

## Original Article

# Green tea polyphenols inhibit testosterone production in rat Leydig cells

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### Abstract

This study investigated the acute effects of green tea extract (GTE) and its polyphenol constituents, (–)-epigallocatechin-3-gallate (EGCG) and (–)-epicatechin (EC), on basal and stimulated testosterone production by rat Leydig cells *in vitro*. Leydig cells purified in a Percoll gradient were incubated for 3 h with GTE, EGCG or EC and the testosterone precursor androstenedione, in the presence or absence of either protein kinase A (PKA) or protein kinase C (PKC) activators. The reversibility of the effect was studied by pretreating cells for 15 min with GTE or EGCG, allowing them to recover for 1 h and challenging them for 2 h with human chorionic gonadotropin (hCG), luteinizing hormone releasing hormone (LHRH), 22(*R*)-hydroxycholesterol or androstenedione. GTE and EGCG, but not EC, inhibited both basal and kinase-stimulated testosterone production. Under the pretreatment conditions, the inhibitory effect of the higher concentration of GTE/EGCG on hCG/LHRH-stimulated or 22(*R*)-hydroxycholesterol-induced testosterone production was maintained, whereas androstenedione-supported testosterone production returned to control levels. At the lower concentration of GTE/EGCG, the inhibitory effect of these polyphenols on 22(*R*)-hydroxycholesterol-supported testosterone production was reversed. The inhibitory effects of GTE may be explained by the action of its principal component, EGCG, and the presence of a gallate group in its structure seems important for its high efficacy in inhibiting testosterone production. The mechanisms underlying the effects of GTE and EGCG involve the inhibition of the PKA/PKC signalling pathways, as well as the inhibition of P450 side-chain cleavage enzyme and 17 $\beta$ -hydroxysteroid dehydrogenase function.

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## 1 Introduction

Green tea (*Camellia sinensis*) is one of the most commonly consumed beverages worldwide. Its active components are reported to have several biological properties, including cancer chemoprevention,

inhibition of tumour cell growth, antiviral and anti-inflammatory activities [1], antioxidant activity [2, 3] and inhibitory effects on several enzymes, such as aromatase [4, 5], angiotensin converting enzyme [6] and thyroid peroxidase [7]. Dried leaves of *C. sinensis* contain polyphenols (30%–36%), principally flavanols, more commonly known as catechins [8]. The predominant catechins are epigallocatechin-3-gallate (EGCG), epicatechin-3 gallate (ECG), epigallocatechin (EGC) and epicatechin (EC).

The effects of catechins on the male reproductive system have been described. Epidemiological and

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laboratory studies suggest an association between diet and androgens that can alter prostate cancer risk [9–11]. It has been shown that parenteral injection of EGCG can suppress human prostate and breast tumour growth in athymic mice [12] and reduce the weight of testes and accessory reproductive organs, as well the circulating level of luteinizing hormone (LH) and testosterone in the intact rat [13]. Although the antigonadotropic effect of catechins is explained as a secondary effect of EGCG on food intake [13] or on aromatase activity [4, 5], a modulatory function could be present even at the gonadal level.

Currently, there is no evidence for a direct effect of green tea catechins on testicular steroidogenesis or on the enzymes involved in androgen production. Although the involvement of the protein kinase A (PKA) and protein kinase C (PKC) signalling pathways in testicular androgen production is well known [14, 15], it is unclear whether green tea catechins modulate these pathways in Leydig cells. There is evidence that EGCG and other flavonoids can modulate the PKC [16, 17] and PKA signalling pathways in other animal models [16, 18]. The aim of this study was to investigate the direct *in vitro* effects of green tea extract (GTE) and its purified catechins on the basal and the PKA- and PKC-stimulated testosterone production by rat Leydig cells.

## 2 Materials and methods

### 2.1 Materials

Hank's balanced salt solution (HBSS) and Medium 199 were obtained from Gibco (Grand Island, NY, USA). Collagenase (Type I), soybean trypsin inhibitor, leupeptin, phorbol 12',13-dibutyrate (PDBu), human chorionic gonadotropin (hCG), N<sup>6</sup>,2'-*O*-dibutyryl adenosine 3',5'-cyclic monophosphate (dbcAMP), EC, EGCG from green tea, 3-(4',5-dimethylthiazol-2-yl)-2',5-diphenyltetrazolium bromide (MTT), dimethylsulphoxide (DMSO), 22(*R*)-hydroxycholesterol and 4-androstene-3',17-dione were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Bovine serum albumin (BSA, fraction V) was obtained from Miles (Naperville, IL, USA). LH releasing hormone (LHRH) was purchased from Peninsula (San Carlos, CA, USA). Percoll was purchased from Pharmacia (Uppsala, Sweden).

