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Original article

Effect of genistein on the gene expressions of androgen generating key enzymes StAR, P450scc and CYP19 in rat ovary

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Abstract

In this investigation, the effects of genistein (GEN) on the expression of steroidogenic genes such as steroidogenic acute regulatory protein (StAR), side-chain cleavage enzymes (P450scc) and cytochrome P450 aromatase (CYP19) were assessed. For this study, forty young female Sprague Dawley (SD) rats at aged 2-3 months (200±20 g) and forty aged female SD rats aged 10-12 months (490±20 g) were selected. Also, based on weight they were divided into a negative control group (NC), three different GEN dose groups, which received GEN of 15, 30, 60 mg/kg, and a positive control group (PC). The experiment lasted 30 days. Concentrations of serum hormones were determined by Enzyme-linked immunosorbent assay (ELISA). Gene and protein expressions of StAR, P450scc and CYP19 were determined by Real-Time PCR and western blot techniques. It was observed that 30-60 mg/kg GEN could increase the expression of androgen generating key enzymes in the young rat ovary. GEN also significantly increased progesterone and E_2 levels in the serum of aged rats and reduced the levels of LH and FSH in the serum of both young and aged rats. Compared with young rats, the effect of GEN on the ovary of aged rats was stronger and a lower dose of GEN (15 mg/kg) showed an obvious effect on these indicators. GEN influenced both estrogen level and indicators associated with estrogen and androgen transformation processes, which indicates that GEN can impair the growth and maturation of the ovary.

Key words: genistein, steroidogenic acute regulatory protein, side-chain cleavage enzymes, cytochrome P450 aromatase, rat ovary

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Introduction

Follicular development and maturation is a very complex process. Follicular atresia in various developmental stages is a sign of premature ovarian failure. Growth factors and cytokine interactions are under the control of related hormones. Ovary function is regulated by the synergistic effect of estrogen and androgen. Follicle stimulating hormone (FSH) and Luteinizing hormone (LH) are secreted by the pituitary gland and both are important hormones for follicular development, oocyte maturation and steroidogenesis regulation (Gu et al. 2010).

The effect of bioactive substances on the generation of the key enzyme of androgen is one of the important aspects to be carefully considered. Under the influence of some of the factors, follicular stromal cells and granulosa cells can produce androgens. In this process, there are three key enzymes i.e. StAR, P450scc and aromatase are involved in granulosa cells. In the last step of estrogen biosynthesis, the CYP19 gene is one of the most important factors which can change androgens into estrogens. Adjusting the expression of aromatase CYP19 can alter the rate of estrogen production and intervene in the levels of estrogens *in vivo* (Cheshenko et al. 2008). The StAR plays an important role in the metabolism of the conversion of cholesterol to steroid. StAR protein discovered by Clark (Clark et al. 1994) was generally thought to be the long-sought and elusive rate-limiting protein during steroidogenesis (Ferguson et al. 1963, Garren et al. 1965, Crivello et al. 1980). Due to the action of P450scc, cholesterol synthesise pregnenolone, which is the first step and also is the rate-limiting step of steroidogenesis. Steroid hormones play very important medicinal roles in the process of immune regulation and they synthesize estradiol under the actions of CYP19 and CYP17. For this reason, StAR, P450scc and CYP19 are the rate-limiting enzymes which promote the synthesis of estrogen in the body. Thus, any abnormality of an enzyme in the synthesis pathway of a steroid hormone can lead to abnormality in the synthesis of hormone, which may result in the disorder of hormone secretion (Wang 1997). Genistein (GEN) is an isoflavone that has received a great deal of attention over the last few years owing to its potential in preventing the most currently prevalent chronic diseases such as cardiovascular disease, osteoporosis and hormone related cancers (McCarty 2006). GENs are considered to be phytoestrogens as they have been shown to bind to trans-activated estrogen receptors and to induce gene expression (Kuiper et al. 1998). The molecular structure of GEN is similar to estradiol. There are two phenolic hydroxyl residues in its relative ambi-molecular poles, which can cause an estrogen-like effect by binding to the estrogen receptor. It is well known that there is a close relationship between the chemical structure and the biological activity of bioactive compounds. Thus, the structural modification of GEN might alter its biological activity. The initial speculation about its efficacy was based on its estrogen-like properties and earlier studies showed that the chemically synthesized structural drug derivatives of GEN and ipriflavone, exerted skeletal benefits (Ruchun et al. 2008). In recent years, phytoestrogen supplements have become attractive as safer alternatives to estrogen, and their efficacy has been investigated in clinical trials. In our previous study, we confirmed that GEN could regulate the levels of hormones in the ovary of young female rats and the related proteins and genes such as Bcl-2, Bax and P450mRNA in ovarian granulosa cells (Chi et al. 2010). On this basis, the synthesis of StAR can regulate the delivery of cholesterol to the inner mitochondrial membrane for P450scc-mediated conversion to pregnenolone. These proteins (StAR, P450scc and CYP19) may prove to be appropriate targets for GEN, and the androgen generating key enzymes during follicular development were chosen as the research target and Western blot and Real-time PCR techniques were carried out to detect the protein and mRNA expression of StAR, P450sccm and CYP19. At the same time, changes in the levels of sex hormones such as estrogen, FSH and LH in the rat serum were studied. Moreover, a comparison of the regulation effects of GEN on steroidogenic and androgen generating key enzymes in young and aged rats were studied as discussed below.

