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In Vitro Growth of Preantral Follicle and Maturation of Intrafollicular Oocyte from Aged Mice

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ABSTRACT This study aimed to recover the ovarian function through *in vitro* culture of preantral follicles from aged mice. First, we isolated the preantral follicles from ovaries of sixty-seven-week old B6D2F1 mice with decreased fecundity to know how many follicles were present in them, which was 6 preantral follicles including 2 primary, 2 early secondary and late secondary follicles from 8 aged mice. It was confirmed that a few follicles (~2) were present in aged mice through histological analysis compared to adult mice as control. The 9 days of *in vitro* culture of preantal follicles showed *in vitro* growth and induced maturation after treatment with hCG (2.5 IU/mL) and EGF (5 ng/mL). Cumulus cells in the cumulus-oocyte complexes (COCs) were removed using hyaluronidase and oocytes at the germinal vesicle (GV) and GV breakdown (GVBD) were obtained from preantral follicle culture of aged mice *in vitro*. In conclusion, these observations demonstrated that there still were a few preantral follicles in the ovaries of 67 week-old mice, which we were able to culture *in vitro* and oocytes were obtained from them. This study proposed an *in vitro* culture system using preantral follicle as a therapeutic strategy for fertility preservation in humans for assisted reproductive medicine.

Keywords: aged mice, fertility preservation, *in vitro* culture, preantral follicle

INTRODUCTION

Ovarian follicles are the fundamental functional tissue units of the organ, each containing an oocyte and associated somatic cells. In humans, it has been well-known that females are born with a maximum number of follicles (~1 million) or oocytes that are not only non-renewable, but also undergo degeneration with time with a sharply decreased oocyte quality after the age of 35 (Hassold et al., 2001; Woodruff et al., 2008; Barnett et al., 2009; Broekmans et al., 2009; Choi et al., 2013). Less than 1% of them are used during female's reproductive life until menopause and the rest scheduled for degeneration never participate in making eggs. Therefore, it is of importance to culture the ovarian follicles *in vitro* to obtain large quantities of fertilizable oocytes later to preserve the fertility of women who may want to delay childbearing or in case of aggressive medical treatments such as radiation and chemotherapy and for endangered species and invaluable genetic resources (Jeruss et al., 2009; Donnez et al., 2010; Laven et al., 2016; Wang et al., 2018). However, several methods have been successfully developed for *in vitro* culture of ovarian follicles in most of young (10-14 day-old) and adult (8 week-old) mice. However, there are

only a few preclinical studies in aged mice to recover the ovarian function from unexplained infertility incidences such as premature ovarian failure (POF) and polycystic ovary syndrome (PCOS). Here, we show that there are a few preantral follicles in the ovaries of 67 week-old aged mice with decreased fecundity and oocytes can be derived from them using this *in vitro* culture system. Therefore, this study provides important guidance for establishing *in vitro* follicle culture system to recover the fertility in humans and preservation of endangered species and invaluable genetic resources.

MATERIALS AND METHODS

Animals and materials

Sixty-seven week-old B6D2F1 of aged group and 8 week-old B6D2F1 mice of adult group exclusively were provided by Koatech (Pyeoungtack, Korea). All procedures for animal management, breeding, and surgery were conducted in accordance with the standards of the CHA Research Institute of CHA University. All procedures described herein were reviewed and approved by the Institutional Animal Care and Use Committee of CHA University and were performed in accordance with Guiding Principles for the Care and Use of Laboratory Animals. Leibovitz L-15, lyophilized penicillin-streptomycin solution and a-MEM-glutamax medium and fetal bovine serum (FBS) were purchased from Gibco and Corning, respectively. Unless otherwise specifically noted, all other chemicals were purchased from Sigma.

Isolation of preantral follicles

Preantral follicles were isolated using the mechanical method from ovaries of 67-week old female B6D2F1 mice. The ovaries were placed in a 2 mL Leibovitz L-15 medium supplemented with 10% (v/v) heat-inactivated FBS and 1% (v/v) penicillin-streptomycin at 37°C in 5% CO_2 air. Preantral follicles were retrieved by using two 30G needles to mechanically break the extracellular matrix between follicles in the ovarian tissue. Preantral follicles of various types (and sizes in diameter) were obtained, including primary (75-99 μm in diameter), early secondary preantral (100-125 μm), late secondary preantral (126-150 μm) follicles.

Tissue collection and H&E staining

8-week-old adult C57BL/6 mice were provided by Orient Bio (Gapyeong, Gyeonggi, Korea). 67 weeks and 8 weeks old female ovaries were fixed 10% formalin (Sigma-Aldrich) for histology. Fixed tissues were washed, dehydrated, and embedded in Paraplast (Merck KgaA, Darmstadt, Germany). Paraffin-embedded tissues were sectioned using a microtome, stained with hematoxylin and eosin (H&E) (Sigma-Aldrich), and observed by light microscopy.

