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The *in vitro* effect of chick embryo extract on mice pre-antral follicles

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Abstract

Chick embryo extract (CEE) contains a variety of growth factors which may improve *in vitro* follicle growth. Therefore, the effect of CEE on mouse pre-antral follicle culture was evaluated. Different percentages of CEE (0, 0.50%, 1.00%, 5.00% and 10.00%) were added to culture medium. Hence, the osmolarity of media was measured. Pre-antral follicles with diameter of 120-150 µm were isolated from 12-14 days old mouse ovary and cultured for 12 days. After culture, the maturation rate was assessed. Granulosa cells viability was evaluated using MTT test and estradiol levels were evaluated using related radio-immunoassay (RIA). Genes expression (BMP15 and ALK6) was also evaluated. The osmolarity of media and granulosa cells viability were the same in all groups. Estradiol level in group with 10.00% CEE was significantly decreased compared to the control group. After 12 days culture, the percentage of antral follicles development was significantly higher in the group with 5.00% CEE compared to control group. The percentage of metaphase II and germinal vesicle breakdown oocytes was significantly higher in group 5.00% CEE compared to control group. The expression of BMP15 gene in antral follicles in 5.00% CEE and control groups was significantly lower compared to pre-antral follicles. However, the expression of ALK6 gene in antral follicles in 5.00% CEE and control groups was not significantly different compared to pre-antral follicles. The increasing effect of CEE on follicle viability with keeping normal gene expression indicates that addition of proper percentage of CEE to culture media improves culture conditions, making it a possible choice to be used as a follicular growth enhancer in infertility clinics.

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Introduction

In vitro follicle culture development has a major impact on both ovarian physiological researches and clinical trials.[1] In the clinical aspects, optimization and development of a proper follicle culture system help to improve clinical assisted reproduction, specially for cancer survived young women faced pre-mature ovarian failure due to gametotoxic effects of chemotherapy.[2] Recently, ovarian cryopreservation has been more interesting subject for future fertility of patients, which is due to success in fertility preservation using ovarian tissue transplantation technique.[3,4] In addition, ovarian cryopreservation is the only option for young patients with no partner, women that cannot undergo hormone stimulation and for patients that possibly there is a risk of malignancy reintroduction for them with ovarian tissue transplantation. Therefore, follicle culture system is necessary to increase fertility chance for those patients.

In 1992, Nayodu and Osborn have introduced a culture system able to support *in vitro* growth of intact pre-antral mouse follicles.[5] Pre-antral follicles are supposed to be a potential source of fertile oocytes,[6] however, developmental competence of *in vitro* grown follicles is lower than *in vivo*

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grown ones relating to deficiencies of *in vitro* condition compared to *in vivo* one.[7,8] Several *in vitro* culture systems have been developed to support mouse pre-antral follicle development and high-quality oocytes production to develop *in vitro* and produce offspring.[9-12] These systems not only are potentially applicable to grow human follicles but also are applicable to physiological studies,[1] transgenic research and endangered species preservation as well as studies on folliculogenesis mechanism.[13,14] Isolation method affects follicle quality and culture efficiency. Pre-antral follicle isolation is possible mechanically, enzymatically or through a combination of these two methods. Isolation of pre-antral follicles using collagenase and DNase results more follicles; however, this method can damage follicles and reduce *in vitro* survival rate compared to mechanical isolation due to damages to theca layer.[15,16] Another important critical item affecting follicle growth efficiency is the improvement of follicle microenvironment during *in vitro* growth to reduce possible damages such as apoptosis. Oocyte within follicle is surrounded by somatic cells such as granulosa cells with developmental and endocrine functions.[17] These somatic cells produce activin, inhibin, thecal differentiation factor and fibronectin supporting higher pre-antral follicle survival rate.[18] Therefore, proper microenvironment that can protect granulosa cell survival helps more efficient follicle culture.

