ARTICLE
Journal of Cellular Biochemistry 110:333–342 (2010)

Journal of Cellular Biochemistry

Effects of Catechin, Epicatechin and Epigallocatechin Gallate on Testosterone Production in Rat Leydig Cells

Po-Ling Yu,[1,2] Hsiao-Fung Pu,[2] Sung-Yun Chen,[2] Shyi-Wu Wang,[3**] and Paulus S. Wang[2,4*]

[1] *Department of Surgery, Taipei City Hospital, Taipei 10431, Taiwan, Republic of China*

[2] *Department of Physiology, School of Medicine, National Yang-Ming University, Taipei 11221, Taiwan, Republic of China*

[3] *Department of Physiology and Pharmacology, College of Medicine, Chang-Gung University, Taoyuan 33333, Taiwan, Republic of China*

[4] *Department of Medical Research and Education, Taipei City Hospital, Taipei 10431, Taiwan, Republic of China*

ABSTRACT

Catechins have been reported to have many pharmacological properties such as the effects of anti-oxidative, anti-inflammatory, anti-carcinogenic, anti-ultraviolet, and reduction of blood pressure as well as glucose and cholesterol levels. However, the effect of catechins on the reproductive mechanism is still unknown. In the present study, the effects of catechins on testosterone secretion in rat testicular Leydig cells (LCs) were explored. Both in vivo and in vitro investigations were performed. Purified LCs were incubated with or without catechin (CCN), epicatechin (EC), epigallocatechin gallate (EGCG, 10^{-10}–10^{-8} M) under challenge with human chorionic gonadotropin (hCG, 0.01 IU/ml), forskolin, SQ22536 (an adenylyl cyclase inhibitor), 8-bromo-adenosine 3′:5′-cyclic monophosphate (8-Br-cAMP), A23187 (a calcium ionophore), and nifedipine (10^{-5} M), respectively. To study the effects of catechins on steroidogenesis, steroidogenic precursors-stimulated testosterone release was examined. The functions of the steroidogenic enzymes including protein expression of cytochrome P450 side chain cleavage enzyme (P450scc) and steroidogenic acute regulatory (StAR) protein were investigated and expressed by Western blotting. Catechins increased plasma testosterone in vivo in male rats. In vitro, low-dose concentration of catechins increased gonadotropin releasing hormone (GnRH)-stimulated luteinizing hormone (LH) release by anterior pituitary gland and hCG-stimulated testosterone release by LCs of male rats. These results suggested that catechins stimulated testosterone production by acting on rat LCs via the mechanism of increasing the action of cAMP, but not P450scc, StAR protein or the activity of intracellular calcium. EC, one of the catechins increased the testosterone secretion by rat LCs via the enzyme activities of 17β-hydroxysteroid dehydrogenase (17β-HSD). J. Cell. Biochem. 110: 333–342, 2010.

KEY WORDS: CATECHIN; EPICATECHIN; EPIGALLOCATECHIN GALLATE

Tea is the most consumed beverage worldwide. In the manufacture of tea, the tea leaves are crushed to catalyze the oxidation of catechins in a process known as "fermentation," by which tea are classified into non-fermented green tea, partially fermented Oolong tea and complete fermented black tea. Of all the tea originated from the leaves of the plant Camellia sinensis, 78% is black tea, which is common consumed in the Western people, 20% is green tea which is consumed in Asia such as China and Japan, and 2% is Oolong tea which is used in southern China [Kuriyama et al., 2006]. Thirty-six percent of green tea is composed by tea polyphenols, in which 80% is composed by flavonoids [Chung and Landau, 2000]. The major contents of flavonoids are catechins, for example, CCN, EC, epicatechin gallate (ECG), epigallocatechin (EGC), and EGCG (Fig. 1). Many investigations showed that catechins contained more OH bases structurally, the stronger in preventing damage by free radicals. Accordingly, the anti-oxidative potency between catechins are EGCG > ECG > EGC > EC = CCN [Mukhtar and Ahmad, 1999; Frei and Higdon, 2003]. In addition to anti-oxidative effect, the green tea extracts (GTEs) have been reported many functions including anti-cancer effect [Yang et al.,

Shyi-Wu Wang and Paulus S. Wang contributed equally.

*Correspondence to: Dr. Paulus S. Wang, Department of Physiology, School of Medicine, National Yang-Ming University, Shih-Pai, Taipei 11221, Taiwan, Republic of China. E-mail: pswang@ym.edu.tw

**Correspondence to: Dr. Shyi-Wu Wang, Department of Physiology and Pharmacology, College of Medicine, Chang-Gung University, Taoyuan 33333, Taiwan, Republic of China. E-mail: swwang@mail.cgu.edu.tw

Received 15 October 2009; Accepted 20 January 2010 • DOI 10.1002/jcb.22541 • © 2010 Wiley-Liss, Inc.
Published online 1 April 2010 in Wiley InterScience (www.interscience.wiley.com).

