Combination of L-Ascorbic Acid and 2-Mercaptoethanol Reduces the Damaging Effect of Hydrogen Peroxide During in Vitro Maturation of Nili Ravi Buffalo Denuded Oocytes

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***Abstract*—The objective of this study was to evaluate the anti-oxidative effect of L-ascorbic acid (AsA) and 2-mercaptoethanol (2-ME) on *in-vitro* maturation of Nili Ravi buffalo cumulus enclosed oocytes (CEOs) and cumulus denuded oocytes (CDOs). The medium without any supplementation was used in control group. In treatment groups the medium was supplemented in following combinations (i) 100 <mu>M H_2O_2 (ii) 100<mu>M H_2O_2 + 25 <mu>M 2-ME + 250 <mu>M AsA (iii) 100<mu>M H_2O_2 + 25 <mu>M 2-ME (iv) 100<mu>M H_2O_2 + 250 <mu>M AsA. In absence of H_2O_2 the degeneration at 24 hrs was lower. The combined additives reduced the degeneration of oocytes and increased the proportion of MII CDOs to similar levels to those achieved by CEO. Cumulus cells protect oocytes from oxidative stress. The supplementation of medium with 100<mu>M H_2O_2 + 25 <mu>M 2-ME + 250 <mu>M AsA, for the culture of denuded oocytes effectively counteracts the oxidative stress.**

***Index Terms*—in vitro maturation, reactive oxygen species; MII, cumulus cells, denuded oocytes.**

I. Introduction

There has been an increasing interest of *in vitro* production of buffalo embryos for research and commercial purpose [1]. The *in vitro* maturation (IVM) system is designed to mature the oocyte in the absence of the follicle.

The *in vitro* maturation conditions are inferior to those found *in vivo* as indicated by low intracellular concentrations of glutathione (GSH), inadequate maturation of oocytes [2] and deleterious effects of reactive oxygen species (ROS), which subsequently may alter embryo development [3]. The plasma membranes of oocytes are affected by oxidants because a significant proportion of esterified polyunsaturated fatty acids (PUFA) in phospholipid of the plasma membrane which are particularly sensitive to oxidative reactions. Oxidative stress can occur if the production of reactive oxygen species (ROS) reach to a lethal level that may lead to oocytes deterioration and thereby affect reproductive outcomes [4]. The imbalance between the production of reactive oxygen species (ROS) and their elimination by the host's defense antioxidant systems is termed as oxidative stress and this leads to a toxic effect by damaging the cell and its components [5]. It is reported that in vivo, reactive oxygen species in follicular fluid deteriorates oocyte quality and reduces reproductive outcome [6]. An increased level of ROS has been detected during in vitro maturation and in embryos cultured in vitro [7], [8]. Imbalance between ROS and antioxidants can cause oxidative stress [9]. The ratio of which could change with increased level of ROS, or decrease in antioxidants defense mechanism [10], [11]. This increase ROS level on the other hand disrupt cellular redox circuits, result in disturbance in cellular process and damages cellular macromolecules. [12]. Hydrogen Peroxide can damage genomic DNA able to diffuse through hydrophobic membranes and produce more reactive species [13], [14]. Hydrogen peroxide (H_2O_2) released by one of the cells may, like exogenous H_2O_2, act as a chemical signal inducing the onset of the dying process in its neighboring cells [15]. The damaging effects of ROS may be protected by antioxidants [16]. Recent reports have shown that addition of cysteamine and b-mercaptoethanol to IVM media improved the cytoplasmic maturation of oocytes and embryo development by increasing GSH synthesis [17]-[19]. Antioxidant vitamins and glutathione may help to reduce oxidant damage [20], [21]. The natural antioxidants which are composed of catalase, glutathione reductase, glutathione peroxidase and superoxide dismutase, prevents the damaging of cellular structure by neutralizing excessive ROS. They cause reduction of hydrogen peroxide to alcohol and water [22]. It is

