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Brief communication (Original)

Effects of daidzein on testosterone secretion in cultured immature mouse testis

Liuping Zhang[a], Genbao Shao[a], Yaoqian Pan[b]
[a]*Department of Biology, School of Medical Science and Laboratory Medicine, Jiangsu University, Zhenjiang, Jiangsu 212013,* [b]*College of Animal Science, Henan Institute of Science and Technology, Xinxiang, Henan, China*

Background: Daidzein is a major isoflavone in soybeans. Several in vivo studies have showed that daidzein can affect immature male testosterone production. However, whether daidzein has direct action on immature male testis is unknown.
Objective: We investigated the effects of daidzein on testosterone secretion in 3-day-old and 21-day-old mouse Leydig cells with organotypic culture model.
Materials and Methods: The testes were exposed to different concentrations (10^{-7} to 10^{-4} M) of daidzein for 72 h with medium changed every 24 h. From 72 to 75 h of culture, 100 ng/ml human chorionic gonadotropin (hCG) was added. The testosterone production was determined, and the related mechanisms of daidzein action were also evaluated by measuring the mRNA levels of steroidogenic acute regulatory protein (StAR), cholesterol side-chain cleavage enzyme (P450scc), and 3β-hydroxysteroid dehydrogenase (3β-HSD-1) involved in testosterone biosynthesis.
Results: The results revealed that in the presence of 100 ng/ml hCG, 10^{-7} to 10^{-5} M daidzein had no significant effect on testosterone secretion in cultured 3-day-old mouse testis. But 10^{-4} M daidzein significantly increased testosterone concentration ($p < 0.05$). Daidzein in range of studied doses had no obvious influence on testosterone production in cultured 21-day-old mouse testis. RT-PCR results showed that 10^{-4} M daidzein had obvious influence on the mRNA levels of StAR, P450scc and 3β-HSD-1 in cultured 3-day-old mouse testis ($p < 0.05$).
Conclusion: These results suggest that daidzein mainly influences neonatal mouse testis function, and the influence is partially related to the upregulation of StAR, P450scc, and 3β-HSD-1 mRNA levels.

Keywords: Daidzein, immature mouse Leydig cells, P450scc, testosterone, StAR, 3β-HSD-1

Daidzein is a major isoflavone in soybeans, and the most common form of phytoestrogen. Several in vitro and in vivo studies have revealed that daidzein exerts beneficial effects such as stimulation of male testosterone production [1] and growth [2], inhibition of environmental estrogen-induced proliferation of human breast carcinoma cells [3], modulation of hepatic lipid metabolism [4], and improvement of cardiovascular risk factors [5].

However, exposure to isoflavones, including daidzein, during fetal and childhood development can have effects on male reproductive function as mainly suggested by in vivo studies. For example, exposure of juvenile rats to daidzein in relatively large amounts could impair erectile function and decrease plasma testosterone levels in adulthood [6]. Soy formula milk could decrease marmoset monkey blood testosterone levels [7]. Male rats were given different concentrations of soy isoflavones from gestational day 12 until weaning at day 21 postpartum. The results showed that low dose increased serum and cultured Leydig cell testosterone levels and high doses decreased serum testosterone production at day 21 postpartum [8]. Recent studies have shown that high-dose soy isoflavones administered to rats from prenatal life to sexual maturity induced changes in the morphology of the seminiferous epithelium and significantly decreased testicular testosterone concentration. However, there were no significant changes in the number of spermatozoa in the epididymis and the serum testosterone concentrations

Correspondence to: Liuping Zhang, Department of Biology, School of Medical Science and Laboratory Medicine, Jiangsu University, Zhenjiang, Jiangsu 212013, China.
E-mail: jsuniversity12@163.com

[9]. Furthermore, there is a report that perinatal exposure of male rats to isoflavones induced proliferative activity in Leydig cells [10]. However, pharmacokinetic complications and secondary effects may make it difficult to clarify the direct actions of isoflavones on the target tissues. Therefore, it is important to study the direct effects of daidzein through in vitro experiments.

To date, there is no report to our knowledge regarding whether daidzein acts directly on immature mouse testis and influences testosterone secretion. In addition, testosterone biosynthesis is involved in a series of biochemical processes and is mediated by several key proteins, such as steroidogenic acute regulatory protein (StAR) [11-13], cholesterol side-chain cleavage enzyme (p450scc), and 3β-hydroxysteroid dehydrogenase (3β-HSD) [11]. However, it is whether daidzein influences testosterone biosynthesis by influencing the expression of these proteins has hitherto remained unknown.

