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Effects of Daidzein on Testosterone Synthesis and Secretion in Cultured Mouse Leydig Cells*

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ABSTRACT : The objective of this work was to study the direct effects of daidzein on steroidogenesis in cultured mouse Leydig cells. Adult mouse Leydig cells were purified by Percoll gradient centrifugation, and the cell purity was determined using a 3β-hydroxysteroid dehydrogenase (3β-HSD) staining method. The purified Leydig cells were exposed to different concentrations (10^{-7} M to 10^{-4} M) of daidzein for 24 h under basal and human chorionic gonadotropin (hCG)-stimulated conditions. The cell viability and testosterone production were determined, and the related mechanisms of daidzein action were also evaluated using the estrogen receptor antagonist ICI 182,780 and measuring the mRNA levels of steroidogenic acute regulatory protein (StAR), cholesterol side-chain cleavage enzyme (P450scc), and 3β-HSD-1 involved in testosterone biosynthesis. The results revealed that daidzein did not influence cell viability. Daidzein increased both basal and hCG-stimulated testosterone production in a dose-dependent manner, and this effect was statistically significant at concentrations of 10^{-5} M and 10^{-4} M daidzein ($p<0.05$). ICI 182,780 had no influence on daidzein action. RT-PCR results revealed that 10^{-5} M and 10^{-4} M daidzein did not exert any obvious influence on the mRNA level of P450scc in Leydig cells. However, in the presence of hCG, these concentrations of daidzein significantly increased the StAR and 3β-HSD-1 mRNA levels ($p<0.05$), but in the absence of hCG, only 10^{-5} M and 10^{-4} M daidzein up-regulated the StAR and 3β-HSD-1 mRNA expression ($p<0.05$), respectively. These results suggest that daidzein has direct effect on Leydig cells. Daidzein-induced increase of testosterone production is probably not mediated by the estrogen receptor but correlates with the increased mRNA levels of StAR and 3β-HSD-1. (**Key Words :** Daidzein, Testosterone, StAR, P450scc, 3β-HSD-1, Mouse Leydig Cells)

INTRODUCTION

Daidzein is one of the major isoflavones in soybeans, and it is the most common form of phytoestrogens. Several *in vitro* and *in vivo* studies have revealed that daidzein exerts beneficial effects such as stimulation of male growth (Wang et al., 2002), modulation of hepatic lipid metabolism (Choi et al., 2006), improvement of preovulatory follicles development (Liu et al., 2007), antioxidative property (Jiang et al., 2007), anti-cancer (Messina et al., 1994) and neuroprotective effect (Zhao et al., 2002). In addition, it is reported that daidzein has the function to reduce the risk of osteoporosis (Toda et al., 1999), heart disease (Anthony et al., 1996) and menopausal symptoms (Anderson et al., 1999) by binding to estrogen receptors (ERs), including estrogen receptor α (ERα) and estrogen receptor β (ERβ).

However, the functions of isoflavones, including daidzein, in the male reproductive system are controversial. For example, adult male rats were fed diet containing about 600 μg/g isoflavones for 5 weeks, plasma testosterone and androstenedione levels were significantly lower compared with animals fed the phytoestrogen-free diet (Weber et al., 2001). Exposure of juvenile and adult rats to 20 or 100 mg/kg daidzein for 90 days impaired the apomorphine-induced penile erection and decreased the plasma testosterone concentration (Pan et al., 2007; Pan et al., 2008). Contrary to these inhibitory effects, male rats in the perinatal period exposure to 1,000 ppm isoflavones elevated serum and testicular testosterone levels at 90 days of age (Akingbemi et al., 2007). Furthermore, there are reports that short or long period of feeding with soybean products rich in daidzein and other isoflavonoids did not have obvious

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influence on the global testosterone levels, semen quality, and sperm counts in male macaques and humans (Habito et al., 2000; Mitchell et al., 2001; Perry et al., 2007). These contradictory results of phytoestrogens for testicular function mostly come from the *in vivo* studies. Pharmacokinetic complications and secondary effects may make it difficult to clarify the direct actions of isoflavones to the target cells. Therefore, it is important to study the direct effects of daidzein through *in vitro* experiment.