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reported that ascorbate is concentrated in granulosa cells, theca cells, luteal cells, and oocytes. The beneficial role for ascorbic acid in protecting spindle structures of MII mouse oocytes and chromosomal alignment against hydrogen peroxide-induced damage [23]. It is suggested that the effect of vitamin C is associated mainly with its capability to promote ooplasmic maturation during IVM. Previous studies have shown that AsA enhances porcine oocyte developmental competence [24], from Metaphase I (MI) to Metaphase II (MII) stage and prevents fragmentation of cumulus cells [25]. During in vitro maturation of mouse, hamster, pig and cattle the synthesis of GSH has been reported [26]. The large amounts of GSH in the cumulus cell of hamster was found to synthesize [27] and the depletion of which causes an increase in concentration of hydrogen peroxide as well as DNA lesions in bovine embryos [28]. In addition, the thiol compound β-mercaptoethanol (β-ME) has been reported to increase blastocyst formation rate of IVM, IVF bovine embryos.

Buffalo oocytes matured in medium supplemented with 2-mercaptoethanol increases intracellular glutathione level [29]. and similarly increase MII stage oocytes [30]. In general, cumulus expansion and oocyte maturation is regulated by follicle-stimulating (FSH) hormone and (LH) hormone [31], [32]. Cumulus expansion and oocyte maturation is induced by EGF-like factors produced by granulosa after the LH surge [33]. To understand the mechanisms that regulate expansion of cumulus will thus lead to improvement of conditions for mammalian oocytes to be matured and fertilized in vitro [34]. The cumulus enclosed oocytes (CEO) have a higher content of intra cellular GSH than cumulus denuded oocytes (CDO) [35], [36]. In bovine the CDO can be matured and fertilize *in vitro* up to the 8- to 32-cell stage, morula, or blastocyst stage [37].

ROS such as hydrogen peroxide may induce aging process of oocytes and increase production mediate oocytes dysfunction [38], [39]. Therefore, the objective of this study was to examine the protective effects of 2-mercaptoethanol (2-ME) and AsA on cumulus enclosed and denuded ooocytes of Nili Ravi buffalo cultured *in vitro*

II. Methods and Materials

A. Chemicals and Reagents Used

Ascorbic acid (Merck Germany; hydrogen peroxide was the product of BDH. TCM-199, 2-Mercaptoethanol, Estradiol, Bovine serum albumen (BSA) Glutamine were purchased from Sigma Chemical Co. (St Louis, MO, USA). Freshely prepared solutions were 100 <mu>M hydrogen peroxide, 25 <mu>M 2-mercaptoethanol and 250 <mu>M ascorbic, before in vitro culturing of oocytes the pH of all the culture media was adjusted.

B. Oocyte Collection

The ovaries of slaughtered buffaloes were collected from slaughter house and immediately transported to the laboratory in 0.9 % (w/v) NaCl which contained 50 µg/ml streptomycin, and 100 U/ml penicillin. The follicular fluid was collected from the small antral follicles by aspiration method using a 5 ml syringe with an 18-gauge needle. The contents were collected in a beaker containing PBS, BSA (0.4%), Penicillin (100 U/ml) and glutamine (0.1 mg/ml). The sedimentation was allowed for 15 minutes only the intact (cumulus enclosed oocytes) CEO with multilayer cumulus were utilized for this study. In experiments requiring denuded oocytes, cumulus cell was removed by gentle pipetting. Then the CEOs and CEOs were placed separately in 4-well plates by mouth pipette. Cumulus oocytes complexes and denuded oocytes were washed three time in TCM-199 supplemented with glutamine (0.1/mg), BSA (0.4%), 50 gentamicin (µg/ml) by transferring them between the 4-wells by using mouth pipette and leaving all debris behind.

C. In Vitro Culture of Oocytes

The oocytes were cultured in TCM-199 containing recombinant human FSH (0.05 IU/ml; Organon,Oss, The Netherlands), estradiol (1 µg/ml), BSA (0.4 %), gentamicin (50 µg/ml). The solution was filtered by 0.22 µm Millipore filter and pH value maintained at 7.4. Each washed group of 15 CEO and 15 CDO was separately placed in droplets (100 µl) of the basic medium covered with mineral oil. The maturation of oocytes was carried in CO_2 incubator (5% CO_2) at 37 ^{O}C with for 24 h.