Organotypic culture is useful for studies of the effects of xenobiotics on testis development [14] and the regulation of steroidogenesis and gene expression [15]. Two successive populations of Leydig cells (fetal population and adult population) arise during normal testicular development. In mouse, the adult population begins to differentiate 4 days after birth [16]. Formation of adult Leydig cells involves transformation of mesenchymal-like cells to Leydig cell progenitors by day 21 [17]. The present study was thus designed to characterize the effects of daidzein on testosterone production in 3-day-old and 21-day-old mouse Leydig cells using an organotypic culture model and to investigate the related mechanisms of daidzein action.

Materials and methods

Chemicals

Daidzein (minimum 98%), Ham's F12 medium, Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, and dimethyl sulfoxide were purchased from Sigma-Aldrich (St. Louis, MO, USA). The testosterone radioimmunoassay kit was obtained from the Beijing SINO-UK Institute of Biological Technology (Beijing, China). Total RNA isolation kit was purchased from Invitrogen (Carlsbad, CA, USA). All the reagents used for RT-PCR were from Promega (Madison, WI, USA).

Animals

Adult male and female Kunming mice (60 to 90 days old) were used for this study. Animals were maintained under constant conditions of light (12 h daylight: 12 h darkness) and temperature (between 22 and 24°C), with free access to soy-free diets and water. Males were caged with females. Neonates were killed by cervical dislocation on day 3 and day 21 postpartum, and their testes were immediately removed.

All experimental animal use and experimental design for this study were approved by the Chinese Association for Laboratory Animal Sciences.

Organ culture and treatment

Testes were cultured on Millipore (Bedford, MA) filters (pore size: 0.45 μm) as previously described [18]. Briefly, testes were cut into small pieces (6 pieces for 3 days postpartum (dpp), 3 mm^3 for 21 dpp), and all the pieces from the same testis were placed on a 25 mm Millipore filter. The filters were floated on 1.5 ml (3 dpp) or 2 ml (21 dpp) of phenol red-free Ham's F12/DMEM culture medium with 10% charcoal-stripped fetal bovine serum and antibiotics in tissue culture dishes and incubated at 37°C in a humidified atmosphere containing 95% air/5% CO_2. After 24 h culture, the testes were treated with various doses of daidzein (10^{-7} to 10^{-4} M) for 72 h. The medium was changed every 24 h. We added 100 ng/ml human chorionic gonadotropin (hCG) to all the medium for the last 3 h of culture (72 to 75 h), the medium and testes were then collected for testosterone measurements and RT-PCR, respectively.

Testosterone measurements

Testosterone levels were measured using a testosterone radioimmunoassay kit. The sensitivity of the assay was 20 pg/ml. Intra- and interassay variations were below 7.4% and 9.5%, respectively, for both assays.

Total RNA extraction and semi-quantitative RT-PCR

Total RNA was extracted from the cultured testes using a TRIzol RNA isolation kit. We mixed 2 μg RNA with 2 μl of oligo (dT) and heated the mixture at 65°C for 5 min. The mixture was then cooled on ice for 5 min. We then added 1 μl M-MLV reverse transcriptase, 5 μl dNTP, 2 μl RNasin, and 5 μl 5× M-MLV RT buffer to the mixture. A final volume of 25 μl was achieved using diethyl pyrocarbonate-treated water. Reverse-transcription was then performed

