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Green tea extract affects porcine ovarian cell apoptosis

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ABSTRACT

Green tea is a commonly used beverage and green tea extract is a common dietary herbal supplement manufactured into different over-the-counter products. The aim of this *in vitro* study was to examine the steroid hormone secretion (progesterone and 17-β estradiol), proliferation and apoptosis of porcine ovarian granulosa cells after addition of green tea extract. Granulosa cells were incubated with green tea extract at five doses (0.1, 1, 10, 100 and 200 μg/ml) and the release of hormones by granulosa cells was assessed by EIA after 24 h exposure. The presence of proliferation and apoptotic markers was assessed by immunocytochemistry. Secretion of steroid hormones was not affected by green tea extract at all the doses in comparison to control. Also, markers of proliferation (PCNA and cyclin B1) were not affected by green tea extract. However, the highest dose (200 μg/ml) of green tea extract used in this study increased the accumulation of apoptotic markers caspase-3 and p53 in granulosa cells. In conclusion, our results indicate the impact of green tea extract at the highest dose used in this study on ovarian apoptosis through pathway that includes activation of caspase-3 and p53. Potential stimulation of these intracellular regulators could induce the process of apoptosis in ovarian cells.

1. Introduction

Green tea (*Camellia sinensis* L., Theaceae) leaves contain 26% fibres, 15% proteins, 2–7% lipids, and 5% vitamins and minerals. They also contain secondary metabolites such as pigments (1–2%), polyphenols (30–40%), of which at least 80% are flavonoids, and methylxanthines (3–4%) [1–3]. Green tea and green tea extract are reported to have beneficial effects on improving cancers including ovarian and prostate cancers [4]. Feeding supplementation with green tea has been used in livestock industry, including calves [5] and pigs [6]. The unique property of green tea catechin polyphenols has the potential to improve reproductive health and poses green tea as an important research area [7]. Researchers have postulated the role of green tea and green tea extract in female reproduction and fertility using porcine [8], bovine [9], ovine [10], rat [11] and mouse models [12]. Granulosa cells play a central role in ovarian steroidogenesis [13]. The growth and differentiation of ovarian follicles are governed by hormones, growth factors and intracellular regulators [14–17]. Steroid hormone secretion by ovarian granulosa cells is believed to ensure a receptive environment for the implantation and development of the early embryo [18]. Progesterone is essential for normal ovarian cycles and contributes to regulation of ovarian follicular development and remodeling [19,20]. Estradiol is another steroid essential for keeping the oocytes in meiotic arrest [21] and for fertilization competence of oocytes [22]. Cell proliferation is the amount of cells in culture or in the body that can be divided. The protein involved in cell proliferation include PCNA [14,23,24], which is localized in cell nucleus [25]. The process of ovarian cell proliferation, growth and development also involves proliferation related peptide cyclin B1 [14,26]. Apoptosis is a process of eliminating unnecessary cells from body through programmed death of cells [26], which can be detected with the help of marker proteins such as caspase-3 and p53 [27,28]. The aim of this *in vitro* study was to examine the steroid hormone (progesterone, 17β-estradiol) secretion and the presence of markers of cell proliferation (PCNA and cyclin B1) and apoptosis (caspase-3 and p-53) after addition of green tea extract to granulosa cells.

2. Materials and methods

2.1. Preparation of green tea extract and analysis of catechins, flavan-3-ols and phenolic acids

Briefly, packaged leaves of low-foam Chinese green tea *Chun Mee* were grounded mechanically into fine powder in liquid nitrogen and

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10 g powder was extracted with 100 ml distilled water by boiling under reflux for 30 min. The extract was filtered and evaporated to dryness to yield the dry extract (yield: 42%) [29]. Catechins, flavan-3-ols and phenolic acids were analyzed by HPLC. Samples were separated on a Phenomenex Synergi 4 u Fusion-RP80 column with detection at 280 nm and 345 nm using an HPLC-UV detector (Waters Instruments, MN, USA). The mobile phase consisted of 1% acetic acid in water (A) and 100% acetonitrile (B). The gradient increased linearly from 0% B to 10% B (v/v) at 10 min, 13% B at 30 min, 16% B at 65 min, 33% B at 81 min, and 90% B at 85 min, and stayed at 90% B at 90 min. Peaks were identified and measured [30]. All samples were run in duplicate.