### 2.2 GTE preparation

GTE was prepared according to Wang *et al.* [19] with slight modifications. Five grams of dry green tea leaves were infused for 5 min in 100 mL of boiling saline (90°C), allowed to cool to room temperature and then filtered. The resulting clear solution is similar to tea brews consumed by humans. The amount of solid matter present in this infusion was determined by drying samples (10 mL) in an oven overnight at 100°C and weighing the dry residue. The dry weight was determined to be 69.2 mg for 5 g of green tea leaves, making the concentration of this infusion 0.692% (w/v). The extracts were freshly prepared on a daily basis. This preparation is comprised of approximately 27% catechins, 8.0% caffeine and 0.4% theobromine, and EGCG represents ≥ 50% of total catechins [19].

### 2.3 Animals and Leydig cell-enriched preparation

Adult (70–80 days old) male Wistar rats weighing 200–300 g were kept in a controlled environment (temperature 25–29°C; lights on from 05:00 to 18:00) with free access to standard laboratory chow and tap water. Isolation and purification of rat Leydig cell-enriched preparations were performed as described by Hedger and Eddy [15, 20]. The rats were killed by ether anaesthesia and the testes were quickly removed and decapsulated. The decapsulated testes were incubated in an enzyme solution of 0.5 mg mL<sup>-1</sup> collagenase, 0.2 mg mL<sup>-1</sup> soybean trypsin inhibitor and 5 µg mL<sup>-1</sup> leupeptin in Hank's balanced salt solution containing 0.1% BSA (HBSS/BSA), pH 7.4, in a shaking water bath (20 min, 90 Hz, 34°C). The dispersed testes were suspended in a final volume of 50 mL HBSS/BSA and the dissociated tubules were allowed to settle (5 min). The supernatant was filtered and washed with 5 mL HBSS/BSA. The filtered cell suspension was centrifuged (150 × *g*, 15 min, 20°C), and the pellet was re-suspended in 5 mL HBSS/BSA, loaded onto the top of a discontinuous Percoll density gradient (20%, 35%, 43%, 68% and 90%) and centrifuged at 800 × *g* for 30 min at 20°C. Cells in the 43%–68% interface (specific gravity: 1.0640–1.0960 g mL<sup>-1</sup>) were collected, washed twice with M199 containing 0.1% BSA, re-suspended in M199/0.1% BSA and used immediately for the experiments.

### 2.4 Cell viability

To assess the effects of GTE and EGCG on cell viability, the Trypan blue exclusion and MTT reduction assays were used. These assays are widely used screening methods to measure plasma membrane integrity and

active mitochondrial function, respectively. The Trypan blue assay was performed after incubating cells for 3 h with the different doses of GTE or EGCG. Cells were incubated with Trypan blue (0.5%) for 20 min and the resulting percentage of blue cells, indicating a capture of the colourant due to plasma membrane rupture, were counted. Normal cell viability was considered to be 90%–95% colourless cells. The MTT assay is based on the reduction of a soluble pale yellow tetrazolium dye to water-insoluble purple formazan crystals in living cells [21]. Cells ( $0.3 \times 10^6$  per mL) were incubated in polypropylene tubes with GTE ( $69.2 \mu\text{g mL}^{-1}$ ) or EGCG (10, 50 and  $100 \mu\text{g mL}^{-1}$ ) for 2 h at  $34^\circ\text{C}$  under 95%  $\text{O}_2$  and 5%  $\text{CO}_2$ . After this time, the cells were washed twice with M199 to remove the GTE/EGCG and then resuspended in 100  $\mu\text{L}$  of fresh M199. Subsequently, 25  $\mu\text{L}$  of MTT in PBS ( $5 \text{ mg mL}^{-1}$ ) was added to the cell suspensions followed by 3 h of incubation at  $37^\circ\text{C}$ . This procedure avoids a direct reaction between the polyphenols and the MTT. It is known that polyphenols can reduce MTT in lieu of living cells, so results obtained may not reflect the true cell viability and instead mask the effects of polyphenols [22]. After this incubation period, the cell suspensions were centrifuged at  $400 \times g$  for 5 min. The supernatant was removed and 100  $\mu\text{L}$  DMSO was added to each tube to dissolve formazan crystals overnight. The dissolved pellet was transferred to microplates and the absorbance was measured at 630 nm in an ELISA reader. Appropriate controls were included (i.e., medium plus MTT without cells or cells treated with the toxic reagent saponin 0.1%). The animal care committee of the Federal University of Pernambuco (Recife, PE, Brazil) approved all treatments.