at 42°C for 1 h. Subsequent PCR amplification was performed at a reaction volume of 25 µl. The primer sequences used for StAR, P450scc, 3β-HSD-1, and hypoxanthine phosphoribosyl-transferase (HPRT) (internal control) were based on previously published sequences [19, 20]. The primer sequences for StAR were 5′-TGTCAAGGAGATCAAGGTCCTG-3′ (forward) and 5′-CGATAGGACCTGGTTGATG AT-3′(reverse). The primer sequences for P450scc were 5′-AGGTGTAGCTCAGGACTTCA-3′ (forward) and 5′-AGGAGGCTATAAAGGACA CC-3′ (reverse). The primer sequences for 3β-HSD-1 were 5′-ACTGCAGGAGGTCAGAGCT-3′ (forward) and 5′-GCCAGTAACACACAGAA TACC-3′ (reverse). The primer sequences for HPRT were 5′-CTTGCTCGAGATGTCATGAAG-3′ (forward) and 5′ -GTTTGCATTGTTTTACCAGTG-3′ (reverse). The conditions of cDNA amplification for StAR, P450scc, 3β-HSD-1 and HPRT were as follows: denaturation at 94°C for 5 min; followed by 40 cycles, each consisting of denaturation at 94°C for 30 s, annealing at 54°C for 30 s and extension at 72°C for 35 s; finally followed by an additional extension step at 72°C for 10 min. The sizes of the PCR products were determined by agarose gel electrophoresis (1.2%) and stained with ethidium bromide, using a 100-bp DNA ladder as the standard. The expected sizes were 310 bp (StAR), 370 bp (P450scc), 565 bp (3β-HSD-1), and 290 bp (HPRT). Semiquantification of PCR products was performed using a computer-assisted image analysis system (AlphaImager 2200 and AlphaEase FC software package, version 3.2.1, Alpha Innotech Corp, San Leandro, CA, USA) and normalizing StAR, P450scc, and 3β-HSD-1 gene products to HPRT bands.

Statistical analysis

Data are described as mean ± SEM. Data of testosterone concentrations were analyzed by one-way ANOVA followed by a Dunnett *t* test. Data regarding mRNA levels were analyzed using an independent-samples *t* test. Statistical analyses were performed using SPSS 13.0 for Windows. Differences of $p < 0.05$ were considered significant.

Results

Effects of daidzein on testosterone production in cultured 3-day-old mouse testis

The influence of daidzein on hCG-stimulated testosterone production in cultured 3-day-old mouse testis is illustrated in **Figure 1**. We found that 10^{-4} M daidzein significantly increased testosterone concentration ($p < 0.05$); while lower doses of daidzein had no significant effect. We infer that the effect of daidzein on testosterone production is dose dependent.

Effects of daidzein on testosterone production in cultured 21-day-old mouse testis

The influence of daidzein on hCG-stimulated testosterone production in cultured 21-day-old mouse testis is illustrated in **Figure 2**. The results revealed that daidzein in range of studied doses had no obvious influence on testosterone secretion ($p > 0.05$). We infer that the effect of daidzein on testosterone production is age related.

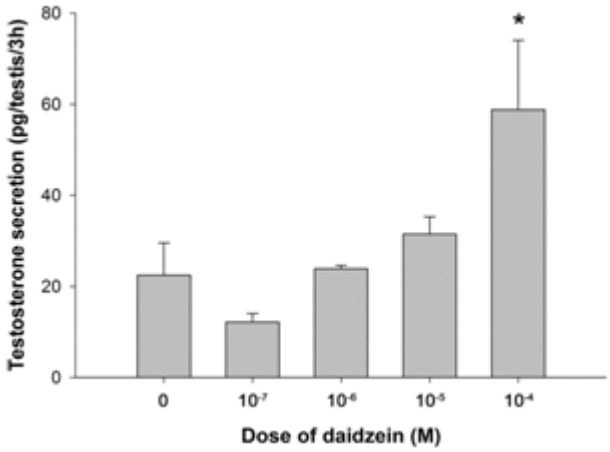

Figure 1. Effects of daidzein on testosterone secretion in cultured 3-day-old mouse testis. The testosterone concentrations in the media were measured by radioimmunoassay. Data are expressed as the mean ± SEM of three separate experiments. *$p < 0.05$

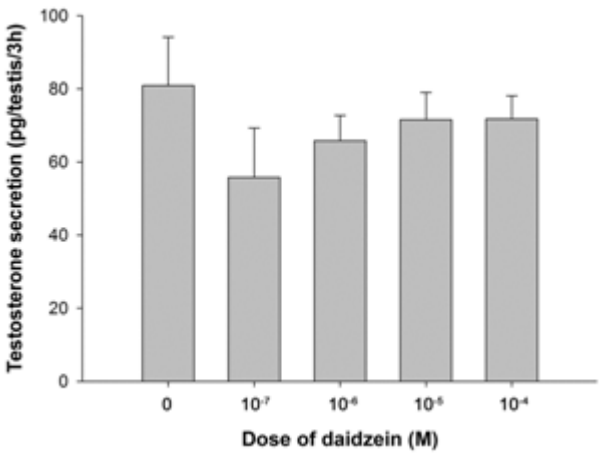

Figure 2. Effects of daidzein on testosterone secretion in cultured 21-day-old mouse testis. The testosterone concentrations in the media were measured by radioimmunoassay. Data are expressed as the mean ± SEM of three separate experiments.