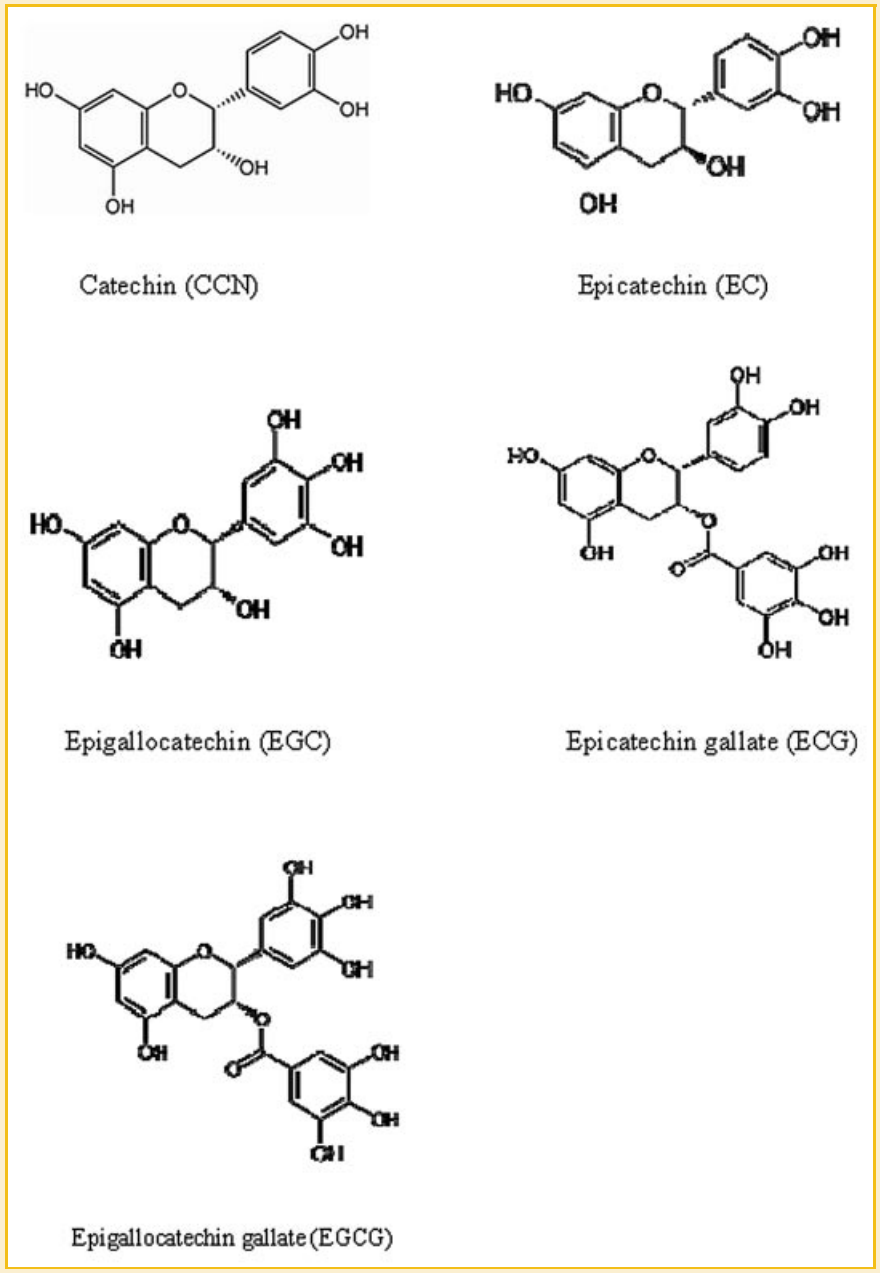


Fig. 1. Chemical structures of catechins in green tea.

2009], protection of cardiovascular system [Basu and Lucas, 2007], reduction of blood glucose and cholesterol [Hara and Honda, 1990; Mukhtar and Ahmad, 1999], anti-ultraviolet [Nomura et al., 2001] and anti-inflammatory [Shapiro et al., 2009] effects. However, the effects of GTEs on endocrine system especially on the testosterone production was seldom studied [Kao et al., 2000]. There was evidence that green tea polyphenols inhibited testosterone production in rat Leydig cells (LCs) in vitro by inhibitions of the PKA/PKC pathways, P450 side chain cleavage enzyme (P450scc) and 17β-hydroxysteroid dehydrogenase (17β-HSD) [Figueiroa et al., 2009]. The aim of this study was to investigate both in vivo and in vitro that the effects of major components of GTEs, that is, CCN, EC, and EGCG on the steroidogenesis of rat LCs and the involving mechanisms, for example, cAMP pathway, P450scc, and StAR protein.

MATERIALS AND METHODS

IN VIVO STUDY

Animals. Male Sprague–Dawley rats weighing 300–350 g were housed in a temperature controlled room ($22 \pm 1^{\circ}C$) with 14 h of artificial illumination daily (06:00–20:00 h) and given food and water ad libitum. Male rats, which were divided into 8 teams with

6–7 rats in each, were catheterized via the right jugular vein [Wang et al., 1994]. Twenty hours later, they were injected with saline (1 ml/kg) as vehicle, CCN (1 μg/ml/kg), EC (1 μg/ml/kg), EGCG (1 μg/ml/kg), human chorionic gonadotropin (hCG, 5 IU/ml/kg), hCG + CCN, hCG + EC, and hCG + EGCG via the jugular catheter. Blood samples (0.3 ml each) were collected at 0, 30, 60, 90, 120, 180, 240, and 480 min after the challenge. An equal volume of heparinized saline was injected immediately after each bleeding. Plasma was separated by centrifugation at 8,000*g* for 3 min. The concentration of testosterone in each plasma sample was measured by radioimmunoassay (RIA) [Wang et al., 1994].

IN VITRO STUDY

Preparation of anterior pituitary gland. After decapitation, the anterior pituitary gland was excised, bisected, and put in tube containing 1 ml Locke's solution. The tube was set in a 37°C water bath and aerated with 95% O_2 and 5% CO_2 for 90 min.

Effects of GTEs on the basal and GnRH-stimulated LH release by anterior pituitary gland of male rats. After incubation for 30 min with GTEs (10^{-9} and 10^{-5} M) in the presence or absence of gonadotropin releasing hormone (GnRH, 10 nM), the tissue was weighed and the medium was collected to measure the concentration of luteinizing hormone (LH) by RIA [Wang et al., 1994].

Preparation of rat testicular interstitial cells (TICs). The method of preparation of TICs followed the procedure described by Tsai et al. [1996]. Briefly, five decapsulated testes were added to a 50 ml polypropylene tube containing 5 ml preincubation medium and 700 μg collagenase. The preincubation medium was composed of 1% bovine serum albumin (BSA) in Hank's balanced salt solution (HBSS), with *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid (HEPES, 25 mM), sodium bicarbonate 0.35 g/l, penicillin-G 100 IU/ml, streptomycin sulphate 50 μg/ml, and heparin 2,550 USP K units/l, pH 7.3. The tube was laid horizontally in a 34°C water bath, parallel to the direction of the shaking. Fifteen minutes after shaking at 100 cycles/min, the digestion was stopped by adding 40 ml of cold preincubation medium and inverting the tube several times. The tube was allowed to stand for 5 min and the digest was then filtered through a four-layer fine nylon mesh. Cells were collected by centrifugation at 4°C, 200*g* for 10 min. The cell pellets were washed with deionized water to disrupt red blood cells (RBCs) and the osmolarity was recovered immediately with 10-fold HBSS. Hypotonic shock was repeated twice for disrupting RBCs and the cell pellets containing TICs were resuspended in incubation medium (substitution of HBSS in preincubation medium with Medium 199, and sodium bicarbonate 2.2 g/l).

Preparation of rat LCs. Following the procedure described above, the TICs then added gently to the upper layer of the continuous Percoll gradient. The continuous Percoll gradient (20 ml/dispersion) was made by adding 9 parts of Percoll to 11 parts of 1.8-fold concentrated incubation medium before centrifugation at 20,000*g* for 60 min at 4°C. The mixture of TICs was loaded onto the Percoll gradient and centrifuged at 800*g* for 20 min at 4°C. The LCs layer which were located in the 3–7 ml layer from the bottom was collected, diluted to 10 ml in incubation medium, and then centrifuged at 200*g* for 10 min at 4°C. After repeating the washing steps, the cell pellet was suspended to 10 ml in incubation medium.

LCs COUNTING AND CULTURE

The cell concentration (2×10^5 cells/ml) and viability (over 95%) were determined using a hemacytometer and the trypan blue method. To measure the abundance of LCs in our preparation, 3β-HSD staining method was used [Chiao et al., 2002]. The cells (2×10^5 cells/ml) were incubated with a solution containing 0.2 mg/ml of nitro blue tetrazolium in 0.05 M PBS pH 7.4 at 34°C for 90 min When the blue formazan deposit sites of 3β-HSD activity were developed, the abundance of LCs was determined using a hemacytometer. Macrophages were determined by flow cytometry with fluorescein isothiocynate-conjugated monoclonal antibody (ED1, IgG1; Biosource International, Foster City, CA). Our preparation contained approximately 90% LCs and very few macrophages. LCs were diluted with incubation medium into 1×10^5 cells/ml and aerated with 95% O_2 and 5% CO_2 for 1 h before use.