2.2. Isolation and culture of granulosa cells

Granulosa cells were collected from the ovaries of prepubertal (100–120 days old) Slovakian White gilts following their slaughter at a local abattoir. Ovaries were transported to the laboratory at 4 °C and washed in sterile physiological solution. Follicular fluid was aspirated from 3 to 5 mm follicles and granulosa cells were isolated by centrifugation for 10 min at 200*g*. Cells were then washed in sterile DMEM/F12 1:1 medium (BioWhittaker, Verviers, Belgium), and resuspended in the same medium supplemented with 10% fetal calf serum (BioWhittaker) and 1% antibiotic-antimycotic solution (Sigma, St. Louis, MO, USA) at a final concentration 10^6 cells/ml medium. 1 ml/well of the granulosa cell suspension was dispensed in 24-well culture plates (Nunc, Roskilde, Denmark, for EIA) and 200 µl/well in 16-well chamber slides (Nunc Inc., International, Naperville, USA) for immunocytochemistry. Both the plate wells and chamber slides were incubated at 37 °C and 5% CO_2 in humidified air until 60–75% confluent monolayer was formed (3–5 days), at which point medium was renewed. Further culture was performed in 1 ml culture medium in 24-well plates or 200 µl/medium in 16-well chamber slides as described previously [14].

After medium replacement experimental cells were cultured in the presence of green tea extract at doses of 0 (control), 0.1, 1, 10, 100 and 200 µg/ml. Just before the addition to the cells, green tea extract was dissolved first in DMSO (concentration 10 mg/ml) and then in culture medium. The maximal concentration of DMSO in culture was 0.1%. This amount of DMSO was added to the cells of control group.

After 24 h of culture with or without green tea extract, the medium was removed. The cells in chamber slides were washed in ice-cold PBS (pH 7.5), fixed in paraformaldehyde (4% in PBS, pH 7.2–7.4; 60 min), dehydrated in alcohols (70%, 80%, 96%; 10 min each) and held at 4 °C in preparation for immunocytochemistry. The medium from the 24-well plates was gently aspirated and frozen at −24 °C to await EIA.

2.3. EIA

Concentrations of progesterone and 17-β estradiol were determined in duplicate in the incubation medium by EIA as described previously [31]. All EIAs were validated for use in samples of culture medium. For progesterone, intra- and interassay coeficients of variation did not exceed 4% and 9.3%, respectively. For 17-β estradiol, intra- and interassay coeficients of variation did not exceed 9% and 10%, respectively.

2.4. Immunocytochemistry

After fixation and washing in PBS for 5 min, the cells were incubated in blocking solution (1% of goat serum in PBS) at room temperature for 1 h to block nonspecific binding of antiserum. Afterwards, the cells were incubated with monoclonal antibodies against either of the markers of proliferation (PCNA and cyclin B1), or markers of apoptosis (caspase-3 and p53) (Santa Cruz Biotechnology, Inc., Santa Cruz, USA; dilution 1:500 in PBS) for 2 h at room temperature. For the detection of binding sites of the primary antibody, the cells were incubated in secondary swine antibody against mouse IgG labeled with horse-radish peroxidase (Sevac, Prague, Czech Republic, dilution 1:1000) for 1 h. Positive signals were visualized by staining with DAB-substrate (Roche Diagnostics GmbH, Manheim, Germany). Following DAB-staining, the cells on chamber slides were washed in PBS and covered with a drop of Glycergel mounting medium (DAKO, Glostrup, Denmark), following which coverslip was attached to a microslide. Presence and localization of PCNA or bax positivity in the cells was detected by DAB-peroxidase brown staining using light microscopy. Cells treated with secondary antibody and DAB but not the primary antibody were used as negative controls. A ratio of DAB-HRP-stained cells to the total cell count was calculated [31].

2.5. Statistics

Each treatment group was represented by 4 wells. Assays for hormone concentration in the incubation medium were performed in duplicates. The values of blank controls (serum-supplemented medium incubated without cells) were subtracted from the specific value determined by EIA in cell-conditioned medium to exclude any non-specific background (less than 10% of total values). Rates of secretion were calculated per 10^6 cells. Each experimental group was represented by 4-well chamber slides. The proportions of cells with specific immunoreactivity were calculated from at least 1000 cells per chamber. The percentage of cells containing antigen in different groups of cells was calculated. Each experiment was performed thrice. Significant differences between the experiments were evaluated using one-way ANOVA followed by paired Wilcoxon-Mann Whitney test (Systat Software, GmbH, Erkhart, Germany). Differences from control at $P < 0.05$ were considered significant.