### 2.5 *In vitro* testosterone secretion

Cells ( $0.3 \times 10^6$  cells per 0.5 mL) were treated for 3 h with M199, GTE ( $6.92$ – $692.0 \mu\text{g mL}^{-1}$ ), EGCG ( $5$ – $200 \mu\text{g mL}^{-1}$ ) or EC ( $200 \mu\text{g mL}^{-1}$ ) in the absence or presence of hCG ( $1 \text{ mIU mL}^{-1}$ ), dbcAMP ( $1 \text{ mmol L}^{-1}$ ), LHRH ( $10^{-7} \text{ mol L}^{-1}$ ), PDBu ( $200 \text{ nmol L}^{-1}$ ) or androstenedione ( $1$ – $100 \mu\text{mol L}^{-1}$ ) in a shaking water bath (60 Hz,  $34^\circ\text{C}$ ) under an atmosphere of 95%  $\text{O}_2$  and 5%  $\text{CO}_2$ . It is noteworthy that a cup of GTE (100 mL) usually contains  $50$ – $150 \text{ mg mL}^{-1}$  of tea polyphenols [23]. To study the reversibility of the inhibitory effect of GTE and EGCG, cells were preincubated for 15 min with M199 GTE ( $69.2 \mu\text{g mL}^{-1}$ ) or EGCG ( $100 \mu\text{g mL}^{-1}$ ), washed with fresh medium, allowed to recuperate for 60 min, and then incubated for 2 h with  $0.5 \text{ mIU mL}^{-1}$

hCG,  $10^{-7} \text{ mol L}^{-1}$  LHRH,  $20 \mu\text{mol L}^{-1}$  22-hydroxycholesterol or  $10 \mu\text{mol L}^{-1}$  androstenedione. This 15-min preincubation period was chosen because it was sufficient to detect an inhibitory effect of GTE or EGCG on testosterone production similar to that seen after 3 h incubation (data not shown). At the end of the second incubation, the cells were centrifuged and the supernatant was collected and stored at  $-20^\circ\text{C}$  until used for testosterone measurement by radioimmunological analysis (RIA).

### 2.6 RIA and statistical analysis

Testosterone was measured directly (without extraction) in the incubation medium by a charcoal–dextran RIA method [24] that employs [ $^3\text{H}$ ]-testosterone as tracer and a primary antiserum raised in rabbits in our laboratory against testosterone-3-(0-carboxymethyl) oxime:BSA. Intra- and inter-assay coefficients of variation were 8.1% and 15.1%, respectively. The testosterone antibody showed  $< 0.1\%$  cross-reactivity with androstenedione, dehydroepiandrosterone, androsterone,  $17\alpha$ -hydroxyprogesterone,  $\beta$ -estradiol and estrone. None of the substances tested interfered with the assays. The data from the different analyses were reported as the mean  $\pm$  SEM. of triplicate determinations and were representative of results obtained in at least two similar experiments. One-way ANOVA and Dunnett tests were used to examine differences among control and GTE/EGCG/EC-treated cells. *P*-values less than 0.05 were considered to be statistically significant.

## 3 Results

### 3.1 GTE and EGCG inhibit basal and hCG-stimulated testosterone production

The modulatory effects of GTE and its pure constituents (EGCG and EC) were tested by incubating Leydig cell-enriched preparations with GTE or EGCG followed by determination of testosterone levels in the incubation medium. Cells were incubated with various concentrations (three different orders of magnitude) of GTE or EGCG. In Figures 1 and 2, Leydig cells were treated with and without GTE or the catechins EGCG and EC, respectively, in the absence or presence of maximum stimulation by hCG (indirect PKA activator). GTE and EGCG both produced an inhibitory effect on basal and stimulated testosterone production. At  $6.92$ ,  $69.2$  and  $692 \mu\text{g mL}^{-1}$ , GTE inhibited basal testosterone levels by  $27.5\% \pm 4.8\%$ ,  $60.5\% \pm 3.9\%$  and  $93.1\% \pm 0.2\%$ ,