Materials and Methods

Animals and test compounds

The experiments ware performed on 40 young female SD rats aged 2-3 months and 40 aged female SD rats aged 10-12 months. The animals were purchased from the Harbin Medical University experimental animal technology company (Harbin Medical University, Harbin, China. Animal qualification number: SCXK (Hei)-2013-0004). They were kept in individual batteries of cages at a neutral temperature (18-20°C), relative humidity (45-65%) and illumination (12 h light, 12 h darkness). Young and aged rats were respectively divided into five groups with ten animals in each: a negative control group (NC), L-Gen (L), M-Gen (M) and H-Gen (H) groups which received 15, 30, 60 mg of genistein, respectively, per kg of body weight, per day and a positive control group (PC, received 0.5 mg of Diethylstilbestrol per kg, per day). Diethylstilbestrol (DES) is a synthetic estrogen and we selected it as the positive

control. As the content of genistein in isoflavone was about 2%~3%, the conversion factor between human (70 kg) and rat (250 g) was 7, the effective dose of genistein was about 0.14 mg~21 mg, and we also read other authors' articles for reference, in this way the doses of genistein (15, 30 and 60 mg/kg) were determined. GEN was dissolved in dimethylsulfoxide and was administered to the rats by gavage. The treatment lasted for 30 days. To consider the effects of estrus cycle on gene expression in the ovary, a vaginal smear was used to determine estrus cycle. We drew rat blood from the abdominal aorta during the estrum period for the determination of the sex hormone. This period was equivalent to menstruation and the corpus luteum degenerated.

All animal experiments were approved by the Committee on Animal Care of the College of Animal Science and Veterinary Medicine of Heilongjiang Bayi Agricultural University and according to accepted veterinary medical practice. The rats were anesthetized using ether and ovaries removed immediately for further analysis, and adipose tissues on the ovarian surface were removed under a microscope. The ovaries were then snap frozen in liquid nitrogen and stored at -80°C until RNA isolation.

Genistein (4′,5,7-trihydroxyisoflavone) was purchased from the Sigma company (Sigma-Aldrich, USA; >99.9% purity); estradiol valerate was purchased from the Hefei Jiu Lian Pharmaceutical Company (HeFei, China).