Chick embryo extract (CEE) is prepared from whole chick embryos with components including hormones, growth factors and other proteins which are critical for cell culture.[19] It has been used for cultivation of some types of stem cells including neural crest and neuroepithelial stem cells.[19,20] Furthermore, CEE has been reported to increase human muscle stem cells proliferation.[21] It has been indicated that CEE could increase the immunity system function and lymphocytes proliferation.[21] The number of growth factors identified in CEE is constantly increasing and the combined effect of these growth factors on cell growth improvement during in the *in vitro* culture is being investigated.[22] The CEE can be considered as a cheap supplement that may provide a proper microenvironment for proper *in vitro* follicle growth. Follicle culture media has been supplemented with variety of additives such as serum supplements and growth factors.[23] Also, there are many types of gene expression showing functionality.

Therefore, considering CEE growth factors, its improving effects on cell proliferation and possibly on *in vitro* follicle culture were investigated by evaluation of granulosa cell survival, steroid hormone in media and genes expression (BMP15 and ALK6).

Materials and Methods

Chemicals. All chemicals were purchased from Sigma (St. Louis, USA), unless otherwise indicated.

Experimental animals. Animal experiments were performed according to the Declaration of Helsinki and the Guiding Principles in the Care and Use of Animals (DHEW Publication, National Institutes of Health, 80e23). The ethical code of this study was 94000133, and the start and end dates were September 23, 2015, and September 21, 2016. Female NMRI mice were purchased from Razi Institute, Tehran, Iran and kept at the temperature of 20.00 - 25.00 ˚C and 50.00% humidity in light-controlled condition (12L:12D photocycle) with sterile food and water. Eggs were purchased from Institute of Animal Science (Tehran, Iran) and incubated for nine days in a standard incubation condition (37.50 ˚C and 60.00% humidity).

Preparation of CCE and addition to media. After sterilization of the incubated eggs surface with ethanol, they were broken and all components of eggs were poured into sterile 100 mm dishes. Embryos were separated from other components using scissors and forceps and washed with phosphate-buffered saline to remove blood. Subsequently, embryos were transferred to another sterile 100 mm dish containing α-minimal essential medium (α-MEM; Gibco, Paisley, UK) (1.00 mg tissue weight mL^{-1}). For homogenization, embryos were blended in media using scissors, transferred to 50.00 mL tube and kept in – 70.00 ˚C overnight. In the next step, it was melted at room temperature and centrifuged at 33000 rpm for 30 min following complete melting. The supernatant was collected immediately, filtered and stored at – 70 ˚C for long term usage.[19]

Osmolarity assay. The osmolarity of culture medium is essential for optimal growth and development of follicle and any variation in medium osmolarity can have detrimental effect on follicle viability and development. In this study, osmolarity of the culture medium containing different concentrations of CEE was evaluated using an osmometer (Gonotec GmbH, Rinteln, Germany).

Follicle isolation. Female mice, 12-14 days old, were sacrificed and ovaries were immediately transferred to dissection medium including α-MEM supplemented with 10.00% fetal bovine serum (FBS: Gibco), 100 μg mL^{-1} penicillin and 50 μg mL^{-1} streptomycin under mineral oil to prevent evaporation and severe pH and temperature fluctuations. Pre-antral follicles were isolated by mechanical dissection of ovaries under a stereo-microscope (Sz61; Olympus, Tokyo, Japan) using 27-gauge needles. Intact follicles with a diameter of 120-150 μm, having one or two visible layers of granulosa cells with central and round oocytes, were selected for *in vitro* culture.

Experimental groups. Follicles were randomly divided into five experimental groups treated with different percentages of CEE (0.50%, 1.00%, 5.00%, and 10.00%) and a control group without CEE during 12 days culture.

