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Influence of Meiotic Stages on the Survival and Development Ability of Vitrified-Warmed Buffalo Oocytes

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Abstract: The present study was undertaken to investigate the effect of meiotic stages as germinal vesicle (GV), germinal vesicle breakdown (GVBD) and metaphase II (MII) during *in vitro* maturation (IVM (on the survival, maturation and developmental ability of vitrified-warmed buffalo oocytes. Oocytes with compact cumulus cells were cultured *in vitro* for 0, 6 and 24 h. Oocytes were vitrified in VS1: 1.5 M ethylene glycol (EG) + 1.5 M dimethyl sulfoxide (DMSO) for 45 s (step one). After this initial exposure, oocytes were transferred to VS2: 3 M EG + 3 M DMSO in a holding medium for 25 s (step two). After warming, oocytes which had been vitrified at 0 and 6 h of IVM were again cultured to complete the 24 h IVM period then fertilized *in vitro*. The survival rates of oocytes vitrified and warmed at 0, 6 and 24h IVM decreased ($P < 0.05$) as compared to the rates for unvitrified control oocytes. Survivability was higher ($P<0.05$) with IVM for 24 h prior to vitrification than those in IVM for 0 and 6 h. The percentages of oocytes reaching telophase I or metaphase-II stages were lower in oocytes cryopreserved in all the groups compared with the control. However, among the vitrified oocytes, the highest maturation rate was seen in oocytes vitrified at 24 h. The cleavage rate of non-vitrified oocytes (control) was higher ($P<0.01$) than that recorded in any of the vitrified groups. 0ocytes matured for 24 h prior to vitrification had a higher cleavage rate than those in IVM for 0 and 6 h. Development into blastocyst decreased ($P<0.01$) in all vitrification groups compared to the control. There was no significant difference in blastocyst rates among the three vitrification groups. In conclusion, these results indicated that vitrification of buffalo oocytes may be affected by their maturation stage *in vitro* and that developmental competence to blastocysts of cleaved oocytes following vitrification is impaired.

Key words: Buffalo · Meiotic stages · Oocyte · Vitrification

INTRODUCTION

Cryopreservation is a desirable option for generating a readily available source of unfertilized oocytes for both biological and commercial reasons. Vitrification has been attempted by several workers with variable success in mouse [1], human [2], bovine [3-5], swine [6], equine [7] and buffalo [8, 9] oocytes. Studies have been performed to investigate the best conditions to preserve viability of vitrified oocytes [10]. Researchers have highlighted the influence of meiotic stage on the viability of oocytes following vitrification and thawing [11-13]. Meiotic stage at the time of vitrification affects the survival of mammalian oocytes [14]. Variable sensitivity of oocytes to cooling procedures during cryopreservation has been related to the cell cycle stage during meiosis [15].

Numerous studies have been conducted to determine optimal meiotic stages for oocyte vitrification, yet the results are inconclusive. Maturation stages ranging from GV [13], GVBD [16] through MII [11] have been described as the most favorable stage for vitrification. In contrast, a few previous studies indicated that meiotic stage does not influence the ability of a oocyte to survive after cryoprservation [7, 17]. The current study was designed to investigate the effect of meiotic stages as GV, GVBD and MII during *in vitro* maturation (IVM (on the survival, maturation and development ability of vitrified-warmed buffalo oocytes.

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MATERIALS AND METHODS

Chemicals: Chemicals for *in vitro* fertilization technique including fetal calf serum and tissue culture medium (TCM 199) were obtained from Gibico BRL (Grand Island, New York, USA). Sodium heparin, sodium caffeine benzoate, bovine serum albumin fraction V and fatty acid free, mineral oil, orcin stain, chemicals constitute of Brackett and Oliphant (BO) medium, modified phosphate buffer saline (MPBS), ethylene glycol and dimethyl sulfoxide were obtained from Sigma chemicals company.

