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Impact of *in vitro* fertilization by fresh and frozen semen on developmental competence and cryotolerance of buffalo embryos

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Summary

Production of high quality embryos *in vitro* needs an efficient *in vitro* fertilization (IVF). Seminal origin is one of the important factors that affects the success of *in vitro* embryo production. So our goal was to determine the effect of using fresh and frozen semen in fertilization on developmental competence and cryo-survival of buffalo embryos. Buffalo oocytes were matured and fertilized *in vitro* by fresh and frozen semen. After embryos evaluation, good quality morula and blastocysts were vitrified using 0.25 ml straws and the post-warmed viability was assessed by further culture for 24 h. There was no significant difference in cleavage rate between embryos derived from fresh and frozen semen, whereas the rate of embryo development to the morula ($P<0.05$) and blastocysts ($P<0.01$) stages was significantly decreased in embryos derived from frozen compared to fresh semen. After warming the vitrified embryos, there was no significant difference between embryos derived from fresh and frozen semen in the percentages of morphologically viable embryos. However, 24 h after culture, the rate of morphologically normal and survived embryos was increased ($P<0.05$) in embryos derived from fresh compared to the frozen semen. In conclusion, in buffalo, the use of fresh semen could improve the rate of embryo development and their crytolerance compared to the frozen semen.

Key words: Buffalo, Embryo vitrification, Fresh and frozen semen, *In vitro* fertilization

Introduction

Semen evaluation by *in vitro* fertilization (IVF) (Mahmoud *et al*., 2004; Scholkamy *et al*., 2009) combined with sperm morphology assessment (Abdel Dayem *et al*., 2009) are useful techniques for predicting bulls' fertility. Sperm motility, morphology, and intact acrosomes percentage are the highest predictive index for IVF success (Zhang *et al.*, 1998).

In spite of the efforts to improve the *in vitro* embryo production in Egyptian buffalo, the rates of development are still low (Mahmoud and Nawito, 2005; El-Naby *et al*., 2016; El-Naby *et al*., 2017). Failure of fertilization and/or embryonic mortality of seminal origin or after artificial insemination (AI) (Gordon, 1996; Saacke *et al*., 2000) has been recognized as a potential source of breeding losses. Impaired sperm quality lowers the rate (Shoukir *et al*., 1998) and quality (Janny and Ménézo, 1994) of developed blastocysts, and eventually causes pregnancy failure (Sanchez *et al*., 1996).

The ability to successfully cryopreserve embryos with minimal loss in viability is an essential prerequisite for the success of assisted reproductive techniques. However, human embryo cryopreservation leads to 30-40% reduction in the implantation potential (Edgar *et al*., 2000). Few studies have been conducted to compare fertilizing ability of fresh and frozen semen (Pugh *et al*., 1991; Lehloenya *et al*., 2010), but no studies have been reported in buffalo to compare the cryotolerance of embryos produced from fertilization by fresh and frozen semen. So, the present work was carried out to evaluate the effect of semen type (fresh and frozen) on developmental ability and cryotolerance of buffalo embryos.

Materials and Methods

Semen collection and cryopreservation

Semen was collected by artificial vagina maintained at 42-45°C from four proven fertile buffalo bulls at the Teaching Farm of Faculty of Veterinary Medicine, Benha University, Moshtohor, Kaliobia governorate, Egypt. Semen was evaluated for volume, motility, viability, and concentration. Semen of good quality was diluted with milk extender to provide a concentration of 50×10^6 spermatozoa/ml. Extended semen was slowly cooled to 5°C (within 2 h), packed into 0.5 ml polyvinyl straws (Minitub, Germany) and equilibrated for 2 h. After equilibrium time, the straws were racked horizontally and frozen in the vapor, 4 cm above liquid nitrogen, for 10 min before being stored in liquid nitrogen. After 24 h of storage, the straws were thawed for 30 s in a water bath at 35-37°C and were evaluated post-thawing.

Fresh semen preparation

On the day of fertilization, fresh semen samples were collected by the artificial vagina. Semen was evaluated for the individual progressive motility, livability, normality, and concentration. Good semen samples were washed and diluted for fertilization.

