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The Effects of Antioxidants on the Culture of Mouse Preantral Follicles *In Vitro*

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ABSTRACT

In order to investigate the effects of antioxidants on the culture of mouse preantral follicles *in vitro*, we examined the effects of taurine, glutathione and catalase on their growth and maturation. Addition of taurine was not effective on the survival of preantral follicles. However, metaphase II rates of oocytes within preantral follicles were significantly higher in 1 mM treated group than in control and 10 mM treated group ($p<0.05$). Glutathione did not improved the rates of survival and metaphase II oocytes. However, metaphase II rates of oocytes progressively decreased with increasing glutathione concentration. Catalase also showed that the rates of survival and metaphase II oocytes progressively decreased with increasing concentration. Especially, all of preantral follicles cultured in medium containing 100 IU/ml catalase were degenerated. These results suggest that low concentraion of taurine, as an antioxidant, have positive effect on the culture of mouse preantral follicles *in vitro*.

(Key words : Mouse preantral follicles, Taurine, Glutathione, Catalase)

INTRODUCTION

The ovarian preantral follicles are a large and potentially valuable source of oocytes that could be used for clinical, agricultural and zoological purposes. If oocytes are harvested in greater numbers from preantral follicles and grown *in vitro*, this would have various advantages. However, for use of oocytes from preantral follicles, the ability to bring preantral follicular stage with immature oocytes to maturity *in vitro* is prerequisite. The development of preantral follicle culture system that can potentially produce large quantities of oocytes of uniform developmental status will significantly advance the assisted reproduction techniques. Additionally, it may make possible the preservation and long-term storage of the female germ plasm (Gutierrez *et al*., 2000).

In vitro culture techniques for preantral follicles have been improved to assist investigation of the mechanism of oocyte and follicular development. Under appropriate conditions, meiotically incompetent oocytes from preantral follicles can grow to final size and complete nuclear maturation *in vitro* (Cortvindt *et al*., 1996; Eppig *et al*., 1998; Kim *et al*., 1999; Nayudu and Osborn, 1992; Park *et al*., 2013; Park *et al*., 2013; Zhang *et al*., 2012). In general, A large variety of additives, such as serum or serum supplements, gonadotrophins and growth factors, have been employed for medium supplementation. Furthermore, studies for culture of mouse preantral follicles have demonstrated successful growth and maturation as well as fertilization and development of oocytes from follicles cultured *in vitro* (Cortvrindt *et al*., 1996; Eppig and Downs, 1989; Kim *et al*., 2004; Nayudu and Osborn, 1992). On the other hand, one of the problems encountered in the culture system has been oxidative stress and its adverse effects on oocytes and embryos (Gardner, 2008; Tatemoto *et al*., 2000). Oxidative stress has been known as excessive production of reactive oxygen species (ROS) or imbalance between the production of ROS and antioxidant defense system (Talebi *et al*., 2012). To alleviated this situation, many studies have been accorded to the addition of antioxidants to the culture system (Hossein *et al*., 2007; Wu *et al*., 2011).

However, studies concerning the effect of antioxidants on the culture of mouse preantral follicles *in vitro* have not been accomplished. Thus, the objective of present study was to examine the effects of taurine, glutathione and catalase on the growth and development of the mouse preantral follicles through their supplementation in culture medium.

MATERIALS AND METHODS

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Collection of Ovaries

The ovaries were aseptically removed from 12-day-old ICR female mice. Intact ovaries were transferred into Leibovitz L-15 medium (Gibo-BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibo-BRL).

Isolation of Preantral Follicles

The ovaries were immersed into Leibovitz L-15 medium containing 1 mg/ml collagenase (Type 1A; C-2674, Sigma, St.Louis, MO, USA) and 0.2 mg/ml DNase I (DN-25, Sigma) for 20 minutes at 37℃ and repeatedly drawn in and out of the pipette until the ovaries were dissociated into individual follicles. The preantral follicles (100～120 μm in diameter) to be cultured were selected based on the following criteria: i) intact round follicular structure with two to three layers of granulosa cells; ii) the oocyte had to be visible, round and centrally located within the follicle. All selected follicles were pooled and randomly divided over the culture condition under study.