***In vitro* culture of pre-antral follicles.** Isolated pre-antral follicles were cultured individually in 60 mm dishes (Becton Dickinson, Temse, Belgium) containing 20.00 µL droplets of α-MEM supplemented with 5.00% fetal bovine serum (FBS; Gibco), 100 mIU mL^{-1} recombinant follicle-stimulating hormone (rFSH or Gonal-f: Serono, Aubonne, Switzerland), 1.00% insulin, transferrin and selenium mix (ITS mix; 5.00 µg mL^{-1}, 5.00 µg mL^{-1} and 5.00 ng mL^{-1}, respectively; Gibco), 100 µg mL^{-1} penicillin and 50 µg mL^{-1} streptomycin as a base medium under mineral oil and incubated at 37 ˚C in a humidified atmosphere of 5.00% CO_2 in air for 12 days. Half of the medium was replaced with fresh media every other day. Follicles were monitored at day 1, 4, 6, 8, 10 and 12 of *in vitro* development using invert microscope (model:TS100, Nikon, Melville, USA) and survival rate of follicles at each stage was recorded.

***In vitro* ovulation induction.** On day 12, the induction of ovulation was performed with the replacement of total volume of droplets with fresh medium containing 1.50 IU mL^{-1} human chorionic gonadotropin (hCG; Organon, Oss, Netherlands). Then, 14-16 hr later, cumulus cells were removed mechanically by pipetting for observation of maturation status and oocytes at germinal vesicle (GV), germinal vesicle breakdown (GVBD) and metaphase II (MII) stages were counted.[6]

Granulosa cells survival rate analysis by MTT assay. Pre-antral follicles were isolated and transferred to 96 wells (TPP; Trasadingen, Schaffhausen, Switzerland) containing Dulbecco's modified Eagle's medium F12 (DMEM F12; Gibco) culture medium, supplemented with 5.00% FBS, 100 IU mL^{-1} penicillin and 100 g mL^{-1} streptomycin sulfate and cultured overnight for adhesion and expansion in 96 well. Adhered follicle and its granulosa cells were treated with 200 mL of fresh medium with different dosages of the extract. After 48 hr, previous media was discarded and fresh 200 µL media containing 20.00 µL of the MTT solution was added. After 3 hr, the supernatant was removed and the cells were treated with 150 mL per well dimethyl sulphoxide (DMSO) for 10 min. Absorbance at 570 nm was recorded using an ELISA plate reader.

Estradiol hormones measurement. During 12 days of follicle culture, 10.00 µL of media was removed from each droplet containing growing follicle and 10.00 µL fresh media was added. Removed media was collected from all follicles in each treatment during 12 days of follicle culture and stored at – 20 ˚C. After defreezing, estradiol level was measured using commercially available radio-immunoassay kits included the IBL (Hamburg, Germany) kit with a sensitivity of 9.70 pg mL^{-1} and a total precision of < 10.00% (% coefficient of variation; CV) and the Demeditec Diagnostics GmbH (Kiel, Germany) kit with a sensitivity of 0.04 ng mL^{-1} and a total precision of < 10.00% CV.

Primer design for RNA isolation and real-time PCR. It was carried out using Primer Design Software (version 6.24; Primer Biosoft, Palo Alto, USA). Primers sequences and gene bank accession number are presented in Table 1. The RT-PCR primers and the real-time PCR primers were designed based on the sequences in GenBank (Table 1) and synthesized by Invitrogen (Paisley, UK). The GAPDH was used as an internal control to normalize all of the threshold cycle values (Table 1).