Reagents. Bovine serum albumin, HEPES, HBSS, medium 199, sodium bicarbonate, penicillin-G, streptomycin sulphate, heparin, collagenase, nitro blue tetrazolium, CCN, EC, EGCG, hCG, GnRH, forskolin, 8-Br-cAMP, A23187, nifedipine, androstenedione, 25-OH-cholesterol, and pregnenolone were purchased from Sigma Chemical Co. (St. Louis, MO). SQ22536 was purchased from Research Biochemical International (Natick, MA). The anti-P450scc antibody and anti-steroidogenic acute regulatory (StAR) antibody were kindly provided by Dr B.C. Chung [Hu et al., 1991] and Dr. D.M. Stocco [Lin et al., 1998], respectively.

Effects of GTEs on the basal and hCG-stimulated testosterone secretion by rat LCs. After preincubation for 1 h, LCs suspensions (1×10^5 cells/ml) were centrifuged at 100*g* for 10 min and then incubated for 1 h with GTEs (10^{-11}–10^{-5} M) in the presence or absence of hCG (0.01 IU/ml). At the end of incubation, 0.5 ml ice-cold gelatin-phosphate buffer saline (GPBS) were added and immediately followed by centrifugation at 100*g* for 10 min at 4°C. The supernatant fluid was stored at −20°C until analyzed for testosterone by RIA.

Effects of GTEs on testosterone release by rat LCs treated with basal, forskolin, SQ22536, and 8-Br-cAMP. After preincubation for 1 h, LCs suspensions (1×10^5 cells/ml) were centrifuged at 100*g* for 10 min and then incubated for 1 h with GTEs (10^{-10}–10^{-8} M) in the presence or absence of forskolin (an activator of adenylyl cyclase, 10^{-5} M), SQ22536 (an adenylyl cyclase inhibitor, 10^{-5} M), or 8-Br-cAMP (a membrane permeable analog of cAMP, 10^{-5} M). At the end of incubation, 0.5 ml ice-cold GPBS were added and immediately followed by centrifugation at 100*g* for 10 min at 4°C. The supernatant fluid was stored at −20°C until analyzed for testosterone by RIA.

Effects of GTEs on the basal, A23187, and nifedipine-stimulated testosterone secretion by rat LCs. After preincubation for 1 h, LCs suspensions (1×10^5 cells/ml) were centrifuged at 100*g* for 10 min and then incubated for 1 h with GTEs (10^{-10}–10^{-8} M) in the presence or absence of A23187 (a calcium ionophore, 10^{-5} M) or nifedipine (a L-type calcium channel blocker, 10^{-5} M). At the end of incubation, 0.5 ml ice-cold GPBS were added and immediately followed by centrifugation at 100*g* for 10 min at 4°C. The supernatant fluid was stored at −20°C until analyzed for testosterone by RIA.

Effects of GTEs on the basal and androstenedione-increased testosterone secretion by rat LCs. After preincubation for 1 h, LCs suspensions (1×10^5 cells/ml) were centrifuged at 100*g* for 10 min and then incubated for 1 h with GTEs (10^{-10}–10^{-8} M) in the presence or absence of androstenedione (a steroidogenic precursor, 10^{-8} M). At the end of incubation, 0.5 ml ice-cold GPBS were added and immediately followed by centrifugation at 100*g* for 10 min at 4°C. The supernatant fluid was stored at −20°C until analyzed for testosterone by RIA.

Effects of GTEs on the substrate (25-OH-cholesterol) and the product (pregnenolone) of P450scc in rat LCs. After preincubation for 1 h, LCs suspensions (1×10^5 cells/ml) were centrifuged at 100*g* for 10 min and then incubated for 1 h with GTEs (10^{-8} M) in the presence or absence of 25-OH-cholesterol (10^{-5} M). At the end of incubation, 0.5 ml ice-cold GPBS were added and immediately followed by centrifugation at 100*g* for 10 min at 4°C. The supernatant fluid was stored at −20°C until analyzed for pregnenolone by RIA.

Effects of GTEs on P450scc and StAR protein expression in rat LCs. After preincubation for 1 h, LCs suspensions (1×10^5 cells/ml) were centrifuged at 100*g* for 10 min and then incubated for 1 h with GTEs (10^{-8} M) in the presence or absence of hCG (0.01 IU/ml). At the end of incubation, 0.5 ml ice-cold GPBS were added and immediately followed by centrifugation at 100*g* for 10 min at 4°C. The supernatant fluid was stored at −20°C until analyzed for P450scc and StAR protein expression by Western blotting.

RIA OF LH

The concentration of LH in media was determined by RIA as previously described [Tsai et al., 2003]. The sensitivity was 47 pg per assay tube for LH. The intra- and interassay coefficients of variability were 2% (n = 6) and 4.5% (n = 8), respectively.

RIA OF TESTOSTERONE

The concentration of testosterone in the medium was determined by RIA as described previously [Wang et al., 1994; Tsai et al., 1996]. With anti-testosterone serum no. W8, the sensitivity of testosterone RIA was 2 pg per assay tube. The intra- and interassay coefficients of variation (CV) were 4.1% (n = 6) and 4.7% (n = 10), respectively.

RIA OF PREGNENOLONE

The concentration of pregnenolone in media was determined by RIA as previously described [Yu et al., 2009]. The sensitivity of the pregnenolone RIA was 16 pg per assay tube. The intra- and interassay CV were 2.3% (n = 6) and 3.7% (n = 4), respectively.

GEL ELECTROPHORESIS AND WESTERN BLOTTING

The Western blotting method has been reported previously [Yu et al., 2009]. LCs were extracted on ice with homogenization buffer, pH 8.0, containing 1.5% Na-Lauroylsacrosine, 2.5 mM Tris-base, 1 mM EDTA, 0.68% phenylmethylsulfonyl fluoride (PMSF), and 2% proteinase inhibitors using an ultrasonic sonicator (model XL 2020; Heat Systems, Inc, Farmingdale, NY). The protein samples were separated using 12% SDS–PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (NEN Life Science Products, Boston, MA) using a Trans- Blot SD semi-dry transfer cell (170-3940; Bio-Rad, Hercules, CA). The membranes were washed in TBS-T buffer (0.8% NaCl, 0.02 M Tris-base, and 0.3% Tween-20, pH 7.6) for 5 min and then 120-min incubation in blocking buffer (TBS-T buffer plus 5% non-fat dry milk). These membranes were incubated with anti-StAR protein antibody (1:1,000, rabbit) or anti-P450scc antibody (1:2,000, rabbit) in blocking buffer overnight at 4°C. After three washes with TBS-T buffer (15, 5, and 5 min intervals), the membranes were incubated for 1 h with secondary antibodies (anti-rabbit IgG-HRP, 1:6,000 dilution; anti-mouse IgG, 1:8,000 dilution). The unbounded antibodies were washed four times (15, 5, 5, and 5 min intervals) with TBS-T buffer, and then the band for StAR protein or P450scc was visualized by chemiluminescence (ECL reagent Kit, Amersham, UK). The P450scc and StAR protein signals were calibrated by β-actin signal.

