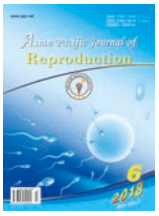

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Effect of buffalo bull breeds on developmental competence and vitrification of *in-vitro* produced embryos

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ABSTRACT

Objective: To assess effect of buffalo bull breed on the development and cryotolerence of the *in vitro* produced embryos. **Methods:** Three types of frozen semen were adopted; Egyptian, Italian and cross-bred (Egyptian-Italian) breeds were used for *in-vitro* fertilization and vitrification of their embryos. Oocytes were collected from buffalo ovaries and matured *in vitro* for 24 h, then they were fertilized using the three semen breeds. The produced embryos of morula and blastocysts were vitrified using ethylene glycol and dimethyl sulfoxide then evaluated for their viability after warming. **Results:** The cleavage and blastocysts rates significantly declined in oocytes fertilized by Egyptian ($P<0.01$) than in Italian ($P<0.05$) and crossbred ($P<0.05$) frozen semen. After embryo vitrification, there were no significant differences among the three breeds in the percentages of morphologically viable embryos evaluated directly after warming and at 24 h post-culture. **Conclusions:** The *in vitro* fertilization response to frozen-thawed semen varies between breeds; however, the resistance of produced embryos to the damage effect of vitrification does not vary.

1. Introduction

Many efforts have been conducted to genetically improve buffalo species by using *in vitro* fertilization and embryo transfer, taking the advantage of superior animals' production. In Egypt, trials for the introduction of foreign buffalo breed (Italian breed) were performed with the aim to improve the genetic makeup of the Egyptian buffaloes for economic traits[1,2]. This act has been encouraged through cross breeding with Italian semen by General Organization of Veterinary Services since 2012. The success of crossbreeding programs needs to be evaluated regularly by assessing the *in vivo* fertility[3]. However, studies about fertility evaluation of crossbred buffalo bull are limited[4] and no research has been conducted on *in-vitro* fertility determination.

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Male is an important factor influencing the reproductive efficacy of the herd. Seminal origin has been recognized as a source of lower fertilization in buffalo[5]. Therefore, the progress in improving reproductive efficiency can be achieved by estimating males' fertility[6]. *In vitro* fertilization is an important technique for the evaluation of male fertility *in vitro*[7].

The rate of embryo development in buffalo is still low[8], in spite of continuous working to improve embryo production in this species[9,10]. Moreover, the *in vitro* survival rates of vitrified embryos are reasonable in buffaloes[11-13] due to high chilling sensitivity and high lipid content[14]. In this study we worked to verify our hypothesis that the buffalo breed could influence the fertility indices of *in-vitro* fertilization, and the resistance of developed embryo to low temperature. Therefore, the present study is aimed to evaluate the fertility potential of Italian, Egyptian and their crossbred semen *in vitro*, and cryotolerence of *in-vitro* produced buffalo embryos.

2. Materials and methods

2.1. Semen source

Frozen Egyptian buffalo semen was prepared and kindly provided by Theriogenology Department, Faculty of Veterinary Medicine farm, Benha University. Cross-bred (Egyptian-Italian) semen was purchased from Abassia Frozen Semen Centre, General Organization for Veterinary Services. Italian frozen semen was purchased from Aton Company for agency and trade imported from Centro Tori Chiacchierini, Perugia, Italy.

2.2. Oocyte recovery and selection

Buffalo ovaries were taken to the laboratory in usual saline supplemented with 100 μg/mL streptomycin sulfate plus 100 IU/mL penicillin and retained at 32 ℃. Oocytes were collected from 2-5 mm follicles in phosphate buffer saline containing 3% bovine serum albumin (BSA) fraction Ⅴ, streptomycin sulfate and penicillin. Oocytes with healthy layers of cumulus cells and homogeneous cytoplasm were chosen under a stereo zoom microscope for maturation and fertilization *in vitro*.

2.3. In vitro oocytes maturation

Oocyte maturing was carried out according to Mahmoud *et al*[15] with slight adjustments. Briefly, 10-20 oocytes were cultured in 100 μL small drop of tissue culture medium (TCM)-199 added with 10% fetal calf serum, 50 μM cysteamine and 50 μg/mL gentamycin sulfate. The drops were covered with mineral oil and pre-created in a humidified 5% CO_2 atmosphere at 38.5 ℃ for at least of 2 h. The oocytes were placed into the small drops and grew in the humidified 5% CO_2 atmosphere at 38.5 ℃ for 24 h.

2.4. In vitro fertilization and culture

The procedures were carried out according to Darwish *et al*[16]. Frozen semen straws were melted in the water bath at 37 ℃ for 30 s, washed by centrifugation (800 × *g* for 10 min) in BSA-free Brackett and Oliphant (BO) medium[17] with 10 μg/mL heparin plus 2.5 mM caffeine. The sperm pellets were watered down with BO medium enclosing 20 mg/mL BSA to amend the concentration to 12.5×10^6 sperm/mL. Matured oocytes without cumulus cells were washed with BO medium containing 10 mg/mL BSA and were introduced into 100 μL drops of sperm suspension (5-10 oocytes/droplet). The spermatozoa and oocytes were incubated for 5 h at 5% CO_2, 38.5 ℃, and 95% humidity. Oocytes groups of 10-20 were again cultured for 6-7 d with previously prepared co-culture droplets of maturation media.