RNA extraction and cDNA synthesis for follicle samples. The 5.00% CEE group was selected based on findings showing that follicle growth and antrum formation were higher in 5.00% CEE group compared to other CEE concentrations. Thus, the levels of gene expression in 5.00% CEE and control groups were examined. The RNA extraction and cDNA synthesis were performed for fresh pre-antral follicles (n = 40), antral follicles (n = 40) grown *in vitro* with the effect of 5.00% CEE and antral follicles (n = 40) without CEE as a control. Total RNA was extracted from all collected pre-antral and antral follicles using RNeasy micro kit (Qiagen, Hilden, Germany). The quantity of RNA was evaluated using a spectrophotometer (WPA; Biochrom, Cambridge, UK) at a wavelength of 260 and 280 nm. Reverse transcription was performed for 250 ng of total RNA and cDNA was converted using Elite DT based on RevertAid™ H Minus First Strand cDNA synthesis kit (Fermentas, Leon-Rot, Germany) according to the manufacturer's instructions. For analysis of BMP15 and ALK6 genes expression, real-time PCR amplification was performed in a mixture containing 2.00 µL cDNA and 8.00 µL PCR mix (1.00 µL forward primer mix, 1.00 µL reverse primer mix, 1.00 µL dH_2O and 5.00 µL power SyBER green PCR master mix). Each reaction mixture included qPCR was performed on Applied Biosystems StepOne (Thermo Fisher Scientific, Paisley, UK) according to a protocol including three stages as follows: Stage 1: 95 ˚C for 10 min, stage 2: 95 ˚C for 15 sec (by 40 cycles) and stage 3: 60 ˚C for 1 min. The reaction was performed in triplicate for each sample. The no template control was run

Table 1. Gene accession number, primer sequence and product size.

Genes	Accession No.	Primer pair (5 3)	Product length (bp)
BMP15	NM-009757	F: AAATGGTGAGGCTGGTAA R: TGAAGTTGATGGCGGTAA	148
ALK6	NM-007560	F: CGCTATATGCCTCCAGAA R:TCCACTATACCTCCAGAAAC	137
GAPDH	NM-008084.3	F: GACTTCAACAGCAACTCCCAC R:TCCACCACCCTGTTGCTGTA	119

concurrently with the original sample. The relative expression of each gene was obtained through Applied Biosystems StepOne software (version 2.1; Thermo Fisher Scientific).

Statistical analysis. All statistical analyses were performed using SAS software (version 9.1; SAS Institute, Cary, USA). The osmolarity, granulosa cells viability, estradiol level, and follicular development were analyzed by one-way ANOVA. Also, *t*-test was performed for analysis of oocyte maturation and gene expression. Data were expressed as mean ± SEM. Significant values ($p < 0.05$) were further analyzed with the Tukey test to determine significant differences.

Results

Osmolarity. Osmolarity parameter in media with the presence of all different examined percentages of CEE was similar to control group.

Follicle growth and oocyte maturation. Totally, 1167 pre-antral follicles with a mean diameter of 120 ± 8.60 µm were isolated. The development of follicles after 1, 4, 6, 8, 10 and 12 days is shown in Table 2. There were no significant differences in the percentage of developed follicles between all experimental and control groups after 1, 4, 6 and 8 days of development. However, on day 12, a significant increase was observed in the percentage of developed follicles in the group treated with 5.00% CEE compared to groups treated with other experimented percentages and control group ($p < 0.05$; Table 2).

The highest number of antral follicles was obtained after 12 days in the group treated with 5.00% CEE. Table 3 shows that there were no significant differences in the percentage of GV, GVBD and MII oocytes generated from fully grown follicles in culture media with 5.00% CEE compared to control group. But, with 5.00% CEE in culture media, the percentage of MII+GVBD oocytes was significantly higher than the control group ($p < 0.05$; Table 3).

Granulosa cells viability. Viability evaluation using MTT test showed no significant differences in the percentage of dead granulose cells with the presence of each experimented dosages of CEE in media and base media without CEE presence.

Estradiol level. The level of estradiol was not significantly different in treated groups with different percentages of CEE (0.50%, 1.00%, and 5.00%) compared to control group. While, a significant decrease was seen in estradiol level in group treated with 10.00% CEE compared to other experimented percentages of CEE and control group ($p < 0.05$).

Gene expression. Comparative gene expressions of antral follicles in control and 5.00% CEE groups are represented in Figure 1. Level of BMP15 expression was significantly decreased in antral follicles of control and 5.00% CEE groups compared to fresh pre-antral follicles. There was no significant difference in ALK6 expression in fresh pre-antral follicles compared to antral ones treated with 5.00% CEE and control group (Fig. 1).