Effects of daidzein on mRNA expression of StAR, P450scc, and 3□-HSD-1

RT-PCR results are showed in **Figure 3**. The results demonstrated that 10^{-4} M daidzein had obvious influence on the mRNA levels of StAR, P450scc, and 3β-HSD-1 in cultured 3-day-old mouse testis ($p < 0.05$). This suggests that daidzein-induced increase of testosterone production is partially related to the upregulation of StAR, P450scc, and 3β-HSD-1 mRNA levels.

Discussion

In the present study, an organotypic culture system was used to detect the effects of daidzein on testosterone production in immature mouse Leydig cells. The results showed that daidzein enhanced hCG-stimulated steroidogenesis of 3-day-old mouse Leydig cells at concentration of up to 10^{-4} M. This is contradictory to many in vivo experiments showing that isoflavones, including daidzein, decrease testosterone concentration [6, 7, 9]. It has been reported that isoflavone, including daidzein can exert inhibitory feedback on the hypothalamus–pituitary–gonadal axis [21]. Daidzein can be metabolized to equol by gut microflora [22, 23], which is found at high levels in the blood of experimental animals, and can modulate reproductive function [24]. These features are possible explanations for the observed in vitro–in vivo dichotomy, at least in part.

Many in vivo and in vitro experiments have shown that exposure of fetus and neonate to potent estrogens decreases male testosterone production and causes reproductive system abnormalities [25-27]. However, it is worth noting that estrogen concentrations in these experiments are high. For example, diethylstilbestrol doses reached several hundred micrograms per kilogram of body weight during in vivo experiments and 4×10^{-6} M in tissue culture. While lower doses of estrogens can regulate spermatogenesis [28, 29], promote the Leydig cell development, and testosterone production [26], daidzein has only weak estrogenic activity and its affinity with estrogen receptors is 10^{-3} less than that of estrogen [30, 31]. Therefore, the effect of a high dose of daidzein on testosterone production in cultured 3-day-old mouse testis probably simulates lower doses of estrogen. While lower doses of daidzein have a very weak estrogen-like effect and are not sufficient to influence testosterone production. This hypothesis is supported by experiments that demonstrated that exposure of neonatal rats to high doses of octylphenol and bisphenol A (weak estrogens) had no side effects on testis and contrarily increased testosterone production and simulated the effects of low doses of diethylstilbestrol on advancing the normal onset of pubertal spermatogenesis [26].

This study found that daidzein in range of studied doses had no obvious influence on testosterone production in cultured 21-day-old mouse testis. Possible reasons are that the production of endogenous estrogens within testis masks the daidzein effects [32] or other mechanisms by which the testis is insensitive to exogenous estrogen.

It has been reported that phytoestrogens including daidzein can modulate the endocrine system by altering

