Asian Pacific Journal of Reproduction

Journal homepage: www.apjr.net

Document heading doi: 10.12980/apjr.6.20170406

Effect of melatonin and/or cysteamine on development and vitrification of buffalo embryos

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ARTICLE INFO

Article history:
Received 10 March 2017
Received in revised form 16 April 2017
Accepted 20 April 2017
Available online 1 July 2017

Keywords:
Melatonin
Cysteamine
Buffalo
Oocyte
Maturation
Vitrification

ABSTRACT

Objective: To assess the effects of melatoninand/or cysteamineon *in vitro* maturation, culturing and post-warming of buffalo embryos. **Methods:** Buffalo oocytes were classified into control, cysteamine (50 μM), melatonin (10 ng/mL) and cysteamine (50 μM) + melatonin (10 ng/mL) treatment groups. In experiment 1, previous treatments were added during *in vitro* maturation and culturing of buffalo oocytes. **Results:** Cleavage and blastocyst rates were significantly (P<0.05) increased in melatonin treated group (70.5±0.9 and 12.8±1.0, respectively), however this effect was potentiated when combinedwith cysteamine (74.0±1.7 and 14.8±1.7, respectively). In experiment 2, the treatements were added in maturtaion, culturing as well as post-warming culture media. Embryos at 7 d were vitrified.Viability assessement directly after warming showed significant increase (P<0.05) in cysteamine, melatonin and their combination groups (76.8±2.8, 80.0±2.1 and 83.3±1.7, respectively) than control (65.8±2.4); but the viability after 24 h post-warming was the best in cysteamine + melatonin combination group (61.4±2.1). **Conculsions:** Enriching maturation, culturing and post-warming media of buffalo oocytes and embryos with melatonin and/or cysteamine have significantly beneficial effects on oocyte developmental competence as well as embryos vitrification procedure outcomes which in turn resulting in enhancement of commercial buffalo embryo production.

1. Introduction

The embryo implantation potential might be dropped to 40% due to side effects of cryopreservation[1]; this drop was mainly due to reduction in the cryopreserved embryos viability; which lagged behind that of *in vivo* derived embryos[2,3]. The reasons of this variation were partially understood. Recently, new researches prove that *in vitro* culture conditions affect oxidative homeostasis by increasing ROS exposure or lessening antioxidant protection resulting in poor quality of embryo developmental competence[4,5]. Furthermore, certain opinions reflected it to the cryopreservation process which promotedreactive oxygen species (ROS) production and accumulation[6,7]. Vitrification is rapidefficient technique for embryoscryopreserving[8]. However, successful vitrification involves certain critical points such as: freezing temperature, vitrification medium volumes[9,10], presence or absence of the antioxidants and cryoprotectants that are important detrimental factors during vitrification process[11]. It had been stated that variation in all preceding factors subjected the cryopreserved embryos to become more amenable to the deleterious effects of ROS[12,13]. So, it is important to protect live cells from oxidative stressors during culturing processes. A new approach is to supply the culture medium with antioxidant compounds. In between, Melatonin as indole participates in regulating mammal's biological clock[14]. This indole amine is multifunctional and universal hydrophilic and hydrophobic

