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Effect of the 2-Mercaptoethanol on Nili Ravi Buffalo Oocytes During *In Vitro* Maturation

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Abstract: The present study was conducted to investigate the effect of 2-Mercaptoethanol (β-ME) on Nile Ravi buffalo oocytes. Cumulus Enclosed Oocytes (CEOs) and Cumulus Denuded Oocytes (CDOs) were cultured for 24 hrs in TCM-199 supplemented with Recombinant Human Follicular Stimulating Hormone (RhFSH), 0.4% Bovine Serum Albumin (BSA), oestradiol and Gentamycin. Nili Ravi buffalo oocytes with or free from cumulus cells were exposed to 25 μM 2-ME. Meiotic maturation and degeneration of oocytes was assessed at different maturation time (0, 12, 24 h). CDOs treated with 25 μM 2-ME resumed meiosis and proportion of MII oocytes were significantly ($P < 0.05$) increased where as CDOs matured in control medium with out 25 μM 2-ME did not show any significant increase in MII oocytes proportion as the maturation time was extended to 24 h ($p<0.05$). Similarly CDOs treated with 25 μM 2-ME significantly reduced the proportion of degenerated oocytes (7.31 %) against control group in which 21.4 % CDOs degenerated. In present study it was observed that cumulus cells are involved in protection against degeneration as CEOs treated in control medium the degenerated oocytes high significantly ($p<0.05$) decreased to 7.40 % as compared to CDOs in which 21.4 % oocytes were found to be degenerated. In present study the proportion of MII oocytes was significantly increased to 81.81 % when CEOs were treated with 25 μM 2-ME against CDOs treated with 25 μM 2-ME after 24 hrs of culture. These results indicates that exposure to 25 μM 2-ME promote the development of the Nili Ravi buffalo CDOs and CEOs and cumulus cells are involved in protection against degeneration of oocytes.

Key words: *In vitro* maturation, mercaptoethanol, oocyte, MII

INTRODUCTION

The *In vitro* Maturation (IVM) system is designed to increase the chance of oocyte maturation occurring without the presence of the follicle. IVM portion of the -*in vitro* system is designed to closely resemble that which occurs *in vivo*. Currently, the conditions *in vitro* are inferior to those found *in vivo* as evidenced by low intracellular concentrations of glutathione (GSH), inadequate oocyte maturation and the inability to assess/screen for superior oocytes[1]. One factor that is thought to have a negative impact on the *in vitro* development of the embryo is oxidative stress. Reactive Oxygen Species (ROS), including Superoxide anions, hydrogen peroxide and hydroxyl radicals can cause damage to DNA and induce lipid peroxidation which adversely affects membrane structure, fluidity and function[2] Oocyte plasma membranes are susceptible to attack by oxidants because the phospholipid of the plasma membrane contains a significant proportion of esterified polyunsaturated fatty acids (PUFA) which are particularly sensitive to oxidative reactions. An increased level of free radicals has been detected in embryos cultured *in vitro*.[3-5] It is generally accepted that cumulus cells during the maturation period support *in vitro* maturation (IVM) of oocytes to the metaphase-II (MII) stage and are involved in the cytoplasmic maturation needed for developmental competence of post fertilization such as male pronucleus formation in porcine oocytes[6].

Bovine Cumulus Enclosed Oocytes (CEOs) have a higher content of intra cellular GSH than cumulus denuded oocytes (CDOs) (). GSH plays an important role in the redox state of cells and protecting cells against harmful effects of oxidative injury (cattle)[7,8] GSH also provides a reducing environment for oocytes and embryos against oxidative damage. Elevated levels of GSH are reported to have beneficial effects on the oocytes and promotion of the oocyte-spermatozoon complex to develop a MPN after IVF[9]. It is further demonstrated that GSH functions in DNA and protein synthesis and amino acid transport inside maturing oocytes[10]. A lack of GSH in oocytes prior to fertilization results in the inability to form a MPN after fertilization[11,12]. Synthesis of GSH during oocyte maturation has been reported in the oocytes of mouse, hamster, pig and cattle[9]. Cumulus cells

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were found to synthesize large amounts of GSH in the hamster[1] and pig[13]. An improvement in bovine embryo production *in vitro* by GSH supplementation has been observed[14].

In addition, the thiol compound β-mercaptoethanol (β-ME) has been reported to increase blastocyst formation rate of IVM, IVF bovine embryos. The mechanism of action through which β-ME exerts its effect on embryo development is not yet known. It is possible that β-ME is itself an antioxidant and removes free radicals. Alternatively, it has been proposed that β-ME may act by increasing intracellular GSH[15]. Induction of GSH synthesis in bovine[16] or porcine[12] oocytes with beta-mercaptoethanol and cysteine during *in vitro* maturation improved cleavage rates and embryo development. Conversely, reduced GSH levels contributed to the impaired growth of bovine embryos from oocytes matured in high-glucose media[17].