STATISTICAL ANALYSIS

The treatment means of both in vivo and in vitro studies were tested for homogeneity using analysis of variance (ANOVA), and the difference between specific means was tested for significance using Duncan's multiple range test. A difference between two means was considered statistically significant when P was <0.05.

RESULTS

IN VIVO STUDY

Effects of GTEs on the basal and hCG-stimulated concentration of plasma testosterone in male rats. The concentration of plasma testosterone did not change when rats treated with normal saline (vehicle group) via jugular vein. However, the concentration of plasma testosterone increased significantly 8 h later after treated with CCN (1 μg/ml/kg). The same reaction was also noted when treated with EC (1 μg/ml/kg) and EGCG (1 μg/ml/kg), respectively (Fig. 2a). The concentration of plasma testosterone increased significantly when male rats treated with hCG (5 IU/ml/kg, vehicle group) and this phenomenon sustained for 8 h ($P < 0.01$, Fig. 2b). The same reaction was also noted when treated with hCG plus CCN (1 μg/ml/kg) or hCG plus EC (1 μg/ml/kg). However, the concentration of plasma testosterone increased significantly when treated with hCG (5 IU/ml/kg) plus EC (1 μg/ml/kg) compared to the hCG (5 IU/ml/kg) alone ($P < 0.05$, Fig. 2b) at 2 and 8 h.

IN VITRO STUDY

Effects of GTEs on the basal and GnRH-stimulated LH release by anterior pituitary gland of male rats. The basal LH release by anterior pituitary gland of male rats was stimulated by GnRH ($P < 0.05$, Fig. 3). The LH release by anterior pituitary gland of male rats increased significantly when treated with low-dose concentration of CCN (10^{-9} M) or EC (10^{-9} M), respectively ($P < 0.01$, Fig. 3). But this phenomenon was not noted when treated with low-dose concentration of EGCG (10^{-9} M). While, high-dose concentration of GTEs (10^{-5} M) did not increase the LH release by anterior pituitary gland of male rats in spite of addition of GnRH.

Effects of GTEs on the basal and hCG-stimulated testosterone secretion by rat LCs. GTEs did not increase the basal testosterone secretion by rat LCs; however, hCG (0.01 IU/ml) increased basal testosterone secretion by rat LCs significantly ($P < 0.01$, Fig. 4).

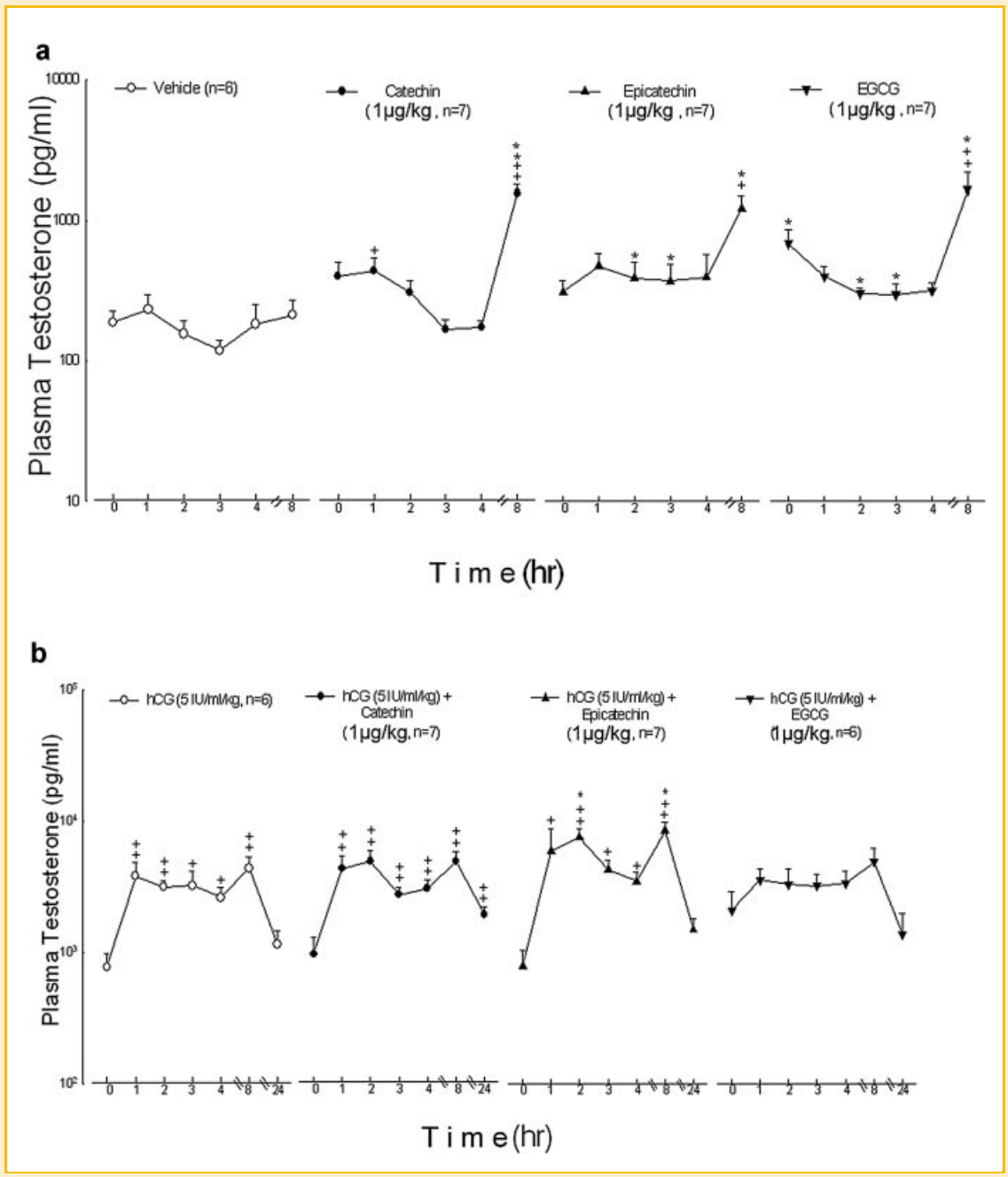


Fig. 2. a: Effect of GTEs on the basal concentration of plasma testosterone in male rats. $^{*}P<0.05$ and $^{**}P<0.01$ compared with vehicle group. $+P<0.05$ and $++P<0.01$ compared with time = 0 min. Each value represents mean ± SEM. b: Effect of GTEs on hCG-stimulated concentration of plasma testosterone in male rats. $^{*}P<0.05$ compared with vehicle group. $+P<0.05$ and $++P<0.01$ compared with time = 0 min. Each value represents mean ± SEM.

The testosterone secretion increased significantly when treated with hCG plus CCN (10^{-9} M), EC (10^{-8} M), or EGCG (10^{-8} M), respectively ($P<0.01$, Fig. 4).