In vitro culture of preantral follicles

Preantral follicle (100-150 μm) with multiple layers of granulosa cells and an intrafollicular oocyte were collected without enzymatic digestion, and the retrieved follicles were placed singly in 10 μL culture droplets and overlaid with washed mineral oil in 60 × 15 mm Falcon plastic Petri dishes (Becton Dickinson, Franklin Lakes, NJ). Preantral follicles were cultured at 37°C containing 5% CO_2 in air in ribonucleoside- and deoxyribonucleoside containing α-MEM-glutamax medium supplemented with FBS (5%, v/v), insulin, transferrin, and selenium (ITS) liquid medium (1%, v/v), recombinant human FSH (100 mIU/mL) and 1% (v/v) lyophilized penicillin-streptomycin solution was added. On the following day, 10 μL of fresh medium was added to each drop, and starting from day 3, half the medium (10 μL) was replaced with the fresh medium every other day.

In vitro maturation of oocytes in antral follicles

To retrieve mature oocytes, preantral follicles were cultured for 9 days and maturation of oocyte was triggered by exposure to hCG (2.5 IU/mL) and epidermal growth factor (5 ng/mL) for 17 h prior to the end of culture. Retrieved oocytes were freed from cumulus cells by mechanical pipetting in L-15 medium supplemented with 200 IU/mL hyaluronidase (Choi et al., 2007). Oocytes at the germinal vesicle (GV) stage were judged by the presence of a clear GV observed under a phase-contrast microscope; GV breakdown (GVBD) oocytes were judged by the disappearance of the clear GV and MII oocytes were judged by the disappearance of the clear GV and the appearance of a characteristic first polar body.

RESULTS

As shown in Table 1, a total of 8 aged mice with decreased fecundity were used to isolate preantral follicles

mechanically. We obtained a total number of 6 preantral follicles including 2 primary (75-99 μm), 2 early secondary (100-125 μm) and late secondary follicles (126-150 μm) according to their size. In addition, two preantral follicles over 150 μm were isolated. As shown in Fig. 1, a preantral follicle was present in the ovaries of aged mice through histological analysis, and folliculogenesis did not occur at all in ovaries of aged mice (Fig. 1A), while we could observe the different stages of folliculogenesis in ovaries of 8 week-old adult mice used as a control (Fig. 1B). As shown in Table 2, 4 preantral follicles were cultured for 9 days *in vitro* and two preantral follicles were developed into antral follicles (Fig. 2A). The 9 days of *in vitro* culture of preantal follicles showed *in vitro* growth and induced maturation after treatment with hCG (2.5 IU/mL) and EGF (5 ng/mL). Cumulus cells in the cumulus-oocyte complexes (COCs) were removed using hyaluronidase and the oocytes at the germinal vesicle (GV) and GV breakdown (GVBD) were obtained from preantral follicle culture of aged mice *in vitro* (Fig. 2B, C). No difference in the morphologies of growing follicles was observed between aged and adult mice groups (Fig. 2A, D). However, a matured oocyte released with the first polar body was derived from the preantral follicles of adult mice (Fig. 2E).

Table 1. A summary of the number of total, primary (75-99 μm), early secondary preantral (100-125 μm), and late secondary preantral follicles (126-150 μm) retrieved per animal by mechanical method from ovaries of 67 week-old mice

Number	Total no. of follicles retrieved	Follicles retrieved by type			Comments
		Primary	Early secondary preantral	Late secondary preantral	
1	2	1	0	0	One follicle (153 μm)
2	0	0	0	0	
3	0	0	0	0	
4	2	0	1	1	
5	1	0	0	1	One follicle (164 μm)
6	2	1	1	0	
7	0	0	0	0	
8	0	0	0	0	
Total	6	2	2	2	

DISCUSSION

The results of this study demonstrate that the ovaries of aged animals with decreased or no fecundity still possess follicles that can support maturation of intrafollicular oocytes after ovarian function has decreased due to aging. This indicated that *in vitro* culture of preantral follicles enables use of the follicles from aged ovaries for

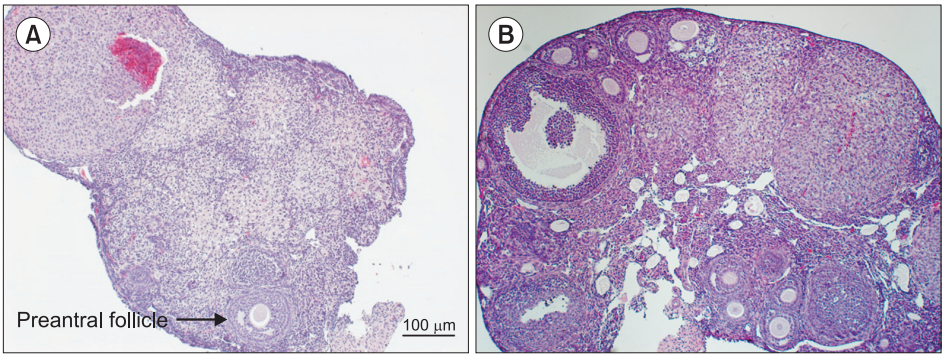

Fig. 1. The histology of from ovaries of 67 week (A) and 8 week-old mice (B). Scale bar: 100 μm (A, B).

