Chlorogenic acid improves functional potential of follicles in mouse whole ovarian tissues in vitro

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Abstract

Background: *Chlorogenic acid* (CGA) is one of the well-known polyphenol compounds involved in several important biological and therapeutic functions. In optimizing a culture system to achieve complete development of follicles, we focus on the effects of CGA supplementation during *in vitro* culture (IVC) on follicular development, oxidative stress, antioxidant capacity, developmental gene expression, and functional potential in mouse cultured ovarian tissue.

Methods and Results: The collected mouse whole ovaries were randomly divided into 4 groups: 1) non-cultured group (control 1): 7-day-old mice ovaries, 2) non-cultured group (control 2): 14-day-old mice ovaries, 3) cultured group (experimental 1): the culture plates contained only the basic culture medium, 4) cultured group (experimental 2): the culture plates contained basic culture medium + CGA (50, 100 and 200 μmol/L CGA). The following evaluations were done for all groups: histological evaluation, biochemical evaluation, the expression of genes related to follicular development and apoptosis by RT-qPCR, and analysis of 17-β-estradiol. The outcomes of the current study showed that supplementation of ovarian tissue culture media using CGA (100 μmol/L) significantly increased the survival, developmental and functional potential of follicles in mouse whole ovarian tissues after 7 days of culture. Furthermore, the result of this study revealed that CGA (100 μmol/L) could suppress oxidative damage and improve the culture system by enhancing the concentration of antioxidant and developmental gene expression.

Conclusion: For the first time, results showed that the supplementation of ovarian tissue culture media using CGA could optimize the culture system.

Introduction

In vitro culture (IVC) system of ovarian tissue is a considerably interesting alternative approach to improve the reproductive potential and preserve female fertility [1]. Many researchers have also been committed to defining the optimal maturation condition (physical and biochemical condition) for the IVC system of ovarian tissue [2–5]. Despite the proposed designation of IVC systems, the improvement of this type of culture system is challenging because the survival rate of follicles, maturation rate of oocytes, and embryo development are still low compared with in vivo [6, 7].

Several studies have clarified that the reasons for these negative effects of *in vitro* culture on follicular development may be related to multiple exogenous factors such as culture medium, oxygen tension, visible light, and handling of the specimen [8]. It is guessed that the IVC systems for ovarian follicles contain a higher oxygen concentration than those of in vivo habitats inside the lumen of the female reproductive tract [9]. The high concentration of oxygen is predominantly associated with IVC conditions that result in the production of oxidative stress (OS) which reportedly poses a threat and damage to biological molecules and leads to poor quality, dysfunction, and cell death of *in vitro* matured oocytes [10].

Many researchers have also been committed to triggering alternative treatment strategies by adding some supplements such as growth factors [11], antioxidants [12], nutrients [13], and ROS scavengers [14] to culture media that help to reduce oxidative stress and improve IVC conditions. Many reports suggest that antioxidant supplementation of IVC media improves oocyte quality by reducing ROS levels and apoptotic factors [15–18].

Chlorogenic acid (CGA) (Fig. 1), the most available caffeoylquinic acid, is one of the well-known polyphenol compounds [19]. A variety of scientific pieces of evidence have clarified several important biological and therapeutic functions of CGA including antioxidant activity, antibacterial, hepatoprotective, cardioprotective, anti-inflammatory, antipyretic, neuroprotective, anti-obesity, antiviral, anti-microbial, and anti-hypertension functions [20]. Some studies have also confirmed the significant antioxidant potential of CGA; besides, it is speculated that CGA can scavenge free radicals [18, 21]. Moreover, it has been demonstrated that supplementation of human hepatoma cell line using CGA limited oxidative-stress-related apoptosis by alleviating ROS production that occurs via increasing intracellular glutathione (GSH) storage [22].

To the best of our knowledge, there is not any information regarding the effects of CGA on cultured ovarian tissue. On the other hand, it is crucial to improve the developmental competence of ovarian follicles because its inefficiency diminishes its application to further stages [23, 24]. Therefore, to optimize a culture system and achieve a complete development of follicle and maturity of the oocyte, we focus on the effects of CGA on follicular development, OS, antioxidant capacity, and developmental gene expression in mouse cultured ovarian tissue. Additionally, this study evaluates the effect of the CGA on the levels of estradiol hormone (as an indicator of ovarian function) in a culture medium to determine the adequacy or functional potential of the ovarian tissue.

Materials & Methods

Animals and Ovarian Tissue

In this original study, ovarian tissues were obtained from 7 and 14-day-old female mice. The mice were bred in standard cages and subjected to conventional and management conditions (12 hours' light/dark cycle and comfortable temperature (22 ± 2˚C) and controlled humidity (40–50%) in the central animal house of Urmia University.

Experimental Design

The collected mice ovaries were randomly divided into 4 groups: 1) non-cultured group (control 1): 7-day-old mice ovaries, 2) non-cultured group (control 2): 14-day-old mice ovaries, 3) cultured group (experimental 1) with culture plates containing only the basic culture medium, 4) cultured group (experimental 2) with culture plates containing basic culture medium + CGA (50, 100 and 200 μmol/L). All groups underwent the subsequent analyses.

In Vitro **Culture of Ovarian Tissues**

The mice (n = 46) were sacrificed by cervical dislocation and their ovaries were isolated and dissected (under sterile conditions) mechanically free of fat and mesentery. For tissue culture, whole ovaries in both experimental groups were cultured individually on culture inserts (Millicell® CM, 0.4 μM pore size, Millipore Corp, Billerica, MA, USA) and placed in 24-well plates containing the basic culture medium that consisted of 400 μL of α-MEM (Gibco, UK) supplemented with 10% FBS, 100 IU/ml penicillin, 100 μg/ml streptomycin, 0.23 mmol sodium pyruvate and 0.22 gr/ml sodium bicarbonate (Abedpour, Salehnia, et al. 2018). All of the cultures were incubated at 37°C in 5% CO_2 for one week.

Administration of Chlorogenic Acid

In cultured groups (experimental 2), CGA (Sigma-Aldrich, St. Louis, MO, USA) powder was dissolved in ethanol solution (Sigma-Aldrich, St. Louis, MO, USA) and directly (after filtering) added into the culture medium (Nguyen, Wittayarat et al. 2018). The best dose of CGA was chosen pending our different evaluations.