Effects of GTEs on testosterone release by rat LCs treated with basal, forskolin, SQ22536, and 8-Br-cAMP. The basal testosterone secretion by rat LCs increased significantly when treated with forskolin (10^{-5} M, $P<0.01$). Meanwhile, forskolin plus CCN (10^{-9} M) increased the basal testosterone secretion ($P<0.05$, Fig. 5 left upper). SQ22536 (10^{-5} M) decreased the basal testosterone secretion by rat LCs significantly ($P<0.01$). However, the inhibitory effect of SQ22536 on the testosterone secretion was blocked by the addition of CCN (10^{-10} M, $P<0.05$, Fig. 5 right upper). 8-Br-cAMP (10^{-5} M) increased the basal testosterone secretion by rat LCs significantly ($P<0.01$). The testosterone secretion increased significantly when treated with 8-Br-cAMP plus CCN (10^{-9} M), EC (10^{-10} M), EGCG (10^{-10} M) ($P<0.05$), or EGCG (10^{-8} M), respectively ($P<0.01$, Fig. 5 bottom).

Effects of GTEs on the basal, A23187, and nifedipine-stimulated testosterone secretion by rat LCs. A23187 (10^{-5} M) increased the basal testosterone secretion by rat LCs significantly

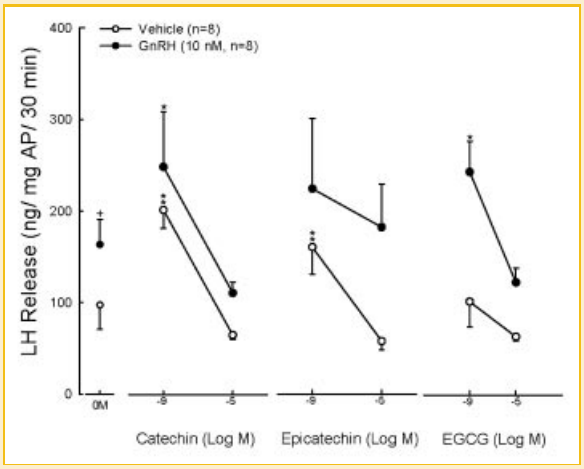


Fig. 3. Effect of GTEs on the basal and GnRH stimulated LH release by anterior pituitary of male rats. $^{*}P<0.05$ and $^{**}P<0.01$ compared with non-GTEs treated GnRH group. $+P<0.05$ compared with 0 M. Each value represents mean ± SEM.

($P<0.01$, Fig. 6 left). However, the effect was not affected by the addition of CCN, EC, or EGCG. Nifedipine (10^{-5} M) decreased the basal testosterone secretion by rat LCs significantly ($P<0.01$, Fig. 6 right). However, the effect was not affected by the addition of CCN, EC, or EGCG.

Effects of GTEs on the basal and androstenedione-increased testosterone secretion by rat LCs. Androstenedione (10^{-8} M) increased the basal testosterone secretion by rat LCs significantly ($P<0.01$, Fig. 7). This effect was not affected by the addition of CCN or EGCG. However, the basal testosterone secretion increased by androstenedione plus EC (10^{-10} M, $P<0.05$ and 10^{-9} M, $P<0.01$, Fig. 7).

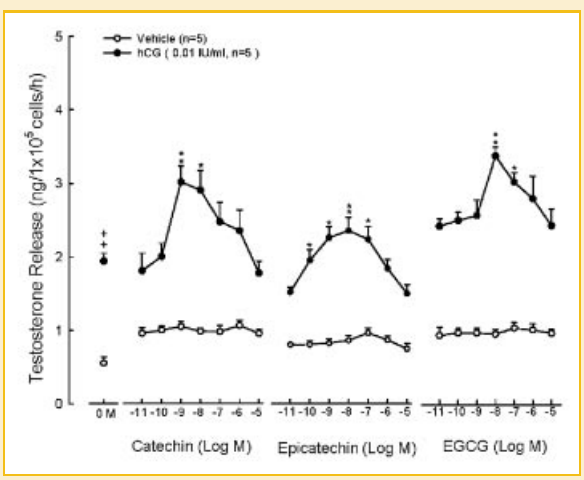


Fig. 4. Effect of GTEs on the basal and hCG-stimulated testosterone secretion by rat Leydig cells. $^{*}P<0.05$ and $^{**}P<0.01$ compared with non-GTEs treated hCG group. $++P<0.01$ compared with 0 M. Each value represents mean ± SEM.

Effects of GTEs on the substrate (25-OH-cholesterol) and the product (pregnenolone) of P450scc in rat LCs. 25-OH-cholesterol (10^{-5} M) increased the secretion of pregnenolone by rat LCs ($P<0.01$, Fig. 8). However, this effect was not affected by the addition of CCN, EC, or EGCG (10^{-8} M). This implicated that GTEs did not affect the activity of P450scc.

Effects of GTEs on P450scc and StAR protein expression in rat LCs. The expression of P450scc and StAR protein in rat LCs did not change after administration of CCN, EC, or EGCG (10^{-8} M) for 1 h (Fig. 9).

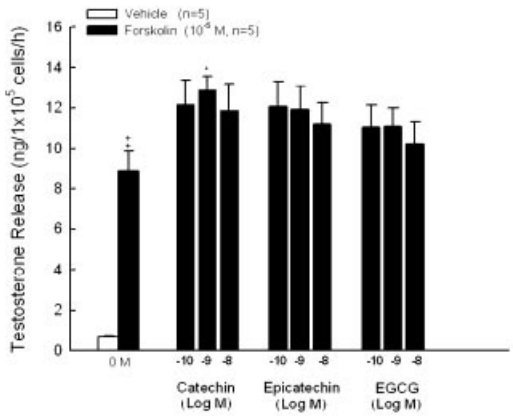
DISCUSSION

IN VIVO STUDY

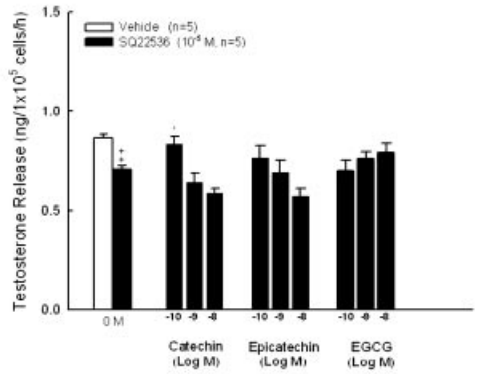
The concentration of plasma testosterone increased significantly 8 h after injection with CCN, EC, or EGCG compared to the vehicle group (Fig. 2a). Different results of GTEs on plasma testosterone in rats were reported in literatures. The plasma testosterone of rats increased by intraperitoneal injection of EC, however, decreased by EGCG [Kao et al., 2000]. The plasma testosterone of rats was increased by oral administration of CCN via the mechanism of aromatase inhibition [Satoh et al., 2002]. Oppositely, the plasma testosterone was decreased with chronic green tea consumption via the mechanism of enhanced aromatase expression [Monteiro et al., 2008]. Because of the similarity of bioactivity and amino acid sequences between LH and hCG, the hCG was used as stimulatory hormone for rat LCs in our study [McFarland et al., 1989]. The concentration of plasma testosterone increased significantly 1 h later when male rats treated with hCG (vehicle group) and this phenomenon sustained for 8 h ($P<0.01$, Fig. 2b). The same reaction was also noted when treated with hCG plus CCN or hCG plus EC but not plus EGCG. Especially, the concentration of plasma testosterone increased significantly when treated with hCG plus EC compared to the hCG alone ($P<0.05$, Fig. 2b). This implicated that EC reinforced the effect of hCG on increasing the plasma testosterone of male rats; while EGCG seemed to inhibit the stimulatory effect of hCG on plasma testosterone. In vivo model suggested that the duration of administration of GTEs only or plus hCG seemed as a crucial point in changing of plasma testosterone concentration in male rats. Accordingly, we hypothesized that the peak concentration of plasma testosterone after treated with GTEs for 8 h inhibited the hCG-stimulated testosterone secretion via the testosterone-LH feedback mechanism (Fig. 10a).

