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In vitro maturation and fertilization of cryopreserved oocytes of Egyptian buffaloes.

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ABSTRACT

The aim of this study was to evaluate the effect of cryopreservation by conventional straws on viability and normality, *in vitro* maturation and fertilization and blastocyst production rate of cryopreserved immature buffalo oocytes. Oocytes were recovered from ovaries of slaughtered buffaloes, cryopreserved in tissue culture medium and thawed. Thereafter, oocytes were *in vitro* matured, fertilized and cultured. The obtained results indicated that viability rate (89.2 vs. 92.7%) and normality percentage (67.7 vs. 80.3%) was lower (P<0.05) for the cryopreserved than fresh oocytes. Percentage of oocytes at stage (*in vitro* maturation rate) was lower (P<0.05) for cryopreserved than fresh oocytes (40.8 vs. 61.7%). Fertilization rate was lower (P<0.05) for cryopreserved than fresh oocytes (38.5 vs. 75.3%). Blastocyst rate relative to total oocytes was lower (P<0.05) for cryopreserved than fresh oocytes (5.2 vs. 23.6%). This study indicated the possibility of cryopreserved immature buffalo oocytes collected from ovaries of slaughtered buffaloes to in vitro mature, fertilize and develop to embryos at blastocyst stage, but at a little extend of that in fresh case.

Keywords: Buffaloes, immature oocyte, cryopreservation, in vitro maturation, fertilization.

INTRODUCTION

Egyptian buffaloes are important livestock animals because of their draft power, hide and milk and meat production. Some attempts gave attention to collection, *in vitro* maturation and *in vitro* fertilization of immature oocytes of Egyptian buffaloes (Shamiah *et al*., 2008). Cryopreserved oocytes led to chilling injury and cellular damage caused by ice crystal formation (Vajta, 2000). The extent of damage in oocytes is varied and may depends on the cryopreservation procedures used (Chen *et al*. 2003). There are various factors affecting viability and survival of the cryopreserved oocytes including type of cryopreservation device, concentration of cryoprotectants, time of exposure, equilibration steps, and dilution steps at warming (Dattena *et al.,* 2000). Also, high warming rates are also important to prevent damage at the time of warming (Seki and Mazur, 2008).

In vitro cleavage and development into morula and blastocyst stage after cryopreservation/thawing were at higher rates in mature oocytes than those cryopreserved at the immature stage (Hammam and El-Shahat, 2005). Cryopreservation of immature oocytes would significantly advance basic research and commercial applications (Candy *et al*. 1994) as indicated in cattle (Suzuki *et al*. 1996) and buffalo (Wani *et al*. 2004) because it may enable a flexible utilization in time and space of live oocytes for research and animal production purposes. At the same time, this technique may provide an

opportunity to replenish gene banks of endangered species and contribute to the genetic improvement of domestic animals. Therefore, *in vitro* maturation (IVM) and fertilization (IVF) of slaughterhouse-derived oocytes, large-scale production of buffalo embryos, is gathering increasing interest for the production and faster multiplication of superior germplasm.

To date no available reports on the effects of in vitro maturation and fertilization of cryopreserved oocytes of Egyptian buffaloes. Aims of this study was to evaluate the successful rate of in vitro maturation and fertilization of immature buffalo oocytes cryopreserved by conventional straws.

MATERIALS AND METHODS

This study was carried out at the Laboratory of Physiology and Biotechnology, Animal Production Department, Faculty of Agriculture, Mansoura University, in cooperation with Animal Production Department, Faculty of Agriculture, Tanta University.

All chemicals used in this study were purchased from Sigma, unless otherwise indicated.

Oocyte recovery:

Ovaries collected from slaughtered buffaloes were placed in NaCl solution (9 mg/ml) containing antibiotics (penicillin, 100 UI/ml and streptomycin sulphate, 100 µg/ml) and maintained at 30 °C until oocyte recovery. The collected ovaries were washed twice in distilled water and once in freshly prepared saline. Ovarian follicles of 2-8 mm in diameter were aspirated using 18-gauge needle connected to a syringe. Follicular fluids were placed in Petri dishes for oocyte collection, and then oocytes were examined under stereomicroscope. Oocytes enclosed in a compact cumulus with evenly granulated cytoplasm were selected and washed three times in Dulbecco's phosphate buffer solution (DPBS) medium.