Evaluation of the Ovarian Follicular Viability using Trypan Blue Staining

The survival rates of the isolated preantral follicles from cultured ovaries (n = 30/each group) were determined using trypan blue staining (0.4%) (Sigma, St. Louis, MO). The follicles were recorded as degenerated or survived: degenerated follicles stained blue, damaged follicles partly stained blue and surviving ones not stained [25].

Morphological Analysis of Apoptosis

For quantification of apoptosis by morphologic criteria, isolated preantral follicles (n = 30/each group in 3 repeats) were stained with both Acridine Orange (AO) and Ethidium Bromide (EB) (Sigma-Aldrich, USA). The follicles were reported as viable (green), early apoptotic (bright orange), and late apoptotic (red) under a fluorescent microscope [26].

Histological Evaluation

For morphological and histological examinations of all groups of the study, first ovaries were fixed in Bouin's solution then sent to a pathological laboratory for sectioning and staining (hematoxylin-eosin or H&E). By using a light microscope, histological evaluation of the follicular stage was performed based on the number of layers and morphology of the granulosa cells surrounding the oocytes, using follicles classified categories as described previously [27].

Ovarian Area

The ovarian morphology was assessed using an inverted microscope every 2 days during the culture period on days 3, 5, and 7 in all groups of study (n = 5/subgroup). The area of ovarian tissues was calculated by the photos of each ovary that were captured with an attached digital 10 MP microscope

camera (D-Moticam 10^{+}) at the same magnification and imported into Image J software (National Institutes of Health, Bethesda).

Hormonal Assay

The concentration of 17 β-estradiol (E_2) was measured in the collected media obtained from all groups of study during the culture period on days 3 and 7 using an ELISA Kit according to the provider's instruction. The analytical sensitivity of the assay was expressed pg/mL for E_2 (MyBioSource, San Diego, California, USA).

Assays for Oxidative Stress Markers

Biochemical assays were carried out for evaluating oxidative stress markers (Malondialdehyde (MDA), glutathione (GSH), and total antioxidant capacity (TAC) in all groups using an ELISA Kit according to the provider's instruction (ZellBio GmbH, Biotechnology company in Lonsee, Germany) [28].

Real-Time RT-qPCR

Gene expression of apoptotic genes (*Bax* and *Bcl-2*), bone morphogenetic protein (*BMP15*), and growth differentiation factor (*GDF9*) were determined by RT-qPCR in ovarian tissues using the Light Cycler 480 with SYBR Green detection and Amplicon Kit (Applied Biosystem). To design PCR primer sequences, we used an online software package Gen Bank (http://www.ncbi.nlm.nih.gov) website and evaluated using the IDT Primer Quest tool – Oligo analyzer (http://scitools.idtdna.com/analyzer/Applications/OligoAnalyzer). The sequences for forward and reverse primers of *Bax*, *Bcl-2*, *BMP15*, and *GDF9* are presented in Table1. The expression level of each gene in an obtained sample was normalized using *β-actin* (as housekeeping genes). Relative expression levels were calculated by the $2^{-\Delta\Delta CT}$ method [28]. All experiments of RT-PCR were replicated three times.

Table 1
List of primers used for qRT-PCR

Accession numbers	Gene	Primer Sequence	PCR product size (bp)
NM-007527.3	BAX	Change forward: CGGCGAAATGGAGATGAACTG Reverse: GCAAAGTAGAAGAGGGCAACC	160
NM-177410	Bcl2	Forward primer: GGTGTTCAGATGTCGGTTCA Reverse primer: CGTCGTGACTTCGCAGAG	135
NM_008110.2	Gdf9	Forward primer: CAAACCCAGCAGAAGTCAC Reverse primer: AAGAGGCAGAGTTGTTCAGAG	164
NM_009757	BMP15	Forward primer: AAATGGTGAGGCTGGTAA Reverse primer: TGAAGTTGATGGCGGTAA	148

Statistical Analysis

Data were analyzed using Statistical Package for the Social Sciences (SPSS) program version 16 (SPSS Inc. Chicago, IL, USA) software. We used Kolmogorov–Smirnov test for data normality. One-way ANOVA followed by post hoc Turkey's test (for homogeneous variances) was performed for multiple comparisons across pairs. The results with normally distributed were expressed as mean ± standard deviation. In this study, differences with a *p*-value less than 0.05 were accepted to be statistically significant at the 95% confidence level.

Results

Phase Contrast Microscopy Observations of Cultured Whole Ovaries

The morphological observations of the ovarian tissues before and after culturing in the presence and absence of CGA are presented in Fig. 2 (A-C). According to phase-contrast microscopy, our morphological results demonstrated that the anterior surfaces of ovaries were swollen and the follicles exhibited outgrowth in the cortical parts of the tissues. These changes were more prominent in the CGA^{+} groups. In addition, the central parts of all cultured ovaries were darker than the marginal parts; this dark area was more visible in the CGA^{-} group (Fig. 2C).

Light Microscopy Observation

Figure 2 also shows the histological morphology of the ovarian tissues in all groups of study using H&E staining in low (D-F4) and high (G-L) magnification. Before starting *in vitro* study, the primordial follicles were mainly seen and located in the cortical part of the control 1 group ovaries. After 7 days of culture, in the cortical parts of the cultured ovaries, the primordial represented a smaller percentage of the total follicle pool, similar to the follicle distribution seen in ovaries of 14-day-old mice. In addition, we detected (in the cortical parts of the cultured ovaries) primary and preantral follicles with normal morphology that contained an oocyte with germinal vesicle and granulosa cells. Close adhesion was visible between the oocyte and granulosa cells in follicles at different developmental stages. The healthy follicles with the normal structure were seen in all examined groups especially in the 100 µmol/L CGA supplemented group. Moreover, degenerated follicles with pyknotic nuclei of the oocytes and disarranged granulosa cells were detected in the central parts of the cultured ovaries. The damaged follicles were observed more visible in the CGA^{-} group.

The Viability of Isolated Follicles

The morphologies of preantral follicles after trypan blue staining are shown in Fig. 3 (A-C). In the presence of different concentrations of CGA (Experimental 2 groups), especially in the 100 µmol/L CGA supplemented group, the survival rates of preantral follicles were significantly increased in comparison with others.