IN VITRO STUDY

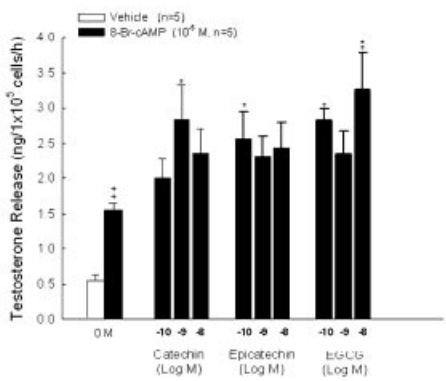
Both GnRH and low-dose concentration (10^{-9} M) of CCN or EC, but not EGCG stimulated the release of LH release from anterior pituitary gland of male rats (Fig. 3). The basal testosterone secretion by rat LCs was not affected significantly by GTEs. However, the basal testosterone secretion by rat LCs increased significantly with the addition of hCG (0.01 IU/ml; Fig. 4). By two experiments mentioned above, we had noted that low-dose CCN (10^{-9} M) and EC (10^{-9} M) stimulated LH release by anterior pituitary gland of male rats most and low-dose CCN (10^{-8} M), EC (10^{-10} M) or EGCG (10^{-8} M) with



Effect of GTEs on testosterone release by rat Leydig cells treated with basal and forskolin (an activator of adenylyl cyclase, 10-5M). * P<0.05 compared with non -GTEs treated forskolin group. ++ P<0.01 compared with vehicle group. Each value represents mean ± SEM.

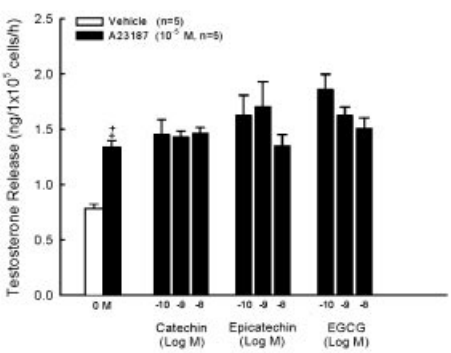


Effect of GTEs on the release of testosterone in rat Leydig cells treated with basal and SQ22536 (adenylyl cyclase inhibitor, 10-5M). * P<0.05 compared with non-GTEs treated SQ22536 group. ++ P<0.01 compared with vehicle group. Each value represents mean ± SEM.

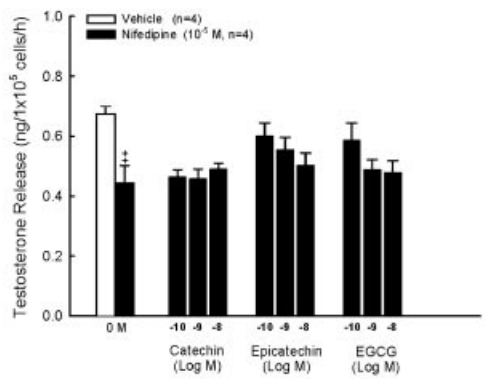


Effect of GTEs on the basal and 8-Br-cAMP (a membrane permeable analog of cAMP, 10-5M) -stimulated testosterone secretion by rat Leydig cells. * P<0.05 and ** P<0.01 compared with non-GTEs treated 8-Br-cAMP group. ++ P <0.01 compared with vehicle group. Each value represents mean ± SEM.

Fig. 5. Effect of GTEs on the basal and cAMP-related chemicals stimulation.



Effect of GTEs on the basal and A23187 (a calcium ionophore, 10^{-5}M) -increased testosterone secretion by rat Leydig cells. ++ P<0.01 compared with vehicle group. Each value represents mean ± SEM.



Effect of GTEs on the release of testosterone in rat Leydig cells treated with basal and nifedipine (L-type calcium channel blocked, 10^{-5}M). ++ P<0.01 compared with vehicle group. Each value represents mean ± SEM.

Fig. 6. Effect of GTEs on the basal and calcium channel chemicals stimulation.

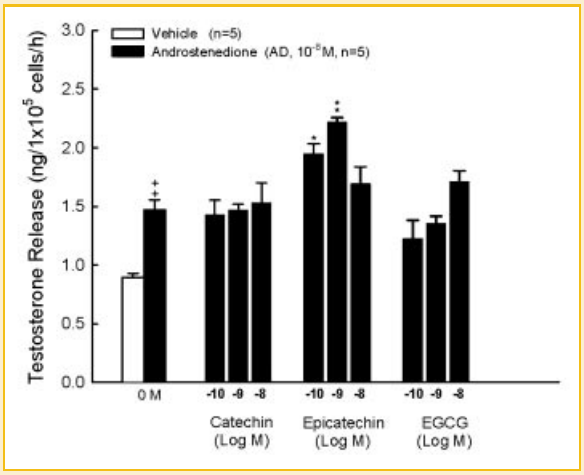


Fig. 7. Effect of GTEs on the basal and androstenedione (a steroidogenic precursor 10^{-8} M)-increased testosterone secretion by rat Leydig cells. $^{*}P<0.05$ and $^{**}P<0.01$ compared with non-GTEs treated androstenedione group. $++P<0.01$ compared with vehicle group. Each value represents mean ± SEM.

addition of hCG made the testosterone secretion from rat LCs reach to peak. The basal testosterone secretion by rat LCs decreased gradually with the increasing concentration of GTEs and the similar declining tendency was also noted with addition of hCG in vitro [Figueiroa et al., 2009]. Oppositely, high-dose GTEs inhibited both of GnRH-stimulated LH release by anterior pituitary gland and hCG-stimulated testosterone releases in LCs of male rats. It would be indicated that hormesis action (a very low-dose of a chemical agent may trigger from an organism the opposite response to a very high dose) was concerned in above observations [Stebbing, 2009].

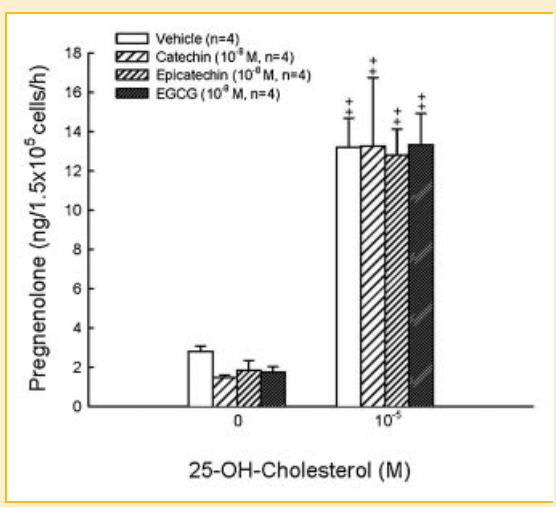


Fig. 8. Effect of GTEs on the substrate (25-OH-cholesterol) and the product pregnenolone) of P450scc in rat Leydig cells. $+P<0.05$ and $++P<0.01$ compared with 0 M. Each value represents mean ± SEM.