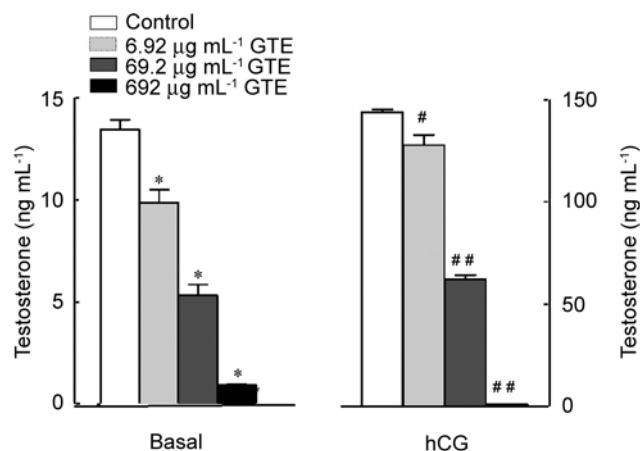


Figure 1. Treatment of Leydig cells with different concentrations of green tea extract (GTE) affects basal and hCG-induced testosterone production. Leydig cells ( $0.3 \times 10^6$  per 0.5 mL) were incubated for 3 h with hCG ( $1 \text{ mIU mL}^{-1}$ ) in the presence or absence of 6.92, 69.2 or  $692 \text{ } \mu\text{g mL}^{-1}$  GTE. Results are the mean  $\pm$  SEM of three determinations repeated in two independent experiments. \* $P < 0.0001$ , # $P < 0.01$ , ## $P < 0.0001$ , compared with the control.

respectively, whereas EGCG at 5, 50 and  $200 \text{ } \mu\text{g mL}^{-1}$  produced  $24.0\% \pm 6.7\%$ ,  $37.9\% \pm 1.4\%$  and  $58.2\% \pm 1.4\%$  inhibition, respectively. The inhibitory effects of 6.92, 69.2 and  $692 \text{ } \mu\text{g mL}^{-1}$  GTE on hCG-stimulated testosterone production were  $11.2\% \pm 6.0\%$ ,  $56.8\% \pm 1.5\%$  and  $99.4\% \pm 0.03\%$ , respectively, whereas the inhibitory effect of 50 and  $200 \text{ } \mu\text{g mL}^{-1}$  EGCG on stimulation with hCG was  $46.5\% \pm 7.8\%$  and  $98.8\% \pm 0.06\%$ , respectively (the  $5 \text{ } \mu\text{g mL}^{-1}$  dose showed no significant inhibitory effect). Exposure to EC at  $200 \text{ } \mu\text{g mL}^{-1}$  did not induce a significant inhibitory effect on basal or stimulated testosterone production. To evaluate whether the effect of GTE or EGCG on testosterone production was due to a decrease in cell viability, the Trypan blue exclusion and MTT reduction assays were used. The Trypan blue exclusion test for cell viability showed that the inhibition was not due to GTE-induced toxicity at the concentrations of  $6.92 \text{ } \mu\text{g mL}^{-1}$  or  $69.2 \text{ } \mu\text{g mL}^{-1}$  or EGCG toxicity at any concentration used. This test showed that at the above-cited concentrations, the percentages of viable cells obtained after 3 h of incubation were comparable between the GTE- or EGCG-treated (91%) cells and the controls (95%). At the maximum concentration of GTE used ( $692 \text{ } \mu\text{g mL}^{-1}$ ), cell viability was 80% of control. This difference (10%–15% fewer viable cells compared with the control group) could have contributed to the observed decrease in testos-