Morphological Analysis of Apoptosis

The representative micrographs of intact (green), late apoptotic (red), and early apoptotic (orange) follicles were presented in Fig. 4 (A-C). The percentages of late apoptotic follicles were significantly increased in the experimental 1 group but this rate was decreased in experimental 2 groups especially in the 100 µmol CGA supplemented group.

Follicular Count

The overall quantitative results of the percentages of normal primordial, primary and preantral follicles and atretic follicles in all groups of the study were compared as summarized in Table 2. At the end of the culture period, the percentage of primordial follicles significantly decreased within the groups treated with 0, 50, and 200 µmol CGA compared to the control group 2 ($p < 0.05$). A normal percentage of primordial follicles was observed in control group 2 and the experimental group received 100 µmol CGA. Moreover, between all experimental groups (0-200 µmol CGA) and control group 2, the number of primary follicles did not show any significant differences ($p > 0.05$). Based on our results, the percentage of preantral follicles was gradually increased concentration-dependently as the concentration of CGA increased up to 100 µmol, but this percentage was reduced at the concentration of 200 µmol CGA (Table 2). In addition, there was a significant decrease in the percentage of preantral follicles in all cultural groups in comparison with in vivo samples. Moreover, the lowest proportion of atretic follicles was observed in control group 2 compared to all experimental groups ($p < 0.05$), while among experimental groups (0-200

μmol CGA), only experimental group 2 treated with 100 μmol CGA showed the lowest rate of atretic follicles compared to the other experimental groups ($p < 0.05$).

Table 2
The percentage of follicles at different developmental stages.

Groups	Normal Follicles	Primordial Follicles	Primary Follicles	Preantral Follicles	Degenerated Follicles
Control 1	2078	1999 (96.19 ± 1.58)	50 (2.40 ± 1.45)	29 (1.39 ± 1.05)	87 (4.10 ± 1.33)
Control 2	1432	799 (55.79 ± 1.75) [a]	148 (10.33 ± 1.45) [a]	290 (20.25 ± 0.85) [a]	195 (13.61 ± 1.45) [a]
Experimental 1 (0 μmol CGA)	1146	459 (40.05 ± 1.46) [ab]	142 (12.39 ± 1.75) [a]	136 (11.86 ± 1.60) [ab]	409 (35.68 ± 1.34) [ab]
Experimental 2 (50 μmol CGA)	1113	491 (44.11 ± 1.75) [abc]	144 (12.93 ± 0.14) [a]	161 (14.46 ± 1.46) [ab]	317 (28.48 ± 0.32) [abc]
Experimental 2 (100 μmol CGA)	1334	711 (53.29 ± 2.42) [acd]	142 (10.64 ± 1.24) [a]	261 (19.56 ± 1.35) [acd]	220 (16.49 ± 0.76) [acd]
Experimental 2 (200 μmol CGA)	1157	583 (50.38 ± 1.08) [abcd]	137 (11.84 ± 1.03) [a]	182 (15.73 ± 0.53) [abce]	255 (22.03 ± 0.53) [abcde]

The Surface Area of the Ovaries

The results of the analysis of ovarian surface area (using Image J software) during the culture period on days 3, 5, and 7 are shown in Fig. 2 (M). Our results demonstrated a significant increase in the surface area in all groups during the culture period compared to the first day of culture ($p < 0.05$). The mean surface area of the ovaries significantly increased in the CGA^+ groups in comparison with their respective non-supplemented group on days 5 and 7 of culture and the surface area was markedly higher in the 100μmol/L CGA-treated group than in other groups of study ($p < 0.05$).

Hormonal Assay

The concentrations of E_2 hormone obtained from collected media on days 3 and 7 of the culture period in cultured groups were compared (Table 3). The results demonstrated a significant difference in the concentrations of E_2 hormone on day 7 of culture compared to the third day of culture. In addition, the concentrations of E_2 hormone on day 7 of culture were significantly different in experimental 2 groups in comparison with the experimental 1 group. This rate was gradually increased concentration-dependently as the concentration of CGA increased up to 100 µmol/L but this rate was reduced at concentrations of 200 µmol/L (Table 3).

Table 3
The Concentration of 17-β-estradiol

Groups	Day 3 of Culture	Day 7 of Culture
Experimental 1 (0 µmol CGA)	3014.52 ± 364.57	9102.37 ± 235.02
Experimental 2 (50 µmol CGA)	3270.47 ± 445.86	9740.38 ± 263.54
Experimental 2 (100 µmol CGA)	3864.12 ± 127.38 [a]	14673.24 ± 366.72 [a]
Experimental 2 (200 µmol CGA)	3655.44 ± 89.57 [a]	12634.61 ± 542.23 [a]

Biochemical Assessment

Table 4 represents the biochemical results obtained from ovarian tissue. Accordingly, the MDA level was significantly higher, whereas the levels of antioxidants such as TAC and GSH were significantly lower in the cultured group when compared to both control groups (p 0.05). However, for all mentioned parameters, no significant difference was observed between experimental group 2, which is treated with 100 µmol CGA, and experimental group 1, which is not received any treatment (0 µmol CGA) ($p > 0.05$). Interestingly, in two experimental groups, 100 and 200 µmol CGA, the levels of MDA and both antioxidants were remarkably decreased and increased, respectively, related to the other experimental groups (i.e. 0 and 50 µmol CGA) (p 0.05). In all experimental groups (0-200 µmol CGA), although the better pieces of evidence were observed in the group treated with 100 µmol CGA, there was no significant difference between the treated groups which were received the highest concentrations (i.e. 100 and 200 µmol CGA) ($p > 0.05$).

Table 4
The oxidative stress parameters in different groups.