Concentrations of serum hormones were determined by Enzyme-linked immunosorbent assay

Concentrations of estradiol, FSH and LH were quantified using an enzyme-linked immunosorbent assay (ELISA). 50 µl of standard diluent was added to the standard wells. The samples were diluted to a final ratio of 1:1 by mixing 50 µl of the sample with 50 µl of diluent and 50 µl of the diluted sample was added to the wells. 50 µl of diluted biotinylated antibody was added to all the wells. Then the plate was then covered and incubated for 1 hour at 37°C. After removing the cover the plate was washed three times. 80 µl of streptavidin-HRP solution were aliquoted into each well, including the blank wells. The plate was then covered and incubated for 30 min at 37°C. The solution was then removed from all the wells, and the samples in the micro well plate were washed according to the corresponding washing step and then immediately proceeded to the next step. 50 µl of substrate A and substrate B were added to each well, and were then incubated for 10 min at 37°C. The enzyme-substrate reaction was stopped by quickly adding 50 µL of 2N H_2SO_4. The absorbance of each well was recorded using a spectrophotometer at 450nm as the primary wavelength and optionally at 620 nm (610 nm to 650 nm is acceptable) as the reference wavelength. Anti – mouse and anti – rabbit were purchased from Cell Signaling (Kyoto, Japan). The catalog numbers of the antibodies were: progesterone (D8Q2J); LH (156-3C11); FSH (9A3); E_2 (D1S5E).

Real-time PCR for StAR arom, P450scc and CYP19 analysis of mRNA levels in the ovary

Total RNA from the follicles was extracted with TRI Reagent according to the manufacturer's protocol. The RNA was used as a template in the synthesis of cDNA. Reverse transcription (RT) was performed using a Master-cycler (Eppendorf, Boston, USA) according to the following thermal profile: (i) 25°C, 10 min; (ii) 37°C, 120 min and (iii) 85°C, 5 min with a High-Capacity cDNA Reverse Transcription Kit. The first strand cDNA was stored at 20°C and subsequently used for the qPCR amplification based on 50 nuclease chemistry using TaqMan1 MGB (minor groove binder) probes according to the procedure. Multiplex qPCR was performed in a 96-well thermocycler (StepOne Plus, Applied Biosystems, Chicago, USA). Assay-on-Demand, TaqMan MGB Gene Expression Kits with specific TaqMan MGB probes designed by Applied Biosystems was used for steroidogenic protein (StAR, P450scc, CYP19A) analysis of mRNA expression. The primers used for real-time RT-PCR were as follows: StAR: forward 5′- TGGCTGCCAAAGACCATCAT −3′ and reverse 5'- TGGTGGGCAGTCCTTAACAC -3′; P450scc: forward 5′- GAGCTGGTATCTCCTCTACCA -3' and reverse 5'- AATACTGGTGATAGGCCACCC -3'; CYP19: forward 5'- CACAAGTTAAGCCCGGTTGC -3′ and reverse 5′- CTGGGAGCACGAACTGAGAG -3′; β-actin: forward 5'- ACCCGCGAGTACAACCTTC -3' and reverse 5'- ATGCCGTGTTCAATGGGGTA -3′.

PCR conditions: Pre-denaturation at 95°C for 10 min; 40 cycles for each sample that comprised an initial denaturation step at 95°C for 15 Sec, annealing at 60°C for 45 Sec, and extension at 60°C for 15 sec (Analysis using ABI Prism 7300 SDS Software).A JY04S Gel imaging system was purchased from the Beijing Oriental Electrophoresis Equipment Company (BeiJing, China); the nucleic acid protein tester was purchased from BioPhotometer Plus (Darmstadt, Germany); the PCR instrument was purchased from Applied Biosystems (MA, USA); the Cracking fluid for protein and protease inhibitors was purchased from Blue Skies Company (ShangHai, China).