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antioxidant[15]. Furthermore, melatonin metabolites also participate in the interactions with ROS; this phenomenon was referred as a scavenging cascade reaction[2]. Melatonin also stimulates the activity of some antioxidant enzymes including glutathione peroxidase and superoxide dismutase[16,17]. This in turn, resulted in reduction in blastocysts apoptosis and the average apoptotic cell numbers/ blastocyst in vitrified two-cell embryos[18]. Furthermore, melatonin prevents protein, DNA damage and lipid peroxidation as it has multiple antioxidant capacities[19]. Additionally, melatonin preserves optimal mitochondrial homeostasis and function by reducing and preventing mitochondrial ROS[20], thereby preventing cell death by limiting apoptotic events[21]. Moreover, melatonin was stated to enhance ROS detoxification during post warming process, where embryos require high metabolic activity to complete the resumption of DNA and protein synthesis[22]. This action may be chiefly effective for cryopreserved *in vitro* produced embryos, considering their poor cryotolerance[23]. Another example of the perfect antioxidants that could be used efficiently in maturation and culture media is cysteamine. Cysteamine is a thiol compound commonly added to IVM medium to improve the oocyte developmental competence through glutathione (GSH) synthesis[24]. GSH play important antioxidant roles besides it had important effects on amino acid transport, DNA and protein synthesis and disulphides reduction[25,26], by acting on delicate cytoplasmic maturation process[27,28]. Consequently, improved fertilization rate, developmental competence and cryoresistance following vitrification process[29]. Generally, vitrified oocytes were significantly of lower glutathione contents and of high ROS levels[30]; Furthermore, buffalo oocytes characterized by high lipid contents, those two factors exposed the oocyte or embryos to become more sensitive to oxidative damage[27]. Considering these beneficial effects of cysteamine and melatonin on oocyte maturation and embryonic development, there is still little work regarding their effects on embryo vitrification. So this study aimed to assess the effect of melatonin and/or cysteamine during *in vitro* maturation, development and post-warming process of buffalo embryos.

2. Material and methods

2.1.Cumulus-oocyte complexes (COCs) collection, selection and in vitro maturation

Buffalo ovaries were collected from the slaughter house in sterile modified Dulbecco's phosphate saline (D-PBS; pH 7.2) containing 100 μg/mL streptomycin and 100 IU/ mL penicillin at 37 ℃. They were washed in D-PBS then in normal saline. Oocytes were aspirated from follicles ranging between 3 and 7 mm in diameter using 18-gauge needle attached to a disposable 10-ml syringe within (3-5) h after the animals were slaughtered. Evenly granulated oocytes surrounded with at least two layers of compact cumulus cells and with homogenous cytoplasm were selected for the experiments. Selected COCs were first washed in sterile D-PBS, and then in IVM medium; IVM was performed in TCM-199 (Gibco) supplemented with 10% calf serum (Gibco) and 50 μg/mL gentamycin (G-1272 Sigma). The COCs were cultured in groups of 10-20 /100 μL drop of IVM medium in Petri-dishes (Nunclone, Roskilde, Denmark) with mineral oil (M-4810, Sigma) overlay for 22 h at 38.5 ℃, 5% CO_2 and 95% humidity. Melatonin (10 ng/mL) and cystamine (50 μM) solutions were prepared in TCM-199, filtered using 0.22-mm filters from Millipore Corporation (Millex GV Co., Cork, Ireland). Melatonin solution was kept at 4 ℃ until use for a maximum of 12 d. In IVM and IVC experiments, melatonin was added after testing the ideal concentration for buffalo oocyte maturation and embryo development that differ than cow[31, 32].

2.2. In vitro fertilization (IVF) and culture

Spermatozoa were treated according to Niwa K and Ohgoda O[33]. Frozen straws were thawed at (35–37) ℃ for 1 min in a water bath. The spermatozoa were washed by centrifugation at 800 g for 10 min in BO medium[34] containing10 μg/mL heparin and 2.5 mM caffeine without BSA (Sigma, St. Louis, MO). The sperm pellets were diluted with BO including 20 mg/mL bovine serum albumin to adjust the concentration of spermatozoa to 12.5×10^6 sperm. Oocytes were washed before introducing to the sperm in BO medium with 10 mg/mL BSA, then added into 100 μL droplets of diluted sperm (about 5–10 oocytes/droplet). The oocytes and spermatozoa were co-cultured at 5% CO_2, 38.5 ℃, 95% humidity for 5 h. After that, the oocytes were washed several times in TCM-199 to remove the attached spermatozoa. Groups of 10-20 oocytes were again replaced with previously prepared co-culture 100 μL droplet consisting TCM-199, 10% serum and 50 μg/mL gentamycin and the chosen concentration of melatonin and cystamine. Cleavage was assessed after 72 h of culture (day 0=day of insemination) and the embryos was recorded at the day 7[35].

