	ACAGGTGCTTATGCTGCAGTGCTG		

Table 2: Re-expansion of buffalo morulae and blastocysts after vitrification and warming at different exposure times (Mean±SE)

Embryo stage	Exposure time (min)	No. of vitrified-warmed embryos	Morphologically normal embryos directly after warming		Post-warming development (expanded morula and blastocysts) after 24 h (viability)	
			No.	(%)	No.	(%)
Morulae	2	104	64	$(60.8 \pm 1.3)^a$	51	$(48.9 \pm 1.1)^a$
	3	98	49	$(49.2 \pm 1.4)^b$	42	$(43.7 \pm 1.9)^b$
	Control (non vitrified)	77	77	$(100 \pm 0.0)^c$	60	$(77.7 \pm 1.7)^c$
Blastocysts (early blastocysts)	2	101	53	$(52.4 \pm 1.5)^b$	47	$(46.4 \pm 0.9)^b$
	3	76	48	$(63.3 \pm 1.0)^a$	39	$(51.3 \pm 1.3)^a$
	Control (non vitrified)	68	68	$(100 \pm 0.0)^c$	54	$(79.9 \pm 1.3)^c$

Values with different superscripts within the same column differ significantly ($P<0.001$). No.: Number

Table 3: Effect of vitrification on sex ratio in buffalo embryos at blastocysts stage

Viability of vitrified blastocysts	Total No. of sexed embryos	Male		Female	
		No.	%	No.	%
Live	29	25	86.2	4	13.8
Dead	32	24	75	8	25
Total	61	49	80.3	12	19.7

No.: Number

the percentages of morphologically normal and re-expansion rates after a 24 h culture, vitrified at day 6 and day 7, respectively (Table 2).

Experiment 2

Multiplex PCR carried out using both male specific and gender-neutral primers revealed that 25 (86.2%) out of the 29 live embryos amplified both 216 and 164 bp DNA fragments and could be assigned, therefore, as male embryos. However, 4 (13.8%) embryos amplified only the gender-neutral fragment (216 bp) and were hence assigned as female. From the 32 dead embryos cultured for 24 h after vitrification and warming, 24 (75%) were male and 8 (25%) were female (Table 3 and Fig. 1). Compared to the expected normal sex ratio *in vivo* (1:1), the male sex ratio increased significantly in both dead and live blastocysts after vitrification ($P<0.01$). Although higher percentages of males were recorded for live compared to dead embryos (Table 3), the difference was not significant for the male sex ratio.

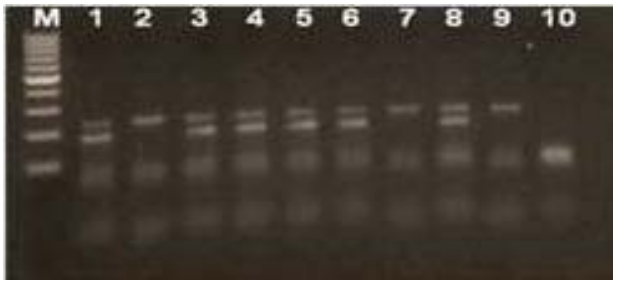

Fig. 1: Ethidium bromide stained agarose gel of multiplex PCR products amplified from vitrified IVF-produced buffalo embryos using both Sat I and II primers, amplifying a gender-neutral sequence (216 bp), and BuRYNI 1 and 2 primers, amplifying a male-specific sequence (164 bp). M: 100 bp ladder DNA size marker. Lane 1: Male positive control, Lane 2: Female positive control, Lanes 3-6 and 8: Male embryos Lanes 7 and 9: Female embryos, and Lane 10: A negative control

Discussion

Vitrification requires high levels of cryoprotectants to achieve glass transition. A successful vitrification procedure requires the optimization of cryoprotectant concentration and exposure time. We tested the buffalo morulae and blastocysts after vitrification and warming by further culturing for 24 h to evaluate their survival and development. Embryo culture can be a useful method to assess viability and to confirm the quality of thawed embryos previously stored in liquid nitrogen (Contreras *et al*., 2008).