3. Results

The contents of catechins, flavan-3-ols and phenolic acids in low-foam Chinese *Chun Mee* green tea extract are shown in Table 1. Granulosa cells formed a monolayer in culture and released steroid hormones progesterone (23.29 ± 3.81, 20.37 ± 2.47, 21.73 ± 2.54, 21.73 ± 1.65, 21.15 ± 3.46, and 21.73 ± 3.75 ng/ml for control, 0.1, 1, 10, 100 and 200 µg/ml doses of green tea extract, respectively) and 17-β estradiol (47.85 ± 5.45, 42.77 ± 5.96, 44.31 ± 4.46, 45.29 ± 7.52, 42.35 ± 5.80, and 41.26 ± 3.97 ng/ml for control, 0.1, 1, 10, 100 and 200 µg/ml doses of green tea extract, respectively). Cultured granulosa cells also contained markers of proliferation (PCNA and cyclin B1) and apoptosis (caspase-3 and p53). Markers of proliferation PCNA and cyclin B1 were localized in the nuclear or perinuclear area, while markers of apoptosis caspase-3 occurred mainly in the cytoplasm and p53 was localized in nuclear area. Some of these parameters were altered under the influence of green tea extract.

Green tea extract, at the experimental doses, did not influence either progesterone or 17-β estradiol output as detected using EIA, even

Table 1
Composition of flavonoids in *Chun Mee* green tea extract.

Compound type	Compound Name	Concentration (mg/g dry weight ± SD)
Flavan-3-ols	Epigallocatechin	62.51 ± 0.07
	Catechin	0.23 ± 0.01
	Epicatechin	5.31 ± 0.20
	Epigallocatechin-3-gallate (EGCG)	45.20 ± 0.05
	Epicatechin-3-gallate	11.14 ± 0.02
Phenolic acids	β-Glucogallin	0.63 ± 0.00
	Galloyl acid	0.84 ± 0.01
	Galloylquinic acid	1.27 ± 0.04
	p-Coumaroylquinic acid	0.72 ± 0.00

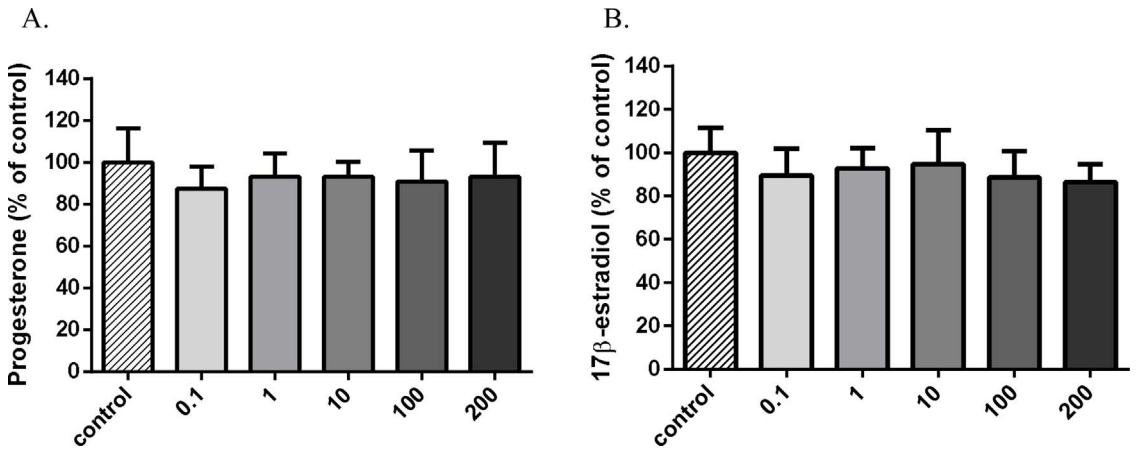

Fig. 1. Influence of green tea extract on release of steroid hormones in cultured porcine ovarian granulosa cells. EIA after 24 h of culture. Each bar represents the mean ± SD progesterone % of control (A) and 17-β estradiol % of control (B).

though the levels were lower than control for all the doses of green tea extract (Fig. 1A and B).

The results of immunocytochemistry showed that the addition of green tea extract at the experimental doses did not have any impact on the number of granulosa cells containing either of the markers of proliferation PCNA or cyclin B1 (Fig. 2A and B). On the other hand, the accumulation of both the markers of apoptosis caspase-3 and p53 in granulosa cells were stimulated by green tea extract at a higher (200 μg/ml) dose (Fig. 3A and B).

4. Discussion

The effect of green tea and its components has generated interest in clinical and research setting for potential molecular targets in the prevention and treatment of various diseases including ovarian cancer [32]. In the present study, cell monolayer formation, release of steroid hormones (progesterone and 17-β estradiol) into culture medium, and occurrence of markers of proliferation (PCNA and cyclin B1) and apoptosis (caspase-3 and p53) indicate that the experimental granulosa cells were suitable for testing and analysis of green tea extract.