Discussion

Several culture systems have been described for *in vitro* growth of female gametes, but the production of competent oocytes has still remained a challenge.[24] As mentioned before, improving effect of CEE has been reported on some types of cells.[19,20] In this survey, effect of CEE presence on *in vitro* growth of follicles was evaluated. One of the most important factors during mammalian *in vitro* cell culture is keeping optimum osmolarity.[25] It has been reported that media osmolarity higher than 300 Osmol L^{-1} has harmful effects on mouse *in vitro* embryo development.[26,27] In his study, osmolarity of media was lower than 300 Osmol L^{-1} and CEE supplementation did not change osmolarity of base media. Therefore, CEE addition to base media during follicle culture has no harmful effect on follicles regarding osmolarity.

Table 2. Effects of chick embryo extract (CEE) on follicle development during 12 days culture.

CEE (%)	Pre-antral follicles	Developing follicles (%)					
		Day 1	Day 4	Day 6	Day 8	Day 10	Day 12
0	247	95.94 ± 0.59	79.75 ± 0.34	72.51 ± 1.84	67.23 ± 1.17	51.80 ± 0.80	26.31 ± 1.37^{b}
0.50	210	95.23 ± 0.67	78.57 ± 1.16	70.47 ± 1.78	67.14 ± 2.02	53.81 ± 1.78	23.81 ± 1.54^{b}
1.00	210	95.68 ± 1.26	76.69 ± 1.24	73.83 ± 2.58	60.46 ± 0.47	53.74 ± 3.68	25.61 ± 2.00^{b}
5.00	290	94.85 ± 0.64	77.33 ± 2.05	75.00 ± 4.08	66.33 ± 2.27	58.71 ± 2.59^{a}	37.62 ± 1.85^{a}
10.00	210	94.28 ± 1.16	75.71 ± 1.16	67.14 ± 2.02	60.94 ± 3.74	48.90 ± 1.16^{b}	26.19 ± 1.78^{b}

Different superscripts indicate significant difference ($p < 0.05$).

Table 3. Gene accession number, primer sequence and product size.

CEE (%)	AF (No.)	GV (%)	GVBD (%)	MII (%)	MII+GVBD (%)
0	65	24.10 ± 4.32	46.81 ± 2.25	29.09 ± 4.28	75.90 ± 0.47^{b}
5.00	109	16.47 ± 1.57	47.00 ± 4.38	36.53 ± 3.17	83.53 ± 1.24^{a}

AF: Antral follicles; GV: Germinal vesicle stage oocytes; GVBD: Germinal vesicle breakdown; MII: Metaphase stage oocytes; MII+GVBD: Meiotic resumption; CEE: Chick embryo extract.

[ab] Different superscripts indicate significant difference ($p < 0.05$).