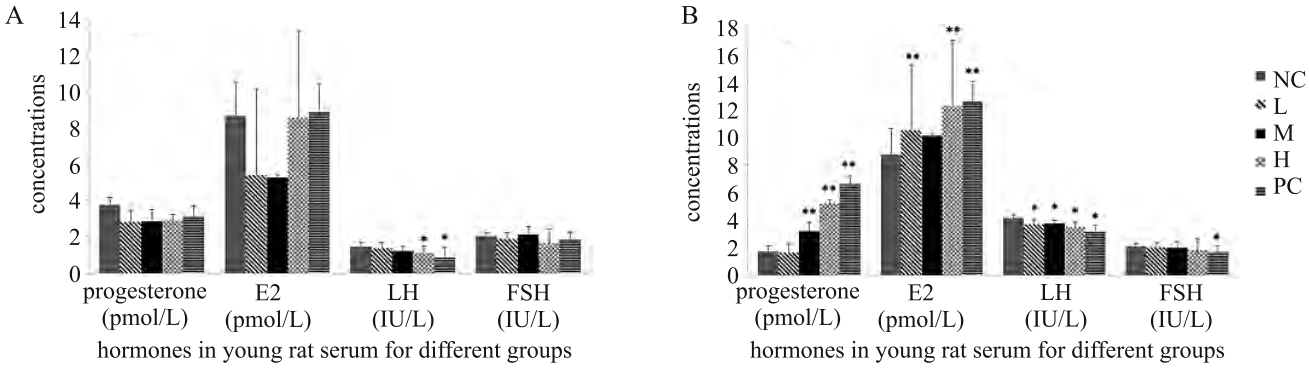

Fig. 1. Level of progesterone, E_2, LH and FSH in young rat (A) and in aged rat (B) serum after 30 days of treatment with genistein. Experimental conditions and treatment procedures are given in Materials and methods. Asterisk means significant control group (* $p<0.05$; ** $p<0.01$), ANOVA test.

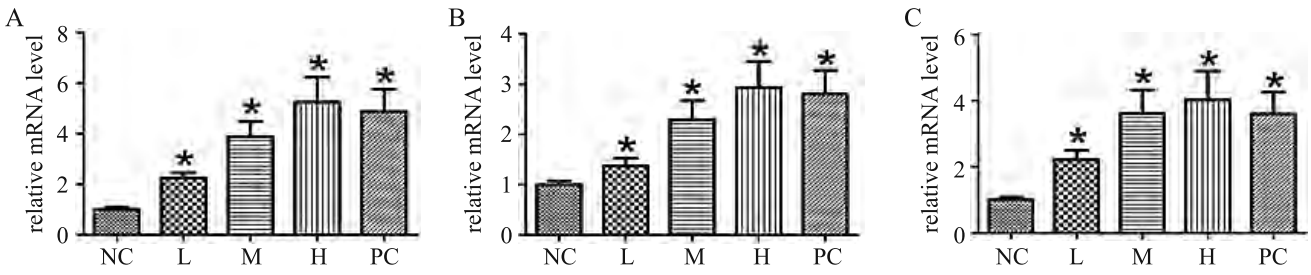

Fig. 2. Expression of StAR (A), P450scc (B) and CYP19 (C) mRNA in young rat ovaries after 30 day exposure to different genistein concentrations (15,30 and 60 mg/kg). mRNA expression levels were quantified using real-time PCR. All values represent means (n=8) ± SEM. Different numbers of asterisk denote a significant difference compared with negative control (* $p<0.05$).