Therefore, low-dose GTEs might be beneficial for reproductive function, especially, in male by this experiment.

The basal testosterone secretion from rat LCs increased by forskolin (an activator of adenylyl cyclase) and 8-Br-cAMP (a membrane permeable analog of cAMP), but decreased by SQ22536 (an adenylyl cyclase inhibitor) significantly (Fig. 5). Different concentrations of CCN exhibited different actions in this model. For example, CCN (10^{-9} M) plus forskolin increased testosterone secretion compared to forskolin alone; CCN (10^{-10} M) blocked the inhibitory effect of SQ22536 on the testosterone secretion (Fig. 5). Moreover, the testosterone secretion increased significantly when treated with 8-Br-cAMP plus CCN (10^{-9} M), EC (10^{-10} M), EGCG (10^{-10} M), or EGCG (10^{-8} M), respectively, compared to rats treated with 8-Br-cAMP alone (Fig. 5). All of the above indicated the cAMP pathway was involved in the mechanism of GTEs to increase the testosterone secretion by rat LCs.

Calcium played a major role in the testosterone secretion by rat LCs in rats [Costa and Varanda, 2007]. In this study, the basal testosterone secretion from rat LCs increased by A23187 (a calcium ionophore) but decreased by nifedipine (L-type calcium channel blocker; Fig. 6). However, both of reactions were not changed significantly by the addition of CCN, EC, or EGCG. These results indicated that the change of the activity of intracellular calcium was not involved in the testosterone secretion regulated by GTEs in rat LCs.

Androstenedione increased the basal testosterone secretion by rat LCs significantly, which was not affected by the addition of CCN or EGCG. However, the testosterone secretion increased by androstenedione plus EC compared to the androstenedione alone (Fig. 7). Our results indicated EC might activate 17β-HSD to increase the testosterone secretion by rat LCs (Fig. 10b). In vivo, women with regular green tea drinkers (weekly or daily) had lower plasma androstenedione than non-regular drinkers [Wu et al., 2005]. Inhibition of androstenedione-stimulated testosterone production in rat LCs was noted by addition of EGCG, but not EC [Figueiroa et al., 2009]. The dosages of GTEs (6.92–692 μg/ml) and EGCG (5–200 μg/ml) used in their studies are larger than ours (1 μg/ml); those are the dosages for tumor cell cytotoxicity reported before [Yang et al., 2000]. We speculated that the decreased secretion of testosterone by addition of EGCG might be as a consequence of declined viability of LCs.

The secretion of pregnenolone induced by 25-OH-cholesterol in rat LCs was not altered by the addition of CCN, EC, or EGCG (Fig. 8). This implicated that GTEs did not affect the activity of P450scc. Also, the expression of P450scc and StAR protein in rat LCs did not change after treated with CCN, EC, or EGCG for 1 h (Fig. 9). These results indicated that GTEs did not affect the expressions of P450scc and StAR protein in rat LCs.

CONCLUSION

Catechins increased plasma testosterone in vivo in male rats. In vitro condition, lower concentrations of catechins increased GnRH-stimulated LH release by anterior pituitary gland and hCG-stimulated testosterone release in testicular LCs of male rats. Our

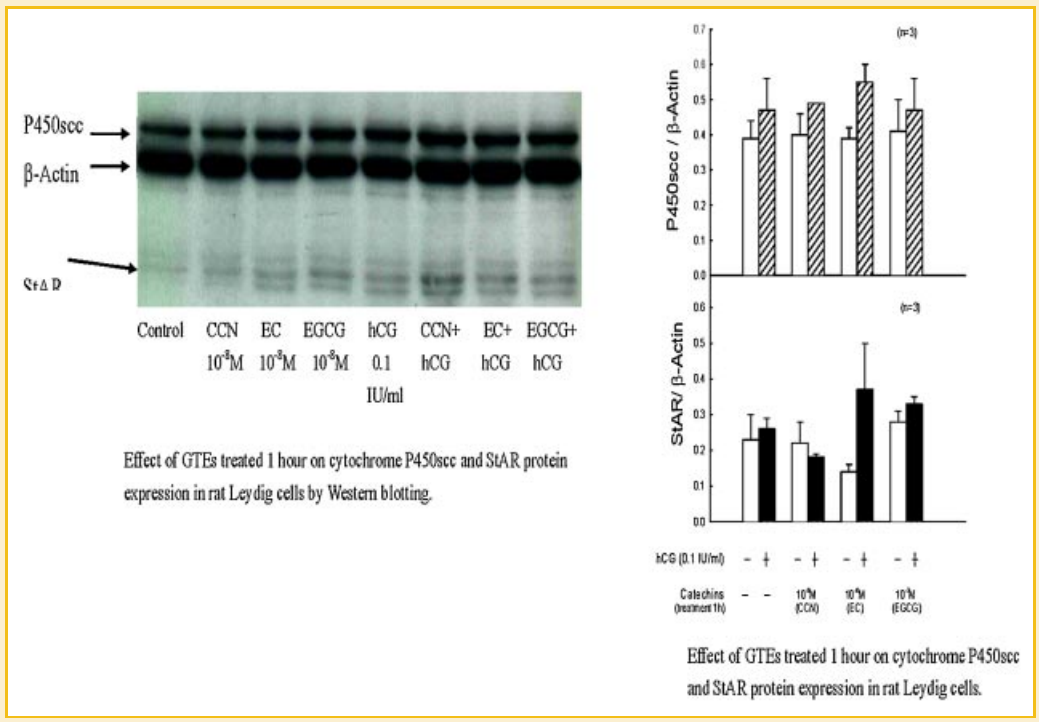


Fig. 9. Effect of GTEs on cytochrome P450scc and StAR protein expression in rat Leydig cells.