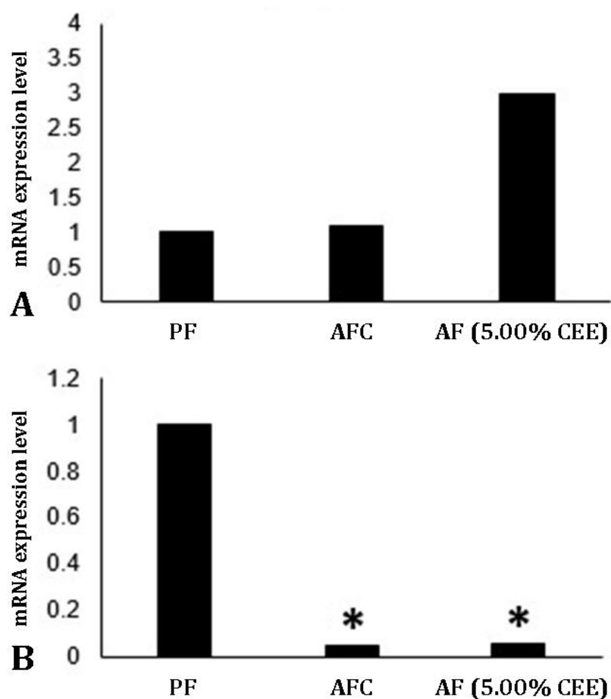

Fig. 1. The expression level of ALK6 (A) and BMP15 (B) genes in pre-antral follicles (PF), antral follicles (AF) generated from control group (AFC) and antral follicles generated from treated follicles with AF (5.00% CEE). Asterisks show significant differences compared to fresh pre-antral follicles ($p < 0.05$).

It has been reported that granulosa cells surrounding oocytes support proper growth of oocytes due to providing nutrients for oocytes.[28] Furthermore, it has been reported that granulosa cells are able to retain their *in vivo* phenotype to keep physiological concentrations of FSH, insulin and insulin-like growth factor-I during *in vitro* follicle growth.[29] The MTT assay showed that the extract has no harmful effect on granulosa cells viability as there were no significant differences in granulosa cells viability rate with or without CEE supplementation.

Estradiol is one of the main steroids that evaluation of its concentration indicates follicular function.[30] In is study, with the addition of 0.50%, 1.00%, and 5.00% CEE, during 12 days of follicle culture, estradiol remained at normal level. However, addition of 10.00% CEE to culture medium caused a significant reduction in estradiol level.

The highest percentage of produced antral follicles at day 12 was observed with the inclusion of 5.00% CEE (Table 2). Also, Table 3 shows that the percentage of MII+GVBD oocytes indicating meiotic resumption under the effect of 5.00% CEE was the highest (Table 3). It has been demonstrated that CEE contains growth factors that can cause different types of cells proliferation.[19-21] Higher antral follicles and a higher percentage of oocytes that were able to start *in vitro* meiotic resumption under the effect of CEE were similar to improving effects on cell culture in previous studies.[19]

The transforming growth factor-β superfamily is the largest family of extracellular signaling proteins.[31] Bone morphogenic protein 15 (BMP15) is a member of this family playing important role in granulosa cells proliferation and follicular growth, especially in the early stages of follicular development. The BMP15 is expressed specifically in oocytes,[32,33] so it is a logical marker to evaluate oocyte function during *in vitro* follicle culture.[3] In this study, BMP15 expression in fully grown follicles under the effect of 5.00% CEE until antral stage and control group was compared to its expression in fresh pre-antral follicles. In treated and control groups, the expression level in antral follicles was significantly diminished compared to fresh pre-antral follicles. It has been reported that BMP15 expression occurs in all stages of folliculogenesis.[34] But, expression level reduces at end stages of follicle development and stops in pre-ovulation stage.[35] The current data also suggest that CEE addition to culture media doesn't change BMP15 expression pattern during *in vitro* growth.

The ALK6 is a BMP15 receptor expressing in granulosa cells and mostly antral follicles.[36,37] The ALK6 gene expression in fully-grown antral follicles treated with 5.00% CEE and control group was not significantly different from fresh pre-antral follicles. During *in vitro* folliculogenesis, survival rate reduction was dramatically elevated at day 10 and day 12 with addition of 10.00% CEE (Table 2). The highest survival rate was seen at day 12 in the group treated with 5.00% CEE and it was significantly different from control and other groups (Table 2). Therefore, the most effective percentage of CEE during *in vitro* follicle growth is probably 5.00% in media, but higher percentage of CEE has adverse effects decreasing survival rate and estradiol level during *in vitro* follicle culture. Furthermore, this research showed that supplementation of a proper percentage of CEE has no adverse effects on granulosa cells viability and it doesn't induce abnormal changes in estradiol level and expression of BMP15 and ALK6 genes.

In conclusion, CEE addition to follicle culture media improves the *in vitro* follicles growth and it could be considered as an easily available and inexpensive supplement for this purpose.

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Conflict of interest

The authors declare that there is no conflict of interest.

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