Groups	MDA (*nmol/mg*)	GSH (*mIU/mg*)	TAC (*μmol/L*)
Control 1	0.425.36 ± 1.66	14.45 ± 3.65	0.654 ± 1.75
Control 2	0.438 ± 2.45	14.26 ± 3.23	0.683 ± 1.23
Experimental 1 (0 μmol CGA)	0.642 ± 3.56 ^{ab}	9.21 ± 2.75 ^{ab}	0.345 ± 1.36 ^{ab}
Experimental 2 (50 μmol CGA)	0.585 ± 2.51 ^{ab}	11.42 ± 1.35 ^{ab}	0.537 ± 1.22 ^{ab}
Experimental 2 (100 μmol CGA)	0.470 ± 2.91 ^{cd}	13.63 ± 3.31 ^{cd}	0.633 ± 1.06 ^{cd}
Experimental 2 (200 μmol CGA)	0.473 ± 2.90 ^{cd}	12.72 ± 3.25 ^{cd}	0.580 ± 0.94 ^{cd}

RT-qPCR Analysis

The expression ratio of apoptotic-related genes (*i.e. Bax and Bcl-2*) and developmental genes (*GDF9* and *BMP15*) to the *β-actin* gene in all studied groups are demonstrated and compared in Figs. 5 and 6. As displayed in Fig. 5, the expression ratio of *the Bax* to *β-actin* gene significantly differed in both the experimental groups compared with the control groups ($p < 0.05$). The mRNA expression of Bax and Bcl-2 and the ratio of Bax/Bcl-2 in the groups received 0 and 50 μmol CGA were significantly different compared to both control groups ($p < 0.05$). On the other hand, there was a significant difference between experimental group 2 received 100 μmol CGA and control 2, experimental 1 (0 μmol CGA), and experimental 2 (50 μmol CGA) groups 200 μmol CGA, for all mentioned parameters. Moreover, in experimental group 2 treated with the highest concentration of CGA (200 μmol CGA), Bax mRNA expression and the ratio of Bax/Bcl-2 were significantly higher in control group 2 and experimental group 2 (100 μmol CGA), and also they were remarkably lower in experimental groups 1 and 2 (i.e. 0 and 50 μmol CGA, respectively) ($p < 0.05$). In this regard, the mRNA expression of Bcl-2 was significantly lower in experimental group 2 treated with 200 μmol CGA than in control group 2 and 100 μmol CGA-treated group, and also it was highly expressed in the last experimental group (200 μmol CGA) compared to the group received 0 μmol CGA (experimental group 1) ($p < 0.05$).

Interestingly, the expression of both development genes, *GDF9* and *BMP15*, was remarkably lower in the groups that received 0 and 50 μmol CGA than in both control groups ($p < 0.05$). In experimental group 2 treated with 100 μmol CGA, their expressions were markedly increased compared to the groups treated with 0 and 50 μmol CGA ($p < 0.05$). On the other hand, in the 200 μmol CGA-treated group compared to the group that received 0 μmol CGA, the mRNA expression of *GDF9* and *BMP15* was significantly increased, and also compared to the group that received 100 μmol CGA, both mRNA expressions remarkably decreased ($p < 0.05$).

Discussion

The present study was the first report that revealed the supplementation of ovarian tissue culture media using CGA could improve *in vitro* growth and development of mouse ovarian follicles by reducing oxidative stress and enhancing antioxidant capacity.

In the current study, we first attempted to determine morphological changes across the groups. Our morphological findings (using H&E, AO/EB, and TB staining techniques) demonstrated that administration of CGA could significantly improve the survival rate of follicles, the proliferation of granulosa cells (GCs), and stimulate the growth of primordial and primary follicles to the preantral follicle stage in parallel with an increase in mean surface area of ovarian tissue. It seems these effects of CGA are dose-dependent because all mentioned parameters were gradually improved during *in vitro* culture up to 100 µmol/L concentration of CGA. However, although the concentration of 200 µmol/L of CGA could significantly change all mentioned parameters, the concentration of 100 µmol/L CGA showed a better function than the 200 µmol/L concentration of CGA.

There is only one related study by Nguyen et al. that indicated the supplementation of porcine oocyte culture media using 50 µmol/L CGA could significantly enhance the maturation, fertilization, and developmental competence of oocytes by preventing apoptosis. They concluded that the application of CGA during oocyte *in vitro* maturation (IVM) has the potential to improve the porcine *in vitro* production (IVP) system [21].

Moreover, in the present study, we employed several complementary techniques in addition to morphological staining. The result of hormonal analysis confirmed morphological findings and demonstrated that the concentration of E_2 was significantly enhanced in the 100 µmol/L CGA supplemented group. This finding is in agreement with Rong Ping Zhou et al. who postulated that CGA exerted estrogenic activity and prevented estrogen deficiency-induced osteoporosis in ovariectomized rats [29]. In addition, Wang et al. indicated that CGA exhibited an estrogen-stimulating effect on ovarian granulosa cells. They expressed that CGA may have the therapeutic potential for the treatment of postmenopausal syndrome [30]. It seems that CGA might stimulate granulosa cells to express cytochrome P450 aromatase genes that convert the androgens to E_2 and promote ovarian follicular development.

Moreover, our biochemical assessment results were in accordance with those of Ali et al. who confirmed that CGA could significantly increase activities of anti-oxidant armory such as SOD (scavenger of the superoxide anion to form H_2O_2), CAT (convertor of H_2O_2 to H_2O), and GSH content [31]. The antioxidant effects of CGA have been also demonstrated by other researchers [31–33], however, they have administered CGA in different tissues and cells. For example, Koriem et al. confirmed that caftaric acid (is the ester form of caffeic acid) enhanced GSH concentrations while it reduced MDA and catalase concentrations in brain, liver, and kidney tissues [34] - but in the present study, we evaluated these criteria in the ovarian tissue for the first time. These observations could be related to the antioxidant effect of CGA and it has important physiological and biological, namely, anti-oxidative and anti-inflammatory effects. It has been discerned that hydroxyl groups (OH) on phenolic acids because of their antioxidant

properties act as positive moieties, associated with the number of hydroxyl groups as follows: tri-hydroxy phenolic acids > di-hydroxy (catechol) > mono-hydroxy [19, 20].

Finally, our molecular findings supported both the histological and biochemical results. Otherwise stated, molecular data amplified the result of developmental ability and functional potential of CGA during IVC of ovarian tissue. Our molecular findings corroborate Dkhil et al's investigation that showed the protective effect of CGA against the injuries induced by sodium arsenite ($NaAsO_2$) in the liver of mice. They demonstrated that, in the presence of CGA, the mRNA levels of the pro-apoptotic markers (*Bax* and *Caspase-3*) significantly decreased, but the mRNA levels of the anti-apoptotic marker (*Bcl-2*) significantly increased [32]. On the other hand, our results showed that CGA up-regulated the expression level of *GDF9* and *BMP15* ($p < 0.05$).