D. Study of Nuclear Phase

The nuclear examination of CDOs was observed at 0 hrs, 12 hrs and then 24 hrs of culture. Examination of CEOs was carried out at 24 hrs of in vitro culture after uncovering the surrounding cumulus cells by pipetting. The morphology of oocytes was observed under a phase contrast microscope at X 20 magnification for nuclear status [40], [41]. The oocytes were categorized as germinal vesicle (GV) stage with single prominent nucleus, germinal vesicle break down (GVBD) stage oocyte that has undergone vesicle breakdown but no polar body and the meta phase II (MII) stage oocyte with an omitted polar body.

E. Experimental Design

1) Experiment 1

The CEO and CDO were cultured in TCM-199 without the addition of additives and hydrogen peroxide (control medium) to evaluate the degree of maturation and degeneration.

2) Experiment 2

It was designed to examine the effect of supplementing the maturation medium with 100 <mu> hydrogen peroxide on meiotic maturation and degeneration of CEO and CDO.

3) Experiment 3

This experiment was conducted to evaluate the degree of protection against the oxidative stress cause by 100 <mu> hydrogen peroxide provided by supplements of 25 <mu> 2-ME and 250 <mu> AsA during the maturation of CEO and CDO.

4) Experiment 4

The protection provided by 25 <mu>2-ME against the stress caused by 100 <mu> hydrogen peroxide during maturation of CEO and CDO was studied in this experiment.

5) Experiment 5

The protection provided by 250 <mu>M AsA against the stress caused by 100 <mu>M hydrogen peroxide during the maturation of CEO and CDO was studied in this experiment.

F. Statistical Analysis

The percentage of MII and degenerated oocytes were examined by percentage t-test between control and treated group. P< 0.05 was considered as significant.

III. Results

1) Experiment 1. Meiotic Maturation of Oocytes Matured in Control Medium

This experiment was carried out in the control medium without the addition of hydrogen peroxide and other antioxidants. Development of MII after 24 h was higher in CEO than CDO (62.9% versus 40.4%; NS). CDO had a significantly higher proportion of degenerate oocytes than CEO (21.4% versus 7.4%: P<0.01) at this time. CDO was in degenerate state 12 h after culture began since 11.9% of these oocytes were degenerate at that time.

TABLE I. Nuclear Status of Nili Ravi Buffalo Cumulus Denuded (Cod) and Cumulus Enclosed (Ceo) Oocytes Cultured in Control Medium at Different Time of Culture Period

Type of oocytes	n	Time	GVBD N (%)	MI N (%)	MII N (%)	DEG N (%)
CDOs	42	0 hrs	41 (97.6)	1 (3.3)	-	-
		12 hrs	10 (23.8)	15 (35.7)	12 (28.5)	5 (11.9)
		24 hrs	5 (11.9)	11 (26.1)	17 (40.4)	9 (21.4)
CEOs	27	24 hrs	-	8 (29.6)	17 (62.9)	2 (7.4)

2) Experiment 2. Effect of 100 <mu>M hydrogen peroxide on meiotic resumption Experiment 2.

Development to MII after 24 h maturation in medium supplemented with hydrogen peroxide alone was higher for CEO than CDO (43.2% versus 30.4%; NS). CEO had a significantly lower proportion of degenerate oocytes than CDO (21.6% versus 45.7%; P < 0.05) in this medium at this time.