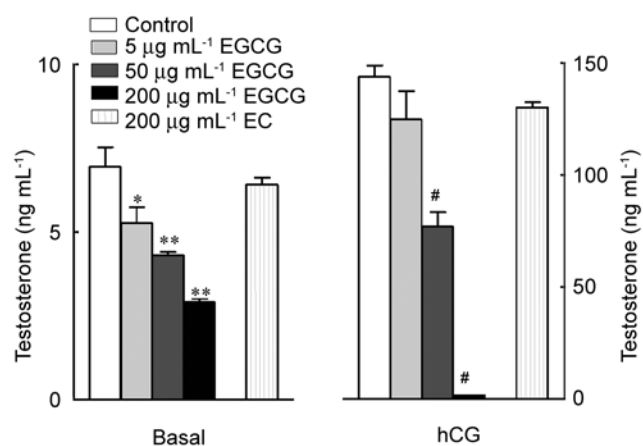


Figure 2. Treatment of Leydig cells with different concentrations of epigallocatechin-3-gallate (EGCG) affects basal and hCG-induced testosterone production. Leydig cells ( $0.3 \times 10^6$  per 0.5 mL) were incubated for 3 h with hCG ( $1 \text{ mIU mL}^{-1}$ ) in the presence or absence of 5, 50 or  $200 \text{ } \mu\text{g mL}^{-1}$  EGCG or  $200 \text{ } \mu\text{g mL}^{-1}$  EC. Results are the mean  $\pm$  SEM of three determinations repeated in two independent experiments. \* $P < 0.05$ , \*\* $P < 0.01$ , # $P < 0.001$ , compared with the control.

one production. For this reason, this concentration was not used in further experiments in this study. When measured by MTT reduction, cell viability was not modified by any concentration of GTE or EGCG used. The two assays for cell viability showed that inhibition of testosterone production was not due to the toxicity of GTE ( $6.92 \text{ } \mu\text{g mL}^{-1}$  or  $69.2 \text{ } \mu\text{g mL}^{-1}$ ) or EGCG ( $10\text{--}100 \text{ } \mu\text{g mL}^{-1}$ ).

### 3.2 GTE and EGCG inhibit testosterone production elicited by activation of PKA and PKC

The action of GTE and EGCG was further investigated in the presence of a direct PKA activator, db-cAMP, and both an indirect and direct PKC activator, LHRH and PDBu, respectively. Table 1 shows testosterone production in the presence of each maximum stimulus alone or with GTE or EGCG. Testosterone production was reduced completely in all groups (to  $< 98\%$ ), regardless of the nature of the stimulus. These results indicate a probable interaction of GTE/EGCG with the signal-transduction process somewhere downstream of PKA and PKC.

### 3.3 EGCG inhibits the stimulatory effect of androstenedione on testosterone production

To further evaluate the effect of EGCG, the steroidogenic precursor androstenedione was used to

Table 1. Effect of GTE or EGCG added together with dbcAMP, LHRH or PDBu on testosterone production in rat Leydig cells preparation.

Additions	Testosterone (ng mL <sup>-1</sup> )			
	Basal	dbcAMP (1 mmol L <sup>-1</sup> )	LHRH (0.1 μmol L <sup>-1</sup> )	PDBu (400 nmol L <sup>-1</sup> )
M199	2.57 ± 0.03	191.70 ± 11.60	5.40 ± 0.40	54.00 ± 5.00
GTE (69.2 μg mL <sup>-1</sup> )	1.03 ± 0.03*	4.20 ± 0.20*	0.44 ± 0.02*	1.47 ± 0.09*
EGCG (100 μg mL <sup>-1</sup> )	1.20 ± 0.06*	2.40 ± 0.06*	0.27 ± 0.05*	1.03 ± 0.03*

Abbreviations: dbcAMP, dibutyryladenosine 3',5'-cyclic monophosphate; EGCG, (-)-epigallocatechin-3-gallate; GTE, green tea extract; LHRH, luteinizing hormone releasing hormone; PDBu, phorbol 12',13-dibutyrate.

\**P* < 0.0001, compared with respective control (M199).

Cells ( $0.3 \times 10^6$  per 0.5 mL) were incubated for 3 h. The data represent the mean ± SEM of triplicate determinations.

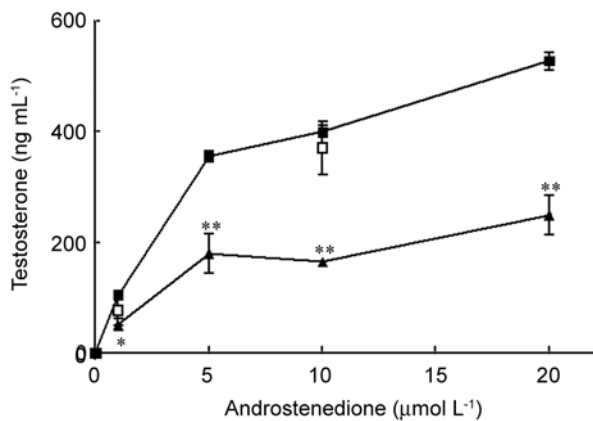


Figure 3. Effects of epigallocatechin-3-gallate (EGCG) on androstenedione-stimulated testosterone production. Leydig cells ( $0.3 \times 10^6$  per 0.5 mL) were incubated for 3 h with androstenedione (1–20 μmol L<sup>-1</sup>) in the absence (black square) or presence of 100 μg mL<sup>-1</sup> EGCG (black triangle) or EC (white square). Results are the mean ± SEM of three determinations repeated in two independent experiments. \**P* < 0.001, \*\**P* < 0.0001, compared with respective androstenedione alone.