GDF-9 and BMP-15 direct the follicle symphony and play important roles in folliculogenesis and oocyte quality [35–37]. Overall GDF-9 has been implicated in follicular development (at the stimulation of the primary follicle to the secondary follicle stage) in parallel with an increase in GCs differentiation [38]. BMP-15 is also another fundamental factor that is implicated in: the promotion of follicle maturation since the primordial gonadotropin independent phases, regulation of GCs sensitivity to Follicle-stimulating hormone (FSH) action, prevention of GC apoptosis, and regulation of the ovulation quota [39–41].

Up to now, there isn't any information about the effect of CGA on the expression level of developmental genes; this study is the first report that revealed enhancing effects of CGA on the expression level of *GDF9* and *BMP15*. There is little literature and a limited number of resources that analyzed the molecular mechanisms of CGA in culture conditions, but it seems that administration of CGA to the IVC medium regulates these molecular changes by phosphatidylinositol-3-kinase (PI3K/Akt) and mitogen-activated protein kinase (MAPK) signaling pathways. Dai Cheng et al. demonstrated that CGA has a cytoprotective effect against Aluminum, which may bring about damage to the macrophages, possibly through PI3K/Akt and MAPK signaling pathways in RAW264.7 cells [42].

In conclusion, the present study confirmed that the supplementation of ovarian tissue culture media using CGA, especially at 100 µmol/L, could optimize the culture system and improve the survival, developmental and functional potential of follicles in mouse whole ovarian tissues after 7 days of culture. Furthermore, the result of this study (for the first time) revealed that CGA could suppress oxidative damage and improve the culture system by enhancing the concentration of antioxidants and developmental genes expression. But, before the clinical application of CGA, further experiments are appreciated to elucidate the exact mechanism at the molecular level.

Declarations

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paper for submission, also participated in the finalization of the manuscript, and approved the final draft.

Compliance with Ethical Standards:

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Ethical approval: Animal procedures and protocols were conducted in accordance with the Principles of Laboratory Animal Care - ethically approved by the Animal Care and Use Committee in Urmia University of Medical Sciences (UMSU). Approval was granted by the Ethics Committee of Urmia University of Medical Sciences under code No. IR.UMSU.REC.1398.078.

Conflict of interest: The authors declare that they have no conflict of interest.

Authors' contributions

Neda Abedpour designed, directed the project and wrote the paper. Masoumeh Zirak Javanmard, Mojtaba Karimipour, Gholam Hossein Farjah contributed to the design and implementation of the research, to the analysis of the results and to the writing of the manuscript. All authors read and approved the final manuscript.

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Figures

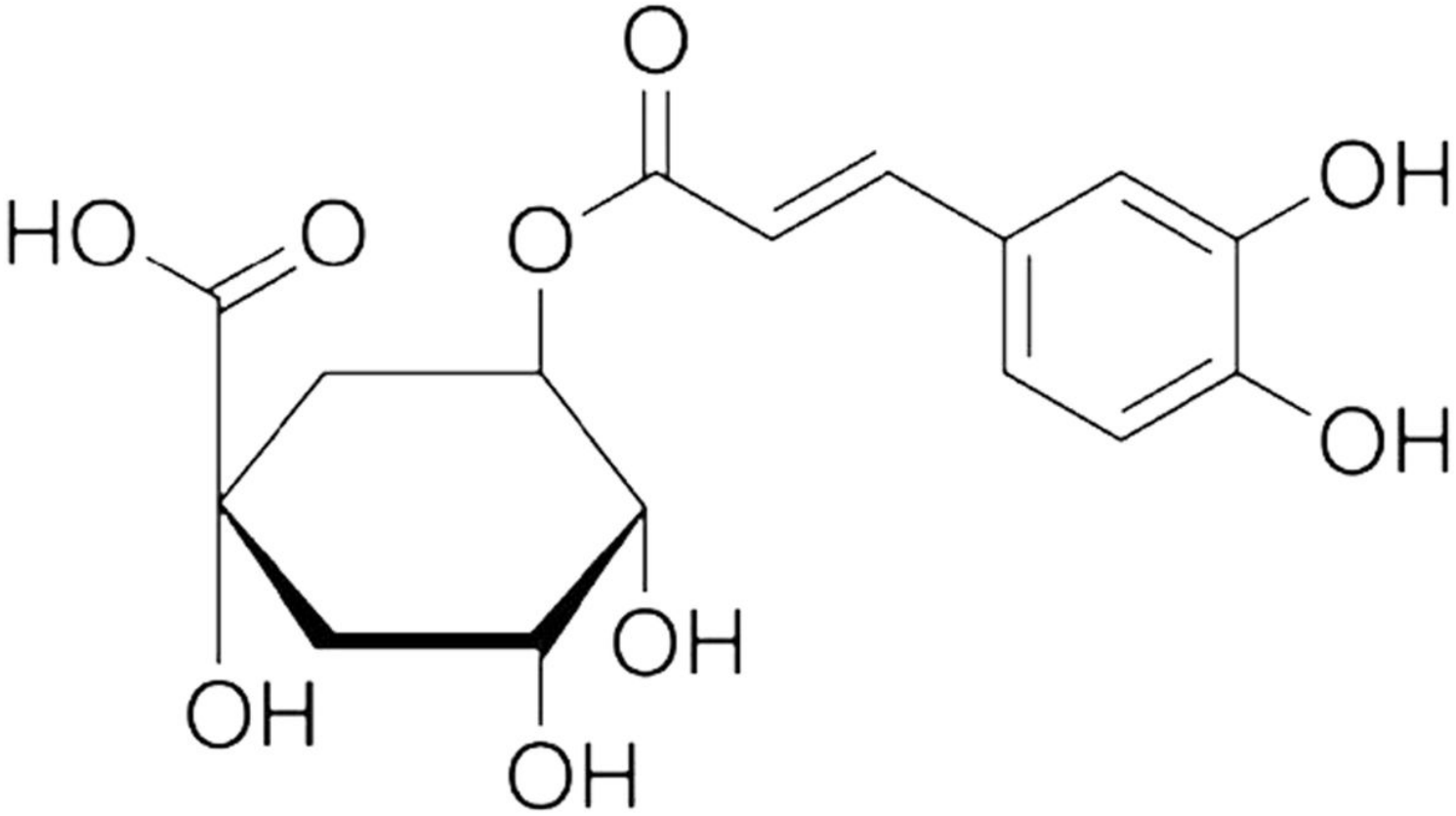

Figure 1

Chemical structure of *Chlorogenic acid* (CGA).