It has been well documented that daidzein has estrogenic activity and is involved in regulating testosterone production by binding to ERs, including ERα and ERβ (Woclawek-Potocka et al., 2005; Akingbemi et al., 2007; Pan et al., 2008), which are widely expressed in the hypothalamus-pituitary-testis axis. However, whether daidzein directly affects testosterone production via testis ERs or whether it influences the hypothalamus-pituitary-testis axis by binding to the hypothalamus and pituitary ERs has not been investigated in detail. In addition, testosterone biosynthesis is involved in a series of biochemical processes and is mediated by several key proteins, such as StAR (Cherradi et al., 1997; Stocco, 1998; Walsh et al., 2000), p450scc, and 3β-HSD (Cherradi et al., 1997). However, it is still unknown whether daidzein influences testosterone biosynthesis by influencing the expression of these proteins. The present study was thus designed to characterize the effects of daidzein on testosterone production in cultured mouse Leydig cells and to investigate the related mechanisms of daidzein action.

MATERIALS AND METHODS

Chemicals

Daidzein (minimum 98%), ICI 182,780, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Ham's F12 medium, Dulbecco's modified Eagle's medium (DMEM), fetal calf serum and hCG were purchased from Sigma-Aldrich (St. Louis, MO, USA). Beta nicotinamide adenine dinucleotide (β-NAD+), etiocholanolone and dimethyl sulfoxide were from Sigma Chemical Co. (St. Louis, MO, USA). Percoll was from Pharmacia, Inc. (Piscataway, NJ, USA). Collagenase type I was obtained from Gibco (Grand Island, NY, USA). The testosterone radioimmunoassay (RIA) Kit was obtained from the Beijing SINO-UK Institute of Biological Technology (Beijing, China). All the reagents used for RT-PCR were from Promega (Madison, WI, USA).

Animals

Adult male Kunming mice (60 to 90 days of age) were used for this study. The mice were approved by the Chinese Association for Laboratory Animal Sciences. 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 provided in glass bottles.

Isolation and purification of primary Leydig cells

The primary Leydig cells were isolated and purified as described by Klinefelter et al. (1987) and Chemes et al. (1992) with some modifications. Briefly, the mice testes were placed in a prechilled 1:1 mixture of Ham's F12 medium and DMEM with 4.5 g/L glucose, 1.2 g/L sodium bicarbonate, 15 mM HEPES, 100 U/ml penicillin, and 100 U/ml streptomycin, and transported on ice. The testes were then decapsulated and mechanically dispersed in the medium. This was then treated with a solution of 0.05% collagenase (type I) in the culture medium mentioned above, under a constant agitation at 34°C for 30 min. Following this digestion procedure, the collagenase solution was diluted four times with culture medium, and separated from tissue fragments by filtration through a stainless-steel mesh. The cells were then collected by centrifugation (1,500 r/min for 10 min) and resuspended in 2 ml of the culture medium mentioned above. Leydig cells were purified with a discontinuous four-layer Percoll density gradient (21%, 26%, 37%, and 60%). The gradient was centrifuged at 3,000 r/min for 30 min at 4°C. The interface between 37% and 60% was collected and washed with medium to remove Percoll. The cell viability was determined using the trypan blue dye exclusion test (Tennant, 1964), and it was observed that more than 90% of the cells were viable. The cells were counted using a hemocytometer, and the purity was assessed by histochemical staining for 3β-HSD using 1 mg/ml etiocholanolone as the enzyme substrate (Klinefelter et al., 1987). It was observed that more than 95% of the cells were stained positive for Leydig cells.

