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THE EFFECT OF GREEN TEA EXTRACT - EPIGALLOCATECHIN GALLATE (EGCG) ON PORCINE OVARIAN GRANULOSA CELL

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ABSTRACT

The aim of our study was to elucidate the potential effect of green tea substance on basic ovarian functions. For this purpose, we examined the action of green tea bioactive molecule, epigallocatechin gallate (given at doses 0, 1, 10, 100 μg/mL), on cultured porcine ovarian granulosa cell functions - proliferation, apoptosis and steroidogenesis. Accumulation of PCNA (marker of proliferation), BAX (marker of apoptosis) and the release of steroid hormones (progesterone and testosterone) were analysed by immunocytochemistry and RIA respectively. It was observed that epigallocatechin gallate addition decreased the percentage of proliferative (PCNA-positive) cells at all used doses (1, 10 and 100 μg/mL). The percentage of apoptotic (BAX-positive) cells was increased at the highest used dose (100 μg/mL), but not a lower doses. Epigallocatechin gallate stimulated progesterone release (at 10 μg/mL but not at 1 and 100 μg/mL) and diminished testosterone release (at 1 μg/mL but not at 10 and 100 μg/mL) by porcine granulosa cells. Our results suggest a direct effect of epigallocatechin gallate on proliferation, apoptosis and steroidogenesis in porcine ovaries. Taken together, these data suggest that green tea molecule epigallocatechin gallate can negatively affect reproductive (ovarian) functions – suppress ovarian cell proliferation, promote their apoptosis and alter release of steroid hormones.

Keywords: epigallocatechin gallate, proliferation, apoptosis, progesterone, testosterone

INTRODUCTION

Tea has been cultivated and consumed for many years. Green tea is obtained from the leaves and the leaf buds of the plant *Camellia sinensis*. Cold or hot drink of it is the second most widely consumed beverage in the world, second only to water **(Yang *et al.*, 2002)**. The main components of green tea are polyphenols. 50-80% of polyphenols are represented by special flavonoids - catechins, especially epigallocatechin-3-gallate (EGCG) **(Fukai, *et al.*, 1991; Khan *et al.*, 2006).** Long term consumption of green tea may influence the incidence of obesity, diabetes, and cardiovascular disease **(Kao *et al.*, 2000)**.
EGCG has a pronounced growth inhibitory effect on cancer cells, but not on normal cells **(Chung *et al.*, 2003)**. This flavonoid exhibits antibacterial activity **(Blanko *et al.*, 2003)**, prevents neural cell death **(Reznichenko *et al.*, 2005)** and induces chromosomal damage in lymphoblastoid cell lines **(Sugisawa and Umegaki, 2002)** .
This catechin can induced reductions in the levels of sex steroids hormones; has possible negative effects on reproductive efficiency **(Kao *et al.*, 2000)**, on granulosa cell functions **(Basini *et al.*, 2005)** and *in vitro* fertilization of swine (**Spinaci *et al.*, 2006)**. This flavonoid modulates protein kinase C activity **(Levites *et al.*, 2002)** inhibits various activities of proinflammatory cytokines **(Ahmed *et al.*, 2002; Han, 2003; Li *et al.*, 2004)** in human cells. EGCG treatment diminished the levels of growth hormone, leptin, insulin-like growth factor I and prolactin on male rats **(Kao *et al*, 2000)**.
Cell proliferation is the amount of cells in culture or in the body can be divided. The extent of DNA synthesis is marker for proliferation **(Wyllie *et al.*, 1998)**. Involving the protein to cell proliferation include PCNA **(Tomanek and Chronowska, 2006)**. This protein is localized in the cell nucleus **(Makarevich *et al.*, 2000; Nahryzny and Lee, 2001)** and located in granulosa cells of gilts **(Sanislo *et al.* 2001)**. EGCG addition supresses proliferation of porcine granulosa cells **(Basini *et al.*, 2005a,b)**, human ovarian carcinoma cell proliferation **(Huh, *et al.*, 2004, Spinella *et al.*, 2006)**.
Apoptosis is programmed death of cells. This process eliminates unnecessary and useless cells from the body **(Wyllie *et al.*, 1998)**. Apoptosis is supported by group of caspases, which include BAX **(Zwain and Amato, 2001)**. This protein is localized especially in mitochondria **(Markström *et al.*, 2002)** and located in granulosa cells of gilts **(Sanislo *et al.* 2001)**. The number of apoptotic cells is increased in human ovarian carcinoma cells **(Huh, *et al.*, 2004, Spinella *et al.*, 2006)**.
Progesterone (P4) is an ovarian steroid produced by ovarian granulosa cells **(Kolesárová *et al.*, 2010a,b; Medvedová *et al.*, 2011)** and *corpus luteum* **(Gregoraszczuk, 1992; Gregoraszczuk, 1997)** of pigs and contributes to regulation of ovarian follicular development and remodelling **(Mahajan, 2008)**. It is a local paracrine or autocrine factor regulating luteal function **(Gregoraszczuk, 1992; Gregoraszczuk, 1997)**. This progestin is essential for normal ovarian cycle of females **(Hagan *et al.*, 2009)**. Another hormone produced in ovary is testosterone (T) **(Delort *et al.*, 2009)**. T is steroid hormone as well as P4 are necessary as a precursor for the synthesis of estrogen **(Mindnich *et al.*, 2004; Sirotkin, 2011)**. Androgens, primarily testosterone, are promoting proliferation of follicular cells, recruitment and development of ovarian follicles up to preovulatory stage, either stimulate or suppress development of Graafian follicles and their ovulation, increase apoptosis and follicular atresia at different stages of follicullogenesis and promotes oocyte nuclear maturation. Testosterone treatments altered release of progesterone, estradiol, by cultured ovarian cells **(Sirotkin *et al.*, 2003)**.
The results about effect of EGCG are poor and datas are limited mainly on studies of cancer and non-ovarian cells. **Basini *et al*. (2005a,b)** found inhibited proliferation and P4 production after EGCG addition. We wanted to refute or confirm the results of previous study. Effect of apoptosis and T release due to EGCG has not been studied in porcine granulosa cells.
The aim of our study was to research the effect of EGCG treatment at doses 1, 10 and 100 μg/mL on accumulation of markers of proliferation (PCNA) and apoptosis (BAX) and secretory activity (steroid hormones of P4 and T) of porcine granulosa cells (GCs) *in vitro.*