TABLE II. Effect of Adding 100 <mu> Hudrogen Peroxide to the Maturation Medium on the Resumption of Meiosis and Degeneration of Cumulus Enclosed (ceo) and Cumulus Denuded (cdo) Nili Ravi Buffalo Oocytes

Type of oocyte	n	Time	GV n (%)	GVBD n (%)	MI n (%)	MII n (%)	Deg n (%)
CDOs	46	0 h	9 (19.5)	37 (80.4)	-	-	-
		12 h	-	13 (28.2)	14 (30.4)	5 (10.8)	14(30.4)
		24 h	-	6 (13.0)	5 (10.8)	14 (30.4)	21 (45.6)
CEOs	37	24 h		4 (10.8)	9 (24.3)	16 (43.2)	8 (21.6)

3) Experiment 3. Effect of 25 <mu>M 2-Mercaptoethanol and 250 <mu> L-ascorbic acids in the presence of hydrogen peroxide on meiotic resumption

The results for experiment 3 showed that the addition of 25 <mu>M 2-ME and 250 <mu>M AsA to maturation media supplemented with hydrogen peroxide had the effect of minimizing the differences between the treatments. The proportion of oocytes achieving MII was 55.6% for CEO and 57.9% for CDO after 24 h maturation in this medium. There was lower proportion of degenerate oocytes in the CEO treatment (11.1%) than the CDO treatment (15.8%) after 24 h culture but the difference was not significant.

TABLE III. Effect of Adding 25 <mu> M 2-Mercaptoethenalol, 250 <mu> M L-ascorbic Acid and 100 <mu> M Hydrogen Peroxide to Maturation Medium on the Meiotic Resumption and Degeneration of Nili Ravi Buffalo Cumulus Enclosed (ceo) and Cumulus Denuded (cdo)

Type of oocyte	n	Time	GV n (%)	GVBD n (%)	MI n (%)	MII n (%)	Deg. n (%)
CDOs	38	0 h	6 (15.7)	32 (84.2)	-	-	-
		12 h	1 (2.6)	6 (15.7)	13 (34.3)	12 (31.5)	6 (15.7)
		24 h	-	5 (13.15)	5 (13.1)	22 (57.8)	6 (15.7)
CEOs	18	24 h	-	3 (16.6)	3 (16.6)	10 (55.5)	2 (11.1)

4) Experiment 4. Effect of 25 <mu>M 2-mercaptoethanol in the presence of 100 <mu>M hydrogen peroxide on meiosis resumption

When the sole additive to the hydrogen peroxide supplemented maturation medium was 25 <mu>M 2-ME, the CEO had a higher proportion of MII (62.5%) and lower proportion of degenerate oocytes (12.5%) than for CDO (52.7% and 26.1%) after 24 h culture. These differences were not significant.

TABLE IV. Effect of Adding 25 <mu> M 2-Mercaptoethenalol and 100 <mu> M Hydrogen Peroxide to Maturation Medium on the Meiotic Resumption and Degeneration of Nili Ravi Buffalo Cumulus Enclosed (ceo) and Cumulus Denuded (cdo)

Type of oocyte	n	Time	GV n (%)	GVBD n (%)	MI n (%)	MII n (%)	Deg. n (%)
CDOs	46	0 h	13 (28.26)	33 (71.73)	-	-	-
		12 h	-	7 (15.2)	17 (36.9)	14 (30.4)	8 (17.3)
		24 h	-	3 (6.2)	7 (15.2)	24 (52.1)	12 (26.0)
CEOs	24	24 h	-	2 (8.3)	4 (16.6)	15 (62.5)	3 (12.5)

5) Experiment 5: Effect of <mu>M L-ascorbic acid in the presence of 100 <mu>M hydrogen peroxide on meiotic resumption

Adding 250 <mu>M AsA alone to the maturation medium containing hydrogen peroxide had a similar effect to using 25 <mu>M 2-ME (Experiment 4). After 24 h culture the proportion of CEO at the MII stage was 50.0% and the proportion of degenerate oocytes was 12.5%. In the culture of CDO at the same time, the proportions of MII and degenerate oocytes were 39.0% and 26.8%, respectively. The differences between the treatments were not significant.

