Reproductive Biology xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Reproductive Biology

journal homepage: www.elsevier.com/locate/repbio

Green tea extract affects porcine ovarian cell apoptosis

Shubhadeep Roychoudhury[a,b], Marek Halenar[b], Katarina Michalcova[b], Sangeeta Nath[a], Miroslava Kacaniova[c], Adriana Kolesarova[b,*]

[a] *Department of Life Science and Bioinformatics, Assam University, Silchar 788 011, India*
[b] *Department of Animal Physiology, Faculty of Biotechnology and Food Sciences, Slovak University of Agriculture in Nitra, Nitra 949 76, Slovak Republic*
[c] *Department of Microbiology, Faculty of Biotechnology and Food Sciences, Slovak University of Agriculture in Nitra, Nitra 949 76, Slovak Republic*

ARTICLE INFO

Keywords:
Green tea
Porcine ovarian granulosa cells
Steroid hormones
Proliferation
Apoptosis

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

* Corresponding author.
E-mail address: adriana.kolesarova@uniag.sk (A. Kolesarova).

https://doi.org/10.1016/j.repbio.2018.01.007
Received 29 November 2017; Received in revised form 11 January 2018; Accepted 18 January 2018
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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 200g. 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

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