2.3. Embryo vitrification and warming

The vitrification solutions were prepared in TCM 199 + 20% fetal calf serum. Embryos were vitrified in 1.75 M EG + 1.75 M DMSO for 2.5 min (step one). Then, in 3.5 MEG + 3.5 M DMSO for 45 s (second step) in 0.25 mL straws. Immediately, straws were cooled in liquid nitrogen vapor for 1 min before storing in liquid nitrogen for 1 wk. For warming, straws were held in air for 10 s, placed in water at 37 ℃ for 30 s, and flicked four to six times to mix columns.

Then, embryos were washed in 0.5 M galactose for 5 min at room temperature. Finally, the embryos were washed five times in TCM plus 5% FCS and cultured at 38.5 ℃, 5% CO_2 for further 24 h[36].

2.4. Survival assay

Embryos were examined morphologically after warming and the viability was evaluated by culturing for 24 h. The embryos developed to advanced stages, were considered to be survived. The morulae that developed into more advanced stage (blastocysts) and the blastocysts that re-expanded were considered as surviving[36].

2.5. Experimental design

Experiment 1: Effect of melatonin and/or cysteamine supplementation to IVM and IVC media on oocyte and embryo development. Oocytes were classified into four groups; control, 50 µM cysteamine, 10 ng/mL melatonin and 50 µM cysteamine + 10 ng/mL melatonin. The cysteamine and melatonin were added during the maturation and development of oocytes.

Experiment 2: Effect of melatonin and/or cysteamineon viability of vitrified embryos. All good embryos at day 7 were vitrified using mixtures of DMSO and EG in TCM-199 with two concentrations of cryoprotectants (3.5 and 7 M). The four groups of oocytes were matured and cultured as in the first experiment, and then the cysteamine and melatonin were added also at the post-warming culture media.

2.6. Statistical analysis

Three replicates per group were statistically analyzed by ANOVA using SPSS version 16.0 statistical software. A comparison of means was carried out by Duncan's Multiple Range test. Differences were considered to be significant at P<0.05 level.

3. Results

3.1. Effect of melatonin and/orcysteaminesupplementation to culture media on embryo development

Table 1 showed that the cleavage rates were significantly increase in melatonin (P<0.01) and cysteamine + melatonin (P<0.001) than control group. Moreover, there were significantly increase in blastocyst rates for melatonin (P<0.01) and cysteamine + melatonin (P<0.001) compared to control group. There were no significant differences in percentage of cleavage and blastocyst between melatonin and cysteamine + melatonin groups.

Table 1

Effect of melatonin and/or cysteamine on buffalo embryo development (nean±SE).

Treatment	No. of inseminated oocytes	Cleavage No* (%)	Blastocysts No* (%)
Control	210	126 (60.2±2.4^{c})	16 (7.4±1.2^{c})
Cysteamine	204	134 (66.1±1.9^{bc})	18 (9.1±1.2^{bc})
Melatonin	232	164 (70.5±0.9^{ab})	30 (12.8±1.0^{ab})
Cysteamine + Melatonin	176	130 (74.0±1.7^{a})	26 (14.8±1.7^{a})

*Values with different superscripts within the same column differ significantly (P< 0.01 - P< 0.001). No. = number.

3.2. Effect of melatonin and/orcysteamine on viability of vitrified embryos.

The viability of vitrified buffalo embryos directly after warming was significantly increase in cysteamine (P<0.01), melatonin (P< 0.01) and cysteamine + melatonin (P<0.001) than control group. But there were no significant differences among the cysteamine, melatonin and cysteamine + melatonin groups in viability directly after warming (Table 2). Furthermore, there was significant increase in viability of embryos after 24 h post warming in cysteamine + melatonin (P<0.001) than other groups including the control. But, there were no significant differences among control, melatonin and cysteamine groups in viability 24 h post warming.

