Effects of cysteamine during *in vitro* maturation on viability and meiotic competence of vitrified buffalo oocytes

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Summary

The aims of the present study were to assess the effects of cysteamine as an anti-oxidant on the rate of *in vitro* maturation (IVM) of buffalo oocytes (experiment 1), and their viability and nuclear status following vitrification (experiment 2). Immature oocytes with compact cumulus cells obtained from the ovaries of slaughtered animals were harvested and then cultured in the maturation medium with no cysteamine (control) or 50 μM cysteamine (treated). Oocytes were vitrified in vitrification solution 1 (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 were evaluated morphologically and then cultured for a further 2 h in the cysteamine-supplemented or control maturation media. The oocytes were evaluated morphologically, stained with trypan blue for viability evaluation. The maturation rate of oocytes was higher ($P<0.05$) for IVM media with cysteamine compared with controls. There was no significant difference in morphology, survivability and maturation rate between the two vitrification groups (cysteamine-treated and untreated groups) but the morphology, survivability and percentages of metaphase-II oocytes in both groups of vitrified oocytes were lower compared with their respective controls. In conclusion, the addition of cysteamine to the maturation medium improved nuclear maturation of buffalo oocytes but had no positive effect on their cryoresistance during vitrification.

Key words: Buffalo oocytes, Cysteamine, Meiotic maturation, Vitrification

Introduction

In vitro oocyte or embryo culture results in higher O_2 concentrations than *in vivo* environments, leading to increased reactive oxygen species (ROS) levels (Bedaiwy *et al*., 2004). Reactive oxygen species such as O_2 are able to diffuse and pass through cell membranes and alter most types of cellular molecules such as lipids, proteins and nucleic acids that can affect the early development of embryos (Kitagawa *et al*., 2004). Living organisms possess natural protective factors known as ROS scavengers (anti-oxidants) that counteract the negative effects of ROS. These anti-oxidants include enzymes such as superoxide dismutase, which eliminates O_2, catalase and selenium-dependent glutathione (GSH) peroxidase, which transforms H_2O_2 into H_2O and O_2 as well as lipid- and water-soluble anti-oxidants such as vitamins C and E, and uric acid (Kitagawa *et al*., 2004). However, during *in vitro* oocyte and embryo culture, the levels of anti-oxidants are lower than *in vivo* because the oocytes or embryos do not benefit from the maternal anti-oxidant protection. The addition of an anti-oxidant(s) to the culture medium may, therefore, impinge on the *in vitro* oocyte maturation and ensuing embryo development.

Various thiol compounds, especially cysteine and cysteamine, are commonly added to *in vitro* maturation (IVM) media to support GSH synthesis, and to improve the developmental competence of oocytes. The precursor of GSH in oocytes is cysteine which may play an important role as an anti-oxidant supplement to *in vitro* culture systems (Ali *et al*., 2003). Low molecular weight compounds such as β-mercaptoethanol and cysteamine enhance cysteine mediated GSH synthesis in bovine embryos (Gasparrini *et al*., 2006; Sadeesh *et al*., 2014). The addition of cysteamine to maturation medium enhanced the GSH synthesis and improved oocyte maturation by protecting the oocytes from oxidative stress and probably by acting on the delicate process of cytoplasmic maturation (Gasparrini *et al*., 2000; Kobayashi *et al*., 2006). However, the thiol compounds such as cysteamine could have different effects when added to embryo culture medium, depending on the concentration used, species and type of oocyte (*in vitro*- and *in vivo*-derived oocytes) (De Matos *et al*., 2002; Balasubramanian and Rho, 2007).

Vitrification is simple, rapid and has proven to be more effective than slow-cooling methods to cryo-preserve mammalian oocytes (Mahmoud and El-Sokary, 2013; Mahmoud *et al*., 2013, 2014). Vitrification technique involves the addition of relatively high concentrations of cryoprotectants (CPAs) and ultra rapid cooling (Vajta, 2000), and has been tested in various species with good results (Yadav *et al*., 2008; Mahmoud and Seidel, 2010; El-Sokary *et al*., 2013; Scholkamy *et al*., 2015). A several variables may affect the outcome of

vitrification of oocytes including the type and concentration of cryoprotectants, stage of development of oocytes, and the presence or absence of cumulus cells (Mahmoud *et al*., 2010a, b).

