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Effects of β-Mercaptoethanol on the Growth of Preantral Follicles and the Maturation of Intrafollicular Oocytes

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ABSTRACT : This study was undertaken to evaluate how β-mercaptoethanol (bME), an exogenous antioxidant, interacts with preantral follicles cultured *in vitro*. Mouse primary or secondary follicles were cultured in glutathione (GSH)-free or GSH-containing medium supplemented with bME of various concentrations, and the growth of preantral follicles, the maturation of intrafollicular oocytes and preimplantation development after parthenogenesis were monitored. In experiment 1, 0, 25, 50 or 100 μM bME was added to culture medium supplemented with 100 μM GSH or not. When secondary follicles were cultured in GSH-free medium, no significant change in follicle growth was detected after bME addition. However, exposure to bME in the presence of GSH significantly inhibited both follicle growth and oocyte maturation. Such detrimental effect became prominent in primary follicles and bME strongly inhibited follicle growth in the absence of GSH. In conclusion, there are stage-dependent effects of bME on follicle growth and oocyte maturation, and selective use of antioxidants contributes to establishing an efficient follicle culture system. (**Key Words :** Preantral Follicle, *In-vitro* Culture, β-Mercaptoethanol, Follicle Growth, Oocyte Maturation)

INTRODUCTION

Many studies have been conducted to establish an optimal condition for manipulation of preantral follicles and intrafollicular oocytes in livestock animals, as well as in experimental animals (Eppig and Schroeder, 1989; Donnelly and Telfer, 1994; Eppig and O'Brein, 1996; Telfer, 1996; Wu et al., 2001; Mao et al., 2002; Lenie et al., 2004; Choi et al., 2006; Choi et al., 2007; Lee et al., 2007; Wang et al., 2007; Choi et al., 2008). We have continuously made an effort to develop follicle culture system and subsequent successes to generate viable oocytes by the culture of primary or secondary follicles (Choi et al., 2008; Lee et al., 2008), which were retrieved from juvenile or adult ovaries (Kim et al., 2008), have been made. We then turn our attention to develop standard protocol for follicle culture, which is based on physiological and genetic characteristics of the follicles and their intrafollicular oocytes (Gong et al., 2008; Lee et al., 2008; Moon et al., 2008).

It is known that the addition of antioxidants to culture medium is essential for improving cell survival, as well as for enhancing embryo viability during *in vitro* culture. Various antioxidants such as glutathione (GSH), cysteine, cysteamine and β-mercaptoethanol (bME), which affect indirectly or directly on cell apoptosis (Guerin et al., 2001), have been employed. However, it is not clear whether the use of the antioxidants for the follicle culture is beneficial for improving *in vitro*-survival of preantral follicles and intrafollicular oocytes. Cellular component of the follicles interacts with each other, which involve various biological processes consisting of proliferation, differentiation and cell death (Matzuk et al., 2002; Demeestere et al., 2005). The use of antioxidants modifying apoptotic procedure may be detrimental or beneficial for follicle growth and oocyte maturation, but no report on the action of antioxidants added to follicle culture medium has been made yet.

In our culture system, we have used two antioxidants, bME and GSH for improving cell survival through decreasing of apoptotic cell death under 'redox' state (Gong et al., 2008). GSH directly influences on decreasing cell death, while bME has indirect effect by support to increase intracellular GSH level (Abeydeera et al., 1998; Guerin et al., 2001; Rodriguez-Gonzalez et al., 2003). Through this study, we examined the effects of the addition of bME to culture medium on follicle growth and oocyte maturation in culture. Randomized, prospective design was employed for the series of experiments and the role of bME in follicle

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development was examined in the presence or absence of GSH, an antioxidant having different actions. The results of this study can become one of valuable information for developing a standard protocol for effective culture of preantral follicle.

MATERIALS AND METHODS

Experimental animals

As an experimental animal, F1 hybrid mice produced by mating female C57BL6 mice with male DBA2 mice were employed and primary and secondary follicles were retrieved from 2-week-old prepubertal females. All procedures for animal management, breeding, and surgery followed the standard protocols of Seoul National University, Korea. The Institutional Animal Care and Use Committee Review Board at Seoul National University approved our research proposal in April 2005 (approval number: SNU0050331-02).

