Effects of *Matricaria chamomilla* Extract on Growth and Maturation of Isolated Mouse Ovarian Follicles in a Three-dimensional Culture System

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**Abstract**

**Background:** The aim of this study was to design and assess the effects of hydroalcoholic extract of *Matricaria chamomilla* (MC) on preantral follicle culture of mouse ovaries in a three-dimensional culture system.

**Methods:** Isolated preantral follicles were randomly divided into three main groups: the control group containing 10% fetal bovine serum without MC extract (G1), the first experimental group supplemented with 25 µg/ml hydroalcoholic extract of chamomile (G2), and the second experimental group supplemented with 50 µg/ml hydroalcoholic extract of chamomile (G3).

**Results:** After 12 days of culture, the survival rate ($P < 0.05$), antrum formation ($P < 0.01$), metaphase two oocytes ($P < 0.01$), and the expression of $PCNA$ ($P < 0.05$) and $FSHR$ ($P < 0.05$) genes significantly decreased in G3 as compared with G1. On the other hand, the last day of culture (day 12), the mean diameter of follicles cultured in the medium which was supplemented with 50 µg/ml hydroalcoholic extract of chamomile significantly decreased as compared with the G1 ($P < 0.05$). In addition, the levels of progesterone and dehydroepiandrosterone hormones significantly increased in the medium of G3 relative to G1 ($P < 0.01$), while in the medium of G1, the level of 17β-estradiol was significantly higher than that of other groups ($P < 0.01$). Reactive oxygen species levels of metaphase II oocytes were significantly decreased in G2 as compared with G1 ($P < 0.01$).

**Conclusion:** Adding chamomile extract to culture media appeared to decrease follicular function and development.

**Key words:** Follicular Development; Follicular Maturation; $FSHR$ Gene; $PCNA$ Gene; Steroid Hormones; Toxicology

**Introduction**

For many centuries, herbal medicine, which also called botanical medicine or phytomedicine, has been one of the most important solutions for medical therapy in ancient nations. Ancient Chinese, Egyptians, Iranians, and Indians have used plants’ seeds, roots, leaves, and/or flowers for medicinal purposes. There are several species of herbal plants of which several different combinations exist. Nowadays, many chemical analyses have been carried out on plant extracts to identify various compounds of extract. Many combinations such as sterols, flavonoids, organic sugars, and phenolic compounds were found in the extracts of herbal plants. Furthermore, several studies have shown that medicinal plants could play...
an important role in treating many conditions such as allergies, asthma, eczema, and diabetes mellitus.[6-9] On the other hand, the World Health Organization estimates that 80% of people worldwide are using herbal medicines for their health care.[10] One of the herbal plants is *Matricaria chamomilla* (MC). MC, which is known as chamomile, is a limb of the composite family *Asteraceae* that in the traditional medicine has been used as a drug for treating flatulence, colic, hystera, wounds, and intermittent fever in many countries from Europe to Asia.[7-9] Studies have shown that both lipophilic and hydrophilic forms of chamomile extract are effective for therapeutic activities.[1,10,11]

The most characteristic constituents of chamomile are unstable oil, sesquiterpene lactones, ascorbic acid, and phenol compounds, primarily the flavonoids, apigenin, quercetin, patulin, luteolin, and glycosides.[11] Flavonoids are chemical phenyl benzopyrones which are usually observed in all vascular plants. The benzopyran ring system is a molecular scaffold which can be seen in flavonoid-inherent products and has weak aromatase inhibitory activity.[11] One of the Chamomilla compounds is flavonoid antioxidants that neutralize reactive oxygen metabolites.[12-14] Antioxidants are human made or natural substances that prevent the formation of free radicals and lipid peroxidation. Antioxidants by binding to free radicals neutralize their destructive properties, such as breakdown of body cells and tissue, DNA fragmentation, and membrane lipid peroxidation. On the other hand, in two- or three-dimensional mouse follicle culture systems, follicles are kept under higher concentrations of O₂ in an incubator.[15] During *in vitro* follicle culture, free radicals are continuously produced in aerobic cells; thus antioxidants can lead to removal of free radicals.[16] Phytoestrogens are one of the other important compounds of Chamomilla.[17] It is a naturally occurring plant compound which is considered as an estrogen-like compound that has similar effects to estrogen and progesterone hormones.[18] Experimental and clinical studies which were performed on chamomile concluded that the majority of their pharmacological functions are pertaining to its antioxidant activity which is mostly due to its capability to control the free radicals and/or inhibit lipid peroxidation.[19] In one study, Johari et al.[20] investigated the effects of hydroalcoholic extract of MC in several doses (10, 20, and 40 mg/kg) on the level of production of follicle-stimulating hormone (FSH), luteinizing hormone, estrogen, and progesterone hormones, and also on the changes in ovarian tissue. Moreover, in another study, Johari et al.[21] showed that in all of the doses of MC extract, the levels of estrogen decreased and the levels of progesterone increased; also, the number of ovarian follicles decreased. Farideh et al.[11] reported that treatment with chamomile alcoholic extract decreases the signs of polycystic ovarian syndrome (PCOS) in the ovarian tissue and levels of estradiol hormone. MC extracts due to antioxidant activities have positive effects in the treatments of different diseases, such as PCOS, but repeated use of MC extract or use of high concentration of it may be harmful. Thus, the aim of this study was to assess the effects of MC hydroalcoholic extract on three-dimensional follicle culture because there were no scientific reports of Chamomilla effects in *in vitro* mouse ovarian follicle culture.