Cryopreservtion procedures:

The basal solution used for vitrification was TCM-199 medium supplemented with fetal calf serum (FCS, 20% v/v), penicillin (100 UI/ml) and streptomycin sulphate (100 µg/ml). The procedures employed throughout this experiment were according to (Shamiah *et al*., 2008). The 1st cryopreservation solution contained 10% (v/v) ethylene glycol (EG), 10% (v/v) dimethyl sulfoxide (DMSO) and 0.5 M sucrose. However, the 2nd one contained 20% (v/v) EG, 20% (v/v) DMSO and 0.5 M sucrose in basal medium.

Ten compact oocytes cumulus cells (COCs) were loading into the center between two air bubbles in 0.25 ml plastic insemination straws (IVM L' Aigle, France) using a fine glass capillary pipette. After heat-sealing the straws were plunged immediately into liquid nitrogen (LN2) within 30 sec at room temperature.

Thawing procedures:

After storage for two weeks, the oocytes were warmed by holding the straws for 6 sec in air and then agitating them in water bath at 20 or 25 °C for at least 15 sec. The contents of each straw were emptied into Petri dish and oocytes were transferred to three diluents solution, 0.5 M and 0.25 M sucrose

in TCM-199 supplemented with 10% bovine serum albumin (BSA)), then in TCM-199 supplemented with 10% BSA for 3 min per solution, to remove of intracellular cryoprotectants (Asada *et al*., 2002).

Oocyte evaluation:

Oocyte viability was evaluated morphologically, for oocytes post-cryopreservation as compared to fresh COCs oocytes after collection, based on the integrity of the oolemma and zona pellucida; loss of membrane integrity was obvious upon visual inspection as the sharp demarcation of the membrane disappeared and the appearance of the cytoplasm changed.

The criteria used for assessing the post-thaw morphology of cryopreserved-warmed oocytes were as follows: normal oocytes, with spherical and symmetrical shape with no sings of lysis and abnormal damage oocytes, crack in zona pellucida, oocytes split in two halves, change in shape of oocytes and leakage of oocytes contents (Dhali *et al*., 2000).

***In vitro* maturation *cryopreseved oocyte*:**

Complex tissue culture medium (TCM-199, powder) was dissolved in deionized double distilled water and 50 µg/ml gentamicin was added to the medium. On the day of maturation, TCM-199 medium stock was supplemented with 10% FCS, 20 IU /ml hCG (Pregnyl, Nile, CO. for Pharm. Cairo, Egypt), 1 µg/ml estradiol β_{17}, 20 mmol final concentration of pyruvate, 50 µg/ml gentamicin. The pH value of the medium was adjusted at 7.3-7.4. The medium was filtrated by 0.22-µm millipore filter (Shamiah, 2004).

Each of 500 µl from prepared maturation medium was placed into a four well dishes and covered by sterile mineral oil. Before placing oocytes into culture dishes, the medium was incubated in CO_2 incubator (5% CO_2, at 39°C and high humidity) for 60 minutes. After warming, oocytes were washed three times in each of PBS plus 3% BSA and once in TCM-199. Thereafter, fresh or cryopreserved oocytes were placed in the medium and incubated for 24 h at 38.5°C, 5% CO2 and high humidity. *In vitro* maturation was performed for COCs as cryopreserved/post-thawed oocytes in comparing with fresh oocytes

Fixation, staining and examination of oocytes:

After 24 h as a maturation period, oocytes were washed using PBS containing 1 mg /ml hyaluranidase to remove the cumulus cells. Then, oocytes were washed two times in PBS supplemented with 3% BSA and loaded on clean slide. Slides were placed into fixation solution (3 ethanol: 1 glacial acetic acid) overnight. Thereafter, oocytes were stained with 1 % orcein in 45% acetic acid and examined for maturation under phase-contrast microscopy as the following: Matured oocytes: Chromosome at metaphase II (MII) and polar body exhausted in previtilline space, oocytes with germinal vesicle (GV): Chromosomal in disk in cytoplasmic with intact membrane of nuclei, oocytes with germinal vesicle breakdown (GVBD): Chromosomal in disk in cytoplasmic but intact membrane of nuclei is breakdown, oocytes at metaphase I (MI): the chromosomes are arranged in the metaphase plate and the diploid sets of chromosomes (2n) are fully condensed with absence of polar body component of chromatin mass and degenerated oocytes: Oocytes were vaculated or cytoplasmic shrinked or chromatin condensed (Shamiah, 2004).

***In vitro* fertilization:**

Fresh semen collected by artificial vagina from fertile buffalo bulls was swim-up separated as described by Parrish *et al*., (1986) and diluted to 25×10^6 sperm / ml. Swim up separated sperm diluted with IVF-TALP medium were co-incubated with 20 µg/ml heparin (heparin sodium) for 30 min in CO_2 incubator at 38°C, 5% CO_2 in air and humidified atmosphere.

Matured oocytes were washed three times in TL- HEPES medium (Parrish *et al*., 1989) and twice in fertilization medium (IVF-TALP). Oocytes were inseminated with capacitated semen (1.5×10^6 sperm/ml). Oocytes and spermatozoa were co-cultured in fertilization medium and incubated for 22 h at 38.5 °C, 5 % CO_2 in air with maximum humidity. *In vitro* fertilization was done for *in vitro* mature oocytes (cryopreserved versus fresh oocytes).

Embryo development:

After co-incubation, presumptive zygotes (from cryopreserved and fresh oocytes) were stripped of cumulus cells, washed three times in embryo culture medium, (TCM–199 medium supplemented with 20 mMol Na-pyruvate, 3 mg/ml BSA, and 50 µl/ml Gentamycin sulphate) and cultured in pre-equilibrated embryo culture medium in 4 well Petri dishes and overlaid with sterile mineral oil, then incubated at 38.5 °C, 5% CO_2 with 95% humidity (Eyestone and First, 1989). The medium was replaced every 48 hour. The number of cleaved embryos (divided into four cells or more) was recorded on Day 3 fertilization was Day 0), and blastocysts were counted on Day 8.

Statistical analysis

The experiment was replicated 5 times for survival, maturation and development rates. Data were statistically analyzed T-test after arcsine transformation. The significant differences between means were set at $P<0.05$ (Duncan, 1955).

RESULTS AND DISCUSSION

Effect of preservation on:

viability and normality of oocytes:

Data shown in Table (1) revealed that viability rate of cryopreserved oocytes tended to be higher for fresh than cryopreserved immature oocytes (89.2 vs. 92.7%), but pronounced differences were found in oocyte normality, being significantly ($P<0.05$) higher for fresh (80.3%) than cryopreserved (67.7%) oocytes using spatula and hemi-straws (96 and 95%) than conventional straws cryodevice (80%). Such results indicated losses in viability and normality of oocytes as affected by preservation.

Table (1): Effect of cryopreservation on post-thaw viability and abnormality rates of buffalo immature oocytes.

Item	Cryopreserved oocytes		Fresh oocytes	
	n	%	n	%
Total	111	100	110	100
Degenerated oocytes	12	10.8	8	7.3
Viable oocytes	99	89.2	102	92.7
Morphologically normal oocytes	67	67.7^b	82	80.3^a
Morphologically abnormal oocytes	32	33.2^a	20	19.6^b

[a and b]: Means denoted within the same row with different superecripts are significantly different at P<0.05.

