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Evaluation of Viability and Nuclear Status in Vitrified Mature Buffalo Oocytes

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Abstract: Vitrification is a common method which is successfully used for cryopreservation of gametes and embryosin in several animal species, though subsequent progress is still limited,especially in buffalo. The present study aimed to evaluate the effect of vitrification process on nuclear status in matured buffalo oocytes. Morphology, viability, state of nuclear maturation in meiotic stages and maturation rate (matured oocyte/survived oocyte) were assessed in vitrified matured buffalo oocytes.Immature buffalo oocytes were matured *in-vitro* for 22 hrs. and vitrified at room temperature (25°C) in a mixture of ethylene glycol (EG) and dimethyl sulfoxide (DMSO) with 0.25 ml straw. Vitrification solutions (VS) were VS_1 (1.5M EG+ 1.5M DMSO) and VS_2 (3M EG+ 3M DMSO). Cryoprotectants were added in two steps, with the first step concentration half that of the second (and final) step concentration. After warming, normal oocytes were cultured for further 2 hrs., stained with trypan blue for viability evaluation, then fixed and stained with orcein 1% stain for detection of meiotic stages. Oocytes that reached Telophase I (TI) or Metaphase II (MII) stages were considered matured. Results showed that morphologically normal and viable oocytes were significantly decreased in vitrified oocytes than control. The percentage of oocytes reached TI and MII in un-vitrified (control) group was significantly ($P<0.05$) higher than those vitrified after maturation. The maturation rate was higher in control than vitrified oocytes (76.04±2.29 vs. 66.46±2.25). In conclusion, vitrification is a successful method for cryopreservation of matured buffalo oocytes. However, inspite of higher rates of viability and maturation obtained in current study, it is still lower than un-vitrified oocytes.

Key words: Buffalo % Oocytes % Nuclear status % Vitrification

INTRODUCTION

Cryopreservation is a desirable option for generating a readily available source of unfertilized oocytes for both biological and commercial purposes. Vitrification has been attempted by several workers with variable success in mouse [1], human [2], bovine [3], swine [4], equine [5] and buffalo [6] oocytes. Studies have been performed to investigate the best conditions to preserve viability of vitrified oocytes [7]. Oocytes are the only cells which can form a new individual after fertilization. During maturation, oocytes undergo changes in nuclear status involving existence from diplotene stage of the first meiotic prophase, known as germinal vesicle stage and progression to the metaphase II stage with extrusion of the first polar body [8]. The observation of the oocytes chromosomes at this stage is more reliable mean for defining the *in vitro* maturation progress [9]. Cryoprotectants have been reported not only to induce a disorganization of the spindle microtubules, which in turn results in chromosomal scattering, but also to disrupt the cortical microfilament network. Disruption of the cytoskeleton might be intrinsic to changes in shape and shrinkage that accompany cryopreservation procedures, which in turn might lead to premature release of cortical granules and zona hardening. Also, rewarming may then lead to membrane fusion, cortical granule enzyme release and premature zona hardening [10].

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The cryopreservation of oocytes with slow cooling or vitrification protocol was reported to have deleterious effect on viability. Oocyte survival rate after cryopreservation is affected by both morphological and biophysical factors [11]. The main biophysical factors causing cellular disruption during cryopreservation are intracellular ice formation [12] and osmotic injury [13]. These factors could be easily minimized by adequate cryopreservation techniques using proper cryoprotectant and its concentration [14]. So the current study was designed to evaluate the effect of vitrification process on the morphology, survivability and nuclear status in vitrified matured buffalo oocytes.

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 Chemicals Company.

Oocyte Recovery and Selection: Buffalo (*Bubalusbubalis*) ovaries, collected from local abattoir (El-Moneeb, Giza, Egypt) within 2 hrs. after slaughtering and transported to the laboratory in physiological saline (0.9% NaCl) containing antibiotic (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 (fraction V) and antibiotics (100 µg/ml streptomycin sulfate and 100 IU/ml penicillin). Oocytes were screened using stereomicroscope and those with intact layers of cumulus cells and homogenous cytoplasm were selected for *in-vitro* maturation [15].