Western blot analysis for StAR, P450scc and CYP19 protein levels in the ovary

Protein lysate (Equal amount) from cells was resolved using 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Shanghai Yi Chen Biological Technology Co. Ltd, Shanghai, China) and transferred to PVDF membranes (Millipore). The filters were blocked with TBST buffer containing skim milk 5% and incubated with primary antibodies of StAR (1:1000, rabbit), P450scc(1:1000, rabbit), CYP19(1:1000, rabbit) and β-actin (1:5000, mouse, Sigma) overnight at 4°C. Samples were then incubated with anti-mouse/anti-rabbit (Cell Signaling, 1:2000) secondary antibody, and enhanced chemiluminescence (ECL) (Thermo Fisher) was used to ImageQuant LAS 4000 mini (GE Healthcare Life Sciences, Nanjing, China). Band quantification was performed using Quantity One. An Ezna FFPE RNA Kit, SYBR Green PCR kits and Protein with BCA protein quantitative kits were purchased from Thermo Fisher (MA, USA); the RT-PCR kit was purchased from Fermentas (Vilnius, Lithuania); Sds-page kits, Hematoxylin and eosin were purchased from the Polyene Shanghai Biological Technology Co., Ltd (ShangHai, China).

Statistical analysis

Statistical analyses were performed using ANOVA, followed by Bonferroni post hoc testing using GraphPad Prism 5. Statistical significance was set at $p<0.05$. The data obtained from the experiments were statistically analysed by one-way or two-way ANOVA followed by Duncan's multiple range test using Sigma Stat 2.03 (Systat Software GmbH, Munich, Germany). Log transformations were performed as needed to maintain homogeneity of variance.

Results

Concentrations of progesterone, E_2, LH and FSH in rat serum

As shown in Fig.1, the serum progesterone levels in aged rats were significantly increased in M, H and PC ($p<0.01$). However, there was no change in the levels of progesterone in young rats. The serum E_2 levels in young rats showed a downward trend (Fig. 1A), which suggested that GEN played an anti-estrogenic effect in young female rats and affected their serum sex hormone levels. Compared with the control group, the high dosage group of GEN had significantly increased E_2 levels in the serum of aged rats ($p<0.01$,

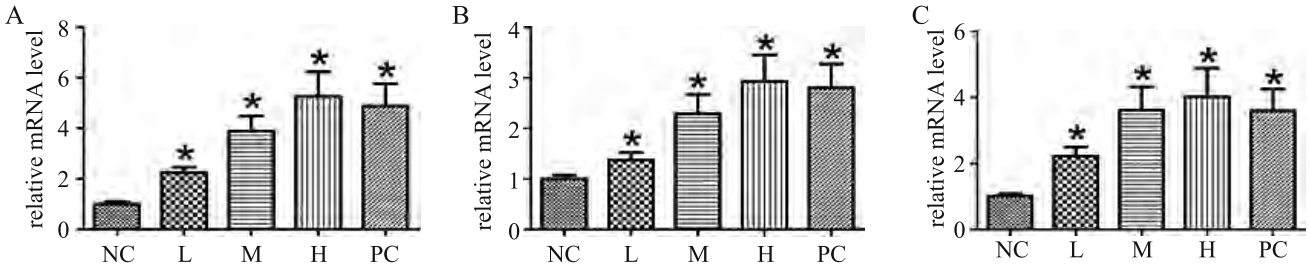

Fig. 3. Expressions of StAR (A), P450scc (B) and CYP19 (C) mRNA in aged rat ovaries after 30 day exposure to different genistein concentrations (15,30 and 60 mg/kg) . mRNA expression levels were quantified using real-time PCR. All values represent means (n=8) ± SEM. Different numbers of asterisk denote a significant difference compared with negative control (* p<0.05).