Isolation of follicles

The ovaries obtained from 2-week-old prepubertal female mice were removed aseptically and placed in Leibovitz's L-15 medium (Gibco Invitrogen, Grand Island, NY) supplemented with fetal bovine serum (FBS; 10%, v/v, heat-inactivated; HyClone Laboratories, Logan, UT). Subsequently, preantral follicles were retrieved mechanically using a 30-gauge needle. Primary follicles (75-99 μm in diameter) and early secondary follicles (100-125 μm) were collected under the guidance of an ocular micrometer of an inverted microscope (TE-2000; Nikon, Tokyo, Japan) at 40× magnification. The follicles were washed three times in 10-μl droplets of Leivovitz's L-15 medium (Gibco Invitrogen) and then cultured at 37°C in an air atmosphere containing 5% CO_2.

In vitro growth of preantral follicles

The early secondary follicles retrieved were placed singly in 10-μl culture droplets overlaid with washed mineral oil in 60×15 mm culture dishes (SPL, Pocheon, Korea). They were then cultured in ribonucleoside- and deoxyribonucleoside-containing α-MEM-glutamax medium (Gibco Invitrogen) supplemented with 5% (v/v) heat-inactivated FBS, 1% (v/v) insulin, transferrin and selenium (ITS) liquid medium, and 100 mIU/ml recombinant human FSH (Organon, Oss, The Netherlands), to which 0, 25, 50 or 100 μM bME (Gibco Invitrogen) and/or 100 μM GSH were added. All medium substrates were purchased from Sigma-Aldrich Corp. (St Louis, MO), unless otherwise stated. On day 1 of culture, an additional 10 μl of fresh medium was added to each droplet, and half of the medium was changed every other day from day 3 to the end of culture.

The primary follicles retrieved were also placed singly in 10-μl culture droplets in 60×15 mm Falcon plastic Petri dishes (Becton Dickinson, Franklin Lakes, NJ). The primary follicle culture medium was consisted of ribonucleoside and deoxyribonucleoside-free α-MEM-glutamax medium supplemented with 1% (v/v) heat-inactivated FBS, 1% (v/v) ITS liquid medium, 100 mIU/ml recombinant human FSH, and 10 mIU/ml LH, to which 0, 5, 10, 50, or 100 μM bME were added. On day 1 of culture, an additional 10 μl fresh medium was added to each droplet and half of a medium was changed on day 3 of culture. The cultured follicles were frequently detached from the bottom of culture dishes by mechanical pipetting and on day 4 of culture, they were transferred into 10 μl droplets prepared by secondary follicle culture medium. Hereafter the same protocol with the secondary follicle culture was employed.

Collection of mature oocytes and parthenogenetic activation

To retrieve mature oocytes, early secondary and primary follicles were cultured for 9 and 11 days, respectively, and maturation of oocyte was triggered by exposure to hCG (2.5 IU/ml, Pregnyl; Organon) and epidermal growth factor (5 ng/ml) 16 h before the end of culture. Cumulus oocyte complexes (COCs) were retrieved from mucified follicles and oocytes were freed from the COCs by mechanical pipetting in M2 medium supplemented with 200 IU/ml hyaluronidase. Oocyte maturation to the metaphase II (MII) stage was monitored by extrusion of the first polar body and expansion of the cumulus cells. To monitoring cytoplasmic maturation of the oocytes, oocytes freed from cumulus cells were activated parthenogenetically by culturing in a Ca^{2+}-free KSOM medium supplemented with $SrCl_2$ (10 mM) and cytochalasin B (5 μg/ml) for 4 h.

Culture of activated oocytes

Modified Chatot, Ziomek, and Bavister (CZB) medium was used for culturing parthenogenetic embryos at 37°C, 5% CO_2 in an air atmosphere. Cleavage and blastocyst formation of oocyte after parthenogenetic activation was monitored under either a stereomicroscope (SMZ-3; Nikon, Tokyo, Japan) or an inverted microscope (Eclipse TE-3000; Nikon) 24 and 120 h after culture, respectively.

Experimental design and statistical analysis

Prospective study was conducted and all preantral follicles retrieved were randomly distributed into each experimental treatment. In experiment 1, early secondary follicles isolated were cultured in GSH (100 μM)-containing or GSH-free culture medium, to which 0, 25, 50 or 100 μM bME was supplemented. The rates of pseudoantrum formation, number of mucified oocytes, number of oocytes developed to the germinal vesicle breakdown (GVBD) and MII stages, and number of oocytes