Table 2

Effect of melatonin and/or cysteamine on viability of vitrified buffalo embryo (mean±SE).

Treatment	No. of vitrified-warmed embryos	Morphologically normal embryos directly after warming No (%)	Post-warming development after 24 h (viability) No (%)
Control	86	56 (65.8±2.4^{b})	39 (47.3±2.6^{b})
Cysteamine	69	52 (76.8±2.8^{a})	29 (52.9±1.6^{b})
Melatonin	270	57 (80.0±2.1^{a})	38 (53.4±0.7^{b})
Cysteamine + Melatonin	76	63 (83.3±1.7^{a})	47 (61.4±2.1^{a})

Values with different superscripts within the same column differ significantly (P< 0.01 - P< 0.001). No. = number.

4. Discussion

In the current work, the rates of cleavage and blastocyst were significantly increased (P<0.01) when melatonin was added to the maturation and culture media of buffalo oocytes. These results came in agreement with El-Raey *et al*, El-Raey *et al*, Manjunatha *et al*, Cebrian-Serrano *et al*[31,32,37,38] who reported beneficial effects of melatonin when supplemented to *in vitro* maturation and culturing media of buffalo and cowembryos. These results were

attributed to the efficient anti-oxidative activity of melatonin. Where, melatonin was found to directly interact with ROS and stimulates the activity of some antioxidant enzymes as superoxide dismutase, glutathione peroxidase, and catalase[16,17]. Also, melatonin prevents lipid peroxidation[39]; protein, and DNA[19,40]. In addition, it was found that melatonin preserve the function of mitochondria through reducing and preventing ROS[20], as well as enhancing mitochondrial distribution activity and patterns during nuclear maturation phase[31], and preventing or reducing apoptosis level[21]. Lately, it had been stated that melatonin can ensure its valuable effects through activation of MTNR1A receptorin both granulosa cells and oocyte[31,32], and MTNR1B receptor only in cumulus cells[31,32,41]. In the present study, viability of buffalo embryos directly after thawing was significantly increased ($P<0.05$) in cysteamine, melatonin and cysteamine+melatonin groups than control. But in cysteamine+melatonin combination group, the viability at 24 h post thawing was the best. It was reported that presence or absence of the antioxidant are important determinable factors during vitrification process[11,30].

Melatonin beneficial effects on buffalo embryo development during post-warming culture were mainly due to oxidative index reduction[23]. It had been reported that ROS detoxifications play a major role in restoring embryo metabolic functions after warming[12]. For the first time, our results demonstrated the synergistic effect between both melatonin and cysteamine as antioxidant. This synergistic effect has essential role for improving buffalo embryo development and cryotolerance. These results came partially in accordance with Manjunatha *et al*[37] who reported that melatonin supplementation to culture medium of buffalo embryos significantly improved its development. Moreover, Kelly *et al*[42] added that the supplementation of cysteamine to the maturation medium improved blastocyst rate of fresh ovine and vitrified bovine oocytes. Also, Oyamada *et al*[29] found that cysteamine improved the development of vitrified bovine oocytes. On the opposite side, Mahmoud *et al*[43] found no effect for cysteamine on the cryoresistance of oocytes during vitrification in spite of improving the *in vitro* oocytes maturation.

The current study showed that the perfect dose for buffalo oocytes maturation and culturing was 10 ng/mL reflecting that melatonin has species/dose dependent effect[31,32]. On conclusion, enriching maturation, culturing and vitrification media of oocytes and embryos with melatonin and/or cysteamine had significantly beneficial effects on buffalo oocyte developmental competence and embryos vitrification procedure outcomes.

Conflict of interest statement

We declare that we have no conflict of interest.

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