support testosterone production (a measure of 17β-hydroxysteroid dehydrogenase [17β-HSD] activity). Androstenedione crosses the cell membrane and moves to the smooth endoplasmic reticulum of Leydig cells in which, after binding to 17β-HSD, it is converted to testosterone. Several doses of androstenedione (1, 5, 10 and 20 μmol L<sup>-1</sup>) were used. These high concentrations were used to obviate the interference of any endogenous precursor. As shown in Figure 3, EGCG decreased testosterone production by approximately 50% at all concentrations of the precursor used. No significant inhibitory effect was observed in cells treated with EC.

### 3.4 Effect of pretreatment with GTE or EGCG on hCG-

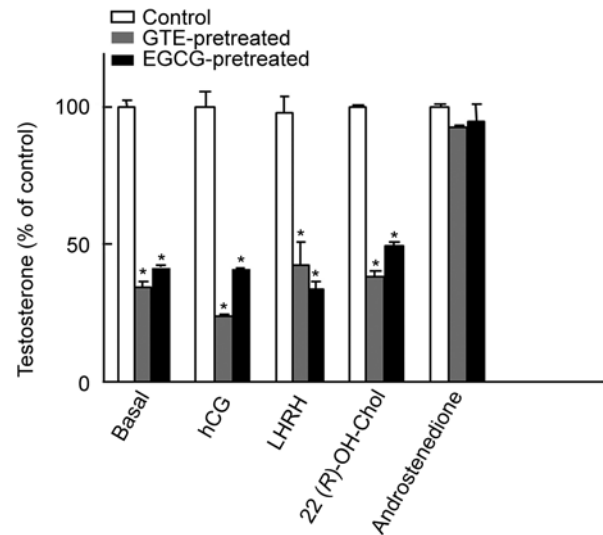


Figure 4. Effect of 15-min pretreatment with green tea extract (GTE) (69.2 μg mL<sup>-1</sup>) or epigallocatechin-3-gallate (EGCG) (100 μg mL<sup>-1</sup>) on testosterone production following a 1-h recovery period in Leydig cells ( $0.3 \times 10^6$  per 0.5 mL). To determine reversibility, after pretreatment, cells were rinsed with fresh M199 and incubated in M199 for 1 h before further challenged with hCG (0.5 mIU mL<sup>-1</sup>), LHRH (0.1 μmol L<sup>-1</sup>), 22(R)-hydroxycholesterol (20 μmol L<sup>-1</sup>) or androstenedione (10 μmol L<sup>-1</sup>) for 2 h. The data represent the mean ± SEM of triplicate determinations.

### or LHRH-stimulated and androstenedione-supported testosterone production

The aim of this experiment was to examine the reversibility of the inhibitory effect exerted by GTE or EGCG on hCG- or LHRH-stimulated and on androstenedione-supported testosterone production. The results shown in Figure 4 reveal that pretreatment with GTE (69.2 μg mL<sup>-1</sup>) followed by a 1-h recuperation period inhibited the responsiveness to subsequent incubation with M199 or stimulation with hCG or LHRH by

65.3%, 76.1% and 57.7%, respectively. Pretreatment with EGCG (100  $\mu\text{g mL}^{-1}$ ) followed by a similar recuperation period decreased the responsiveness to subsequent incubation with M199 or stimulation with hCG or LHRH by 58.4%, 58.7% and 66.6%, respectively. In contrast to the effects observed with hCG and LHRH, pretreatment with GTE or EGCG did not reduce the responsiveness of Leydig cells to androstenedione. Similar results were observed with a 15-min recuperation period (data not shown). These results indicate that the inhibitory effects of GTE or EGCG on the steroidogenic process were reversible only in the androstenedione to testosterone enzymatic step.