Oocyte recovery and selection

Buffalo ovaries collected from abattoir within 2 h of slaughter were transported to the laboratory in normal saline (0.9% NaCl) supplemented with antibiotics (100 µg/ml streptomycin sulfate and 100 IU/ml penicillin) and retained at 30°C. Ovaries were cleaned 3 times with phosphate buffer saline (PBS). Oocytes were collected from 2-5 mm follicles in PBS containing 3% bovine serum albumin (BSA) fraction V, streptomycin sulfate and penicillin. Oocytes with intact layers of cumulus cells and homogeneous cytoplasm were selected under a stereo zoom microscope for maturation and fertilization *in vitro*.

In vitro maturation of oocytes

Oocyte maturation was performed according to Mahmoud *et al*. (2016) with slight adjustments. Briefly, 10-20 oocytes were cultured in 100 µL of TCM-199 supplemented with 10% fetal calf serum (FCS), 50 µM cysteamine and 50 µg/ml gentamycin sulfate. The droplets were covered with mineral oil and pre-incubated in a humidified 5% CO_2 atmosphere at 38.5°C for at least 2 h. The oocytes were placed into the droplets and incubated in the humidified 5% CO_2 atmosphere at 38.5°C for 24 h.

In vitro fertilization and culture

The procedures were carried out according to Niwa and Ohgoda (1988). Frozen buffalo semen straws were thawed in the water bath at 35-37°C for 30 s. Thawed spermatozoa or diluted fresh semen were washed twice by centrifugation (800 × g for 10 min) in BSA-free Brackett and Oliphant (BO) medium (Brackett and Oliphant, 1975) comprising 10 µg/ml heparin and 2.5 mM caffeine. The sperm pellets were diluted with BO medium enclosing 20 mg/ml BSA to amend the concentration of spermatozoa to 12.5×10^6 sperm/ml. After removing the cumulus cells, matured oocytes were washed three times with BO medium containing 10 mg/ml BSA and were introduced into 100 µL droplets of sperm suspension (5-10 oocytes/droplet) under paraffin oil. The spermatozoa and oocytes were cocultured for 5 h at 5% CO_2, 38.5°C, and 95% humidity. Groups of 10-20 oocytes were again cultured for 6-7 days in TCM-199 supplemented with 10% FCS, 50 µM cysteamine and 50 µg/ml gentamycin sulfate (n=6).

Embryo vitrification and warming

The vitrification solutions were prepared in 2.5 mM HEPES and 20% FCS supplemented TCM 199. Good quality embryos were vitrified in 0.25 ml straws following two-steps addition of cryoprotectants. Morula and blastocysts were placed in 1.75 M ethylene glycol + 1.75 M DMSO (step one) for 2-3 min. For the second step, they were placed in 7 M of 3.5 M ethylene glycol + 3.5 M DMSO for 45 s. Immediately, the straws were placed in liquid nitrogen vapor in the goblet for 1 min and then plunged into liquid nitrogen for one month. For warming, straws were held in the air for 10 s, placed in water at 37°C for 30 s, and flicked 4-6 times to mix columns. After warming, embryos were washed in 0.5 M galactose for 5 min at 20-22°C. Finally, the embryos were washed in TCM + 5% FCS and cultured at 38.5°C, 5% CO_2 for a further 24 h.

Survival assay

Embryos were evaluated morphologically after thawing. The viability of morulae and blastocysts were assessed by *in vitro* culture for 24 h. The embryos that developed to more advanced stages, with a clearly visible inner cell mass, were defined as viable (Mahmoud *et al*., 2015b) (n=3).

Statistical analysis

Data were statistically analyzed by Student's t-test using SPSS (version 16) for windows. Differences between fresh and frozen semen were considered to be significant at $P<0.05$.

Results

The motility, livability ($P<0.01$), and normality ($P<0.05$) considerably varied between fresh and frozen semen types (Table 1). The rate of spermatozoa abnormalities non-significantly differed between semen types.

Table 1: Characteristics of fresh and frozen Egyptian local breed semen (mean±SE)

Parameter	Semen type		P-value
	Fresh	Frozen	
Motility	73.00±3.74	48.00±2.71	<0.001
Livability	74.60±4.43	55.00±3.89	<0.01
Normality	82.50±2.53	69.20±4.53	<0.05
Head abnormality	0.50±0.29	3.20±1.16	0.07
Tail abnormality	17.00±2.65	27.60±5.53	0.14

Student's t-test

As shown in the Table 2, the cleavage rate declined from 62.5% in embryos fertilized by fresh semen to 56.8% in frozen semen without significant differences. The rate of embryo development was significantly lower in frozen than fresh semen for both morula ($P<0.05$) and blastocysts ($P<0.01$) stages.