Cell culture and treatment

The purified Leydig cells were plated in 24-well (1×10^5 cells/ml/well) plates in phenol red-free Ham's F12/DMEM culture medium supplemented with 10% charcoal-stripped fetal bovine serum and antibiotics. Culture was performed at 34°C in a controlled humidified atmosphere with 5% CO_2 in air. After 48 h culture, the cells were washed two times with serum-free medium, and then treated with various doses of daidzein (10^{-7}M, 10^{-6}M, 10^{-5}M, and 10^{-4}M) for 24 h with or without hCG (10 ng/ml). At the end of the incubation, the medium was collected from the 24-well plates and stored at -20°C for testosterone measurements, and cells were collected for the measurements of StAR, P450scc and 3β-HSD-1 mRNA. In order to study the effects of an inhibitor of the classical estrogen receptors, ICI 182,780 on testosterone synthesis, the Leydig cells were

cultured in 24-well (1×10^5 cells/ml/well) plates for 48 h, then treated with or without daidzein (10^{-5} M and 10^{-4} M) and/or ICI 182,780 (100 nM) for 24 h in the presence and absence of hCG (10 ng/ml). At the end of the incubation, the medium was collected from the 24-well plates and stored at -20°C for testosterone measurements.

Cell viability test

Cell viability was determined using the MTT assay (Kelce, 1994). Briefly, after cells were treated with daidzein for 24 h, the medium was removed and 100 μl fresh medium containing 0.5 mg/ml MTT was added. After 3 h incubation, the medium was removed, and the cells were then incubated in 100 μl acidified isopropanol. The absorbance was measured with a microtiter plate reader at a test wavelength of 562 nm with a reference wavelength of 650 nm. Percent viability was calculated as (optical density of daidzein treated sample/optical density of control)×100.

Testosterone measurements

The testosterone levels in the incubation medium were measured using a commercial RIA (Beijing SINO-UK Institute of Biological Technology). The sensitivity of the assay was 20 pg/ml. Intra- and inter-assay variations were below 7.4% and 9.5%, respectively, for both assays. The rates of Leydig cell testosterone secretion were normalized to ng/10^6 cells.

Total RNA extraction and semi-quantitative RT-PCR

Total RNA was extracted from mouse Leydig cells using TRIzol regent as per the manufacturer's instructions. 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 (Jin et al., 2000; Akingbemi et al., 2003). The primer sequences for StAR were 5′-TGTCAAGGAGATCAAGGTCCTG-3′ (forward) and 5′-CGATAGGACCTGGTTGATGAT-3′ (reverse). The primer sequences for P450scc were 5′-AGGTGTAGCTCAGG ACTTCA-3′ (forward) and 5′-AGGAGGCTATAAAGG ACACC-3′ (reverse). The primer sequences for 3β-HSD-1 were 5′-ACTGCAGGAGGTCAGAGCT-3′ (forward) and 5′-GCCAGTAACACACAGAATACC-3′ (reverse). The primer sequences for HPRT were 5′-CTTGCTCGAGA TGTCATGAAG-3′ (forward) and 5′- GTTTGCATTGTT TTACCAGTG-3′ (reverse). The conditions of cDNA amplification for basal StAR, P450scc, and HPRT were as follows: denaturation at 94°C for 5 min; followed by 35 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. PCR conditions were similar for basal 3β-HSD-1 cDNA amplification, except that 31 cycles were carried out. The conditions of amplification for the cDNAs of hCG-stimulated StAR, P450scc, 3β-HSD-1, and HPRT were similar to the basal conditions, except that StAR, P450scc, and HPRT underwent 34 cycles of amplification and 3β-HSD-1 underwent 30 cycles. 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 Corporation, CA, USA) and normalizing StAR, P450scc, and 3β-HSD-1 gene products to HPRT bands.

Statistical analysis

Data are described as mean±SEM. Data were analyzed by one-way ANOVA followed by Dunnett's post hoc two-sided *t*-test using SPSS 9.0 for Windows. Differences of $p\leq 0.05$ were considered significant.

RESULTS

Daidzein does not influence Leydig cell viability

In order to answer whether 10^{-7} M to 10^{-4} M daidzein used in this study have cytotoxicity, basal and hCG-stimulated Leydig cell viability was determined. The results showed that daidzein did not influence Leydig cell viability (Figure 1). This suggests that daidzein does not exert toxic effects on Leydig cells.