The 22(*R*)-hydroxycholesterol-induced production of testosterone was also examined to provide further evidence for the involvement of the earlier steps (before and after mitochondrial P450 side-chain cleavage enzyme (P450<sub>scc</sub>)-catalysed conversion of cholesterol to pregnenolone) of the steroidogenic pathway in the GTE/EGCG effect. 22(*R*)-hydroxycholesterol bypasses all signal-transduction pathways and has direct access to mitochondria in which it is a substrate for P450<sub>scc</sub> enzyme [25]. The results shown in Figure 4 show that pretreatment with GTE or EGCG followed by a 1-h recuperation period inhibited the responsiveness of Leydig cells to subsequent incubation with 22(*R*)-hydroxycholesterol by 38.4% and 49.2%, respectively. These results show that GTE and EGCG also reduce the function of the P450<sub>scc</sub> enzyme. However, when we pretreated the cells with lower concentrations of GTE (13.8  $\mu\text{g mL}^{-1}$ ) or EGCG (20  $\mu\text{g mL}^{-1}$ ), a complete reversion of the inhibitory effect of the polyphenols on 22(*R*)-hydroxycholesterol-induced testosterone production was observed, and might even be classified as a stimulatory effect (Table 2).

#### 4 Discussion

In this study, the ability of GTE or individual catechins to inhibit steroidogenesis by rat Leydig cells *in vitro* was examined by quantifying effects on testosterone production. It was found that GTE and EGCG inhibited basal and stimulated testosterone production. This result indicates that GTE and the pure EGCG catechin have direct effects on the testis that are independent of any effects of the drug on the secretion of gonadotropin. The concentrations of GTE used in this study (6.92 and 69.2  $\mu\text{g mL}^{-1}$ ) are within the range of those of pure EGCG (5–100  $\mu\text{g mL}^{-1}$ ). These EGCG concentrations are similar to those used in most of the published *in vitro* tumour cell cytotoxicity studies (4.6–458.4  $\mu\text{g mL}^{-1}$ ) in which the efficacy of inhibition depended on the cell type used ( $\text{IC}_{50}$  = 10–60  $\mu\text{g mL}^{-1}$ ) [1, 26]. Whereas concentrations higher than 4.6  $\mu\text{g mL}^{-1}$  have been reported to reduce cell survival [1, 26, 27], the cytotoxicity assays used to assess our experimental conditions showed that the reduced testosterone production was not a consequence of a decline in Leydig cell viability.

In contrast to GTE and EGCG, pure EC (200  $\mu\text{g mL}^{-1}$ ) failed to produce an inhibitory effect on testosterone production. This result suggests a structure–activity relationship. As the only structural difference between EGCG and EC is the presence of a gallate group at the 3' position in EGCG, we expect that this group is critical for the potent ability of EGCG to inhibit testosterone production. This structure–activity relationship has been observed by other authors [28–30]. As the inhibition was observed with EGCG, but not with EC, the effect cannot be attributed to the antioxidant properties characteristic of both of these catechins.

Table 2. Inhibitory effect of GTE (13.8  $\mu\text{g mL}^{-1}$ ) or EGCG (20  $\mu\text{g mL}^{-1}$ ) on basal or 22(*R*)-hydroxycholesterol-induced testosterone production and its reversion after a 1-h recovery in Leydig cells ( $0.3 \times 10^6$  per 0.5 mL).

	Testosterone (ng mL <sup>-1</sup> )					
	Treated (no recovery)			Pretreated (after 1-h recovery)		
	M199	GTE	EGCG	M199	GTE	EGCG
Basal	0.85 ± 0.05	0.78 ± 0.06	0.68 ± 0.03*	1.07 ± 0.03	1.09 ± 0.03	1.13 ± 0.03
22-OH-Chol	97.50 ± 0.10	70.30 ± 2.25**	65.30 ± 1.25**	75.83 ± 0.83	116.00 ± 4.00**	100.00 ± 5.00**

Abbreviations: EGCG, (–)-epigallocatechin-3-gallate; GTE, green tea extract.

\* $P < 0.05$ , \*\* $P < 0.001$ , compared with respective control (M199).

To determine reversibility, cells were rinsed with fresh M199 after a 15-min pretreatment with GTE/EGCG and incubated in M199 for 1 h before further challenge with 22(*R*)-hydroxycholesterol (22-OH-Chol : 20  $\mu\text{mol L}^{-1}$ ) during 2 h. The data represent the mean ± SEM of triplicate determinations.