***In vitro* Maturation (IVM) of Oocytes:** Oocyte maturation was carried out as described by Mahmoud [16]. Briefly, the recovered oocytes were cultured in groups of 10 or 20 in 100 µl droplets of maturation medium (TCM-199) supplemented with 10 % fetal calf serum, 50µM cysteamine and 50 µg/ml gentamycin sulfate. Droplets were covered with mineral oil and pre-incubated for a minimum of 2 hrs. in a humidified 5% CO_2 atmosphere at 38.5°C. The oocytes were placed into the droplets and incubated for 22 hrs. in a humidified 5% CO_2 atmosphere at 38.5°C.

Evaluation of Nuclear Maturation by Fluorescence Microscope: After 22 h IVM, COCs were subjected to removal of the surrounding cumulus cells by gentle pipetting with a fine capillary tube. Oocytes were stained with probidium iodide (100 µg/ml) and examined under a fluorescence microscope for nuclear status. Oocytes that reached TelophaseI (TI) or MetaphaseII (MII) stages were considered matured.

Vitrification and Warming: Oocytes were exposed to two-step addition of cryoprotectants [17]. Briefly, oocytes were exposed to VS_1 (1.5 M EG + 1.5 M DMSO) for 45 sec. and VS_2 (3 M EG + 3 M DMSO) for 25 sec. The holding media 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 VS_2 and separated by air bubbles, sealed with straw plugs, pre-cooled by exposure to liquid nitrogen (LN_2) vapor for at least 60sec and dipped vertically in LN_2 thereafterfor two months. Straws transferred rapidly in less than 5 sec.; to prevent zona fracture [18]; to a water bath at 35-37°C for 20 sec. for thawing and the expelled oocytes were equilibrated for 5 min.in 0.5 Mgalactose solution [19] in TCM-199 for one-step dilution to remove the cryoprotectants. Oocytes were washed 4-5 times in fresh washing medium and cultured in IVM medium for 24hrs.

Evaluation of Oocytes Morphology after Vitrification-Thawing: Oocyte normality was directly evaluated by an inverted microscope as it was described formerly [20]. Normal oocytes were verified with their spherical and symmetrical shape and absence of lysis signs, membrane damage, swelling, vacuolization, degeneration or leakage of the cellular contents.Abnormal oocytes were characterized by a ruptured zonapellucida or vitelline membrane or having fragmented cytoplasm with signs of degeneration. The normal oocytes were cultured for further 2 hrs. to complete the maturation.

Assessment of Vitrified-Thawed Oocytes Viability by Trypan Blue: Oocyte viability in control (24h-IVM, un-vitrified) andvitrified (2 h post-thawing) was assessedby staining with 0.4% (w/v) trypan blue and examination under an inverted phase-contrast microscope. Exclusion of dye by COCs is an indicative of viability. Viable oocytes with viable or dead cumulus were considered viable while dead oocytes with viable or dead cumulus were considered dead.

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Evaluation of Nuclear Maturation by Orcein Stain: At the end of the culture period, oocytes were subjected to chromosome slides were prepared according to the procedure described by Tarkowski [21]. Briefly, cumulus cells were removed mechanically by gentle pipetting, oocyte was transferred to 1% hypotonic sodium citrate solution for 10 min. and placed on a microscope slide with a minimal amount of hypotonic solution. Three drops of methanol/glacial acetic acid (3:1) fixative were dropped onto the oocytes before staining with 1% orcein stain. The state of nuclear maturation was determined as described earlier by Mahmoud [22]. Oocytes that reached TI or MII stages were considered matured. TI was defined as completion of separation of two chromosome sets and MII was haploid set of chromosomes after emission of first polar body.

Statistical Analysis: Data were presented as mean ±S.E.,tabulated and analyzed for significance by Student *t*-testusing SPSS (*ver.*14) statistical software. Differences were considered to be significant at P<0.05.