Table 2. *In vitro* follicle growth and maturation of intrafollicular oocytes derived from preantral follicles between aged and adult mice

Group	No. of follicles cultured	No. (%)[a] of antral follicles	No. of COCs	No. (%)[b] of oocyte at the stage of		
				GV	GVBD	MII
Aged mice	4	2 (50%)	2 (50%)	1 (50)	1 (50)	0
Adult mice	45	39 (87)	39 (87)	4 (10)	34 (87)	1 (3)

COCs, cumulus-oocyte complex; GV, germinal vesicle; GVBD, germinal vesicle breakdown; MII, metaphase II.

[a]Percentage of antral follicles out of total follicles cultured.

[b]Percentage of GV, GVBD, MII oocytes out of total COCs retrieved.

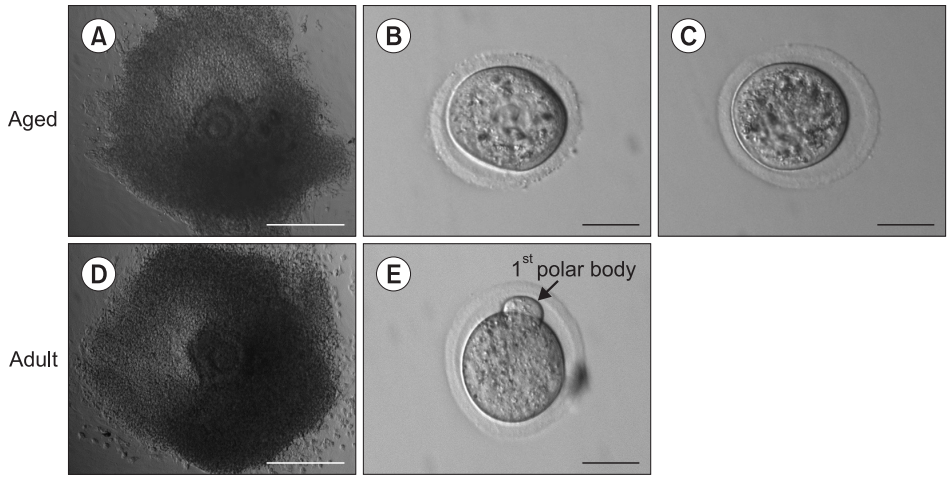

Fig. 2. Typical micrographs showing the development of preantral follicle retrieved mechanically from aged mouse ovaries of 67 week-old and adult mouse of 8 week-old during in vitro culture. Preantral follicle could develop through the nonphysiologically to antral stage (A, D; Day 9). After further IVM, Cumulus cells in the COC were removed using hyaluronidase to obtain oocyte. Oocytes at the germinal vesicle (GV) stage were judged by the presence of a clear GV (B) observed under a phase-contrast microscope; GV breakdown (GVBD; C) oocytes were judged by the disappearance of the clear GV; MII oocytes (E) were judged by the disappearance of the clear GV and the appearance of a characteristic first polar body. Scale bar: 100 μm (A-C), 40 μm (B, C, E).

reproduction. However, the number of preantral follicles cultured was much less as mice over 52 weeks of age are a limited in a commercial animal supplying company and are also expensive. Thus, further studies are needed with more number of mice. To get mature oocytes from preantral follicles of aged mice, we need to develop an optimized culture medium using a combination of hormones such as follicle stimulating hormone (FSH) and luteinizing hormone (LH), growth factors such as epidermal growth factor (EGF), insulin-transferrin-selenium (ITS), and fetal bovine serum (FBS) that play a vital role in developing preantral follicles *in vitro* (Wu et al., 2007). Furthermore, unlike the conventional two-dimensional (2D) culture, a three-dimensional (3D) culture system is suggested as an alternative to improve the efficiency of *in vitro* growth of preantral and MII oocytes because the biomaterials and tissue engineering techniques used in 3D culture system will maintain the follicular structure and support intercellular communication between the different cell compartments within the follicle (Woodruff et al., 2011; Choi et al., 2014; Filatov et al., 2015; Higuchi et al., 2015). In conclusion, the *in vitro* culture system with preantral follicles reported in the present study will provide a therapeutic strategy for fertility preservation in humans or endangered species and breeds with decreased fecundity.

CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

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