Buffalo oocytes are likely to be more sensitive to oxidative damage due to high lipid content (Gasparrini *et al*., 2000). Moreover, vitrified oocytes had significantly lower GSH content and higher H_2O_2 level (Somfai *et al*., 2007). The addition of cysteamine to maturation media improved the blastocyst formation rate of fresh ovine and vitrified bovine oocytes (Kelly *et al*., 2005). On the other hand, Oyamada and Fukui (2004) found that cysteamine had no positive effect on nuclear maturation, but improved fertilizability, developmental competence and cryoresistance of vitrified bovine oocytes. In the buffalo, Singhal *et al*. (2009) reported that supplementing the maturation medium with IGF-1 + cysteamine significantly improved the *in vitro* production of buffalo embryos but there is a paucity of data on the effects of cysteamine-enriched culture media used prior to vitrification of buffalo oocytes. Hence, the specific aim of this study was to assess the effects of cysteamine on the rate of *in vitro* oocyte maturation as well as viability and chromosomal status of vitrified buffalo oocytes.

Materials and Methods

Media preparation

Holding media

TCM 199 containing 2.5 mM HEPES + 20% fetal calf serum (FCS, Gibco BRL).

Washing media and maturation media

TCM 199, 10% FCS and 50 μg/ml gentamycin sulfate.

Vitrification media

VS1: 1.5 M EG + 1.5 M DMSO (step one) and VS2: 3 M EG + 3 M DMSO (step two).

Oocyte recovery and selection

Ovaries were collected from slaughtered buffalo within 2 h of slaughter. Ovaries were transported to the laboratory in physiological saline (0.9%, w/v, NaCl) with 100 μg/ml streptomycin and 100 IU/ml penicillin at 30°C. At the laboratory, ovaries were washed three times in phosphate-buffered saline (PBS) and the oocytes were aspirated from 2- to 5-mm follicles with a 20-gauge needle containing PBS with 3% bovine serum albumin, fraction V, 100 μg/ml streptomycin, and 100 IU/ml penicillin. Oocytes were collected under stereomicroscope and oocytes with intact layers of cumulus cells and homogenous cytoplasm were selected.

In vitro maturation of oocytes

Oocyte maturation was carried out as described previously (El-Naby *et al*., 2013). Briefly, about 10 or 20 oocytes were cultured in 100-μL droplets of TCM-199 supplemented with 10% fetal calf serum and 50 μg/ml gentamycin sulfate. Oocytes were cultured in the IVM medium with no cysteamine (control) or 50 μM cysteamine (treated) (Gasparrini *et al*., 2000). The oocytes were incubated for 22 h in a humidified 5% CO_2 atmosphere at 38.5°C.

Vitrification and warming

All vitrification solutions (VS) were prepared using the holding medium. Manipulation of oocytes and vitrification procedures were performed at room temperature (25°C). Vitrification solutions for mature oocytes were: 3 M ethylene glycol + 3 M dimethyl sulfoxide. Cryoprotectants were added in 2 steps, using half the final concentration for the first step. Mature oocytes plus their cumulus cells were exposed to VS1 for 45 s and VS2 for 25 s. All procedures, from the exposure of cumulus-oocyte complexes to the final vitrification solution to placing the loaded straws in liquid nitrogen (LN) vapor, were done within 45 s. After that, oocytes were immediately loaded in 0.25-ml mini-straws (I.M.V; Orsay, France) in the middle column of the vitrifying solution separated by air bubbles. Ten oocytes were loaded in each straw, pre-cooled in LN vapor for at least 1 min and dipped in LN. All oocytes were kept in LN for a 1-2 h. Straws were warmed in air for 5 s to prevent zona fracture (Subramaniam *et al*., 1990). Then they were transferred rapidly to a water bath at 35-37°C for 20 s. The content of the straws was expelled into an empty plastic dish (Nuclon 153066; Inter-Med, Roskilde, Denmark), and the oocytes were kept in 0.5 M galactose solution for 5 min at 37°C in TCM-199 for one-step dilution to remove the cryoprotectant. Oocytes were washed 4 to 5 times in fresh washing media (TCM-199, 10% FCS and 50 μg/ml gentamycin sulfate) at 37°C.

Assessment of oocyte morphology after vitrification-warming

Post-thawing morphological appearance was evaluated under an inverted microscope immediately after thawing and after the 2-h incubation. Oocytes with spherical and symmetrical shape with no sign of lysis, membrane damage, swelling, vacuolization, degeneration or leakage of the cellular content were considered normal while oocytes with a ruptured zona pellucida or ruptured vitelline membrane or with fragmented cytoplasm were degenerated (Mahmoud *et al*., 2013).