RESULTS

Mature oocytes with complete or partial cumulus cells were selected, matured *in vitro* for 22hrs andtheir nuclear status was determined by probidium iodide as shown in Fig. 1. Morphological evaluation of *in-vitro* matured vitrified buffalo oocytes directly after thawing and furtherculture for two hours are shown in Table 1 and Fig. 2. The percentage of normal oocytes significantly (P<0.05) decreased in vitrified oocytes than control. Also, the survivability of oocytes was significantly (P<0.001) decreased with vitrification.

The stages of nuclear maturation in control and vitrified oocytes as determined by orcien staining and microscopic examination are shown in Table 2. Cytogenetic analysis of 209 control and 167 vitrified oocytes showed a wide range of meiotic configurations: Metaphase-I, Anaphase-I, Telophase-I and Metaphase-II (Fig. 3). The percentage of oocytes reached TI and MII in control oocytes was significantly (P<0.05) higher than those vitrified after maturation. The mean maturation rate in controloocytes (76.04±2.29) was higher than oocytes exposed to vitrification (66.46±2.25).

Table 1: Morphological evaluation and survivability directly after thawing and two hours culture of vitrified mature buffalo oocytes

		Morphologically. Normal	Survivability 2hrs. post-thawing
Oovytes culture	No. oovytes examined	No %	No %
Vitrified	255	226 (89.33±1.8)	170 (75.29±0.8)
Control (un-vitrified)	245	242 (98.60±0.7)*	214 (88.50±0.4)**

(*P <0.05-** P <0.001)

Table 2: Effect of vitrification on nuclear status of *in-vitro* matured buffalo oocytes

		Stage of nuclear maturation						
Oocyte type	n	Germinal vesicle o. (%)	GVBD No. (%)	Metaphase I (MI) No. (%)	Anaphase I (AI) No. (%)	Metaphase II (MII) No. (%)	Telophase I (TI) No. (%)	Maturation rate (TI+MII) No. (mean± S.E.)
Control	209	3 (15.6)	5 (2.7)	5 (2.7)	6 (2.9)	123 (58.4)	37 (17.4)	160(76.04±2.29)*
Vitrified	167	0 (13.1)	5 (10.1)	6 (3.2)	9 (5.7)	82 (48.2)	35 (20.6)	117 (66.46±2.25)

n: number of oocytes, GVBD: Germinal Vesicle Break Down.Maturation rate (matured oocyte (TI+MII)/survived oocyte) with superscript (*) indicated a significant difference between verified and control oocytes at P<0.05.

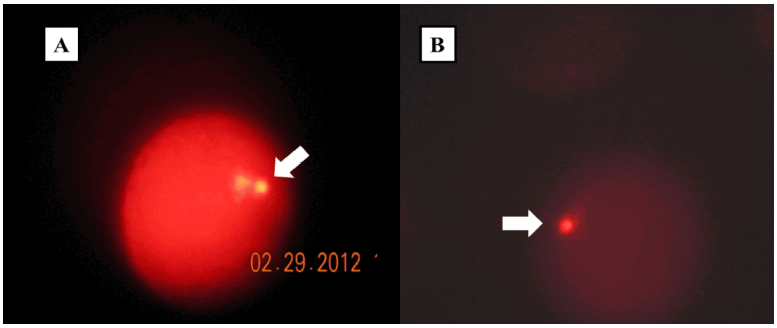

Fig. 1: Buffalo oocytes at Telophase I (A) and Metaphase II (B) stages stained with probidium iodide and visualized under fluorescence microscope (×400).

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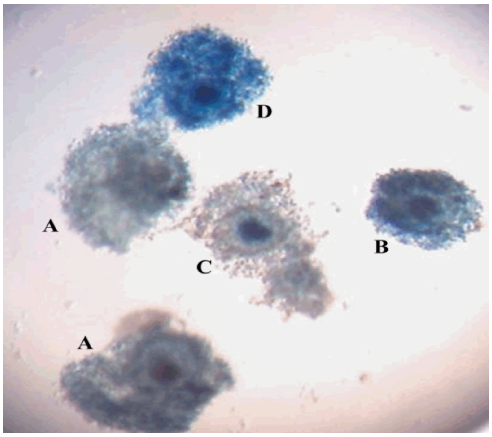

Fig. 2: Morphological evaluation of *in-vitro* matured vitrified buffalo oocytes stained by trypan blue. (A) viable oocyte and viable cumulus cells, (B) viable oocyte and non-viable cumulus cells, (C) non-viable oocyte and viable cumulus cellsand (D) non-viable oocyte and non-viable cumulus cells.