Oocyte Recovery and Selection: Buffalo ovaries were collected from abattoir within 2 h of slaughter. The ovaries were transported to the laboratory in physiological saline (0.9% NaCl) containing antibiotic (100 μg/ml streptomycin sulfate and 100 IU/ml penicillin) maintained at 30EC. 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 (BSA), fraction V and antibiotics (100 μg/ml streptomycin sulfate and 100 IU/ml penicillin). Oocytes were screened using a stereo zoom microscope. The oocytes with intact layers of cumulus cells and homogenous cytoplasm were selected for the study [18].

***In vitro* Maturation (Ivm) of Oocytes:** Oocyte maturation was carried out as previously described [19]. 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 and 50 μg/ml gentamycin sulfate. The droplets were covered with mineral oil and pre-incubated for a minimum of 2 h in a humidified 5% CO_2 atmosphere at 38.5°C. The oocytes were placed into the droplets and incubated for 0, 6, or 24 h in a humidified 5% CO_2 atmosphere at 38.5°C.

Vitrification and Warming: Oocytes were exposed to two-step addition of cryoprotectants [20]. Briefly. Oocytes were exposed to VS1 (1.5 M EG + 1.5 M DMSO) for 45 s and then transferred to VS2 (3 M EG + 3 M DMSO) and exposed for 25 s. The holding media was TCM 199 containing 2.5 mM HEPES + 20% fetal calf serum. Oocytes (5 to 10) were then immediately loaded in 0.25 ml straws in the middle column of the vitrifying solution (VS2) separated by air bubbles. The straws were sealed with polyvinyl alcohol powder and precooled by keeping them in liquid nitrogen (LN2) vapor at a height of about ~5 cm from the level of LN2 for at least one min. The straws were then dipped vertically in LN2. The time between the contact of oocytes with VS2 and cooling of loaded straws in LN2 vapor did not exceed 40 s. Oocytes were kept in LN2 for two months. During thawing, the straws were warmed in air for 5 s for preventing zona fracture [21] and then transferred rapidly to a water bath at 35 to 37EC for 20 sec. The contents were expelled into an empty plastic dish and the oocytes were allowed a 5-min equilibration in 0.5 M galactose solution [22] in TCM-199 for one-step dilution to remove the cryoprotectants. Oocytes were washed 4 to 5 times in fresh washing medium and cultured in IVM medium for 24 h. Non-vitrified oocytes that cultured for 24 h were served as control.

Survival of Oocytes after Vitrification-Warming: Oocyte normality was evaluated by their post-thaw morphological appearance under an inverted microscope. The criteria used for assessing morphology [8] were as follows: normal-oocytes with spherical and symmetrical shape with no sign of lysis, membrane damage, swelling, vacuolization, degeneration or leakage of the cellular contents; abnormal-oocytes with a ruptured zona pellucida or ruptured vitelline membrane or having fragmented cytoplasm with signs of degeneration.

Evaluation of Nuclear Maturation: At the end of the culture period, oocytes were subjected to chromosome slides were prepared according to the procedure described by Tarkowski [23]. Briefly, cumulus cells were removed mechanically by gentle pipetting. Each oocyte was transferred into 1% hypotonic sodium citrate solution for 10 minutes and then placed on a microscope slide with a minimal amount of hypotonic solution. Three drops of fixative (methanol: acetic acid, 3:1) were dropped onto the oocytes. Subsequently, the fixed material was stained with 1% orcein stain. The state of nuclear maturation was determined as described earlier by Mahmoud [24]. Oocytes that reached telophase I or metaphase II stages were considered matured. Telophase I was defined as completion of separation of two chromosome sets and MII was haploid set of chromosomes after emission of first polar body.