***In Vitro* Growth and Maturation of Preantral Follicles**

The *in vitro* growth (IVG) medium was α-minimal essential medium (αMEM; Gibo-BRL) supplemented with 100 mIU/ml follicle stimulating hormone (FSH, Metrodin-HP; Sereno, Aubonne, Switzerland) and 10 mIU/ml luteinizing hormone (LH; Sigma) and 5% FBS (Kim *et al.*, 2012). The preantral follicles were cultured on Transwell-COL membrane inserts (3.0 μm pore size, 24.5 mm diameter; Costar, Cambridge, MA, USA) in 6-well cluster dishes to prevent the loss of structural integrity between the oocyte and granulosa cells (Eppig and Schroeder, 1989). The preantral follicles were cultured for 10 days at 37℃ in 5% CO_2 in air. Half of the medium was changed every 2 days. After 10 days of growth *in vitro*, preantral follicles allowed to mature for 16～18 hours in medium supplemented with 1.5 IU/ml hCG (Profasi, Sereno).

Measurement of Oocyte Diameter

After maturation, oocyte-cumulus cell complexes from preantral follicles were collected and adherent cumulus cells were removed by pipetting in Leibovitz L-15 medium containing 0.1% hyaluronidase. The maturation status and the diameter of the oocytes, excluding the zona pellucida, were examined with an ocular micrometer attached to an inverted microscope.

Experimental Design

In order to investigate the effects of antioxidants on the survival and maturation rates of the preantral follicles *in vitro*, the mouse preantral follicles were cultured in IVG medium containing taurine (0, 1, 2.5, and 5 mM), glutathione (0, 1, 2.5, and 5 mM) and catalase (0, 1, 10, and 100 IU/ml) for 10 days and further cultured in α MEM medium supplemented with 5% FBS and 1.5 IU/ml hCG for 16～18 hours to induce meiotic maturation.

Statistical Analysis

The statistical significance of the data was analyzed using Students *t*-test and the chi-square test. Statistical significance was established at the $p<0.05$ level.

RESULTS

Effect of Taurine on the Culture of Mouse Preantral Follicles

In order to examine the effect of taurine, mouse preantral follicles were cultured in the medium supplemented with the different concentration of taurine. As shown in Table 1, the survival rates of preantral follicles following the *in vitro* growth and maturation was not difference among the treatment concentrations. The metaphase II rates of oocytes was significantly higher in 1 mM concentration (49.5%) than in control (35.8%) and 2.5 mM (36.2%) concentration ($p<0.05$). And also, the mean diameter of matured oocytes was not difference among the each concentration groups.

Table 1. Effect of taurine on the *in vitro* growth and maturation of mouse preantral follicles

Concentration (mM)	No. of follicles cultured	No.(%) of oocytes survived	GV(%)	GVBD(%)	MetaII(%)	Oocyte diameter (μm)
0	95	64(67.4)	12(12.6)	18(18.9)	34(35.8)[a]	69.0±1.5
1	105	75(71.4)	14(13.3)	9 (8.6)	52(49.5)[b]	68.9±1.6
2.5	105	70(66.7)	22(21.0)	10 (9.5)	38(36.2)[a]	68.3±1.6
5	105	74(70.5)	19(18.1)	15(14.3)	42(40.0)	69.2±1.7

[a,b] : $p<0.05$.

Table 2. Effect of glutathione on the *in vitro* growth and maturation of mouse preantral follicles

Concentration (mM)	No. of follicles cultured	No.(%) of oocytes survived	GV(%)	GVBD(%)	MetaII(%)	Oocyte diameter (μm)
0	98	71(72.4)	20(20.4)	11(11.2)	40(40.8)	69.1±1.8
1	98	66(67.3)	17(17.3)	10(10.2)	39(39.8)	68.8±1.6
2.5	98	69(70.4)	26(26.5)	8 (8.2)	35(35.7)	69.1±2.0
5	98	58(59.2)	19(19.4)	9 (9.2)	30(30.6)	68.5±1.9

No significant differences were detected among treatment groups.

Table 3. Effect of catalase on the *in vitro* growth and maturation of mouse preantral follicles

Concentration (IU/ml)	No. of follicles cultured	No.(%) of oocytes survived	GV(%)	GVBD(%)	MetaII(%)	Oocyte diameter (μm)
0	130	31(74.6)[a]	24(18.5)	11 (8.5)	62(47.6)[a]	71.1±2.6
1	130	94(72.3)[a]	21(16.2)	10 (7.7)	63(48.5)[a]	70.8±2.3
10	130	86(66.2)[a]	23(17.7)	13(10.0)	50(38.5)[a]	71.4±2.1
100	130	0 (0.0)[b]	0 (0.0)	0 (0.0)	0 (0.0)[b]	ND[*]

[a,b] : $p<0.0001$, [*]ND : Not determined.