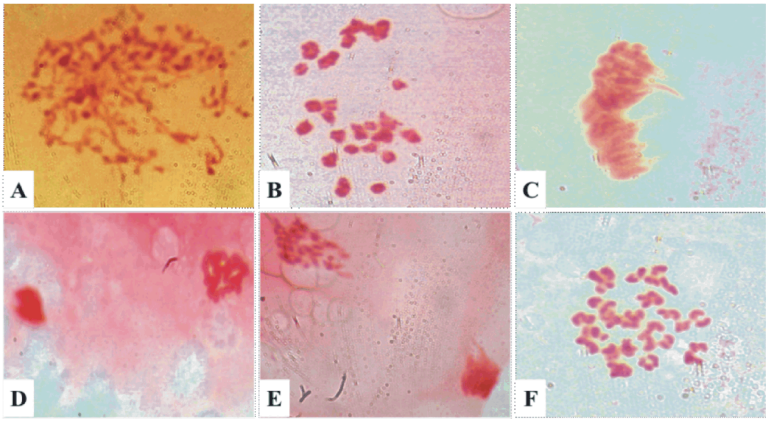

Fig. 3: Buffalo oocytes at germinal vesicle break down with a condensation of chromatin (A), Metaphase I stage (B), anaphase I stage with complete homologous segregation of chromosomes (C), Telophase I stage with two groups of equally spread homologous chromosomes (D), Metaphase II. Note the normal haploid number and the first polar body chromosomes have undergone degeneration (E). Metaphase II stage (F).

DISCUSSION

Oocytes meiosis is very sensitive to endogenous and exogenous factors that could result in oocytes with chromosomal abnormalities. They might be affected by temperature reduction, cryoprotectant concentration and *in vitro* culture [23]. Cytogenetic analysis in the present study showed a wide range of meiotic configurations in Metaphase I, Anaphase I, Telophase I and Metaphase II. During maturation, oocytes undergo changes in nuclear status that involve exit from diplotene stage of the first meiotic prophase, known as germinal vesicle stageand progression to the metaphase II stage with extrusion of the first polar body [8]. The observation of the oocytes chromosomes at this stage is more reliable mean for defining the *in vitro* maturation progress [9].

The survival rate of vitrified-warmed oocytes observed in the present study was lowas compared with control. This in accordance with data reported previously in buffalo oocytes [17, 24-26]. In fact, all oocytes and embryos suffer considerable morphological and functional damage during cryopreservation, but the extent of the injury as well as differences in post-thaw survival and developmental rates may be highly variable depending on the species, developmental stage and origin. Oocytes are particularly difficult to be cryopreservedsuccessfully and thus resulted in low rates of blastocyst production after thawing, fertilization and culture [28]. This problem may be due to the large size of oocytes whichconsequently have a low surface to volume ratio, making it more difficult for water and cryoprotectants to move across the cell plasma membranes.

In the current work, maturation rate; represented by the percentage of oocytes reached TI and MII; decreased in vitrified than control group. It is well known that vitrification causes several ultra-structural [28] and structural alterations, including damages of the meiotic spindle apparatus in the oocytes [29]. Disorganization of meiotic spindles might lead to chromosomal dispersion, aneuploidy or polyploidy of *in-vitro*matured cryopreserved oocytes, as well as failure of normal fertilization and development [30]. The evidence of these structural alterations provides the basis for developing new strategies to improve the efficiency of cryopreservation in cattle and buffalo species [31].

In conclusion, vitrification is a successful method for cryopreservation of matured buffalo oocytes butstill has adverse effects on the viability and percentage of oocytes reached Metaphase II.

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