With respect to the vitrified embryos, there were no significant differences between fresh and frozen semen in the percentages of morphologically viable embryos evaluated directly after warming (Table 3). However, after 24 h culture, morphologically normal and survival rates of vitrified/warmed embryos significantly ($P<0.05$) increased in embryos produced from fertilization by fresh than frozen semen.

Table 2: Developmental competence of buffalo oocytes fertilized by fresh and frozen semen (mean±SE)

Parameter		Semen type		P-value
		Fresh	Frozen	
Total inseminated oocytes	No.	144	137	
Cleavage	No.	91	79	NS
	(%)	62.5 ± 3.2	56.8 ± 2.0	
Morula	No.	60	48	<0.05
	(%)	41.6 ± 1.8	34.3 ± 2.0	
Blastocyst	No.	27	19	<0.01
	(%)	18.0 ± 0.5	13.4 ± 1.1	

Student's t-test, replicates number = 6, NS: Non-significant

Table 3: Cryotolerance of buffalo embryos produced from fertilization with fresh and frozen semen (mean±SE)

Parameter			Semen type		P-value
			Fresh	Frozen	
Total vitrified-warmed embryos		No.	43	37	
Morphologically normal viable embryos	At 0 h post-warming	No.	35	28	NS
		(%)	80.6 ± 3.3	74.9 ± 2.5	
	24 h post-warming	No.	27	20	<0.05
		(%)	62.5 ± 1.4	53.6 ± 2.1	

Student's t-test, replicates number = 3, NS: Non-significant

Discussion

In the current study, no significant differences were identified in the cleavage rate between fresh and frozen semen. Similar results were recorded in ovine (Pugh *et al*., 1991; Lehloenya *et al*., 2010). However, in this work, the rate of morula (P<0.05) and blastocyst (P<0.01) development significantly decreased in frozen than fresh semen. Similarly, Romao *et al*. (2013) documented that IVF by fresh semen improved ovine embryo developmental competence by increasing blastocyst quality. In this respect, Januskauskas and Žilinskas (2002) reported that the fertility after AI with bovine frozen semen is lower than that obtained with fresh semen. As referred, the sperm viability reduced by more than 50% during cryopreservation process (Watson, 1995). Cryopreservation induces oxidative stress in buffalo semen through decreasing the activity of antioxidant enzymes (El-Sisy *et al*., 2007). Spermatozoa motility and live sperm percentage drop by freezing up to 42.5% and 61.8%, respectively (Mahmoud *et al*., 2015a). Membrane integrity (Mahmoud *et al*., 2013), as well as nuclear DNA fragmentation (Mukhopadhyay *et al*., 2011), decrease significantly in fresh vs. frozen semen.

The viability evaluation after vitrification of *in vitro* produced embryos is one of the critical factors in embryo production (Mahmoud, 2010; Mahmoud and Seidel, 2010). *In vitro* survival rates of vitrified embryos are acceptable in buffaloes (Mahmoud *et al*., 2015b), probably due to high chilling sensitivity and lipid content in buffalo embryos that impeded the progress of cryopreservation (Gasparrini, 2002). In our study, frozen semen significantly reduced survival rates of morula and blastocyst than fresh semen after 24 h of culture. Romao *et al*. (2013) noticed higher expansion rates after 3 h of culture for warm edovine embryos derived from fresh than frozen semen, this could be attributed to the change in spermatozoa characteristics due to freezing. Abd El-Fatah *et al*. (2008) and Mahmoud *et al*. (2015a) found a significant increase in DNA damage detected by comet assay in buffalo frozen semen than fresh.

To our knowledge, there are no reports in buffalo comparing embryo cryotolerance after IVF using fresh and frozen semen. In spite of freezing and thawing that decrease the viability of buffalo semen (El-Sisy *et al*., 2007; Mahmoud *et al*., 2013), the frozen semen is widely used in IVF to prevent disease transmission which balances the advantage of fresh semen (Englert *et al*., 1989).

In conclusion, our data provide reliable information about the importance of semen type as fresh semen on *in vitro* embryo production and their cryotolerance. Better development and viability were detected in embryos derived from fresh semen.

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Conflict of interest

All authors have no conflict of interest to declare.

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