Effect of Glutathione on the Culture of Mouse Preantral Follicles

In order to examine the effect of glutathione, mouse preantral follicles were cultured in the medium supplemented with the different concentration of glutathione. As shown in Table 2, the survival rates of preantral follicles following the *in vitro* growth and maturation was lower than in 5 mM concentration (59.2%) when compared to 1 mM (67.3%) and 2.5 mM (70.4%) concentration. The metaphase II rates of oocytes was progressively decreased with increasing glutathione concentration in the medium (39.8, 35.7 and 30.6%). However, the mean diameter of matured oocytes was not difference among the each concentration groups.

Effect of Catalase on the Culture of Mouse Preantral Follicles

In order to examine the effect of catalase, mouse preantral follicles were cultured in the medium supplemented with the different concentration of catalase. As shown in Table 3, the survival and metaphase II rates of preantral follicles following the *in vitro* growth and maturation were not difference between the control (47.6 %) and 1 IU/ml concentration (48.5%). However, some decrease of the survival and metaphase II rates were found in 10 IU/ml concentration (38.5%). Particularly, it was found that all of preantral follicles, in 100 IU/ml treated group, were degenerated. The mean diameter of matured oocytes was not difference among the each concentration groups.

DISCUSSION

It is now well established that preimplantation embryo development *in vitro* is more successful when the embryos are cultured with low concentrations of oxygen (Batt *et al.*, 1991; Legge and Sellens, 1991; Li and Foote, 1993). The normal atmospheric oxygen tensions of 20% (5% CO_2 and 95% air) induce oxidative stress with concomitant generation of free oxygen radicals (Freeman and Crapo, 1982; Legge and Sellens, 1991). These free radical species are highly reactive in biological systems where they damage cell membranes, proteins and nucleic acids (Freeman and Crapo, 1982). However, these free radical species are reduced by a battery of enzyme and a number of antioxidants which can quench free radical activity (Freeman and Crapo, 1982; Li *et al.*, 1993).

On the other hand, Eppig and Wigglesworth (1995) reported that the development of growing oocytes was severely impaired by elevation of the concentration of gaseous oxygen used in the culture system. Recently, it was reported that culture of mouse preantral follicles in the presence of alpha-liporic acid (ALA), is an antioxidant, decreased ROS levels, and improved the developmental competence of preantral follicles *in vitro*

(Talebi *et al.*, 2012). Thus, in the present study, we examined the effects of antioxidants such as taurine, glutathione and catalase, on the culture of mouse preantral follicles, using embryo effective concentrations.

Taurine is found in high concentration in female reproductive tract fluid and embryos (Miller and Schultz, 1987; Schultz *et al.*, 1981). And, taurine serves as a antioxidant (Li *et al.*, 1993), chelating agent (Nasr-Esfahari *et al.*, 1992) and osmolyte (Thurston *et al.*, 1981). Glutathione (GSH) is a major intracellular free thiol that has important biological functions during cellular proliferation, amino acid transport, synthesis of protein and DNA and cellular protection against oxidative stress (Meister and Anderson, 1983). It has been reported that GSH synthesis during oocyte maturation is beneficial to pronuclear formation and subsequent development (Yosida *et al.*, 1993). Catalase is known that rapidly reduces H_2O_2 to H_2O and molecular O_2 (Li *et al.*, 1993). It has been shown to decrease H_2O_2 in embryos, and to improved the development of mammalian embryos (Nasr-Esfahani and Johnson, 1991; Orsi and Leese, 2001).

The results from this study showed that in embryo effective concentration of antioxidants, taurine only had promoting effect but not in the glutathione and catalase. Especially, all of preantral follicles were degenerated in 100 IU/ml concentration of catalase. This result may be due to harmful effect of catalase on the proliferation of granulosa cells, because granulosa cells surrounding oocytes were degenerated when high concentration of catalase was added in culture medium.

In conclusion, this study suggested that only taurine, as an antioxidant, had significant effect on the culture of mouse preantral follicles *in vitro*. However, further study are needed to examine the developmental competence of oocytes grown in medium supplemented with antioxidants following *in vitro* fertilization.

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