In our experiments, the release of progesterone was not affected by green tea extract. Our results revealed a decreasing trend of progesterone after green tea addition in comparison to control ($P > 0.05$). Progesterone is considered a marker of ovarian follicle luteinization [35]. EGCG, a green tea component, reportedly inhibited progesterone release by granulosa cells after EGCG treatment (5 and 50 μg/ml EGCG) [33]. On the contrary, Kadasi et al. reported increased progesterone secretion by granulosa cells at a low dose (10 μg/ml EGCG) but not at higher doses [34]. The differences in the effects observed could be explained by differences in green tea components (EGCG by Basini et al. [33] and Kadasi et al. [34] vis-a-vis green tea extract in our study) and varied source of ovarian cells (Basini et al. [33] used mature porcine ovaries, while Kadasi et al. [34] and the present study worked with ovarian granulosa cells from prepubertal pigs).

Similar to progesterone, the release of 17-β estradiol was not influenced by green tea extract. On the other hand, Bassini et al. [33] reported that EGCG reduces estradiol secretion by granulosa cells at doses of 5 and 50 μg/ml. Estradiol is a marker of meiotic arrest of oocytes [35]. Green tea may modify estrogen metabolism or conjugation and thus influence breast cancer risk [36]. Our results revealed a decreasing trend of 17-β estradiol was noted after green tea addition in comparison to control. The differences were not statistically significant. Hormone production is generally affected by flavonoids by decreasing the activity of steroidogenic enzymes [37], particularly aromatase [38], which plays a fundamental role in estradiol biosynthesis in granulosa cells.

In our study, green tea extract addition did not influence accumulation of both the markers of proliferation PCNA and cyclin B1. On the other hand, Kadasi et al. reported that the green tea component EGCG decreased the percentage of cells containing PCNA at all experimental doses (1, 10, 100 μg/ml EGCG) [34]. In another study, green tea component was also found to inhibit proliferation of granulosa cells at a dose of 50 μg/ml EGCG. It was further postulated that EGCG from green tea can negatively affect reproductive performances by inhibiting

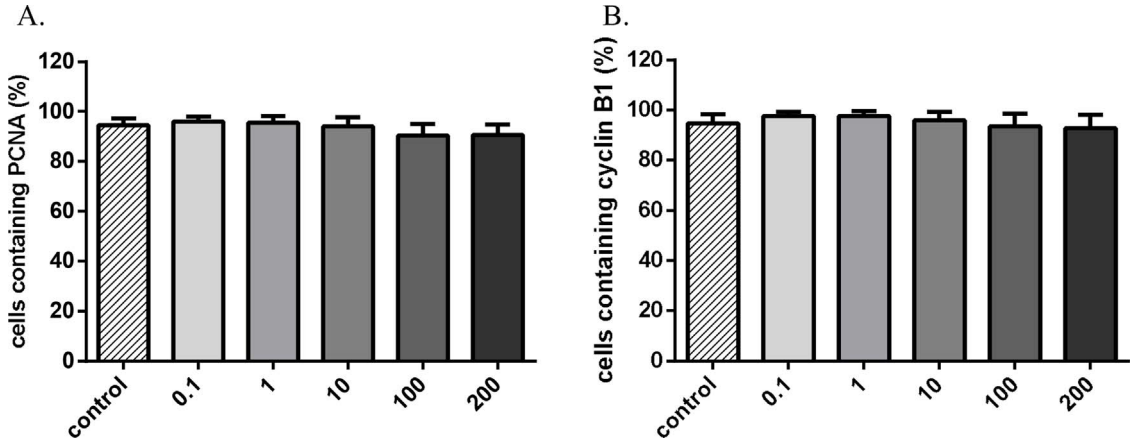

Fig. 2. Influence of green tea extract on accumulation of markers of proliferation in cultured porcine ovarian granulosa cells. Immunocytochemistry after 24 h of culture. Each bar represents the mean ± SD cells containing PCNA % (A) and cyclin B1 % (B).