Higher doses of daidzein enhance testosterone production

The influence of daidzein on basal and hCG-stimulated testosterone production in Leydig cells is illustrated in Table 1. The results showed that 10^{-5} M and 10^{-4} M daidzein significantly increased testosterone concentrations in the medium with or without hCG ($p<0.05$); while lower doses of daidzein had no significant effect. These infer that the effect of daidzein on testosterone production is dose dependent.

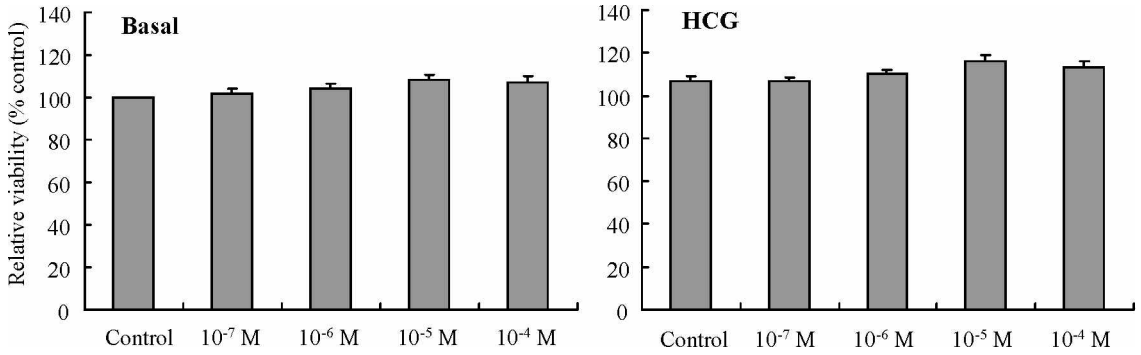

Figure 1. Effects of daidzein on mouse Leydig cell viability. Cells were incubated with different concentrations (10^{-7} M, 10^{-6} M, 10^{-5} M and 10^{-4} M) of daidzein for 24 h in the presence and absence of hCG (10 ng/ml), and cell viability was then determined by MTT assay. Data are expressed as the mean±SEM of three separate experiments, each performed in replicates of six.

Table 1. Effects of daidzein on testosterone production in cultured mouse Leydig cells[a]

Daidzein levels (M)	Testosterone production (ng/10^6 cells/24 h)	
	Basal	HCG
0	41.23±2.64	115.70±6.66
10^{-7}	37.57±2.85	114.87±4.56
10^{-6}	42.33±3.24	122.53±7.30
10^{-5}	52.43±2.25*	141.83±6.78*
10^{-4}	55.10±2.48*	149.70±5.54*

[a] Leydig cells were incubated with different concentrations (10^{-7} M, 10^{-6} M, 10^{-5} M, and 10^{-4} M) of daidzein for 24 h in the presence and absence of hCG (10 ng/ml), and testosterone concentrations were then measured by RIA. Data are expressed as the mean±SEM of three separate experiments, each performed in triplicate. * Represents $p<0.05$ as compared to the control.

Table 2. Effect of the estrogen receptors inhibitor ICI 182,780 on testosterone synthesis[a]

Treatments	Testosterone production (ng/10^6 cells/24 h)	
	Basal	HCG
CON	40.53±2.75	108.13±6.68
ICI	38.67±4.01	104.73±4.31
10^{-5} M Da	52.40±1.95*	135.63±7.19*
10^{-5} M Da+ICI	52.30±2.51*	135.37±5.30*
10^{-4} M Da	55.73±3.42*	142.23±8.31*
10^{-4} M Da+ICI	53.50±1.75*	141.07±7.12*

[a] Leydig cells were incubated with different concentrations (10^{-5} M and 10^{-4} M) of daidzein and/or ICI 182,780 (100 nM) for 24 h in the presence and absence of hCG (10 ng/ml), and testosterone concentrations were then measured by RIA. Data are expressed as the mean±SEM of three separate experiments, each performed in triplicate. CON = Control; ICI = ICI 182,780; Da = Daidzein; * Represents $p<0.05$ as compared to the control.