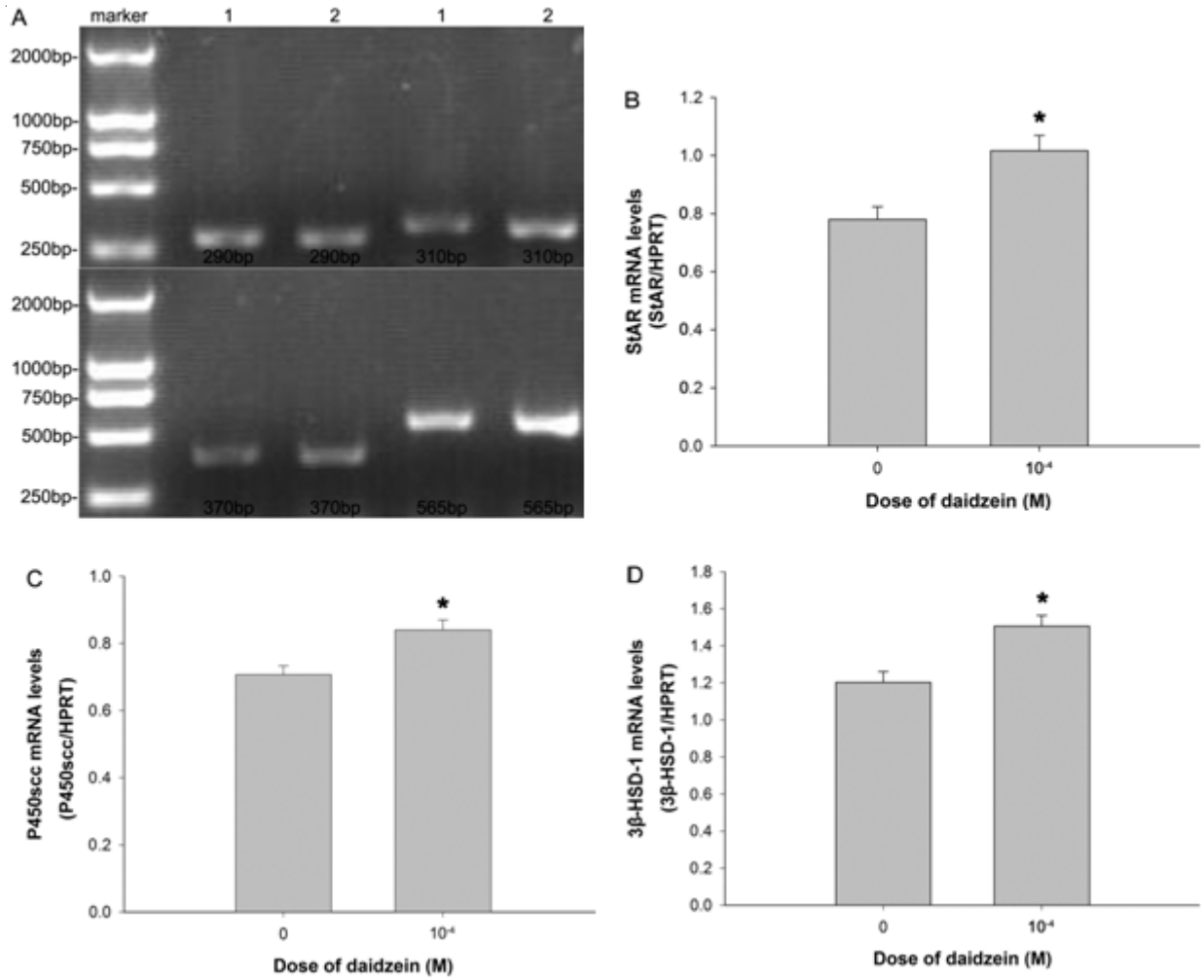

Figure 3. Effects of daidzein on mRNA expression of StAR, P450scc, and 3β-HSD-1 in cultured 3-day-old mouse testis. The mRNA levels of StAR, P450scc, and 3□-HSD-1 were analyzed by RT-PCR and densitometry. HPRT (290 bp) was used as an internal control. **A:** The sizes of the PCR products (310 bp, StAR; 370 bp, P450scc; and 565 bp, 3β-HSD-1). Lanes 1–2: control and 10^{-4} M daidzein, respectively. **B–D:** The results of semiquantification of the PCR products. The panel is representative of the ratio of StAR, P450scc, and 3□-HSD-1 mRNA to HPRT mRNA. Data are expressed as the mean ± SEM of three separate experiments. *$p < 0.05$

the gene expression or activity of the enzymes involved in steroidogenesis [33], and the key enzymes involved in steroidogenesis are important targets for phytoestrogens [34]. It is well known that testosterone is synthesized from cholesterol. The conversion of cholesterol to testosterone involves series of key steroidogenic proteins, such as StAR protein, cytochrome P450scc, and 3β-HSD [11-13]. The rate-limiting step in the synthesis of testosterone is the transfer of cholesterol to pregnenolone via P450scc; StAR mediates this rate-limiting step in steroidogenesis [35]. Whereas the conversion of pregnenolone to progesterone is mediated by 3β-HSD. This enzymatic action is essential for the production of all active steroid hormones [36]. The results of the present study suggest that daidzein at 10^{-4} M significantly increases hCG-stimulated transcriptional activity of StAR, P450scc, and 3β-HSD-1 in cultured 3-day-old mouse testis, which parallels testosterone production. We conclude that the higher testosterone level is caused, at least in part, by the increased expression of StAR, P450scc, and 3β-HSD-1 mRNA.

Conclusion

In summary, data from the present study indicate that high concentrations of daidzein increase hCG-stimulated testosterone production in cultured 3-day-old mouse testis, and this enhancing effect is partially

related to the upregulation expression of StAR, P450scc, and 3β-HSD-1 mRNA. More studies are required to determine whether the effect of daidzein is mediated by other factors.

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