2.5. Embryo vitrification and warming

The vitrification solutions were prepared in TCM 199 and 20% fetal calf serum supplemented. Good quality embryos were vitrified in 0.25 mL straws following two-steps addition of cryoprotectants. For the first step, morula and blastocysts were placed in 1.75 M ethylene glycol + 1.75 M dimethyl sulfoxide for 2-3 min. For the second step, they were put in 3.5 M ethylene glycol + 3.5 M dimethyl sulfoxide for 45 s. Immediately, the straws were dropped into the goblet in liquid nitrogen vapor for 1 min then plunged into liquid nitrogen for one month. For warming, straws were held in the air for 10 s, placed in water at 37 ℃ for 30 s, and flicked 4-6 times to mix columns. After warming the straws , embryos were washed in 0.5 M galactose for 5 min at 20–22 ℃. Finally, the embryos were washed in TCM + 5% fetal calf serum and cultured at 38.5 ℃, 5% CO_2 for further 24 h.

2.6. Survival assay

After thawing, embryos were evaluated morphologically. The viability of morulae and blastocysts were assessed by *in vitro* culture for 24 h. The embryos developing to advanced stages, with visible inner cell mass, were defined as viable[12].

2.7. Statistical analysis

Data were statistically studied by ANOVA using SPSS version 18.0. Comparison between means was performed by the test of Duncan's Multiple Range. Differences among breeds were believed to be significant at $P<0.05$. Data were shown as mean±standard error (Mean±SE).

3. Results

As shown in the Table 1, the cleavage rate significantly declined in embryos produced and fertilized by Egyptian (P<0.01) than in Italian (P<0.05) and crossbred (P<0.05) frozen semen. No significant difference was found in percentage of morula among the three breeds. The rate of blastocysts was significantly lower in Egyptian (P<0.01) than in Italian (P<0.05) and crossbred (P<0.05) semen.

With respect to the vitrified embryos, there were no significant differences among the three breeds semen in the percentages of morphologically viable embryos evaluated directly after warming and after 24 h of culture (Table 2).

4. Discussion

Due to the critical role played by the Egyptian buffaloes in agricultural economy, there is a tendency toward genetic improvement of the animal productivity through application of assisted reproductive techniques, such as artificial insemination and embryo transfer. In the present study, the cleavage and blastocysts rates significantly deceased in oocytes fertilized by Egyptian semen than Italian and crossbred frozen semen. A high correlation has been found between *in vitro* fertilization results and field non-return rates[18]. The *in vitro* fertility rate, an imperative parameter for the evaluation of frozen–thawed semen quality[19], is affected by sperm quality[20] and breeds[21]. The differences in freezability and pregnancy rates between Egyptian and Egyptian-Italian buffalo semen have been studied[3,4]. The variations in pregnancy rates are attributed to various factors including the bull factors[22] due to the differences in metabolic activity of sperm cells[17]. In this context, Ward *et al*[23] showed that the bull has a significant impact on the quality of blastocyst produced *in vitro*. In this respect, Mahmoud *et al*[4] reported that in buffalo the percentage of acrosome integrity and pregnancy rate were significantly much higher in crossbred (Egyptian-Italian) than Egyptian bulls.

Vitrification as well as slow freezing is the most communal means for embryo cryopreservation, which aims to cool the cells to temperature below freezing temperature followed by storage for prolonged periods with minimal loss of viability[24]. In the present study, while all oocytes were collected from slaughtered local buffalo breed, the changes in the vitrified-thawed embryos of the different bull breeds, in terms of the percentages of morphologically viable embryos evaluated directly after warming and 24 h post culture, did not reach statistical significant level. This might be related to the fertilized oocyte cryotolerence, not to the bull effect. A substantial loss of total lipids, a crucial controller of spermatozoa freezability, has been noticed during the process of capacitation and acrosome reaction in buffalo bulls' semen[25]. There are no reports in buffalo comparing embryo cryotolerance after *in vitro* fertilization using Egyptian, Italian and their crossbred semen. Probable bull effect could appear in their daughters' oocytes as a latent effect of the genetic change. Former studies verified that the bovine oocytes are highly sensitive to low temperature and the exposure to cryoprotective agents. Studies in pigs showed that the embryo donor essentially impacts the *in vitro* development and the number of cells of blastocysts after vitrification and warming[26]. The lower lipid content of the embryo inhibits apoptosis and improves the embryo cryosurvival after vitrification[27].

In conclusion, the response of frozen-thawed semen to fertilization *in vitro* varies between breeds, but the cryotolerence of produced embryos is not different.

Table 1

Developmental competence of buffalo oocytes fertilized by Egyptian, Italian and crossbred semen.

Semen source	Total number of inseminated oocytes	Cleavage		Morula		Blastocyst	
		n	%	*n*	%	*n*	%
Egyptian	115	71	61.3 ± 2.6[b]	46	39.8 ± 1.5	13	11.4 ± 1.7[b]
Italian	117	72	71.2 ± 2.5[a]	34	43.1 ± 2.2	19	19.3 ± 1.6[a]
Crossbred	100	70	70.2 ± 1.0[a]	42	42.2 ± 2.7	19	17.7 ± 0.9[a]

Percent was shown as Mean ± SE. *n*: the overall number in 4 replicates. a, b values within column differ at P<0.05, P<0.01, respectively.

Table 2

Morphologically normal embryos (viable) produced from fertilization with Egyptian, Italian and crossbred semen.

Semen source	Number of vitrified-warmed embryos	Directly after warming		24 h post-warming	
		n	%	*n*	%
Egyptian	47	34	72.7 ± 2.1	25	53.6 ± 2.0
Italian	50	39	80.4 ± 2.9	30	59.8 ± 0.8
Crossbred	41	30	73.8 ± 2.0	24	59.2 ± 4.8

Percent was shown as Mean ± SE; *n*: the overall number in 3 replicates

Conflict of interest statement

There is no conflict of interest for all authors.

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