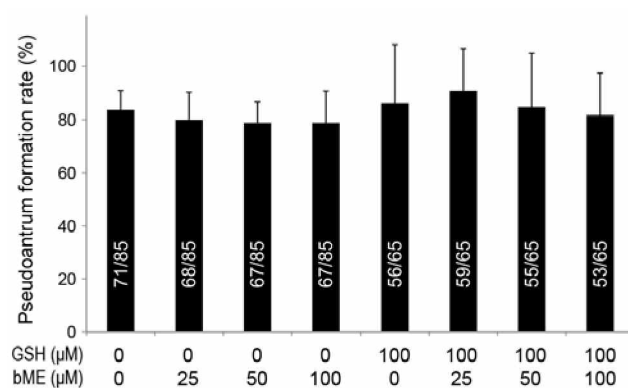

Figure 1. *In-vitro* growth of cultured early secondary follicles. The number of follicles, which formed pseudoantral structure, was measured at day 9 and the values were represented as the percentage of the number of total cultured follicles. No differences were detected among the treatments.

activated (cleaved) and developed to blastocysts after activation were examined. In experiment 2, effects of bME addition (0, 5, 10, 50 or 100 μM) to GSH-free culture medium on growth of primary follicles and maturation of intrafollicular oocytes were monitored. A generalized linear model (PROC-GLM) in a Statistical Analysis System (SAS) program was employed for statistical analysis. One-way ANOVA followed by the least-square method was conducted when the parameter had model effect. Significant differences among treatments were determined where the P value was less then 0.05.

RESULTS

The preantral follicles were retrieved from total 34 two-week-old female mice, from which 600 early secondary follicles (mean 33/animal) and 279 primary follicles (mean 17/animal) were recruited for *in vitro*-culture.

Experiment 1: Effects of antioxidant on secondary follicle growth and oocyte maturation

As shown in Figure 1, no significant treatment effect was detected in the follicle growth to the pseudoantral stage and the rate of development was within the range of 79 to 91%. However, the development to the pseudoantral stage was the highest after the combined addition of 100 μM GSH and 25 μM bME. Based on these results, we redesigned the treatment groups to add 0, 25, 50, 100 μM bME in the presence (Table 1) and the absence (Table 2) of 100 μM GSH. In the presence of GSH, bME did not change

Table 1. Effect of the addition of β-mercaptoethanol (bME) to glutathione (GSH)[a]-containing follicle culture medium on the meiotic maturation and embryonic development of intrafollicular oocytes in early secondary follicle culture

bME concentration (μM)	No. of follicles cultured	No. (%)[b] of mucified COCs	No. (%)[c] of oocytes developed to the stage of			No. of oocytes provided for activation[d]	No. (%)[e] of oocytes	
			GV	GVBD	MII		Cleaved	Developed to blastocyst
0	65	61(94)	61(100)	57(93)[fg]	42(69)	42	21(50)	9(21)[f]
25	65	62(95)	62(100)	59(95)[fg]	43(69)	43	21(49)	2(5)[g]
50	65	56(86)	56(100)	55(98)[f]	44(79)	44	23(52)	3(7)[g]
100	65	59(91)	59(100)	51(86)[g]	37(63)	37	22(59)	2(5)[g]

COC = Cumulus oocyte complex; GV = Germinal vesicle; GVBD = Germinal vesicle breakdown; MII = Metaphase II.
[a] 100 μM GSH was added. [b] Percentage of the number of cultured follicles. [c] Percentage of the number of mucified COCs.
[d] Parthenogenetic activation was conducted by $SrCl_2$ and cytochalasin B treatment. [e] Percentage of the number of oocytes provided for activation.
[f, g] Different superscripts within the same parameter indicate significant differences among the treatments, $p<0.05$.
The model effects of treatment on the number of mucified COCs, the number of oocytes maturing into GV, GVBD, and MII stage, and the number of oocytes cleaving and developing into blastocysts were 0.2459, 1, 0.0743, 0.3270, 0.7924, and 0.0273, respectively.

Table 2. Effect of the β-mercaptoethanol (bME) addition into glutathione-free follicle culture medium on the meiotic maturation and embryonic development of intrafollicular oocytes in early secondary follicle culture

bME concentration (μM)	No. of follicles cultured	No. (%)[a] of mucified COCs	No. (%)[b] of oocytes developed to the stage of			No. of oocytes provided for activation[c]	No. (%)[d] of oocytes	
			GV	GVBD	MII		Cleaved	Developed to blastocyst
0	85	76(89)	76(100)	68(89)	42(55)	42	24(57)	3(7)
25	85	70(82)	70(100)	61(87)	43(61)	43	26(60)	3(7)
50	85	76(89)	76(100)	62(82)	53(70)	53	29(55)	3(6)
100	85	74(87)	74(100)	64(86)	46(62)	46	32(70)	2(4)

COC = Cumulus oocyte complex; GV = Germinal vesicle; GVBD = Germinal vesicle breakdown; MII = Metaphase II.
[a] Percentage of the number of cultured follicles. [b] Percentage of the number of mucified COCs.
[c] Parthenogenetic activation was conducted by $SrCl_2$ and cytochalasin B treatment. [d] Percentage of the number of oocytes provided for activation.
The model effects of treatment on the number of mucified COCs, the number of oocytes maturing into GV, GVBD, and MII stage, and the number of oocytes cleaving and developing into blastocysts were 0.4772, 1, 0.5556, 0.3357, 0.4781 and 0.9408, respectively.