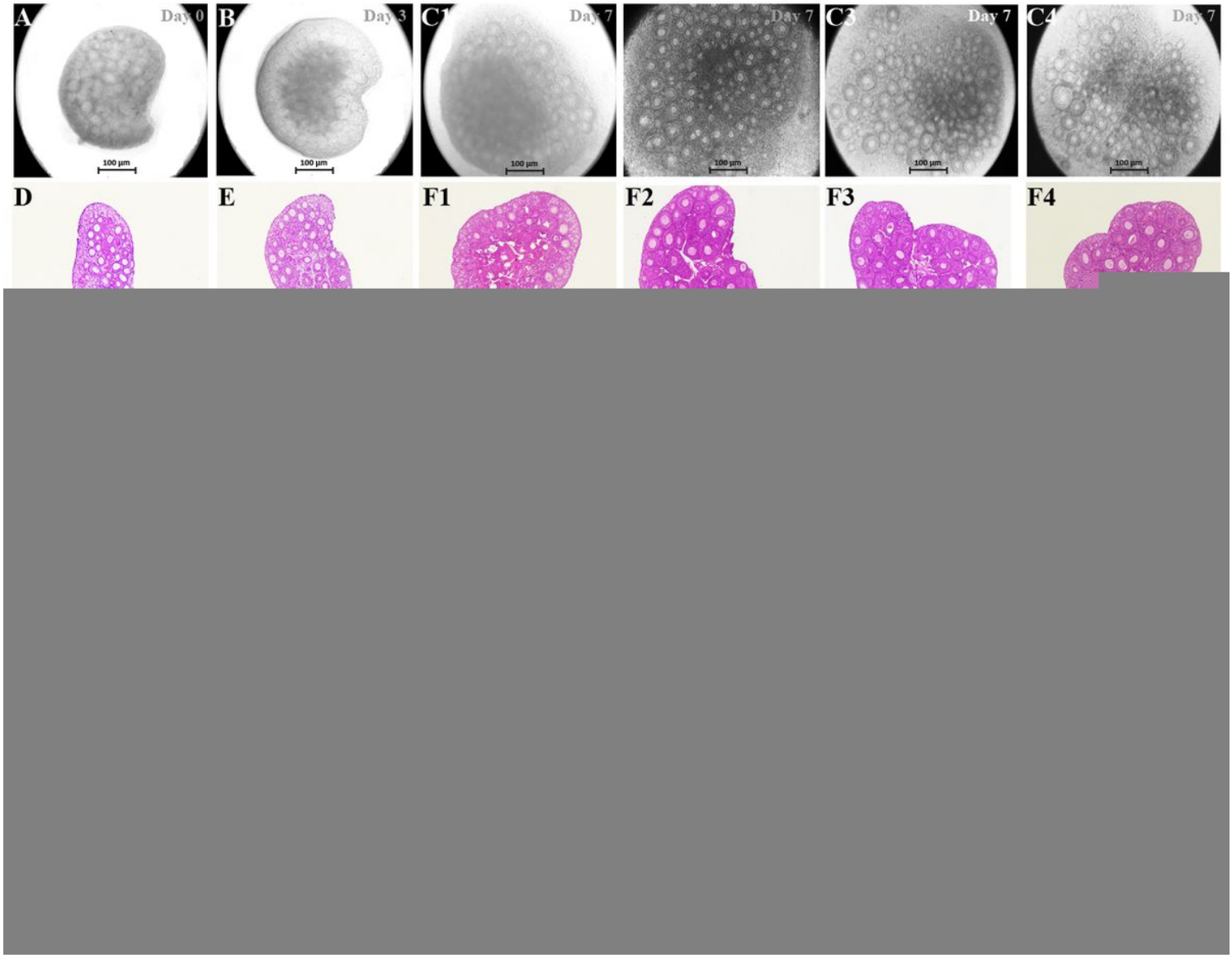

Figure 2

Photomicrographs of mouse ovaries viewed under an inverted microscope on days 0, 3 (A and B), and 7 (C1-C4). D-L: Photomicrographs of mouse ovarian tissues viewed under the light microscope using H&E staining in low (D-F4) and high magnification (G-L): D and G: Control 1 group; E and H: Control 2 group; F1 and I: Experimental 1 group; F2-F4 and J-L: Experimental 2 groups. G: The mean area of mouse cultured ovaries on days 0, 3, 5, and 7 of the culture period.

*: indicates a significant difference with days 0 and 3 ($p<0.05$). **: indicates a significant difference with day 5 ($p<0.05$).

a: indicates a significant difference with other groups on the same day ($p<0.05$).