Assessment of survivability of the vitrified-warmed oocytes by trypan blue

Control (IVM for 24 h but non-vitrified) and vitrified COCs after the 2-h incubation post-thawing were stained with 0.4% (w/v) trypan blue and examined under an inverted microscope. Uptake of dye by COCs was indicative of non-viability and exclusion of dye by COCs was indicative of COC viability.

Evaluation of oocyte nuclear status

Control and vitrified oocytes (matured with and without cysteamine) were prepared for meiotic chromo-

somes evaluation according to the procedure described by Tarkowski (1966). Briefly, cumulus cells were removed mechanically by gentle pipetting. Each oocyte was transferred to 1% hypotonic sodium citrate solution for 10 min and then placed on a microscope slide with a minimal amount of hypotonic solution. For fixation, methanol:acetic acid, 3:1 was dropped onto the oocytes. Subsequently, the slides were stained with 1% orcein. Stages of nuclear maturation were determined according to Mahmoud *et al*. (2010c) and classified as:
(1) Germinal vesicle (GV)
(2) Germinal vesicle breakdown (GVB)
(3) Metaphase-I (M-I)
(4) Anaphase-I (A-I)
(5) Telophase-I (T-I)
(6) Metaphase-II (M-II) stages
Post-thawing maturation rate (number of matured oocytes/number of live oocytes) was also calculated.

Experimental design

Experiment 1

The effect of cysteamine on IVM of buffalo oocytes. Immature oocytes were cultured in maturation media with no cysteamine (control) or 50-µM cysteamine (treated). The nuclear maturation was recorded in both groups.

Experiment 2

The effect of cysteamine on viability and maturation of vitrified buffalo oocytes. The *in vitro* matured oocytes were vitrified in a mixture of 3 M DMSO and 3 M EG. Vitrified oocytes matured in cysteamine-free or in 50-µM cysteamine were compared.

Statistical analysis

Data (5 replicates/group) were subjected to ANOVA using SPSS version 16.0 statistical software. A comparison of means was carried out by Duncan's multiple range test. Student's t-test was performed to compare the two groups, with or without cysteamine. Differences were considered to be significant at $P<0.05$.

Results

Oocyte maturation rates (summarized in Table 1, Figs. 1A-B) were 76.2 ± 0.8% for oocytes cultured in media with cysteamine and 69.2 ± 2.1 SEM % for oocytes cultured without cysteamine ($P<0.05$).

Morphological evaluations of oocytes immediately after thawing and after 2-h culture post-thawing are summarized in Table 2. There were no differences ($P>0.05$) between the two vitrification groups. Moreover, there were no significant differences in the percentage of normal oocytes at 0 h and 2 h post-thawing.

Post-thawing survivability 2 h after culture of control and vitrified groups following trypan blue staining and microscopic examination is shown in Table 3. The survival rate after vitrification was similar for both cysteamine-treated and untreated groups. The percentage