Evidence was found confirming that GSH synthesis occurs intracelluarly during IVM of oocytes and is stimulated by cysteamine, beta-mercaptoethanol and cysteine. This increase in GSH levels during IVM improves embryo development and quality, producing more embryos reaching the blastocyst stage on day 6, those most suitable for freezing. Previous Results demonstrated that cysteamine, but not β-mercaptoethenol, when present during IVM, stimulates sheep embryo development; both cysteamine and β-mercaptoethenol stimulate GSH synthesis; the increase in intracellular GSH is associated with a decrease in peroxide level within oocytes[18].

Prepubertal goat oocytes synthesize GSH during IVM and that thiol compounds increase this GSH synthesis[19]. It is reported that β-ME increases cell number of bovine blastocyst produced *in vitro*[20]. In pig certain concentration of β-ME in IVM medium has beneficial effects on subsequent embryo development and is correlated with intracellular GSH level[11].

The present study was therefore carried out on supplementation of β-mercaptoethanol to TCM-199 medium and its subsequent effect on oocyte maturation and function of cumulus cells during *in vitro* maturation of Nili Ravi buffalo oocytes.

MATERIALS AND METHODS

Reagents and chemicals: TCM-199, 2-Mercaptoethanol, Estradiol, Glutamine, BSA, as well as other chemical were purchased from Sigma Chemical Co. (St Louis, MO, USA), unless otherwise stated.25 μM 2-mercaptoethanol solution was freshly prepared and the pH value of the medium was maintained just prior to incubation of oocytes.

Oocyte collection: Nili Ravi buffalo ovaries, obtained at a local slaughter house, were transported to the laboratory in 0.9 % (w/v) NaCl containing 50 μg mL streptomycin and 100 U mL penicillin G within 2 h of post-slaughter. The follicular contents were recovered by aspiration the visible small antral follicles on the ovarian surface using a 5 mL syringe equipped with an 18-gauge needle. The mixture was collected in a beaker containing PBS containing 0.4 % BSA, 100 U mL Penicillin and 0.1 mg mL glutamine. Sedimentation for 15 min. only the intact CEOs with a compact and multilayer cumulus were used in the study. In experiments requiring denuded oocytes, those CEOs had their cumulus cells removed by gentle pipetting. Subsequently the CEOs CDOs in PBS were placed in separate 4-well plates by narrow bore pipette. Under a phase contrast microscope (Nikon Japan), the oocytes were three time washed in TCM-199 supplemented with 0.1/mg glutamine, 0.4% BSA, 50 μg mL gentamicin. In order to wash the oocytes completely, the oocytes were transferred between the 4-wells by mouth pipette and oocytes were transferred from one well to the next leaving all debris behind.

***In vitro* maturation:** The basic medium for maturation culture of oocytes was TCM-199, supplemented with 0.05 IU mL recombinant human FSH (Organon,Oss, The Netherlands) , 1 μg mL estradiol, 0.4% BSA, 50 ìg mL gentamycine. The pH value of the medium was adjusted to 7.4 and the solution was sterilized by 0.22 μm filter. After washing the oocytes in basic medium, each group of CEOs and CDOs was individually placed into 100 μl droplets of the basic medium covered with mineral oil that was previously equilibrated in CO_2 incubator. The oocytes were placed into micro drops by micropipette which was adjusted to 2 μl and autoclaved tips were utilized. The culture was carried out at 37°C in an atmosphere with 5% CO_2 in air for 24 h.

Examination of the nuclear phase: In all experimental groups the nuclear examination of CDOs was assessed at 0 hrs or one hrs after placing into maturation medium. The same observation was repeated at 12 and 24 hrs of maturation. Nuclear examination of the CEOs was assessed at 24 hrs of *in vitro* culture after removing the surrounding cumulus cells by pipetting.

Oocytes were observed and evaluated morphologically at×20 magnification under a phase contrast microscope, for nuclear status. Which often have been accepted to evaluate the meiotic maturation of mammalian oocytes[20,21,22]. Oocyte with a single prominent nucleus was classified as being in the germinal vesicle (GV), oocyte that has underwent germinal vesicle breakdown but no polar body were classified as germinal vesicle break down (GVBD) stage and oocyte with a polar

body were classified as being in the meta phase II (MII) stage of maturation process.

Experimental design

Experiment 1: In this experiment, oocytes were cultured in TCM-199 without the supplementation of 25 μM 2-Mercaptoethanol was designated control group.