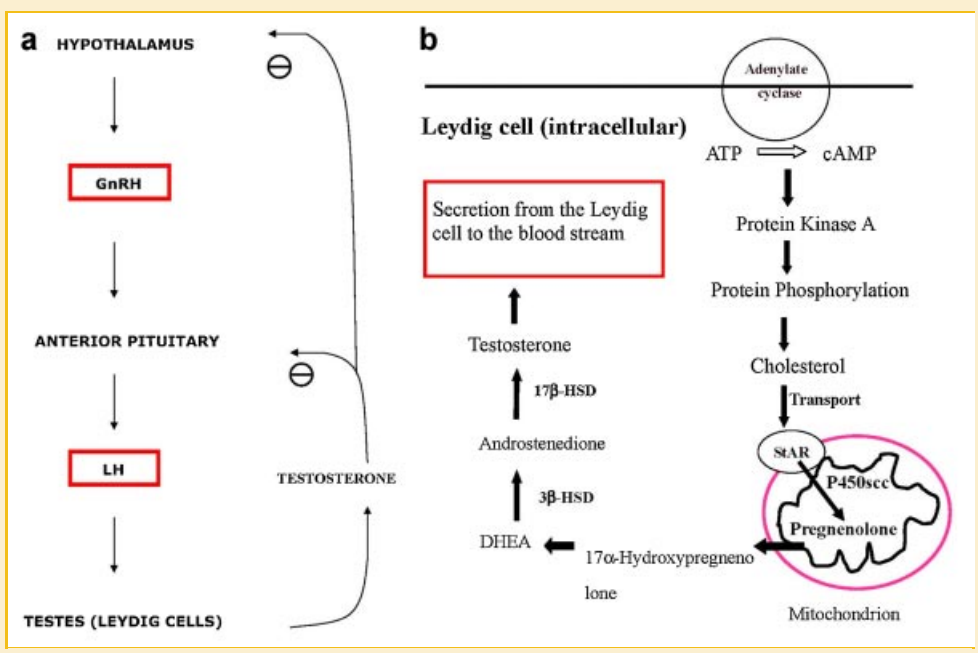


Fig. 10. a: Hypothalamus-pituitary gland-testes axis. b: Cholesterol to testosterone pathway in Leydig cells.

results suggested that first, catechins stimulated testosterone production by acting directly on rat LCs via the mechanism of increasing the action of cAMP, but not P450scc, StAR protein or the activity of intracellular calcium. Second, EC, one of the catechins increased the testosterone secretion by rat LCs via the increase of enzyme activities of 17β-HSD.

REFERENCES

Basu A, Lucas EA. 2007. Mechanisms and effects of green tea on cardiovascular health. Nutr Rev 65:361–375.

Chiao YC, Cho WL, Wang PS. 2002. Inhibition of testosterone production by propylthiouracil in rat Leydig cells. Biol Reprod 67:416–422.

Chung SY, Landau JM. 2000. Effects of tea consumption on nutrition and health. J Nutr 130:2409–2412.

Costa RR, Varanda WA. 2007. Intracellular calcium changes in mice Leydig cells are dependent on calcium entry through T-type calcium channels. J Physiol 585:339–349.

Figueiroa MS, César Vieira JS, Leite DS, Filho RC, Ferreira F, Gouveia PS, Udrisar DP, Wanderley MI. 2009. Green tea polyphenols inhibit testosterone production in rat Leydig cells. Asian J Androl 11:362–370.

Frei B, Higdon JV. 2003. Antioxidant activity of tea polyphenols in vivo: Evidence from animal studies. J Nutr 133:3275S–3384S.

Hara Y, Honda M. 1990. The inhibition of α-amylase by tea polyphenols. Agr Biol Chem 54:1939–1945.

Hu MC, Guo IC, Lin JH, Chung BC. 1991. Regulated expression of cytochrome P-450scc (cholesterol-side-chain cleavage enzyme) in cultured cell lines detected by antibody against bacterially expressed human protein. Biochem J 274:813–817.

Kao YH, Hiipakka RA, Liao S. 2000. Modulation of endocrine systems and food intake by green tea epigallocatechin gallate. Endocrinology 141:980–987.

Kuriyama S, Shimazu T, Ohmori K, Kikuchi N, Nakaya N, Nishino Y, Tsubono Y, Tsuji I. 2006. Green tea consumption and mortality due to cardiovascular disease, cancer, and all causes in Japan: The Ohsaki study. JAMA 296:1255–1265.

Lin T, Hu J, Wang D, Stocco DM. 1998. Interferon-gamma inhibited the steroidogenic acute regulatory protein messenger ribonucleic acid expression and protein levels in primary cultures of rat Leydig cells. Endocrinology 139:2217–2222.

McFarland KC, Sprengel R, Phillips HS, Köhler M, Rosemblit N, Nikolics K, Segaloff DL, Seeburg PH. 1989. Lutropin-choriogonadotropin receptor: An unusual member of the G protein-coupled receptor family. Science 245:494–499.

Monteiro R, Assunção M, Andrade JP, Neves D, Calhau C, Azevedo I. 2008. Chronic green tea consumption decreases body mass, induces aromatase expression, and changes proliferation and apoptosis in adult male rat adipose tissue. J Nutr 138:2156–2163.

Mukhtar H, Ahmad N. 1999. Green tea in chemoprevention of cancer. Toxicol Sci 52:111–117.

Nomura M, Kaji A, He Z, Ma WY, Miyamoto K, Yang CS, Dong Z. 2001. Inhibitory mechanisms of tea polyphenols on the ultraviolet B-activated phosphatidylinositol 3-kinase-dependent pathway. J Biol Chem 276:46624–46631.

Satoh K, Sakamoto Y, Ogata A, Nagai F, Mikuriya H, Numazawa M, Yamada K, Aoki N. 2002. Inhibition of aromatase activity by green tea extract catechins and their endocrinological effects of oral administration in rats. Food Chem Toxicol 40:925–933.

Shapiro H, Lev S, Cohen J, Singer P. 2009. Polyphenols in the prevention and treatment of sepsis syndromes: Rationale and pre-clinical evidence. Nutrition 25:981–997.

Stebbing AR. 2009. Interpreting 'dose-response' curves using homeodynamic data: With an improved explanation for hormesis. Dose Response 7:221–233.

Tsai SC, Chiao YC, Lu CC, Doong ML, Chen YH, Shih HC, Liaw C, Wang SW, Wang PS. 1996. Inhibition by amphetamine of testosterone secretion through a mechanism involving an increase of cyclic AMP production in rat testes. Br J Pharmacol 118:984–988.

Tsai SC, Chiao YC, Lu CC, Wang PS. 2003. Stimulation of the secretion of luteinizing hormone by ginsenoside-Rb1 in male rats. Chin J Physiol 46:1–7.

Wang PS, Tsai SC, Hwang GS, Wang SW, Lu CC, Chen JJ, Liu SR, Lee KY, Chien EJ, Chien CH, Lee HY, Lau CP, Tsai CL. 1994. Calcitonin inhibits testosterone and luteinizing hormone secretion through a mechanism involving an increase in cAMP production in rats. J Bone Miner Res 9:1583–1590.

Wu AH, Arakawa K, Stanczyk FZ, Van Den Berg D, Koh WP, Yu MC. 2005. Tea and circulating estrogen levels in postmenopausal Chinese women in Singapore. Carcinogenesis 26:976–980.

Yang CS, Chung JY, Yang G, Chhabra SK, Lee MJ. 2000. Tea and tea polyphenols in cancer prevention. J Nutr 130:472S–478S.

Yang CS, Wang X, Lu G, Picinich SC. 2009. Cancer prevention by tea: Animal studies, molecular mechanisms and human relevance. Nat Rev Cancer 9:429–439.

Yu PL, Chao HL, Wang SW, Wang PS. 2009. Effects of evodiamine and rutaecarpine on the secretion of corticosterone by Zona fasciculata–reticularis cells in male rats. J Cell Biochem 108:469–475.