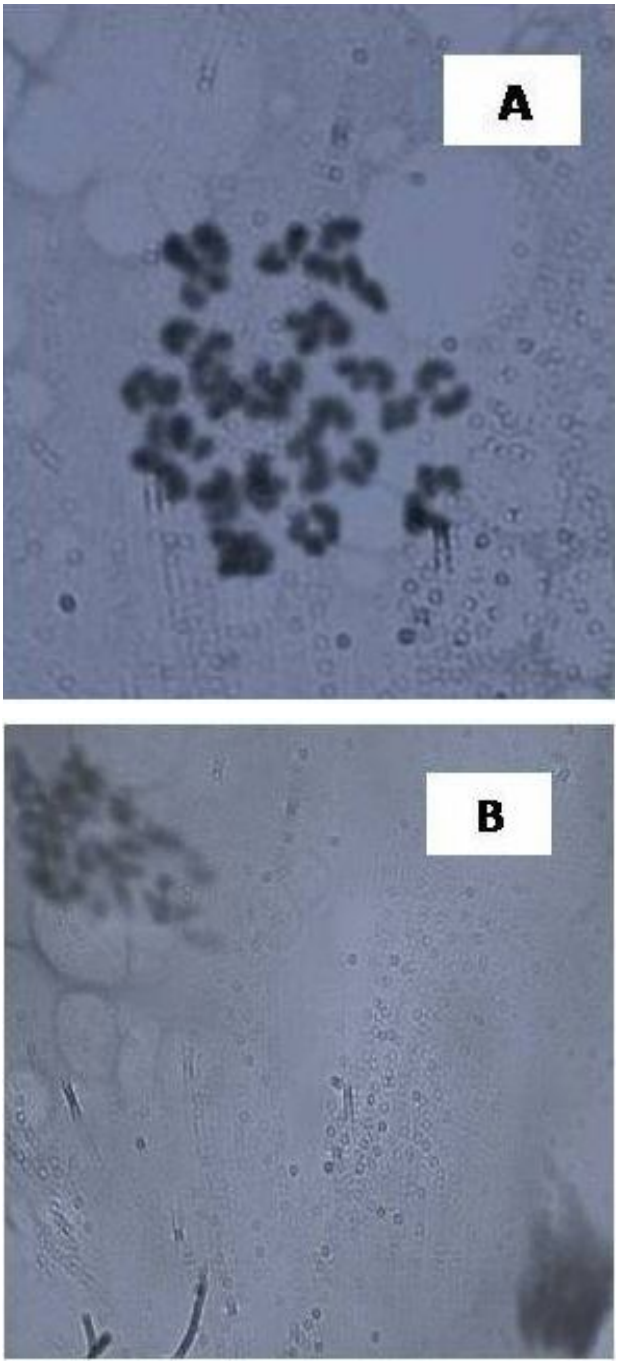

Fig. 1: Mature buffalo oocytes at metaphase-II (A) and telophase-I (B) stages

Table 1: Effect of cysteamine addition to culture media on nuclear maturation of buffalo oocytes (mean±SE)

Culture	No. of examined oocytes	Germinal vesicle No. (%)	Germinal vesicle break down No. (%)	Metaphase-I (M-I) No (%)	Anaphase-I (A-I) No. (%)	Telophase-I (T-I) No. (%)	Metaphase-II (M-II) No. (%)	Mature oocytes (T-I + M-II) No. (%)
With cysteamine	119	21 (17.6)	3 (2.5)	1 (0.8)	3 (2.5)	18 (15.1)	73 (61.3)	91 (76.2±0.8)*
Without cysteamine	110	19 (17.3)	5 (4.5)	2 (1.8)	8 (7.3)	16 (14.5)	60 (54.5)	76 (69.2±2.1)

* $P<0.05$ between mature oocytes with and without cysteamine - Student's t-test

Table 2: Morphological evaluation, immediately after thawing and after 2 h of culture of vitrified buffalo oocytes matured in the presence or absence of cysteamine antio-xidant (mean±SE)

Oocyte culture	No. oocytes vitrified	No. oocytes recovered	Morphologically normal oocytes	
			No. % (0 h)	% (2 h)
With cysteamine	291	273	222 (81.3 ± 1.2)	(78.1 ± 1.9)
Without cysteamine	257	234	189 (80.7 ± 1.5)	(77.9 ± 1.5)

Table 3: Viability evaluation after 2 h of culture by trypan blue staining of vitrified buffalo oocytes matured in the presence or absence of cysteamine anti-oxidant (mean±SE)

Oocyte culture	No. oocytes vitrified	No. oocytes recovered	Viable oocytes VOC+VO	Dead oocytes VC+NV
With cysteamine	251	237	135 (58.2 ± 2.6)[a]	102 (41.8 ± 2.6)[a]
Without cysteamine	223	214	114 (53.7 ± 4.4)[a]	100 (42.9 ± 5.2)[a]
Control (non-vitrified)	195	170	152 (89.5 ± 1.4)[b]	18 (10.4 ± 1.4)[b]

[a, b] Values within column with different superscripts differ (P<0.0001 for viable oocytes, and P<0.001 for dead oocytes). Control averaged over the cysteamine and without-cysteamine groups. VOC: Viable oocyte and viable cumulus cells, VO: Viable oocyte and non-viable cumulus cells, VC: Non-viable oocyte and viable cumulus cells, and NV: Non-viable oocyte and non-viable cumulus cells

of viable oocytes was significantly higher (P<0.0001) in control than both vitrification groups. The percentage of dead oocytes was significantly (P<0.001) higher in two vitrified groups than control one. In spite of the fact that approximately 80% of all oocytes had normal morphology 2 h post-thawing, a large proportion of the oocytes did not survive the freeze-thaw.

The stages of nuclear maturation in control and vitrified oocytes, as determined by orcein staining and microscopic examination, are summarized in Table 4. The percentage of oocytes reaching M-II in the control group was significantly (P<0.0001) higher than of that for vitrified oocytes after maturation in culture media (with or without cysteamine).