The estrogen receptors inhibitor ICI 182,780 has no obvious effect on testosterone synthesis

In order to investigate whether the effect of daidzein on testosterone synthesis was mediated by estrogen receptors, Leydig cells were incubated with different concentrations (10^{-5} M and 10^{-4} M) of daidzein and/or ICI 182,780 for 24 h in the presence and absence of hCG. The results are presented in Table 2. Surprisingly, the combined use of 10^{-5} M and 10^{-4} M daidzein with ICI 182,780, or ICI 182,780 alone did not have evident effects on the testosterone production. This suggests that the action of daidzein on testosterone synthesis is probably not directly mediated via estrogen receptors.

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

RT-PCR results are showed in Figure 2 and 3. The results demonstrated that 10^{-5} M and 10^{-4} M daidzein did not have obvious influence on the mRNA level of P450scc in Leydig cells. However, in the presence of hCG, these concentrations of daidzein significantly increased the StAR and 3β-HSD-1 mRNA levels in Leydig cells ($p<0.05$). But when hCG was absence, only 10^{-5} M daidzein exerted apparent positive effect on the StAR mRNA level in Leydig cells, and 10^{-4} M daidzein up-regulated the 3β-HSD-1 mRNA expression ($p<0.05$).

DISCUSSION

In the present study, MTT assay was used to detect the effects of daidzein exposure on the viability of Leydig cells (Kelce, 1994). The results reveal that daidzein does not influence Leydig cell viability, which excludes the possibility of toxic effects of daidzein impacting on testosterone production in Leydig cells.

Unexpectedly, the RIA results showed that daidzein enhanced both basal and hCG-stimulated steroidogenesis of mouse Leydig cells at concentrations of up to 10^{-5} M and 10^{-4} M. However, many *in vivo* experiments have demonstrated that daidzein decreases testicular and/or serum testosterone concentrations in males (Weber et al., 2001; Wisniewski et al., 2003; Pan et al., 2007; Pan et al., 2008). It has been reported that daidzein can exert inhibitory feedback on the hypothalamus-pituitary-gonadal