**Methods**

**Preparation of hydroalcoholic extract**

In order to prepare the whole-plant chamomile extract, a half kilogram of chamomile flower was dried at 25°C and was protected from direct sunlight. For extraction, after grinding the dried plants, they were dissolved in 2 L of alcohol 96% and then kept at room temperature for 48 h. Over this period, after shaking frequently, the solution was filtered. Then, the solution was centrifuged at 3000 r/min for 5 min. At the end of the process, the resulting solution was poured into an open-top container, and the solvent was evaporated. Some 90 g of a semi-solid extract was obtained from 500 g of chamomile powder. To achieve appropriate concentration, the extract was dissolved in normal saline.[20,21]

**Animals and collection of ovarian follicles**

In this study, 12- to 14-day-old female National Medical Research Institute mice (*n = 40*) were used. They were maintained in the Laboratory Animals of the University at standard conditions (temperature 25°C, 12 h/12 h light/dark cycle, and 55% humidity). Mice were sacrificed by cervical dislocation, and then immediately their bilateral ovaries were dissected free of fat and mesentery using 29G insulin needles and transferred to dissection medium containing α-minimum essential medium (α-MEM, WelGENE) supplemented with 10% fetal bovine serum (FBS) (Gibco, Paisley, UK), 100 IU/ml penicillin, and 100 mg/ml streptomycin. All experimental procedures were conducted according to the Guide for the Care and use of laboratory animals of the Tabriz University of Medical Sciences, Tabriz, Iran.

**Study design**

In this stage, 150–170 µm intact preantral follicles were mechanically isolated from immature mouse ovaries. Then, they were divided into three main groups: G1, the control group containing 10% FBS without MC extract; G2, the first experimental group supplemented with 25 µg/ml hydroalcoholic extract of chamomile; and G3, the second experimental group supplemented with 50 µg/ml hydroalcoholic extract of chamomile. Moreover, the base of the culture medium of follicles for three groups was composed of α-MeM supplemented with 0.33 mmol/L sodium pyruvate, 100 µIU/ml recombinant FSH (or Gonal-f; Serono, Switzerland), 1% insulin (Gibco, Paisley, UK), transferrin (Gibco, Paisley, UK), 100 µg/ml penicillin, 50 µg/ml streptomycin, and FBS 5%. The isolated follicles were cultured for 12 days at 37°C in 5% CO₂. Furthermore, every other day, half of the medium was replaced by fresh medium.[22] At the end of culture period, analysis of development genes (PCNA and FSHR)
and steroid hormones (17-β estradiol, progesterone, and dehydroepiandrosterone (DHEA)) was performed and also the released metaphase II (MII) oocytes in three groups of culture were collected and randomly considered for reactive oxygen species (ROS) assay.

**Three-dimensional in vitro culture of isolated preantral follicles**
The preantral follicle was encapsulated in sodium alginate. Brieﬂy, alginate was dissolved in deionized water at a concentration of 1% (w/v) and was then mixed with activated charcoal (0.5 g of charcoal per gram of sodium alginate). Following charcoal treatment, the prepared sodium alginate solution was sterile ﬁltered through 0.22-µM ﬁlters. Then, aliquots of charcoal-stripped and sterilized sodium alginate were diluted with sterile phosphate-buffered saline (PBS) at a concentration of 0.5% (w/v) at room temperature for experimental study. Each isolated follicle was individually transferred to the 8 µl of alginate solution and then they were slowly immersed in 140 mmol/L NaCl and 50 mmol/L CaCl₂, for 2 min. At the end of the process, all alginate droplets were removed and washed in α-MeM media. Then, they were transferred into 30-mm Petri dishes (SPL Life Science Co., Seoul, South Korea) which were previously ﬁlled with 30 µl of culture medium (each plate was ﬁlled with 25 droplets of 30 µl of culture medium) overlaid with mineral oil (Sigma-Aldrich, Munich, Germany). The isolated follicles were cultured for 12 days at 37°C in 5% CO₂.