Experiment 2: In this experiment, Oocytes were matured in TCM-199 supplemented with 25 μM 2-Mercaptoethanol was designated treated group.

Statistical Analysis: All percentage values of nuclear status among the treatments in each experiment and morphological differences were analyzed by applying t-test. Statistical package,
STATISTICA was used for statistical analysis. Probability level $p<0.05$ was considered as statistically significant.

RESULTS

Meiotic resumption of oocytes matured in control medium: Results of oocytes meiotic maturation are given in Table 1. CDOs matured in control medium showed 97.6 % GVBD and 3.38 % MI stage oocytes at 0 h. At 12 hrs GVBD oocytes were 23.8%. The percentage of MI stage oocytes at 12 hrs was 35.71% and 28.5 % CDOs progressed to MII stage, the incidence of degenerated oocytes was found to be 11.9 % (Table 1).

After 24 h of maturation, no difference was found regarding GVBD, MI and MII oocytes percentage when compared with GVBD, MI and MII oocytes percentages at 12 hrs (Table 1).

CEOs matured in control medium showed 29.62 % MI, There was no significant difference found between the percentages of MII oocytes in either CEOs or CDOs (62.96 % and 40.47, respectively) treated as control after 24 hrs of maturation ($p = 0.072$). The protective effect of the cumulus cells against degeneration of oocytes was observed when CEOs were matured in control medium (without antioxidants), highly significantly ($p = 0.000$) lower percentage of degenerated oocytes (7.40 %) was observed as compared to CDOs (21.4%) matured in control medium after 24 hrs of maturation.

Effect of 2-Mercaptoethanol on Meiosis resumption : CDOs treated with 2-ME showed 92.68 % GVBD and 7.31 % MI stages oocytes at 0 hrs (Table 2). At 12 hrs of maturation the oocytes were progressed to further stages and the percentage of GVBD was decreased to 12.19 %. The proportion of MI stage oocytes was 58.53, 24.39 % MII stage oocytes and 4.87 % were found to be degenerated. Due to the progression of oocytes to further stages the percentage of GVD became 0.00% at 24 h of maturation. The percentage of MI stage oocytes was 43.90% which is statistically not different from the percentage of MI oocyte at 12 hrs ($P=0.0188$). 48.87% CDOs were progressed to MII stage which was significantly ($P=0.024$) different from the percentage of MII stage CDOs at 12 hrs (24.39%).

The results listed in Table 2 show, that CEOs treated with 2-ME showed 13.63% MI, 81.81% MII and 4.54% degenerated oocytes. The Maturation rate of CEOs at the metaphase II stage was significantly ($P=0.013$) increased to 81.81% by treatment with 2-ME, compared with that of CDOs (48.78%) after 24 hrs of maturation. Similarly significant ($P=0.028$) difference was observed among the degenerative oocytes in both CEOs (4.45%) and CDOs (7.31%) after 24 hrs of culturing.

DISCUSSION

The present study revealed that when buffalo CDOs were culture in TCM-199 without supplementation of β-mercaptoethanol (control), they resolved their nuclear membrane and resumed meiosis. In present study when Nile Ravi buffalo CDOs were matured in control medium without antioxidants non-significantly ($p = 0.2518$) difference was found between the percentage of MII stage CDOs on both 12 h (28.5%) and 24 h of maturation (40.47%).

Table 1: Meiotic maturation of CDOs and CEOs cultured in control medium at different incubation time

Type of oocyte	n	Time	GVBD n (%)	MI n (%)	MII n (%)	Degenera-ted n (%)
CDOs		0 h	41 (97.6)	1 (3.38)	-	-
	42	12 h	10 (23.8)	15 (35.71	12 (28.5)	5 (11.9)
		24 h	5 (11.90)	11 (26.19)	17 (40.47	9 (21.4)
CEOs	27	24 h	8 (29.62)	17 (62.96)	2 (7.40)^{b***}	

GVBD, germinal vesicle breakdown; MI, metaphase I; MII, metaphase II, CEOs, cumulus-enclosed oocytes; CDOs, cumulus denuded oocytes, b = When 24 hrs CEOs compared with 24 hrs CDOs, ***$p<0.0$

Table 2: Effect of 2-ME on meiotic resumption and degeneration of Nile Ravi buffalo CEOs and CDOs

Type of oocyte	n	Time	GVBD n (%)	MI n (%)	MII n (%)	Degenera-ted n (%)
CDOs		0 h	38 (92.68)	3 (7.31)	-	-
	41	12 h	5 (12.19)	24 (58.53)	10 (24.39)	2 (4.87)
		24 h	-	18 (43.90)	20 (48.78)^{a*}	3 (7.31)^{ac*}
CEOs	22	24 h	-	3 (13.63)	18 (81.81)^{b**}	1 (4.54)

a = When 24 hrs CDOs compared with 12 hrs CDOs, b = When 24 hrs CEOs compared with 24 hrs CDOs, ac = When 24 CDOs compared with 24 hrs CDOs in control group, **p<0.02, *p<0.05

In contrast when Cumulus-Enclosed Oocytes (CEOs) were matured in TCM-199 medium, most of the CEOs reached to MII stage (62.96%). Our results have some agreement with previous studies that cumulus cells during the maturation period support *In vitro* Maturation (IVM) of oocytes to the metaphase-II stage and are involved in the cytoplasmic maturation needed for developmental competence of post fertilization such as male pronucleus formation in porcine oocytes[6].