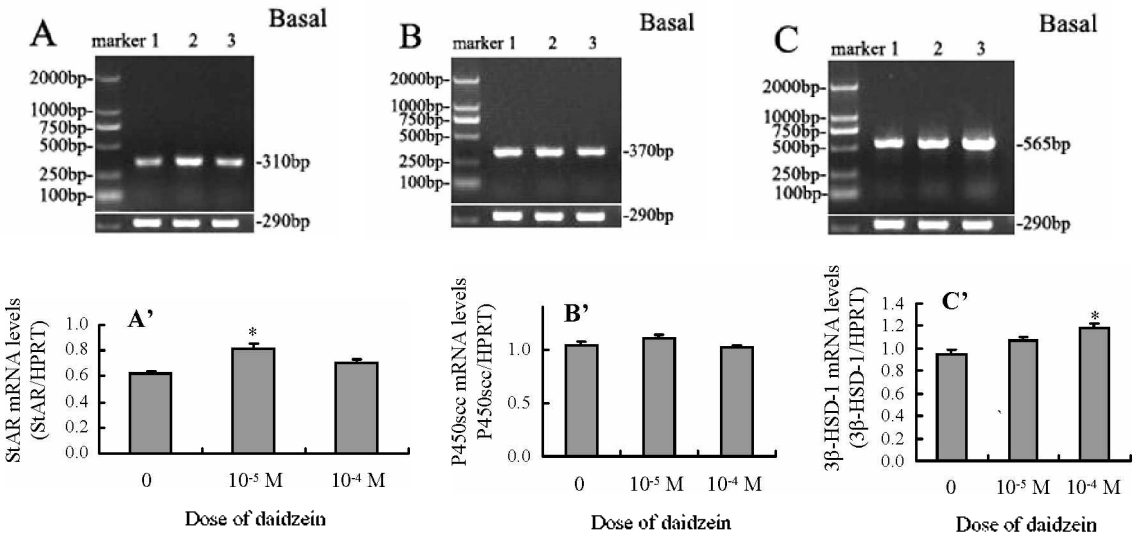

Figure 2. Effects of daidzein on mRNA expression of StAR, P450scc, and 3β-HSD-1 in the absence of hCG. The Leydig cells were incubated with 10^{-5} M and 10^{-4} M daidzein for 24 h, and the mRNA levels of StAR, P450scc, and 3β-HSD-1 were analyzed by RT-PCR and densitometry. HPRT (290 bp) was used as an internal control. (A)-(C) are the sizes of the PCR products (310 bp, StAR; 370 bp, P450scc; and 565 bp, 3β-HSD-1). Lanes 1-3: control, 10^{-5} M daidzein, and 10^{-4} M daidzein, respectively. (A')-(C') are the results of semiquantification of the PCR products. The panel is representative of the ratio of StAR, P450scc, and 3β-HSD-1 mRNA to HPRT mRNA. Data are expressed as the mean±SEM of three separate experiments, each performed in triplicate. * Represents $p<0.05$ as compared to the control.

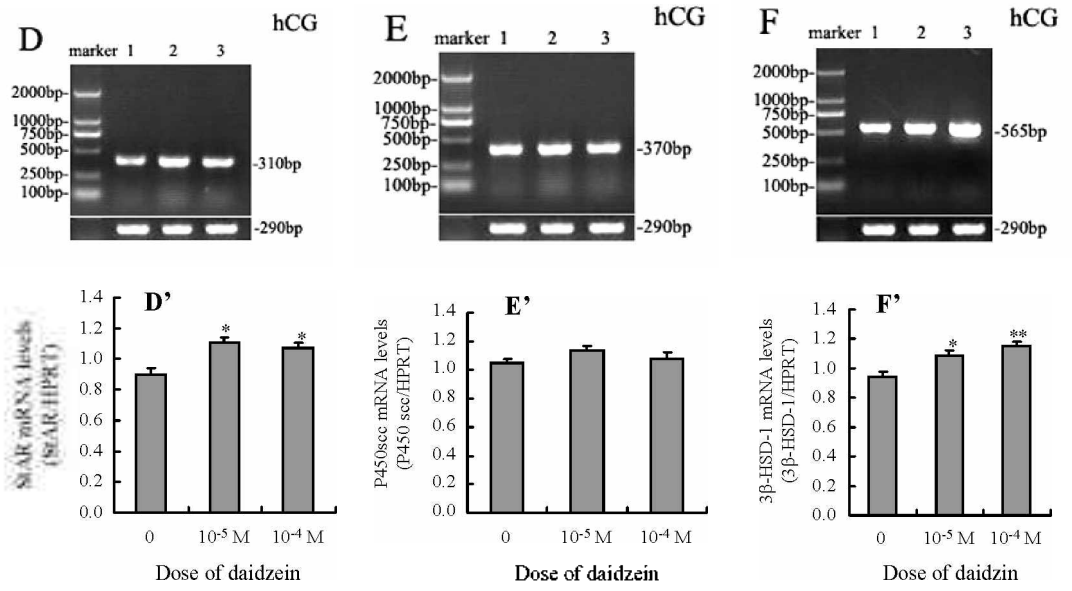

Figure 3. Effects of daidzein on mRNA expression of StAR, P450scc, and 3β-HSD-1 in the presence of hCG (10 ng/ml). The Leydig cells were incubated with 10^{-5} M and 10^{-4} M daidzein for 24 h, and the mRNA levels of StAR, P450scc, and 3β-HSD-1 were analyzed by RT-PCR and densitometry. HPRT (290 bp) was used as an internal control. (D)-(F) are the sizes of the PCR products (310 bp, StAR; 370 bp, P450scc; and 565 bp, 3β-HSD-1). Lanes 1-3: control, 10^{-5} M daidzein, and 10^{-4} M daidzein, respectively. (D')-(F') are the results of semiquantification of the PCR products. The panel is representative of the ratio of StAR, P450scc, and 3β-HSD-1 mRNA to HPRT mRNA. Data are expressed as the mean±SEM of three separate experiments, each performed in triplicate. * Represents $p<0.05$ as compared to the control, ** represents $p<0.01$ as compared to the control.