MATERIAL AND METHODS

Preparation, culture and processing of granulosa cells from ovaries

Granulosa cells were collected from the ovaries of prepubertal Slovakian White gilts, after slaughter at a local abattoir. After aspiration and isolation of granulosa cells, these cells were then washed in sterile DMEM/F12 1:1 medium (BioWhittakerTM, Verviers, Belgium), resuspended in the same medium supplemented with 10% fetal calf serum (BioWhittakerTM) and 1% antibiotic-antimycotic solution (Sigma, St. Louis, MO, USA) at a final concentration 10^6 cells/mL medium. Portions of the cell suspension were dispensed to 24-well culture plates (NuncTM, Roskilde, Denmark, 1 mL suspension/well; for RIA) or 16-well chamber slides (Nunc Inc., International, Naperville, USA, 200 µl/well, 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 the medium was renewed. Further culture was performed in 2 mL culture medium in 24-well plates (medium for RIA) or 200 µl/medium in 16-well chamber slides, (cells for immunocytochemistry) as described previously. After medium replacement experimental cells were cultured in the presence of epigallocatechin gallate (EGCG) (Changsha Sunfull Bio-tech. Co, Hunan China) at concentrations of 0, 1, 10 and 100 µg/mL. EGCG was dissolved in culture medium immediately before their addition to the cells. After two days in culture, the medium from the 24-well plates was gently aspirated and frozen at -24°C to await RIA. After removing the medium from chamber slides, cell were washed in ice-cold PBS (pH 7.5), fixed in paraformaldehyde (4% in PBS, pH 7.2-7.4; 60 min) and held at 4 °C to await immunocytochemistry.

Immunocytochemical analysis

Following washing and fixation, the cells were incubated in the blocking solution (1% of goat serum in phosphate-buffered saline – PBS) at room temperature for 1 h to block nonspecific binding of antiserum. Afterwards, the cells were incubated in the presence of monoclonal antibodies against either PCNA (marker of proliferation) and BAX (marker of apoptosis) (all from Santa Cruz Biotechnology, Inc., Santa Cruz, USA; dilution 1:500 in PBS) for 2 h at room temperature at overnight at 4 °C. For the detection of binding sites of primary antibody, the cells were incubated in secondary swine antibody against mouse IgG labelled with horse-radish peroxidase (Servac, Prague, Czech Republic, dilution 1:1000) for 1 h. Positive signals were visualized by stanning with DAB-substrate (Roche Diagnostics GmbH, Manheim, Germany). Following DAB-staining, the cells on chamber-slides were washed in PBS, covered with a drop of Glycergel mounting medium (DAKO, Glostrup, Denmark); then coverslip was attached to a microslide. Cellular presence and localization of PCNA and BAX positivity in cells was proved on the basis of DAB-peroxidase brown staining. A ratio of DAB-HRP- stained cells to the total cell number was calculated.

Immunoassay

Concentrations of P4 and T were determined in 25-100 µl samples of incubation medium by RIA. The concentrations of P4 and T were assayed using Radioimmunoassay (RIA) according to the manufacturer's instructions. All RIA were validated for use in samples of culture medium.

Statistical Analysis

Significant differences between the experiments were evaluated using Student´s T-test and one/two-way ANOVA followed by paired Wilcoxon-Mann Whitney test, Sigma Plot 11.0 software (Systat Software, GmbH, Erkhart, Germany). Differences from control at $P< 0.05$ were considered as significant.