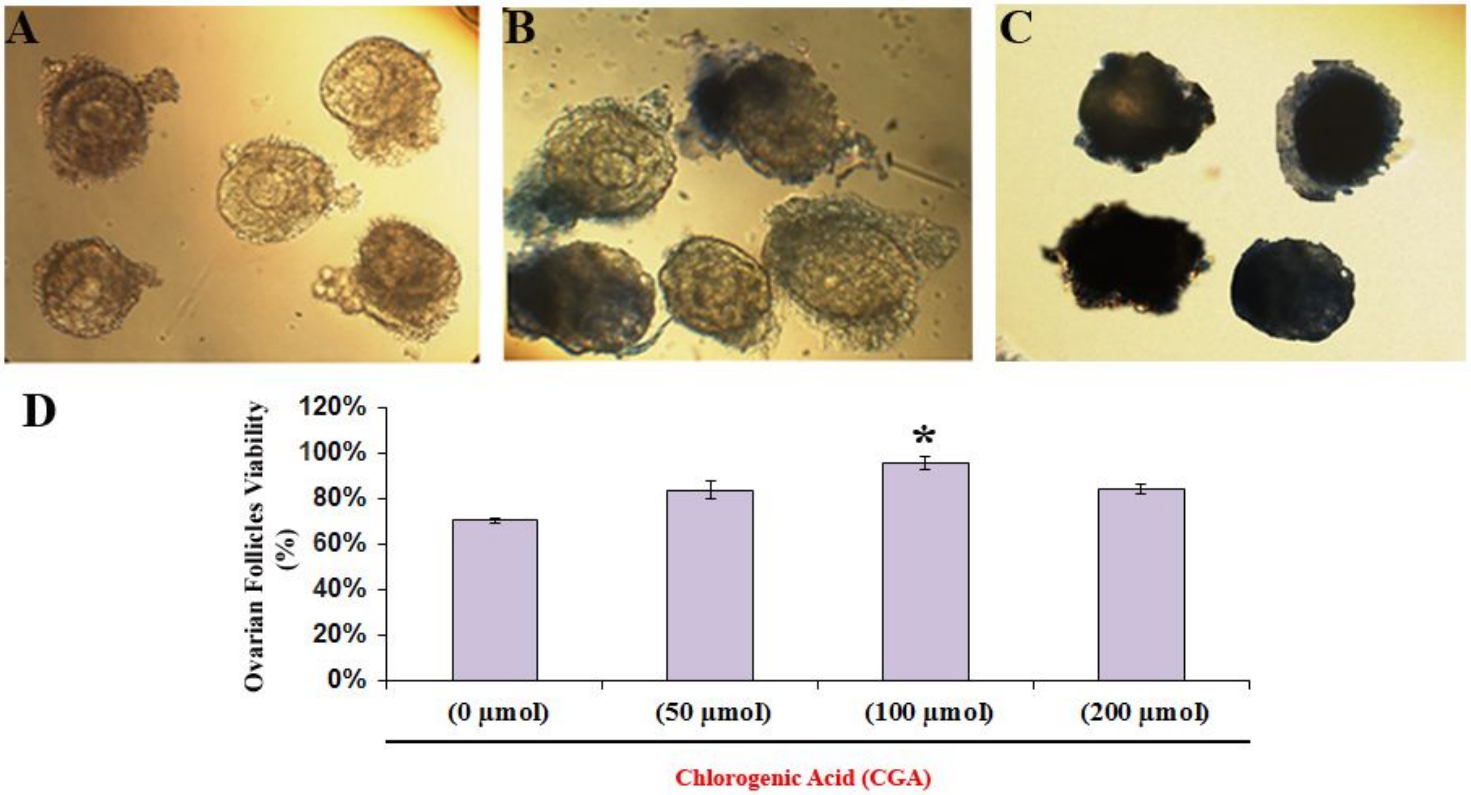

Figure 3

Using TB staining: A) white (unstained) follicles were classified as live (TB-), B) pale blue stained follicles were considered damaged, and C) dead follicles appear dark blue (TB+). Figure D showed the percentage of survival, damaged, and dead follicles in culture groups at different concentrations of CGA. *: significant difference with other groups ($p<0.05$).

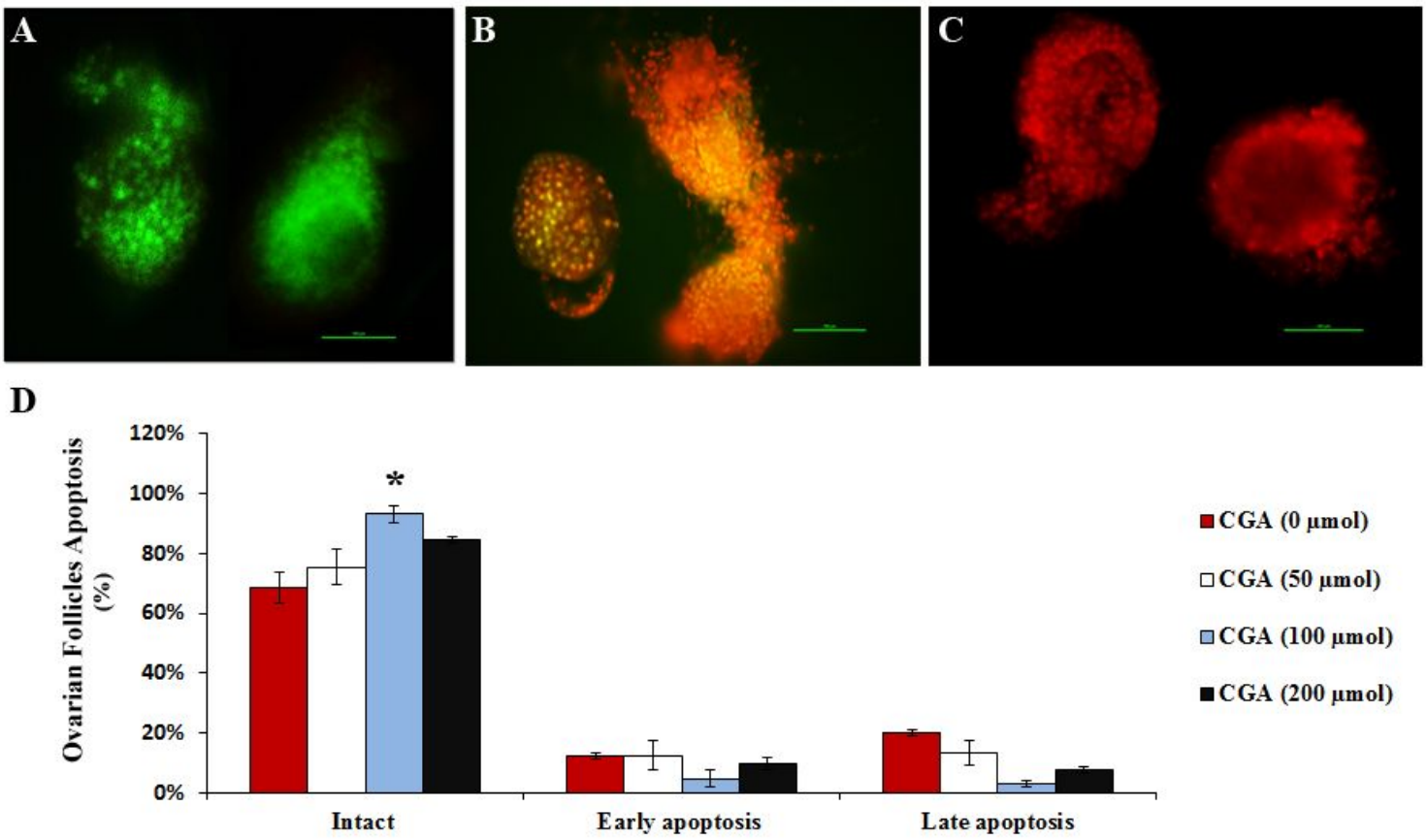

Figure 4

Acridine orange and ethidium bromide (AO/EB)-stained ovarian follicles: A: intact cells (green), B: early apoptotic (bright orange), and C: late apoptotic (red) under a fluorescent microscope. Data in figure D are expressed as the total number of intact and apoptotic cells (early + late) as a percentage of the total. *: significant difference with other groups (P<0.05).