***In vitro* Fertilization and Culture:** Spermatozoa were treated as described by Niwa and Ohgoda [25]. Briefly,

two straws of frozen buffalo semen were thawed in a water bath at 35 to 37°C for 1 min. The spermatozoa were washed twice by centrifugation (800 g for 10 min) in BO medium [26] 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 x10^6 sperm/ml. The oocytes following vitrification, thawing and IVM were washed three times in BO medium containing 10 mg/ml BSA, introduced into 100 µl droplets of sperm suspension (5 to 10 oocytes/droplet) under paraffin oil and co-cultured for 5 h in a humidified 5% CO_2 atmosphere at 38.5 °C. The oocytes were then washed in TCM-199 to remove attached spermatozoa. Groups of 10-20 oocytes were again cultured in previously prepared co-culture 100µl droplet consisting of TCM-199+10% serum. The development of fertilized oocytes was assessed on day 2 (for cleavage; day 0 = day of insemination) and day 7 and 8 (for blastocyst). The rate of blastocyst production was calculated in relation to the total number of inseminated oocytes.

Statistical Analysis: Data were analysed using SPSS for windows, Version 13.0. One-way ANOVA and least significant difference (LSD) as a post-hoc test were performed to compare the means.

RESULTS

Post-thaw survivability of vitrified oocytes was greater (P<0.05) when cultured for 24 h prior to vitrification compared to 0 or 6 h (Table 1).

Nuclear maturation results for control and vitrified oocytes are shown in table 2. The proportion of oocytes reaching MII was greater (P<0.01) in the control as compared to vitrified groups. Among the vitrification groups, the 24 h group was found superior (P<0.01) to 0 or 6 h group.

Cleavage rate of the control oocytes was greater (P<0.01) as compared to vitrified oocytes (Table 3). Among the vitrification groups, cleavage rate was greater (P<0.01) when oocytes were cultured for 24 h prior to vitrification (Table 3).

Blastocyst formation was greater (P<0.01) in the control as compared to vitrified oocytes, but it did not vary significantly among the vitrification groups (Table 3). Nevertheless, blastocyst formation was marginally higher when oocytes were cultured for 24 h prior to vitrification.

Table 1: Post-thaw survivability of vitrified buffalo oocytes that cultured for different duration prior to vitrification

Duration of culture before vitrification (h)	No. oocytes vitrified	No. oocytes recovered	No. morphological normal oocytes (% " S.E)
0 (a)	273	238	193 (81.2 " 0.59)^a
6 (b)	253	224	185 (82.4 " 0.64)^a
24 (c)	234	198	169 (85.2 " 0.46)^b

^{a,b} Values within column without common superscripts differ (p<0.05).

Table 2: Nuclear maturation rate of vitrified buffalo oocytes that cultured for different duration prior to vitrification

Maturation time before vitrification (hours)	No. oocytese xamined	No. oocytes matured (% " S. E)
0 (a)	57	24 (42.1" 0.81)^a
6 (b)	69	31 (45.0 " 0.62)^a
24 (c)	66	36 (55.2 " 0.38)^{ab}
Non-vitrified (d)	113	90 (79.8 " 1.27)^b

^{a,b} Values within column without common superscripts differ (p<0. 01).

Table 3: *In vitro* development of vitrified buffalo oocytes following IVF and culturing

Duration of culture before vitrification (h)	Oocytes fertilized	Cleaved (% " S. E)	Blastocyst (% " S. E)
0 (a)	135	20 (14.7" 0.52)^a	4 (3.0 " 0.24)^a
6 (b)	116	18 (15.5 " 0.38)^a	4 (3.5 " 0.13)^a
24 (c)	102	21(20.7 " 0.61)^{ab}	5 (4.8 " 0.56)^a
Non-vitrified (d)	81	34 (41.9 " 0.58)^b	7 (8.5 " 0.97)^b

^{a,b} Values within column without common superscripts differ (p<0.01).