To investigate the mechanisms of the inhibitory effects of GTE and EGCG on stimulated testosterone production, an established *in vitro* model was used. This model is based on the ability of hCG/dbcAMP or LHRH/PDBu to stimulate testosterone production through mechanisms involving PKA or PKC pathway activation, respectively [14, 15, 31]. These data show that both GTE and EGCG inhibited hCG-, dbcAMP-, LHRH- and PDBu-stimulated testosterone production. These results suggest that GTE and EGCG act directly on Leydig cells to regulate testosterone secretion at the PKA/PKC level and/or at a point downstream of the activation of PKA and PKC.

To further study the effects of EGCG, the enzymatic function of the steroidogenic enzyme that converts androstenedione to testosterone was also examined. The production of testosterone in Leydig cells involves several enzymatic steps [32]. First, the side chain of cholesterol is cleaved to yield pregnenolone by mitochondrial P450scc, the rate-limiting enzyme. A series of steroidogenic enzymes that are located in the smooth endoplasmic reticulum then convert pregnenolone to testosterone, the principal secreted hormone. Pregnenolone is oxidized to progesterone by 3 $\beta$ -hydroxysteroid dehydrogenase (3 $\beta$ -HSD), progesterone is hydroxylated to 17 $\alpha$ -hydroxyprogesterone by 17 $\alpha$ -hydroxylase and 17 $\alpha$ -hydroxyprogesterone is converted to androstenedione by C17-20 lyase. Androstenedione is then reduced to testosterone by 17 $\beta$ -HSD. As we were measuring the concentration of testosterone, the end product of the steroidogenic process, the activity of the final enzyme, 17 $\beta$ -HSD, was specifically examined. To that end, Leydig cells were incubated with the direct precursor of the last metabolic step, androstenedione. The inhibitory effect of EGCG was detected at all concentrations of androstenedione, indicating that the regulation of 17 $\beta$ -HSD is affected by EGCG and contributes to the reduction in the cell's steroidogenic capacity.

This effect of GTE or EGCG on the function of 17 $\beta$ -HSD seems to be independent of the effects on the PKA/PKC signalling pathways and the transfer of cholesterol from the outer to the inner mitochondrial membrane. This was shown by the reversibility of the inhibitory effects of GTE or EGCG on androstenedione-supported testosterone production. In contrast, hCG/LHRH-stimulated and 22(R)-hydroxycholesterol-supported testosterone production continued to be inhibited after a recuperation period after pretreatment with a high concentration of GTE (69.2  $\mu\text{g mL}^{-1}$ ) or

EGCG (100  $\mu\text{g mL}^{-1}$ ). It is possible that the reversibility of androstenedione-supported testosterone production is related to the constitutively high expression levels of 17 $\beta$ -HSD [33]. On the other hand, the results also indicate that the steroidogenic step in the mitochondrial compartment (P450scc function) remained inhibited in these experimental conditions. However, after pretreatment with a lower concentration of GTE (13.8  $\mu\text{g mL}^{-1}$ ) or EGCG (20  $\mu\text{g mL}^{-1}$ ), the inhibitory effects on P450scc function were indeed reversed after a recuperation period, suggesting that inhibition/reversion might be concentration-dependent.

Several studies have addressed the ability of other subclasses of phytochemicals to decrease the activity of a variety of steroidogenic enzymes, including P450scc, P450c17, P450c21, P45011 $\beta$ , 3 $\beta$ -HSD [34–38], aromatase and 17 $\beta$ -HSD [35, 38], and affect testosterone production [39, 40]. Additionally, EGCG has been shown to inhibit ovarian aromatase activity [5] and estradiol and progesterone production by swine granulosa cells [41].

Taken together, these results show that the GTE and its major constituent EGCG inhibit testosterone production by rat Leydig cells *in vitro* and that the inhibitory effects observed with GTE may be explained, at least in part, by the EGCG present in the extract. The results also indicate that the mechanisms underlying these effects involve inhibition of the PKA/PKC signalling pathways, as well as direct or indirect inhibition of both P450scc and 17 $\beta$ -HSD, which are required for hormone synthesis. The inhibition of P450scc activity could explain the observed inhibitory effects of GTE and EGCG on basal testosterone production, which is maintained after a recuperation period of 1 h. Finally, the data presented here might help to explain the decrease in testosterone plasma levels observed in *in vivo* studies with green tea catechins [13].

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