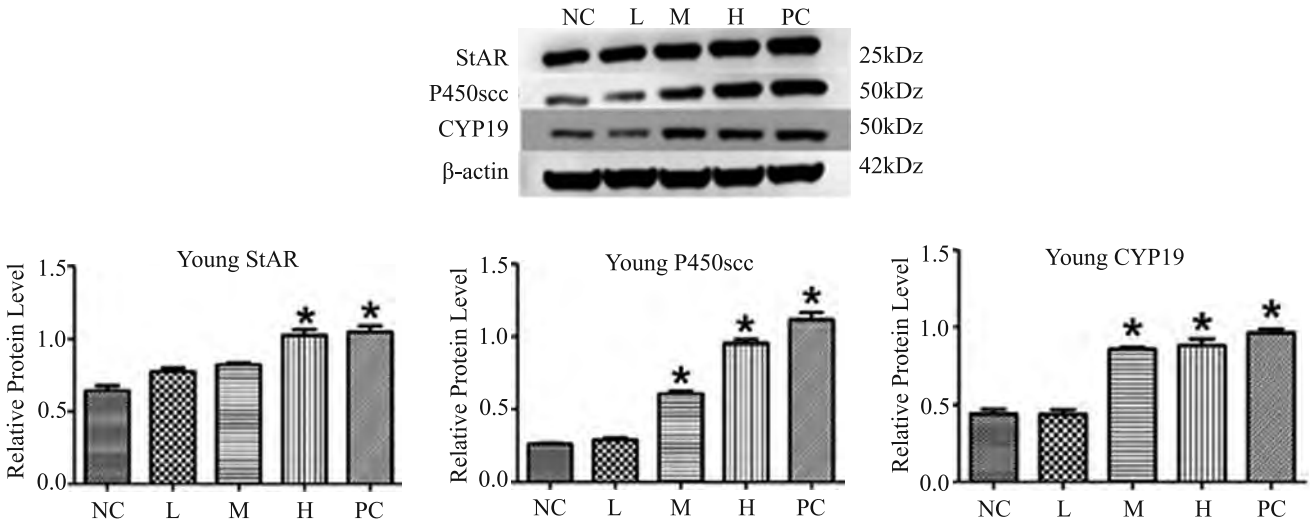

Fig. 4. Expressions of StAR, P450scc and CYP19 protein in young rat ovaries after 30 day exposure to different genistein concentrations (15,30 and 60 mg/kg). Protein expression levels were qualified and quantified using Western blot. All values represent means (n=8) ± SEM. Asterisk denotes a significant difference compared with negative control (* p<0.05).

Fig. 1B). Different doses of GEN could reduce the level of LH and FSH, especially for the level of LH where there was a significant difference (p<0.05).

The effect of GEN on StAR, P450scc and CYP19 gene expression in rat ovary

In the case of young female rats, it seemed to be significant that StAR gene expression was greatly increased with the addition of GEN, especially in middle and high dose groups (relative mRNA level of StAR was 2.731±1.348, 2.410±0.864, p<0.05) (Fig. 2A). The results of P450scc mRNA expression are shown in Fig. 2B, from which it can be seen that there is a significant increase in the levels of P450scc mRNA expression for the middle and high groups (relative mRNA level of P450scc was 2.691±1.229, 3.198±0.859) as compared to the control group (p<0.05). In addition, RT-PCR analysis revealed the stimulatory effect of GEN on the expression of the CYP19 gene (Fig. 2C). Compared to the negative group, the expression of CYP19 mRNA in the GEN dose group increased significantly, especially in the middle and high dose groups (relative mRNA level of CYP19 was 3.348±1.715, 3.153±1.284, p<0.05), and the effect was similar to the positive group.

In the case of aged female rats, the effect of GEN on these indicators was more obvious as compared to young rats. A significant increase in the gene expression of StAR (relative mRNA level of StAR was 2.252±0.585, 3.887±1.691, 5.259±2.767, Fig. 3A), P450scc (relative mRNA level of P450scc was 1.371±0.425, 2.295±1.069, 2.928±1.487, Fig. 3B) and CYP19 (relative mRNA level of CYP19 was 2.216±0.792, 3.613±2.021, 4.019±2.485, Fig. 3C) was noted for the GEN-supplemented group (at the dose between 15 and 60 mg/kg) as compared to the control group (p<0.05).