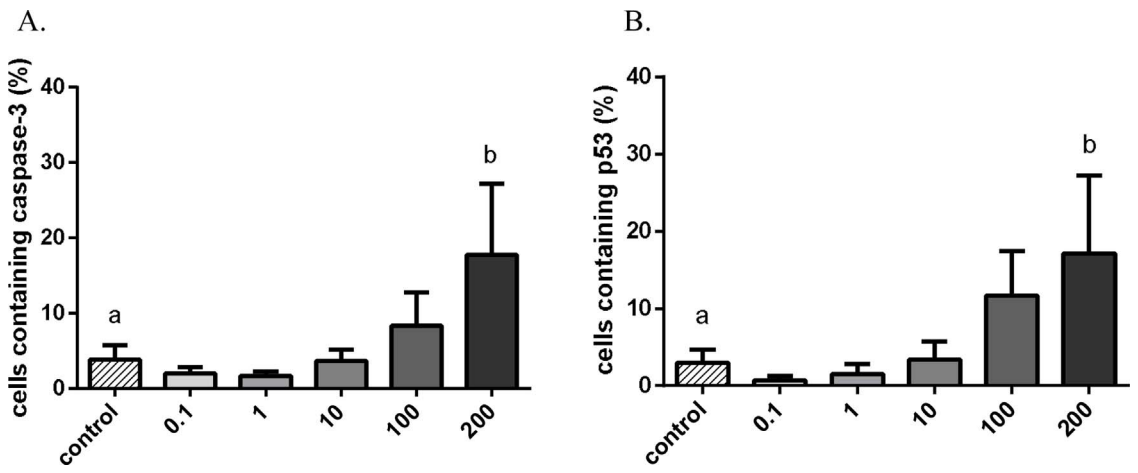

Fig. 3. Influence of green tea extract on accumulation of markers of apoptosis in cultured porcine ovarian granulosa cells. Immunocytochemistry after 24 h of culture. Each bar represents the mean ± SD cells containing caspase-3% (A) and p53 % (B).

proliferation of granulosa cells [33]. Cell culture study involving leukemia cells also revealed anti-proliferative activity of green tea polyphenols [39]. Singh et al. reviewed that EGCG decreases the expression of cyclin D1 [40]. These reports are not in line with our present observations which could be explained mainly by differing sources of green tea used in these studies and its dose-dependent nature of action. Previous studies on consumption of green tea extracts reported their protective effects on cardiovascular system [41], lowerong of blood glucose and cholesterol levels [42], and anti-inflammatory effects [43]. But the use of green tea extract is not free from health risks, such as hepatotoxicity [44]. EGCG, a strong antioxidant, can reduce oxidative stress and plays a role in reducing the incidence of cancer [45], infertility [46], osteoarthritis [47], cardiovascular diseases [41], type 2 diabetes mellitus [48], Parkinson's disease [49], and retinal diseases [50]. It has the ability to chelate metals, *e.g.* iron, copper, chromium and cadmium [51,52]. EGCG reduces Fe(III) and Cu(II) to Fe(II) and Cu (I), respectively. Thus the production of free radicals from the Fenton reaction is inhibited. In our experiment, accumulation of both the apoptotic markers caspase-3 and p53 in granulosa cells was stimulated by green tea extract at the highest dose used in this study (200 µg/ml). Based on our analysis of contents of catechins, flavan-3-ols and phenolic acids in the green tea extract, this dose will correspond to 9.04 µg/ml EGCG. This suggests that green tea extract induces apoptosis in porcine ovarian cells. At a dose of at doses 120.5 µg/ml green tea was reported to increase mRNA expression of both caspase-3 and p53 after 72 h of culture of a human laryngeal carcinoma cell line [53]. EGCG was earlier reported to suppress porcine ovarian function by promoting apoptosis [54]. In mouse model, EGCG was showed to induce apoptosis in blastocysts and retarded early post-implantation development in mice [55]. Cell culture studies involving cancer cells also confirmed the apoptotic nature of this green tea component through caspase-3 activation [56,57], but associated with increased expression of p53 [58]. It was proposed that green tea induces apoptosis in a p53 dependent pathway [53]. On the contrary, Hsu et al. indicated a mitochondria-targeted, caspase 3-executed mechanism of green tea polyphenol-induced apoptosis [59]. Transcription factor p53 mediated apoptosis was reported from porcine ovarian cells [28]. Lin and Tongy suggested that green tea polyphenols can also induce apoptosis through other pathways including activation of caspases in p53 deficient cells [60]. Caspase-3 dependent apoptosis was reported from human granulosa cells [61] and from preadipocytes by EGCG [62].

In conclusion, our results indicate towards dose-dependent impact of green tea extract on ovarian apoptosis through pathway that includes activation of caspase-3 and p53. Potential stimulation of these intracellular regulators could induce the process of apoptosis in ovarian cells.

Conflicts of interest

None.

Acknowledgements

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