axis (Kwon et al., 2001). Daidzein can also be metabolized to equol by the gut microflora (Borriello et al., 1985; Setchell and Adlercreutz, 1988), which is high in blood in experimental animals and can modulate reproductive function (Lund et al., 2004). These factors are possibly partial explanations for the observed *in vitro-in vivo*

dichotomy. However, the present result is also opposite to the *in vitro* results of Akingbemi et al. (2007), which observed that genistein, another major isoflavone, decreased testosterone production in cultured immature rat Leydig cells. The discrepancy is possibly due to differences in animal models, age, and structure between genistein and daidzein.

It has been generally believed that daidzein exerts estrogen-like effects on the male reproductive function by binding to ERα and ERβ (Woclawek-Potocka et al., 2005; Akingbemi et al., 2007; Pan et al., 2008). However, the present results demonstrated that ICI 182,780, an inhibitor of classical estrogen receptors (Hall et al., 2001), did not change the positive effect of daidzein on testosterone synthesis of the cultured Leydig cells in the presence and absence of hCG. It is possible that the dose of ICI 182,780 was lower and could not prevent daidzein action. Higher doses of ICI 182,780 were excluded because of the altered testosterone secretion by itself. Another explanation is that daidzein stimulates testosterone synthesis through a nongenomic mechanism (Wehling, 1997; Falkenstein et al., 2000; Losel et al., 2003; Arshami and Cheng, 2007). Daidzein may also play its function in regulating testosterone synthesis by binding to other receptors *in vitro* as reported by Barnes and Peterson (1995). Therefore, further study is required to elucidate the molecular mechanism of daidzein on testosterone synthesis in cultured Leydig cells.

Furthermore, it has been reported that phytoestrogens including daidzein can modulate the endocrine system by altering the gene expression or activity of the enzymes involved in steroidogenesis (Hilscherova et al., 2004), and the key enzymes involved in steroidogenesis are important targets for phytoestrogens (Sanderson, 2006). It is well known that testosterone is synthesized from cholesterol. The conversion of cholesterol to testosterone involved series of key steroidogenic proteins, such as StAR protein, cytochrome P450scc and 3β-HSD (Cherradi et al., 1997; Stocco, 1998; Walsh et al., 2000). The rate-limiting step in the synthesis of testosterone is the transfer of cholesterol to pregnenolone via P450scc enzyme, StAR protein mediates this rate-limiting step in steroidogenesis (Stocco and Clark, 1996). 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 (Payne and Dale, 2004). The results of the present study suggest that daidzein at concentrations of 10^{-5} M and 10^{-4} M significantly increases hCG-stimulated transcriptional activity of StAR and 3β-HSD-1, which are parallel to the testosterone production. On the other hand, under basal conditions, mRNA levels of StAR and 3β-HSD-1 increased only at 10^{-5} M and 10^{-4} M daidzein, respectively. These results suggest that the action of daidzein on StAR and 3β-HSD-1 mRNA levels is dose and hCG related and a decline of StAR mRNA level at 10^{-4} M daidzein may come along with a contrary StAR protein increase (Akingbemi et al., 2007). No change in the P450scc mRNA expression was observed under both basal and hCG-stimulated conditions. We conclude that the higher testosterone levels are caused partially by the increased mRNA expression of StAR and 3β-HSD-1.

In summary, data from the present study indicate that higher concentration of daidzein directly stimulates both basal and hCG-stimulated testosterone synthesis in cultured mouse Leydig cells, and this stimulating effect is probably not through nuclear estrogen receptor pathway but related to the up-regulation of StAR and 3β-HSD-1 mRNA expression. More studies are required to determine whether the effect of daidzein is mediated by other factors.

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