**Assessment of follicular morphology and diameter**
During the culturing period, the morphology of follicles was checked under an inverted microscope every 48 h. In addition, after the follicular photographs were taken at ×100 magniﬁcation under an inverted microscope with transmitted light and phase objectives (Leica, Bannockburn, IL, USA), measurement of follicular diameter was carried out by ImageJ software (ImageJ, NIH, USA) (n = 30 for each group).

**In vitro ovulation induction**
On day 12 of culture, by adding 1.5 IU/ml human chorionic gonadotropin (hCG; Organon, Netherlands) to the culture media, oocyte maturation and ovulation were induced. The oocytes are considered as germinal vesicle (GV) stage if the GV is visible, but if not, they are recorded as GV breakdown. On the other hand, if a polar body is present in the perivitelline space, the oocytes are classiﬁed as MII. They were assessed 18–24 h after adding hCG to the culture media.

**Hormonal assays**
At the end of the culture period, day 12, the levels of 17-β estradiol (E₂), progesterone (P₄), and DHEA hormones were measured in collected media derived from follicle culture. The levels of E₂ were measured by a Microplate Enzyme Immunoassay kit (monobind, sensitivity = 6.5 pg/ml), of P₄ by an enzyme-linked immunosorbent assay kit (DIAPLUS, sensitivity = 0.1 ng/ml), and of DHEA by a Microplate Enzyme Immunoassay kit (monobind, sensitivity = 0.04 mg/ml). These experiments were minimally done in triplicates.

**RNA extraction and cDNA synthesis for molecular assessment**
In order to evaluate gene expression, some follicles in all of the study groups were collected in three replicates (15 follicles in each replicate) at day 12 of culture. Total RNA was extracted from each group using a TRIzol reagent extraction method (Invitrogen, Paisley, UK). The RNA concentration was determined by spectrophotometry and adjusted to a concentration of 400 ng/ml. Using oligo dT, RNA was reverse transcribed by Moloney murine leukemia virus reverse transcriptase. Sequences for gene-speciﬁc PCNA, FSHR, and GAPDH primers were as follows: PCNA forward: AGGAGCGGTAAACCATAG, PCNA reverse: ACTCTACAACAGGGGCACATC; FSHR forward: CCAGGCTAGTGATGCATC; FSHR reverse: GGCCTCAAACCTCTGAACACT and GAPDH forward: GGAAAGAGCTTAGGCACAT, GAPDH reverse: CTGCCTGACGGCCAGG. GAPDH gene was used as an internal control.

**Real-time reverse transcription-polymerase chain reaction**
Reverse transcription-polymerase chain reaction (RT-PCR) was performed on Applied Biosystems (UK) by SYBR Green quantitative RT-PCR Kit (Sigma-Aldrich). The real-time thermal condition included holding step at 1 cycle at 95°C for 10 min (as an initial denaturation), cycling step, 40 cycles of 95°C for 15 s, 58°C for 30 s, and 72°C for 30 s, and a ﬁnal extension step, the melt curve step, at 95°C for 15 s, 60°C for 1 min, and 95°C for 15 s. Then, quantitative analysis of the genes in two groups was done by Pfaffl method (ΔΔCt, ΔΔCt = ΔCtSample − ΔCtControl). ΔCt = CtTarget − CtGAPDH.

**Reactive oxygen species assay**
ROS levels were measured in the MII oocytes obtained from all in vitro culture groups (n = 24 for each group for three repeats). Eight MII oocytes were pooled in the RNase-free microtube and were washed three times with PBS. In step one that was done in a dark room, pooled MII oocytes were incubated in 40 mmol/L of Tris–HCl buffer at pH = 7.0 containing 5 mmol/L 2′,7′-dichlorodihydroﬂuorescein diacetate (Sigma-Aldrich, Germany) at 37°C for 30 min. In step two, they were washed with PBS and then sonicated at 50 W for 3 min, and immediately centrifuged at 3000 × g for 10 min at 4°C. In the last step, the supernatants were collected and monitored using a spectroﬂuorometer at 488 nm excitation and at 525 nm emissions.