The effect of GEN on StAR, P450scc and CYP19 protein expression in rat ovary

Consistent with Fig. 2, StAR, P450scc and CYP19 protein expression showed a significant increase in the presence of GEN (Fig. 4). The observed results showed that at a dose of 30-60 mg/kg, GEN can increase the expression of androgen generating key enzymes

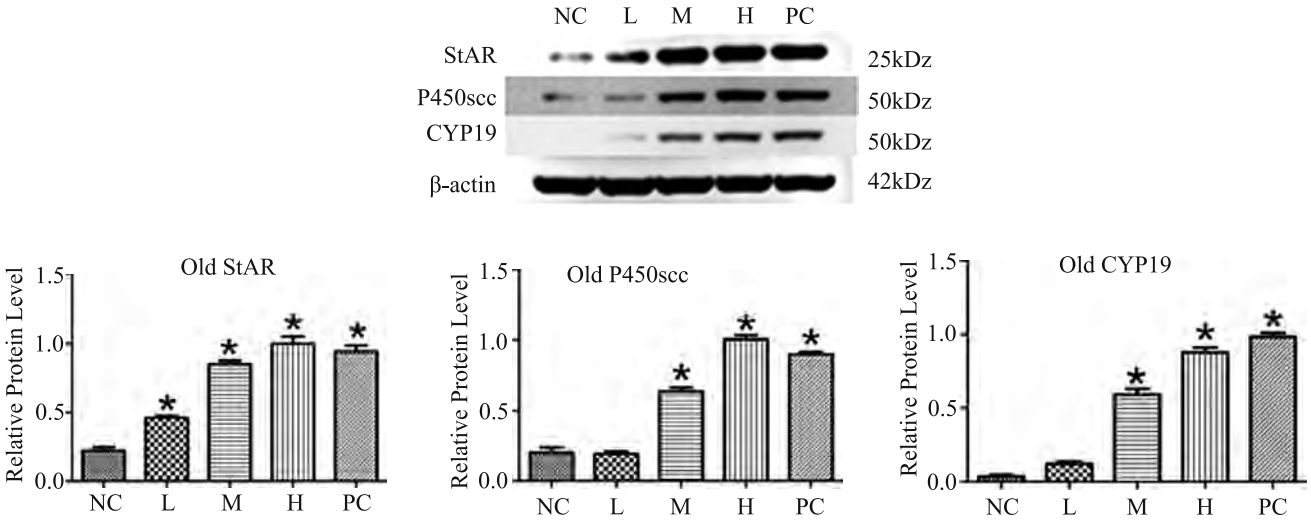

Fig. 5. Expressions of StAR, P450scc and CYP19 protein in aged rat ovaries after 30 day exposure to different genistein concentrations (15,30 and 60 mg/kg). Protein expression levels were qualified and quantified using Western blot. All values represent means (n=8) ± SEM. Asterisk denotes a significant difference compared with negative control (* $p<0.05$).

in young female rat ovaries. It can be seen from the data of aged rats that GEN with a lower dose showed an obvious effect on the protein expression of these indicators (Fig. 5).

Discussion

In order to determine the regulatory effect and molecular mechanism of GEN on ovary, the influence of GEN on the protein and mRNA expression of genes which are involved in the synthesis of steroid hormones (i.e. StAR, P450scc and CYP19) was investigated. To the best of our knowledge, this is the first study that shows the effects of phytoestrogen genistein on steroidogenic gene expression in the rat ovary at different ages.