DISCUSSION

The cryopreservation of oocytes is an open problem as a result of their structural sensitivity to the freezing process. In this study, buffalo oocytes were vitrified at different stages of maturation to assess post-thaw morphology, nuclear maturation and developmental competence. Post-thaw survivability of buffalo oocytes in our study increased with time in IVM prior to vitrification, higher in mature oocytes than GV and GVBD stages. The difference may be due to membrane permeability. Permeability of plasma membrane to cryoprotectant changes according to the meiotic stage of the oocytes [27]. Immature oocytes are less permeable to water and cryoprotectant than matured oocytes [28] and low permeability [29] of plasma membrane has been considered responsible for lower survival and a higher incidence of damage to immature oocytes. In addition, The GVBD to MII stage bovine oocytes are as permeable to ethylene glycol as the later stage embryos, but GV stage oocytes are less permeable [30]. Chilling injury is higher in immature than mature oocytes, probably due to

low membrane stability and susceptibility of the cytoskeleton [31] and differences in the hydraulic conductivity [32]. The increased percentage of morphologically normal oocytes vitrified at 24 h than 0 and 6 h may support the fact that MII stage human [33], bovine [34,4], porcine [35], equine [7] and buffalo [36,12] oocytes evidenced a higher capacity to recover cryopreservation damages than GV stage oocytes.

In our study, maturation rate varied depending on the time COCs remained in the maturation medium prior to vitrification. COCs that were vitrified following 0 or 6 h of IVM prior to vitrification had a lower percentage of oocytes reaching M-II than the control group. The lower maturation rate in oocytes that had spent less time in the maturation medium prior to vitrification may have multifactorial causes. Cumulus cell projections, which control the intercellular communication between oocyte and cumulus cells and in turn affect oocyte maturation [37,38] may have been damaged as vitrification of immature oocytes produced lysis in the cumulus cell (CC). Cryopreservation may damage the ooplasm, which in turn may affect protein synthesis [39]. The cytoskeleton in first meiotic division of immature oocytes is particularly vulnerable to cryodamage, whereas matured oocytes display a more flexible cytoskeleton. This may explain why matured oocytes are less subject to cryodamage [40]. The lower maturation rate in vitrified matured oocytes compared to non-vitrified oocytes may be due to chromosomal aberrations that would have taken place during vitrification owing to alteration in the meiotic spindle [41].

In the present work fewer oocytes cleaved from cryopreserved oocytes compared to controls. The cleavage rate of the 24 h maturation treatment was significantly higher than for oocytes vitrified at 0 and 6 h. The low cleavage rates of the vitrified-warmed oocytes might be due to many factors. The oocytes have relatively complex sub-cellular structure, within which many of the sub-cellular components are particularly temperature sensitive [42] and osmotically and ionically sensitive [43]. Cooling affects spindle fiber integrity [44] and cortical granules [45]. Depolymerization of the spindle fiber is likely to lead to aneuploidy [46] and premature release of the cortical granule vesicles is likely to lead to zona hardening [45]. The zonae of dead, immature bovine oocytes (from frozen ovaries) are penetrated at lower rates than those of *in vitro* matured oocytes [47]. We reported higher cleavage rate in oocytes vitrified at 24 h compared to those vitrified at 0 and 6 h. In this respect, Luna *et al.* [3] recorded that the incidence of diploid metaphase II (abnormal oocytes) was higher in oocytes vitrified before maturation or 8 h after the onset of maturation than mature oocytes.

An especially useful measure of oocyte quality is the developmental ability after cryopreservation. In the present study, viability was tested by the development of these oocytes up to blastocyst stage. We found a significant reduction of blastocyst yield in all vitrified groups compared to the control non vitrified group. Among the vitrified groups, the highest blastocyst rate was obtained from vitrified MII oocyte followed by germinal vesicle breakdown stage. The higher proportion of blastocyst in MII than germinal vesicle stage oocytes is in accordance with Men *et al.* [11] in bovine and El-shahat and Hammam [36] in buffaloes. The poor development in vitrified groups was attributed to the damage in oocytes itself, rather than fertilization failure [11]. In contrast, Barnes *et al.* [16] indicated that oocytes cooled at the germinal vesicle breakdown stage (GVBD) of meiosis cleaved and developed into blastocysts at higher rates than the oocytes cooled at the GV or MII stage. They added that, choosing an intermediate stage, such as (GVBD), may circumvent some of the problems associated with cryopreservation of GV and MII oocytes.

In conclusion, the present study indicates that MII stage of *in vitro* mature buffalo oocyte is able to survive vitrification better than GV and GVBD but their further development is impaired.

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