Method of oocyte cryopreservation affected viability of oocytes and the success of cryopreservation is based on procedures that minimize the formation of intra-cellular ice crystals as affected by cryodevice and the surrounding cryoprotectants. In buffaloes, the cryotop was better than the solid surface vitrification method in term of viable immture oocyte yield after cryopreservation (Liang *et al*., 2012). In bovine, Hajarian *et al*. (2011) mentioned that vitrification of immature bovine oocytes with the cryotop method resulted in higher survival and nuclear maturation rates. In mous, Tsang and Chow (2009) found that reduced exposure time of the embryos to osmotic stress and toxic cryoprotectant leads to a high viability rate of the stored mouse embryos.

***In vitro* maturation:**

Results presented in Table (2) showed that marked effect of cryopreservation on stage of *in vitro* maturation of immature buffalo oocytes. Only percentage of oocytes at metaphase-II (MII) stage or those at both metaphase-I (MI) and MII stages was significantly (P<0.05) higher in fresh than cryopreserved oocytes (40.8 and 54.5 vs. 61.7 and 80.0%, respectively). Percentage od degenerated oocytes showed an opposite trend. However, percentages of oocytes at germinal vesicles (GV) and germinal vesicles breakdown (GVB) were not affected by cryopreservation.

Such results indicated the potentiality of cryopreserved immature oocytes to *in vitro* mature at acceptable rates, but still lower than fresh ones. This was in association with remarkable effects negatively on viability and quality of buffalo oocytes during cryopreservation.

Liang *et al*. (2012) found that cryopreservation method affected *in vitro* maturation of buffalo oocytes. The cryotop was better than the solid surface vitrification method in term of *in vitro* maturation. In bovine, Hajarian *et al*. (2011) mentioned that vitrification of immature bovine oocytes with the cryotop method also resulted in higher survival and nuclear maturation rates.

Table (2): Effect of cryopreservation on *in vitro* maturation rate of cryopreserved buffalo oocytes.

Oocyte stage	Cryopreserved oocytes		Fresh oocytes	
	n	%	n	%
Total	110	100	115	100
Germinal visciles stage	11	10.0	8	6.9
Germinal vesicles breakdown stage	11	10	8	6.9
Oocytes at metaphase I stage	15	13.6	21	18.3
Oocytes at metaphase II stage	45	40.8[b]	71	61.7[a]
Oocytes at MI + MII stages:	60	54.5[b]	92	80.0[a]
Degenerated oocytes	28	25.4	7	6.1

[a, b and c]: Means denoted within the same row with different superecripts are significantly different at P<0.05

***In vitro* fertilization and embryo developmental competence:**

Data presented in Table (3) show more pronounced effects of cryopreservation on fertilizing capacity and developmental competence of *in vitro* matured/cryopreserved buffalo oocytes. Fertilization rate and percentages of embryos at morula and blastocyst stages, relative to total or fertilized oocytes, significantly (P<0.05) decreased by cryopreservation.

Fertilization rate was 38.5 vs. 75.3%, while blastocyst rate was 5.2 vs. 23.6% relative to total number of oocytes.

These results indicated that cryopreserved immature buffalo oocytes could be *in vitro* matured, fertilized and developed but at potential capacity and developmental competence lower than oocytes in fresh case.

Table (3): Effect of cryodevice on cleavage and blastocyst rate of cryopreserved immature buffalo oocytes.

Oocyte stage	Cryopreserved oocytes		Fresh oocytes	
	n	%	n	%
In vitro mature oocytes	96	100	93	100
Unfertilized/degenerated oocytes	59	61.5[a]	23	24.7[b]
Fertilized oocytes	37	38.5[b]	70	75.3[a]
Embryos at morula stage(1)	8	8.3[b]	25	26.9[a]
Embryos at morula stage(2)	8	21.6[b]	25	35.7[a]
Embryos at blastocyst stage(1)	5	5.2[b]	22	23.6[a]
Embryos at blastocyst stage(2)	5	13.5[b]	22	31.4[a]

[a and b]: Means denoted within the same row with different superecripts are significantly different at P<0.05. (1): Relative to total oocytes. (2) : Relative to fertilized oocytes.