**Statistical analysis**
Statistical analysis was carried out by IBM SPSS Statistics 22.0 Software (IBM Corp., Armonk, NY, USA). Data were tested for normality analysis of the parameters with Kolmogorov–Smirnov test. The results were expressed as mean ± standard deviation (SD) and were assessed by one-way analysis of variance. Tukey’s honestly signiﬁcant difference was used for post hoc tests. Differences with P < 0.05 were considered statistically signiﬁcant.
RESULTS

The diameter of cultured isolated preantral follicles

The inverted microscope images of the cultured isolated follicles in the three-dimensional culture system are demonstrated in Figure 1. For all groups of the culture, the mean diameter of follicles at the beginning of culture was 160 ± 5 μm. On day 12 of culture, the mean diameter of cultured follicles was significantly decreased in G3 as compared with G1 (P < 0.05). Moreover, on day 6 of culture, the mean diameter of cultured follicles in the G2 and G3 decreased in comparison to G1, although it was not significant (P > 0.05). Furthermore, in all groups of the study, the size of preantral follicles increased during the culture period [Figure 2].

Follicular developmental rate

The developmental rate of cultured follicles in all groups of the study is summarized in Table 1. At the end of the culture period, day 12, the survival rate of follicles in G3 as compared with other groups was significantly decreased (P < 0.05); therefore a higher percentage of survival rate was observed in G1. Moreover, our results showed that the survival rate between both experimental groups has a significant difference. In addition, the rate of antrum formation of follicles cultured in the medium supplemented with 50 μg/ml and 25 μg/ml hydroalcoholic extract of chamomile was 38.1 ± 1.5% and 42.6 ± 1.5%, respectively. In addition, it was 45.6 ± 2.2% for follicles cultured in G1. Thus, there was a significant difference between G3 and two other groups (P < 0.01). Moreover, the MI rate was significantly decreased in G3 as compared with two other groups (P < 0.01).

Real-time reverse transcription-polymerase chain reaction analysis

In all of the study groups, the relative expression of PCNA and FSHR genes as compared with the housekeeping gene (GAPDH) is shown in Figures 3 and 4, respectively. Real-time RT-PCR results showed that the levels of mRNA for FSHR and PCNA were significantly lower in G3 than that of G1 (P < 0.05).

Hormonal assays

At the end of follicular culture period, half of the medium was collected to investigate the concentrations of E2, P4, and...
DHEA. Their concentrations are summarized in Table 2. Our observations showed that the E2 levels in two experimental groups were significantly decreased as compared with the control groups (P < 0.01). Moreover, the P4 and DHEA levels in G3 and G2 were significantly higher than that of G1 (P < 0.01).

Reactive oxygen species level

There was a significant decrease in the ROS levels of MII oocytes collected from both experimental groups as compared with the control group (P < 0.01). On the other hand, there was no significant difference between G3 and G2 and also between G3 and G1 [Figure 5]. Data of ROS levels are shown as mM H2O2.