RESULTS AND DISCUSSION

Proliferation and apoptosis (Immunocytochemistry)

The results of immunocytochemistry are showed in Tab.1. In our study all used doses of EGCG significantly ($P<0.05$) decreased the percentage of cells containing PCNA.
A number of porcine granulosa cells containing BAX was improved by EGCG treatment at 100 µg/mL, but not lower doses (1 and 10 µg/mL).

Table 1 The percentage of cells containing markers of proliferation (PCNA) and apoptosis (BAX) after EGCG treatment (Imunocytochemistry)

Supplement	% of cells contained	
	PCNA	bax
Control (no additon)	49.86±1.3 (3311)	49.8±1.24 (3939)
EGCG 1 µg/mL	40,42±2,99* (844)	55.83±2.02 (728)
EGCG 10 µg/mL	38.88±3.46* (907)	59.88±3.49 (874)
EGCG 100 µg/mL	36.88±2.53* (909)	67.25±2.85* (908)

All the values represent % of cells containing particular antigen, means ± SEM, *- significant ($P<0.05$) differences with control (cells not treated with ECGC). In the brackets is a number of counted cells.

Our observations are in line with observation of the negative effect of EGCG on proliferation of non-ovarian cells, human ovarian carcinoma and healthy porcine ovarian granulosa cells, **(Huh, *et al*., 2004; Spinella *et al*., 2006; Basini *et al*., 2005a,b)**.
Our datas confirmed positive impact of this catechin on apoptosis of human ovarian carcinoma cells **(Huh, *et al*., 2004, Spinella *et al*., 2006)**. These data suggest that EGCG can directly inhibit proliferation and stimulated apoptosis not only of swine ovarian cells, also other sort of cells and tissues. Furthermore, they suggest the therapeutic effect of ghreen tea on ovarian carcinoma cells, but also its suppressive effect (inhibition of proliferation and stimulation of apoptosis) on healthy ovarian cells.

Steoidogenesis (RIA)

Secretion of steroid hormones was detected by radioimmunoassay (Tab. 2). In our study 10 µg/mL dose of EGCG increased the P4 secretion by porcine ovarian granulosa cells. Other doses 1 and 10 µg/mL did not affect this progestin release.
In the case of secretion of T by porcine ovarian GCs stimulatory effect of EGCG (at the dose 10 µg/mL but not at 10 and 100 µg/mL) was found in our study.

Table 2 The secretion of steroid hormones by porcine ovarian granulosa cells after EGCG treatment (RIA)

Supplement	P4 secretion	T secretion
	ng/10^6cells/day	pg/10^6 cells/day
Control (no additon)	81.20±6.28	344.46±79.20
EGCG 1 µg/mL	63.70±6.47	274.40±12.10*
EGCG 10 µg/mL	230.00±5.00*	270.60±45.90
EGCG 100 µg/mL	101.00±17.80	297.00±50.80

All the values represent P4 or T release, means ± SEM, *- significant ($P<0.05$) differences with control (cells not treated with EGCG).

This study demonstrated effect of EGCG addition on porcine granulosa cells. Our data not correspond the result of **Basini *et al*. (2005a)**, who found inhibited reduced P4 release in porcine granulosa cells after EGCG treatment (at doses 5 and 50 µg/mL). The differences in EGCG effect observed in our experiments and experiments of Basini et al., (2005a) could be explained by different source of cells and different soufce of ovarian cells. **Basini *et al*. (2005a)** applied his study on mature porcine ovaries, while we worked with granulosa cells from young noncyclic swine ovaries.
In our experiment, T release was stimulated by EGCG addition. This is the first finding, that curcumin can influence not only P4 but also androgen output. The causes and physiological significance of EGCG-induced changes in progestagen and androgen release requires further studies. Nevertheless, it might be hypothesised, that reduction in P4 outpout might indicate, that green tea can reduce ovarian cell luteinisation, which is characterised by promotion of P4 production and reduction in P4 derivates – androgens and estrogens **(Sirotkin, 2011)**. Both P4 and T have antiproliferative and proapoptotic properties, therefore they can suppress growth of ovarian follicles **(Sirotkin, 2011)**. Therefore, it can be hypothesized, that EGCG in through promotion of P4 can inhibit porcine ovarian development. This hypothesis was supported by the ability of EGCG to affect markers of ovarian cell proliferation and apoptosis.

CONCLUSION

The present study suggest a possible stimulatory effect of EGCG on the release of progesterone and inhibitory influence on the testosterone secretion, inhibitory impact on proliferation (accumulation of PCNA) and stimulatory influence on apoptosis (accumulation of bax) on granulosa cells of porcine ovaries. Our results suggest a direct effect of EGCG on steroidogenesis, proliferation and apoptosis in porcine ovaries. Our study is the first evidence between EGCG treatment and its increased effect on testosterone release. Taken together, these data suggest that EGCG can suppress porcine reproductive (ovarian) function – suppress ovarian cell proliferation, promote their apoptosis and alter release of steroid hormones.

If such EGCG effects occur in human ovarian cells too, the potentially anti-cancer, but negative anto-reproductive effect of EGCG should be taken into account by green tea consumption.

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