TABLE V. EFFECT OF ADDING 25 <MU> M 2-MERCAPTOETHENALOL AND 100 <MU> M HYDROGEN PEROXIDE TO MATURATION MEDIUM ON THE MEIOTIC RESUMPTION AND DEGENERATION OF NILI RAVI BUFFALO CUMULUS ENCLOSED (CEO) AND CUMULUS DENUDED (CDO)

Type of oocyte	n	Time	GV n (%)	GVBD n (%)	MI n (%)	MII n (%)	Deg. n (%)
CDOs	41	0 h	14 (34.4)	27 (65.8)	-	-	-
		12 h	-	8 (19.5)	18 (43.9)	7 (17.0)	8 (19.5)
		24 h	-	4 (9.7)	10 (24.3)	16 (39.0)	11 (26.8)
CEOs	24	24 h	-	4 (16.6)	5 (20.8)	12 (50.0)	3 (12.5)

IV. DISCUSSION

In this study maturation and degeneration of oocytes was observed in both CDO and CEO state in the presence of different additives. In this study CDOs treated with H_2O_2 showed higher proportion (45.65%) of degeneration and little amount of CDOs progressed to MII stage as compared to control and other treated groups. This deleterious effect of ROS was found very little with those of CEOs (21.62) as compared to CDOs (Table II). These different responses to oxidative stress between CEOs and CDOs may be associated with different ability to synthesize GSH in these oocytes throughout in vitro culture. It is demonstrated that cumulus cells have critical role in protecting oocytes against oxidative stress induced apoptosis through the enhancement of GSH content in oocytes [42], [43]. It is reported that most toxic intermediate is hydrogen peroxide because of its ability to cross membranes freely and to inhibit enzyme activities and cellular function [44]. In light of previous studies, it has been revealed that increased levels of ROS could induce destabilization of maturation, reduce survival factors, trigger mitochondria-mediated apoptosis of oocyte [6]. Previous studies showed that ROS negatively affect spindle integrity and biological function [45] of oocyte and lower meiotic resumption rates [46], that could result in decrease oocyte maturation potential. In our study, we observed that when CDOs and CEOs in the absence of hydrogen peroxide were allowed to mature in control medium, the percentage in degeneration in CDO at 24 hrs is very much low than in the experiment conducted with the addition of hydrogen peroxide. It is reported that exposure of porcine cumulus oocyte complexes (COCs) to ROS reduced the maturation rate and other detrimental effects were not recognized [47].

Denuded porcine oocytes cultured in vitro were highly susceptible to oxidative stress, and degeneration, the length of DNA migration, and caspase-3 activity were significantly increased in CDO compared with those of CDO matured without xanthine oxidase (XOD) recognized [47]. Previous studies have shown the induction of glutathione in bovine [48] and in porcine [47], [49]. The oocytes maturation medium supplemented with GSH precursors, such as cysteamine or cysteine, improved the efficacy of IVM of bovine CDO by increasing the GSH content of the oocytes [50]. To preserve the antioxidant defense system in oocyte during in vitro oocytes/embryo culture and storage, *β*-mercaptoethanol acts as an antioxidant [51]. It is indicated that GSH synthesis is stimulated by β mercaptoethanol associated with a decrease in peroxide levels within oocytes [52], [53]. Since this increase concentration of the glutathione that is induced by supplementation of 2-ME may improve the meiotic competence of oocytes to MII stage. The beneficial effect of adding 250 <mu> M L-ascorbic acids to IVM medium improve the development of porcine CDO from MI to MII and prevent fragmentation of cumulus cell DNA [22].

In this study the additives used in combination with hydrogen peroxide (25 <mu>M ME and 250 <mu>M ASA) improved oocyte maturation and reduced their degeneration.

This study suggests that due to the presence of hydrogen peroxide there was higher damage of CDO compared to CEO. Since CEOs are surrounded by cumulus cells that protect them from oxidative damage produced by hydrogen peroxide. Further it was also revealed in this study that without hydrogen peroxide the maturation rate was high and damage of CDO and CEO was very much low than in the presence of hydrogen peroxide. This study proves that hydrogen peroxide reduces oocyte maturation rate and enhances degeneration of CDO and CEO.

V. CONCLUSION

It was concluded that cumulus cells protect the oocytes from oxidative stress whereas, denuded oocytes are highly susceptible to oxidative stress. However, the addition of 25 <mu>M 2-ME plus 250 <mu>M AsA to maturation medium including 100 <mu>M H2O2 for the culture of denuded oocytes effectively counteracts the oxidative stress.

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