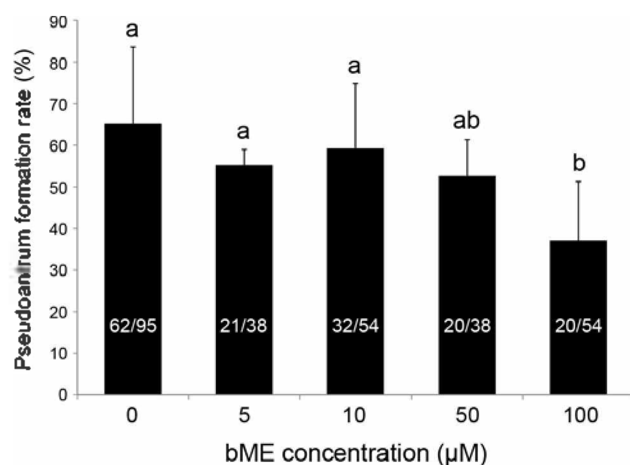

Figure 2. *In-vitro* growth of cultured primary follicles. The number of follicles, which formed pseudoantral structure, was measured at day 11 and the values were represented as the percentage of the number of total cultured follicles. [a, b] Different superscripts indicate significant differences among the treatments, p<0.05. The model effect is 0.0203.

the rates of oocyte maturation to MII, and number of oocytes activated. However, the rates of germinal vesicle breakdown was significantly (p<0.05) decreased at 100 μM and a great (p = 0.0273 in model effect) decrease in blastocyst formation after parthenogenetic activation was detected by bME addition (21% vs. 5-7%). In the absence of GSH, no significant difference in oocyte maturation (55 to 70%), cleavage (55-70%), and blastocyst formation (4-7%) was detected after bME addition.

Experiment 2: Effect of antioxidant on primary follicle growth and oocyte maturation

Significant retardation in both primary follicle growth and oocyte maturation was detected. As shown in Figure 2, less (p = 0.0203) primary follicles formed pseudoantrum after the addition of 100 μM bME than after the addition of 0, 5 or 10 μM bME to culture medium (37% vs. 55-65%). As shown in Table 3, oocyte maturation reduced significantly (p = 0.0198) after the addition of 10, 50 or 100 μM bME than after no addition (26-37% vs. 56%), which showed different response for bME compared with the secondary follicles (Table 2). Number of mucified COCs was also decreased (p = 0.0004) after the addition of 100 μM bME than after no addition (57% vs. 89%). No significant decrease in oocyte activation (17-40%) and development to the blastocyst stage (0-7%) was detected after bME addition.

DISCUSSION

In this study, we hypothesized that antioxidants might have function as autocrine and paracrine factors that influence growth, differentiation and retardation of developing follicles. The results of this study demonstrated that in the presence of GSH, bME is detrimental to follicle development and that there may be an interaction between exogenous antioxidant and developing follicles. In the case of secondary follicles, bME was an inhibitory factor for follicle growth and oocyte maturation in the presence of 100 μM GSH, while no inhibitory effect of bME was detected in the absence of GSH in culture medium. On the other hand, inhibitory role of bME in follicle development became more prominent in primary follicles than in secondary follicle. When primary follicles were cultured *in vitro*, significant retardation in follicle development and oocyte maturation was detected after bME addition even in the absence of GSH. Exogenous antioxidants influence follicle growth and nuclear maturation of intrafollicular oocytes.