A common practice to reduce cryoprotectants toxicity, but not its effectiveness, is to place the cells in a solution of lower strength cryoprotectants first in order to partially load the cells before transferring them to the full-strength cryoprotectants. In our experiments, we tried to reduce the toxicity of higher cryoprotectant concentrations by combining different cryoprotectants and applying a two step exposure to embryos. Many researchers believe that the mixture of cryoprotectants has advantages over solutions containing only one solute (Dhali *et al*., 2000; Manjunatha *et al*., 2009; Mahmoud *et al*., 2010). The combination of DMSO and EG was reported to be the mixture of choice (Mahmoud *et al*., 2010).

In the present study, the morphologically normal and survival (re-expansion) rates following vitrification/ warming were higher in vitrified morulae exposed to the first vitrification solution for 2 min compared to 3 min, while they were lower in vitrified blastocysts exposed to 2 min in the first vitrification solution compared to 3 min. Our results are consistent with those previously reported for vitrification of buffalo embryos by Manjunatha *et al*. (2009). They stated that, the vitrification of morulae and blastocysts in 25% EG + 25% DMSO with an exposure time of 2 and 4 min, respectively, resulted in a better hatching rate. In this respect, Campos-Chillon *et al*. (2006) stated that longer exposure times were favorable for blastocysts and detrimental to morulae, which may correspond with the larger amount of water content in the blastocoele cavity.

In our study, no significant differences were found between the two developmental stages (morulae and blastocystes) in the percentages of morphologically normal and re-expansion rates after the 24 h culture vitrified at day 6 and day 7, respectively. In a study on human embryos comparing the outcome of IVF-ET for embryos frozen at the pronuclear (day 1), cleavage (day 3), or blastocyst stage, Moragianni *et al*. (2010) found no difference between the three in their implantation and clinical pregnancy, multiple and twin pregnancy rates, as well as the male to female ratio. The only difference found was in the post-thaw survival rate where day 3 embryos had lower survival rates than day 1 embryos or blastocysts. In contrast, Manjunatha *et al*. (2009) and Hochi *et al*. (1996) reported that *in vitro*-produced morulae survival rates were lower compared to blastocysts for buffalos and bovines. Moreover, in a study conducted by Campos-Chillon *et al*. (2006) *in vitro*-produced bovine morulae showed poor survival rates.

With respect to the effect of vitrification at the blastocyst stage on the sex ratio of both live and dead

embryos, our findings indicate that although a higher number of live males was observed compared to dead embryos, the difference was not significant. Moreover, the total male sex ratio increased significantly ($P<0.01$) when compared to expected normal sex ratio (1:1) regardless of whether they were dead or alive. This suggests that a greater number of male fetuses result from embryo transfer following *in vitro* fertilization. This observation supports the report of Hasler *et al*. (2000) who maintained that more male calves were born following the transfer of *in vitro*-produced embryos.

The higher survival rate of male blastocysts 24 h post-warming was probably due to the slower development of female blastocysts. These blastocytes are reported by Xu *et al*. (1992) and Gutierrez-Adan *et al*. (2001) to be more often graded as fair or poor under *in vitro* conditions. The enhanced survival of male blastocysts might be related to the fact that male blastocysts have a greater number of cells compared to female blastocysts (Catt *et al*., 1997). By contrast, Beyhan *et al*. (1999) stated that male and female embryos had the same total cell numbers at day 8.

One possible explanation for the enhanced cryosurvival of male blastocysts reported by Nedambale *et al*. (2004) is that male embryos developed faster. Hence, it is reasonable to assume that they also form more cells. In addition, female embryos under *in vitro* culture conditions are known to be more affected by suboptimum conditions of nutrition or the environment than male embryos (Xu *et al*., 1992; Gutierrez-Adan *et al*., 2001). Another important factor affecting sex ratio after vitrification is the embryo quality (Nedambale *et al*., 2004).

In conclusion, both morulae and blastocystes were suitable stages for vitrification in buffalos. The 2 min and 3 min exposure times were found to be the best times for vitrification of morulae and blastocysts, respectively. Viability after vitrification had no significant effect on the sex ratio of vitrified blastocysts, although the number of live male embryos was larger in comparison to dead ones.

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Conflict of interest

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

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