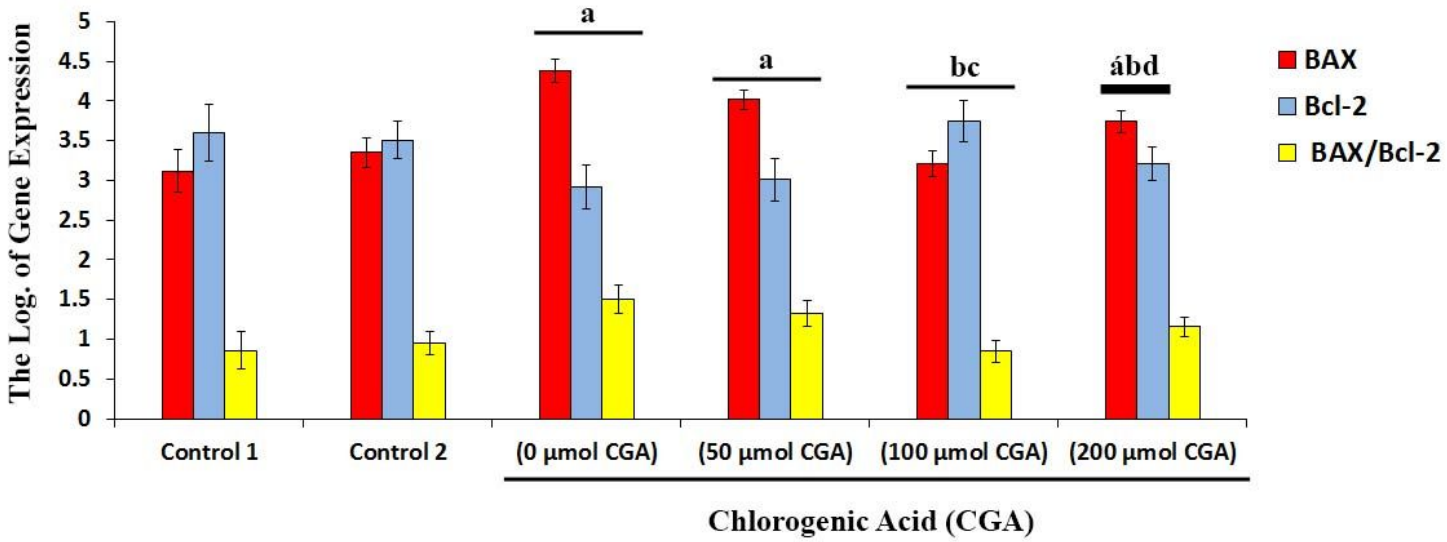

Figure 5

The mRNA expression of apoptotic-related genes in mouse ovarian tissues.

(a): significant difference with both control groups (1 and 2) ($p<0.05$); (á): significant difference with the first control group ($p<0.05$); (b): significant difference with experimental 1 group (0 µmol/L CGA) ($p<0.05$); (c): significant difference with experimental 2 group (50 µmol/L CGA) ($p<0.05$); (d): significant difference with experimental 2 group (100 µmol/L CGA) ($p<0.05$).

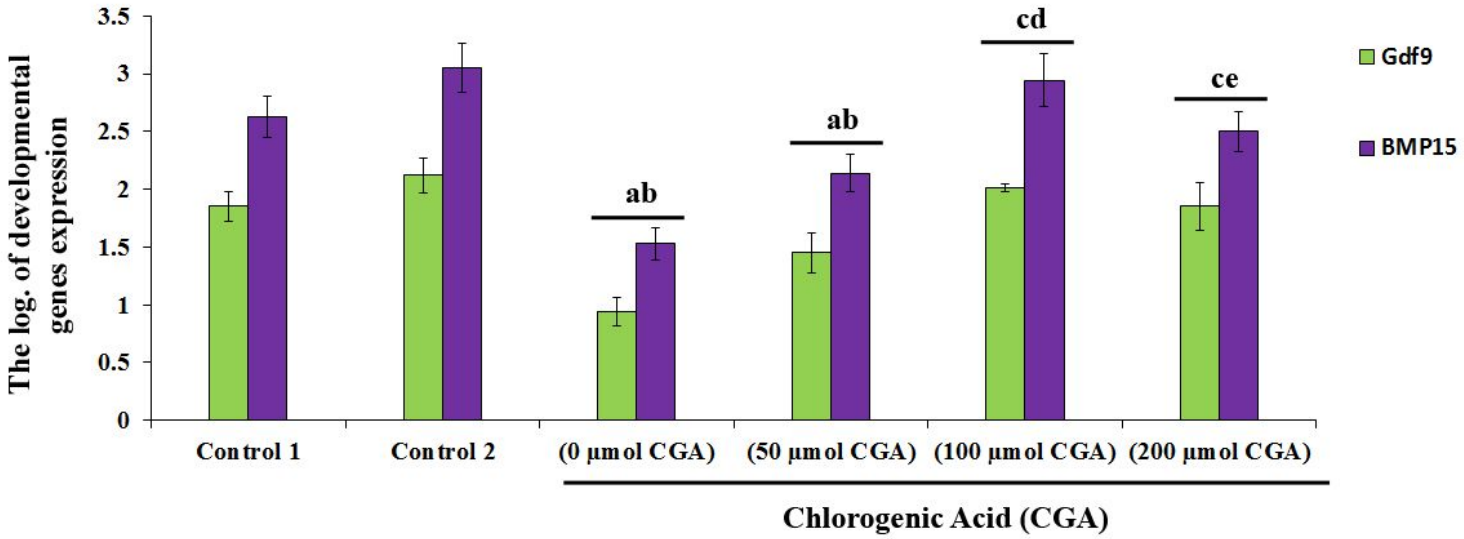

Figure 6

The mRNA expression of developmental genes (GDF9 and BMP15)

(a): significant difference with the 1st control group ($P<0.05$); (b): significant difference with the 2nd control group ($P<0.05$); (c): significant difference with the 1st experimental group (0 µmol/L CGA) ($P<0.05$); (d): significant difference with the 2nd experimental (50 µmol/L CGA) ($P<0.05$); (e): significant difference with the 2nd experimental group (100 µmol/L CGA) ($P<0.05$).