Discussion

Folliculogenesis is a complex and dynamic process in which a number of small primordial follicles which contain an immature oocyte surrounded with somatic cells grow to form preovulatory follicles that enter the menstrual cycle. In the growth and development of follicles, various para- and autocrine factors are involved. On the other hand, for in vitro studying of folliculogenesis and effective factors on it, oogenesis, and/or oocyte–somatic cell interactions, three-dimensional culture system has been developed. Moreover, in this system, factors such as hormones, antioxidant supplements, and herbal extracts can be added to culture media for investigating their effects on folliculogenesis and oogenesis. However, our results showed that the size and diameter of follicles were increased in all of the study groups, while isolated follicles which were cultured in supplemented media containing 25 and 50 µg/ml hydroalcoholic extract of chamomile have shown smaller diameter than that of the control group on days 6 and 12 of culture. Furthermore, only on day 12 of culture, the mean diameter of follicles which were cultured in the medium supplemented with 50 µg/ml MC extract significantly decreased relative to the control group. Capcarova et al. acclaimed that quercetin, one of the compounds of chamomile, had no effect on granulosa cell proliferation. Furthermore, Murray et al. demonstrated that adding ascorbic acid with different concentrations to culture media had no effect on the growth of the follicles. Therefore, it seems that adding hydroalcoholic extract of chamomile to culture media of follicles has no dramatic effects on follicle growth and size. Moreover, data from the present study showed that the survival rate of follicles cultured in the experimental group 2 as compared with the control group had a significant decrease, and also the rates of MII and antrum formation in the experimental group 2 were significantly decreased. On the other hand, the expression levels of FSHR and PCNA were carried out by real-time RT-PCR to investigate the follicular development. FSHR is one of the most important genes which involved in the normal folliculogenesis process. Structurally, it is a G protein-coupled and seven-transmembrane receptor which links to the several pathways, such as adenylate cyclase. It has been reported that the FSHR is expressed in growing follicles. PCNA is a protein with a weight of 36,000 and it has been reported in fetal and
It has been reported that other ROS levels of MII oocyte derived from in vitro the activity of cytochrome p450; therefore, the amount of transformation of cholesterol to pregnenolone by reducing However, it has been reported that phytoestrogens inhibit the androstenedione, testosterone, and estradiol, successively. CYP17A1, the progesterone is converted into the by HSD3B1 converts into the progesterone. On the other hand, in the second pathway, the pregnenolone the testosterone converts into the estradiol by CYP19A1. testosterone by HSD17B1. and subsequently by HSD3B1, DHEA converts into the DHEA by CYP17A1 pregnenolone via two pathways converts into estradiol. and then by CYP11A1, it converts to pregnenolone. The increment of P4 level in the culture media can lead to decrease of estrogen secretion. Moreover, genistein is one of the phytoestrogen compounds of the progesterone hormone increase. Several studies have shown that genistein can inhibit steroidogenic enzymes and steroidogenesis, induce follicular atresia, and decrease oocyte maturation. Patel et al. reported that adding genistein to the culture media inhibited follicle growth, but did not induce follicle atresia and also genistein increased testosterone and DHEA and decreased progesterone levels. Previous studies demonstrated that genistein exposure (50 µM) can inhibit the ability of granulosa cells to produce progesterone and also genistein exposure (1, 18.5, and 185 µM) inhibits estrogen and progesterone levels in cultured porcine granulosa cells. In addition, one study acclaimed that neonatal genistein exposure (50 mg·kg⁻¹·d⁻¹) has no effect on progesterone and testosterone serum levels during pregnancy and before puberty in mice. We guess that hydroalcoholic extract of chamomile at the doses of 25 and 50 µg/ml due to altering the steroidogenesis decreased follicular growth and maturation. The increment of P4 level in the culture media can lead to inhibition of granulosa cell division and, consequently, decrease of estrogen secretion. Although the mechanism of how genistein alters steroid hormones is unknown, Patel et al. reported that 72 h after culture, the STAR and Cyp11a1 gene expressions increased. Two mentioned enzymes are involved in increasing progesterone production. Kang et al. evaluated the effects of quercetin on meiotic maturation of mature oocytes and cumulus cell steroidogenesis. Their results showed that the levels of estrogen and progesterone and also the rates of polar body extraction decreased. Moreover, another study reported that quercetin at the dose of 50 µg/ml did not affect granulosa cell growth while inhibiting progesterone production by granulosa cells and altered estradiol-17β production in a dose-related manner. In the culture of preantral follicles, oxidative stress by inducing an apoptotic mechanism can play an important role in follicular atresia. Our results showed that the ROS level of oocytes in the first experimental group as compared with the control group significantly decreased, but no significant
difference between the second experimental and control groups was observed. ROS are spontaneously generated in the normal metabolism of cells and also in the physiological phenomena against severe infections that lead to cell death. Flavonoids that present in the plant-derived beverages, fruits, and/or vegetables of plant nuts are powerful natural antioxidants that can protect the cells during culture from oxidative stress.\footnote{5,56} Also, several studies have shown that the flavonoids of chamomile not only have antiviral, antiallergic, and anticancer effects, but also have antioxidative effects.\footnote{5,56} On the other hand, it has been suggested that high levels of flavonoids could be toxic to the oocyte.\footnote{49} It has been demonstrated that apigenin decreases oxidative stress-induced damage in osteoblastic cells.\footnote{52} Also, Kang et al.\footnote{53} reported that quer cetin decreased the ROS level in \textit{in vitro} maturation oocytes.

In conclusion, it seems that the high concentration of hydroalcoholic extract of \textit{Matricaria} can inhibit cell proliferation and also decrease the estradiol level. Therefore, these happenings may be related to the high concentration of flavonoids and/or phytoestrogens. Our results demonstrated that adding chamomile extract to the culture media during follicular culture in a three-dimensional culture system appeared to decrease follicular function and development.

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Conflicts of interest

There are no conflicts of interest.

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