In present study the protective role of the cumulus cells resulted into highly significantly (p = 0.001) lower percentage of degenerative oocytes against CDOs, which is in close accordance with the previous results on porcine[23]. This lower percentage of degenerated oocytes might be associated with diminished ROS level and increase concentration of antioxidant enzymes in CEOs. In bovine diminished level of Reactive Oxygen Species (ROS) activity and increase level of glutathione peroxidase, Superoxide dismutase (SOD) and catalase activity were detected in cumulus oocytes, which is lower in denuded oocytes with respect to cumulus[24]. It is demonstrated that cumulus cells have a critical role in protecting oocytes against oxidative stress-induced apoptosis through the enhancement of glutathione (GSH) content in oocytes[23]. This increase concentration of Glutathione protects cell membranes by providing a reducing environment to prevent cell membrane damage from circulating oxidants[25]. Previous studies have shown that high concentration of glutathione is found in CEOs compared to CDOs[26].

In present study Slightly higher percentage of MII oocytes (62.96%) was observed compared to CDOs (40.47%) but no statistically difference was found between the both groups (p = 0.072). The cumulus cells benefit bovine oocyte development either by secreting soluble factors which induce developmental competence or by removing an embryo development-suppressive component from the medium[27]. The present study indicates that cumulus cells protect Nile Ravi buffalo oocytes from degeneration and increase meiotic maturation which is in agreement with previous studies[23,24,25,27].

When the buffalo CDOs were cultured in TCM-199 supplemented with 25 μM β-ME significantly (p = 0.024) increased MII oocytes percentage (48.78%) was observed after 24 hrs of culturing. The β-ME exerted beneficial effect on oocytes protection against degeneration as highly significantly (p = 0.000) lower percentage of degenerated oocytes (7.31%) was observed compared to control group (21.4%). β-ME stimulate GSH synthesis; the increase in intracellular GSH is associated with a decrease in peroxide levels within oocytes[18]. In cattle GSH play an important role in maintaining the redox state of cells and protecting cells against harmful effects of oxidative injury[8]. There was no significant (p = 0.448) difference found among the MII oocytes proportion either in control or β-ME group. However in present study, the β-ME exerts beneficial effect on oocytes protection against degeneration. The protective role of cumulus cells observed during the present study. CEOs matured in TCM-199 supplemented with 25μM β-ME significantly (p = 0.013) increased MII oocytes percentage (81.81%) as compared to CDOs treated with β-ME (48.78%). The present study showed that supplementing the TCM-199 with β-ME accelerates meiotic maturation of Nile Ravi buffalo CEOs and provides protection against degeneration of oocytes. It has been reported that cumulus cells are involved in the cytoplasmic maturation of oocytes followed by the acquisition of developmental competence[28,29,30,31]. Cumulus cells play an important role on the GSH synthesis enhanced by addition of cysteine and cysteamine during bovine oocyte maturation *In vitro*[32]. The beneficial effect of the glutathione is well understood in previous studies, which is major nonproteinous sulfhydryl compound in mammalian cells and is well known to play an important role in protecting the cell from oxidative damage[7]. Cysteamine and beta-mercaptoethanol supplementation of *In vitro* Maturation (IVM) medium has been found to increase intracellular glutathione (GSH) content in oocytes and to improve embryo development and quality in several species[18]. At certain concentrations β-ME in IVM medium has beneficial effects on subsequent embryo development and is correlated with intracellular GSH level in pig oocytes. Higher (p<0.01) level of GSH was found in oocytes matured with 25 μM β-ME[11]. β-ME stimulates GSH synthesis; the increase in intracellular

GSH is associated with a decrease in peroxide levels within oocytes[18]. In the present study it is concluded that 25 μM β-ME increase MII oocytes proportion specially in cumulus enclosed oocytes and protect the oocytes from degeneration caused by the Reactive oxygen species during *In vitro* maturation. Supplementation of β-ME to maturation medium will be beneficial, as most of the oocytes even from dead animal can be utilize for embryo production and for conservation of endangered species.

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