FSH and LH are directly involved in stimulating follicle the development, ovulation, formation of corpus luteum and the synthesis of steroid hormones (Gu et al. 2010). In addition, FSH and LH take part in the recruitment, selection and a series of follicular developmental processes. Serum FSH levels increase with age, and increase in basal FSH levels is associated with diminished ovarian reserves. In this study, GEN had a depressed regulation effect on FSH and LH levels both in young and aged rats, which indirectly reflected the improvement of GEN on the function of the ovary. As age increases, ovarian dysfunction, follicle and estrogen secretions gradually decrease. The pituitary gland causes the level of FSH and LH to increase in response to the lack of estrogen. The estrogen action of GEN can raise the E2 level, thereby lowering LH and FSH levels. Due to different levels of estrogen in young and aged rats, the sensitivity to GEN is different. Thus, our results showed the age-dependent effect of GEN (old rats are more sensitive than young rats). This demonstrates that in young rats, although the circulating level of LH was attenuated by GEN of 60 mg/kg, E2 is insensitive to this treatment. Some of the previous investigations reported similar alterations in this gene expression in the ovary. It has been found that the expression levels of StARmRNA in the corpus luteum of rabbits, sheep and cattle were consistent with that of progesterone in a short period of time (Bosmann et al. 1996, Pescador et al. 1996, Townson et al. 1996). Researchers have also found that the rate-limiting step in acute steroid hormone production is the movement of cholesterol across the mitochondrial membrane by StAR protein, with a subsequent conversion to pregnenolone by P450scc (Sierra 2000, Stocco 2000, Geslin et al. 2004, Augustine 2008). StAR protein and P450scc are synthesized rapidly in response to the stimulation of acute tropic hormone, which occurs regardless of steroidogenic organ or tissue. By the experimental induction of birth defects or knockout in the mitochondria of steroidogenic acute regulatory protein, namely female fetal androgen synthesis and steroid biosynthesis StAR deficiency caused by P450c17, it obviously leads to ovarian dysfunction and androgen deficiency, which continue after birth. P450scc testosterone in the mammalian ovary and testis tissue in the mitochondria is the only conversion of cholesterol into pregnenolone enzyme. Pregnenolone is an important intermediate for the synthesis of progesterone and under the action of P450c17 and P450arom, it is converted to estradiol. The expression of the androgen gene in human ovarian granulosa cells was not clear, whereas the expression of StAR in ovarian granulosa cells after the stimulation of testosterone was not stable and showed fluctuations (Chen 2016). GEN can inhibit both the rupture of neo-

natal rat oocyte nests as well as the apoptosis of oocytes, but it is not clear in adult and early aged rats. Related experiments showed that GEN in the large growth period, mice intervention may lead to reduced fertility, and even cause infertility (Naderi et al. 2003). Overall, the mechanism of GEN in female rats is not clear, and thus the results are not consistent. Our study showed that at a dose of 15 mg/kg, GEN had no significant effect on the ovary of young female rats in the expression of StAR, P450scc and CYP19mRNA. However, when the concentration of GEN reached 30-60 mg/kg, the expression of StAR, P450scc and CYP19mRNA, StAR and P450scc protein in the ovary of young female rats significantly increased. Interestingly the effect of GEN on aged rats was more obvious. It has been found that, although the expression of steroidogenic proteins (StAR, P450scc and CYP19) was upregulated by treatment with GEN, the serum levels of E2 in young rats tend to be decreased by treatment with GEN, which showed that GEN is bidirectional to estrogen regulation. When the level of estrogen in the body is high, GEN may play an anti-estrogen role, thereby reducing the level of estrogen. Owing to this, an analysis in young rats demonstrated that GEN played an anti-estrogen role at a dose range of 15-30 mg/kg.

This clearly indicated that GEN could increase the level of androgen generating key enzyme, promote the conversion of cholesterol to androgen, and indirectly enhance the content of estrogen in female rats. Thus, it could be speculated that GEN might have an effect on the follicular development and maturation in this way. However, further research is needed in order to clarify this hypothesis. Genomics such as transcriptomics, proteomics and metabolomics should be considered as a better method to understand aromatic and non-aromatic androgens as regards their regulation of ovarian function and to further clarify the effects and mechanism of GEN on the function of the ovary.

In conclusion, the data observed from this study clearly demonstrate the effects of GEN on the secretion of sex hormones in the rat ovary. They are at least partly associated with the modulation of StAR, P450scc and CYP19. It is suggested that in the ovary of rats at different ages, GEN may influence the process associated with the transformation of estrogen and androgen by regulating follicular steroidogenesis and estrogen expression, which consequently impair the growth and maturation of the ovary.

Acknowledgements

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