The present study indicated that blastocyst could be produced from immature buffalo oocytes, which is in similarity with the results of Gautam *et al*. (2008). Moreover, immature cumulus compact buffalo oocytes had tolerate cryopreservation stress by vitrification in term of fertilization rate and blastocyst rate as recently reported by Purohit *et al*. (2012) on goats. In accordance with the present results, buffalo oocytes cryopreserved at the mature stage cleaved and developed into morula and blastocyst stage after thawing at higher rates than those cryopreserved at the immature stage (Hammam and El-Shahat, 2005).

The developmental capacity of oocytes could be varied by using different cryodevices, open pulled straws vs. conventional straws in cryopreservation of buffalo oocytes (Sharma and Purohit (2008). Also, Sharma *et al*. (2010) reported that cryopreservation which permitted high cooling/warming rates with less cryo injuries (open pulled straws), was suitable for cryopreservation of matured buffalo oocytes to allow better embryo development up to the blastocyst stage as compared to French mini straw.

Generally, combination of meiotic arrest and cryopreservation of immature buffalo oocytes under the conditions of this study is responsible for serious damages at the cellular level, making several oocytes unable to undergo *in vitro* maturation and subsequent embryonic development to blastocyst stage as compared to fresh oocytes.

Despite the acceptable results of survival and fertilization of cryopreserved immature buffalo oocytes, blastocyst development is required further researches to optimize the efficiency of immature oocyte cryopreservation (Attanasio *et al*., 2010) and attention is needed to prevent cryo-injuries, using different concentrations of cryoprotectants, and cooling

and warming rates, thereby reducing exposure of oocytes to cryoprotectants (de Leon *et al*., 2012).

CONCLUSION

This study indicated the possibility of cryopreserved immature buffalo oocytes collected from ovaries of slaughtered buffaloes to in vitro mature, fertilize and develop to embryos at blastocyst stage, but at a little extend of that in frsh case.

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الانضاج والاخصاب المعملى لبويضات الجاموس المصرى المجمدة.

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الهدف من هذة الدراسة هو تقييم تاثيراستخدام طريقة حفظ البويضات بالتجميد بواسطة القشة العادية على مدى حيوية البويضات والانضاج والاخصاب المعملى ومعدل انتاج الاجنة فى مرحلة البلاستوسيست من بويضات جاموس مجمدة غير ناضجة . حيث تم تجميع البويضات من مبايض الجاموس بعد الذبح . ثم تم تجميدها فى بيئة زراعة اللانسجة ثم تم اسالتها . بعد ذلك تم انضاج هذة البويضات واخصابها وزراعتها . تشير النتائج المتحصل عليها الى ان معدل حيوية البويضات (٨٩.٢ مقابل ٩٢.٧ %) ونسبة البويضات الطبيعية (٦٧.٦ مقابل ٨٠.٣%) كانت اقل فى حالة البويضات المجمدة عنها فى حالة البويضات الطازجة . و نسبة البويضات فى مرحلة metaphase-II(معدل الانضاج المعملى) كانت اقل فى حالة البويضات المجمدة عنها فى حالة البويضات الطازجة (٤٠.٨ مقابل ٦١.٧ %) . وايضا معدل الاخصاب للبويضات المجمدة اقل منه للبويضات الطازجة (٣٨.٥ مقابل ٧٥.٣ %) . كذلك كانت نسبة الاجنة فى مرحلة البلاستوسيست نسبة الى العدد الكلى للبويضات اقل فى حالة البويضات المجمدة عن البويضات الطازجة (٥.٢ مقابل ٢٣.٦ %) . نستنتج من نتائج هذه الدراسة الى ان بويضات الجاموس المجمدة غير الناضجة والتى تم تجميعها من مبايض الجاموس بعد الذبح يمكن ان يتم انضاجها واخصابها معمليا وكذلك من المكن ان تتطور الى اجنة فى مرحلة البلاستوسيست ولكن بدرجة اقل من البويضات الطازجة.