In the process of follicular development, intrafollicular oocytes and follicular granulosa cells significantly interact with each other and this interaction is dependent upon the stage of follicle development. There are paracrine factors

Table 3. Effect of the β-mercaptoethanol (bME) addition into follicle culture medium on the meiotic maturation and embryonic development of intrafollicular oocytes in primary follicle culture

bME concentration (μM)	No. of follicles cultured	No. (%)[a] of mucified COCs	No. (%)[b] of oocytes developed to the stage of			No. of oocytes provided for activation[c]	No. (%)[d] of oocytes	
			GV	GVBD	MII		Cleaved	Developed to blastocysts
0	95	85(89)[e]	85(100)	68(80)	48(56)[e]	48	9(19)	0(0)
5	38	29(76)[e]	29(100)	25(86)	12(41)[ef]	12	2(17)	0(0)
10	54	41(76)[e]	41(100)	34(83)	15(37)[f]	15	6(40)	1(7)
50	38	29(76)[e]	28(97)	20(69)	10(34)[f]	10	3(30)	0(0)
100	54	31(57)[f]	31(100)	24(77)	8(26)[f]	8	3(38)	0(0)

COC = Cumulus oocyte complex; GV = Germinal vesicle; GVBD = Germinal vesicle breakdown; MII = Metaphase II.

[a] Percentage of the number of cultured follicles. [b] Percentage of the number of mucified COCs.

[c] Parthenogenetic activation was conducted by $SrCl_2$ and cytochalasin B treatment. [d] Percentage of the number of oocytes provided for activation.

[e, f] Different superscripts within the same parameter indicate significant differences among the treatments, p<0.05.

The model effects of treatment on the number of mucified COCs, the number of oocytes maturing into GV, GVBD, and MII stage, and the number of oocytes cleaving and developing into blastocysts were 0.0004, 0.1699, 0.5357, 0.0198, 0.4084, and 0.2696, respectively.

and/or cell-to-cell communication to regulate follicle development (Matzuk et al., 2002). It has been known that FSH and LH are the main regulator of follicle growth and oocyte maturation (Demeestere et al., 2005; Yang et al., 2008). However, regulatory role of other factors remains to be unknown, although a lot of studies have evaluated the effects of various factors on follicle growth and endocrinal regulation during folliculogenesis and oocyte maturation to date (Boland and Gosden, 1994; Almahbobi et al., 1995; Liu et al., 1998; Smitz et al., 1998; Hayashi et al., 1999; Durlinger et al., 2001).

An antioxidant bME is a thiol compound and its promoting effect on embryo development have been reported (Takahashi et al., 1993; Caamaño et al., 1996; Lim et al., 1996; Ohboshi et al., 1996; Abeydeera et al., 1998; Tao et al., 2004). In this study, we did not find any promoting effect of bME on follicle growth and oocyte development. The presence of somatic cells may mediate the function of antioxidants on follicle development. Otherwise, physiological difference between intrafollicular oocyte and embryo may be the cause for inhibitory action of bME.

We previously reported the promoting effects of GSH on *in vitro* folliculogenesis (Gong et al., 2008). Extracellular GSH cannot be transported directly within the cells, but they are degraded into some compounds, which can be used for GSH synthesis after transportation into the cells (Rodríguez-González et al., 2003). In addition, exogenous bME is able to increase GSH synthesis by reducing cystine to cysteine (Ishii et al., 1981; Issels et al., 1988) and increased GSH level promotes embryonic development by maintaining intracellular redox state (Takahashi et al., 1993; Abeydeera et al., 1998; Salmen et al., 2005). In this study, we added these two compounds together to follicle culture medium and subsequently tested their effects on *in vitro* folliculogenesis. Our results clearly showed that within the certain range of GSH and bME concentrations, the concomitant addition of these two antioxidants to culture medium did not promote but inhibit follicle growth. In addition, we found the stage-specific action of the antioxidants on the follicle growth and oocyte maturation.

In this study, the development to the blastocyst stage after parthenogenesis was extremely low from 0 to 7% except for one case utilizing bME-free and GSH-containing culture system. One of the reasons for such low rate may result from low quality of oocytes derived from *in vitro*-cultured follicles, while instability of *in vitro*-culture system may be the other cause. Several studies have been conducted to solve this problem through optimization of follicle culture condition, but there are still remains to be unsolved (Cortvrindt et al., 1996; Smitz and Cortvrindt, 2002; Lenie et al., 2004). An attempt to develop an effective follicle culture system will be made in terms of the understanding of the interrelation between oocyte and surrounding somatic cells. In this study, embryo culture system does not influence negatively on blastocyst formation because large number of parthenogenetic oocytes derived from natural ovulation could develop into the blastocyst stage. In different set of study (Gong et al., 2008), *in-vivo* derived mature oocytes obtained by flushing oviducts showed 82% blastocyst formation after parthenogenetic activation.

In conclusion, our results demonstrate that bME may have a potential to increase meiotic maturation of intrafollicular oocytes in secondary follicles cultured *in vitro*. Care should be taken to select antioxidants added to follicle culture medium, which can improve the efficiency of follicle culture system, and use of more than two antioxidants might be detrimental to follicle development.

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