Table 4: Maturation rates of vitrified buffalo oocytes matured in the presence or absence of cysteamine anti-oxidant (mean±SE)

Oocyte culture	No. examined oocytes	No. matured oocytes (%)
Control (non vitrified)	229	167 (72.7 ± 1.8)[b]
With cysteamine	170	98 (57.4 ± 1.3)[a]
Without cysteamine	155	90 (59.6 ± 2.4)[a]

[a, b] values within column with different superscripts differ (P<0.0001). Control averaged over cystamine and without cysteamine group

Discussion

Our results indicate that the addition of cysteamine to maturation media significantly enhanced the maturation rate of buffalo oocytes. This is in agreement with the results by Roushandeh *et al*. (2006) and Roushandeh and Roudkenar (2009) who demonstrated that cysteamine, as an anti-oxidant, acted as a scavenger of free radicals in culture media, improved microtubule organization in M-II oocytes in a dose dependant manner, and subsequently increased the rate of GVBD and M-II during IVM of mouse oocytes. Positive effects of cysteamine treatment during IVM on developmental competence of pig oocytes were also observed (Grupen *et al*., 1995). In contrast, cysteamine supplementation during IVM had no significant effect on bovine oocyte maturation or fertilizability (Balasubramanian and Rho, 2007). However, Oyamada and Fukui (2004) used epidermal growth factor and cysteamine together for IVM and found no positive effect on nuclear maturation but an improvement in the cleavage rate and developmental competence of bovine oocytes, which could be attributed to increased GSH synthesis during IVM. However, the addition of cysteamine was only effective when oocytes were matured under low oxygen tension to stimulate the GSH synthesis; increased production of ROS under high oxygen tension may diminish the intracellular GSH concentrations.

Glutathione is the major sulphydryl compound in mammalian cells, which plays a critical role in protecting the cell from oxidative-stress damages (Gasparrini, 2002). Low molecular weight compounds, such as cysteamine, enhance cysteine-mediated GSH synthesis in bovine embryos (Takahashi *et al*., 1993). Addition of cysteamine to maturation medium enhances GSH synthesis and improves oocyte maturation (Gasparrini *et al*., 2003) probably by acting on the delicate process of cytoplasmic maturation (Gasparrini *et al*., 2000). Moreover, Mukherjee *et al*. (2010) reported that DNA damage in buffalo oocytes and embryos during the course of *in vitro* culture can be partly ameliorated by supplementation of culture media with cysteamine anti-oxidant. Lastly, cysteamine supplementation during *in vitro* embryo production up-regulates the expression of anti-apoptotic and down-regulates the expression of pro-apoptotic genes (Elamaran *et al*., 2012).

In this study, the percentages of viable oocytes and maturation rate were significantly lower in the two vitrification groups than in controls regardless of the presence or absence of cysteamine. This reduction in oocyte viability could be caused by several factors including toxic effects of cryoprotectants and/or ultrastructural damage to the oocytes post-cryopreservation, i.e., deleterious effects on DNA (Scholkamy *et al*., 2015), chromosomes and other cytoplasmic structures (Liu *et al*., 2003). The elevated lipid content of buffalo oocytes may also contribute to low maturation rates, as it has been reported that high

lipid content of oocytes makes them more sensitive to chilling injuries (Ledda *et al*., 2001).

In the present study, there were no differences in morphology, survivability and maturation rate between the two groups of vitrified oocytes that had been matured in the presence or absence of cysteamine before vitrification. Kelly *et al*. (2005) reported that cysteamine improved blastocyst formation rate of fresh ovine and vitrified bovine oocytes. Balasubramanian and Rho (2007) proposed that due mainly to the greater survival rate after chilling of blastocysts produced by IVM-IVF of oocytes matured in media supplemented with cysteamine, vitrification may be a preferred option for the short-term storage and transport of *in vitro* produced bovine embryos. However, Somfai *et al*. (2007) observed that the GSH content decreased significantly and H_2O_2 level was elevated in vitrified oocytes, and that these factors might contribute to the poor developmental competence of vitrified oocytes. Glutathione is known to play an important role in the anti-oxidant defense system of cells as an important scavenger of reactive oxygen species. In summary, the addition of cysteamine to the maturation medium improved nuclear maturation but had no positive effect on the cryoresistance of vitrified buffalo oocytes.

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Conflict of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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