



Influence of green tea constituents on cultured porcine luteinized granulosa cell functions

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ABSTRACT. The aim of the study was to examine the action of green tea polyphenols (GTPP) and their main constituent, epigallocatechin-3-gallate (EGCG), on porcine ovarian granulosa cells. For this purpose, the effect of GTPP and EGCG on cultured porcine ovarian granulosa cell functions, including proliferation, apoptosis, steroidogenesis and response to insulin-like growth factor I (IGF-I) was examined. Proliferation (the accumulation of proliferating cell nuclear antigen (PCNA) and cyclin B1), apoptosis (the accumulation of bax and caspase 3) and the release of steroid hormones (progesterone and testosterone) were evaluated by using immunocytochemistry and enzyme immunoassay. The addition of both GTPP and EGCG reduced the percentage of both PCNA- and cyclin B1-positive cells, increased the proportion of cells containing bax and caspase 3 and stimulated progesterone release. GTPP had a biphasic effect on testosterone output – stimulating at dose 1 µg/ml and inhibiting at doses 10 and 100 µg/ml, whilst EGCG did not affect testosterone secretion. IGF-I, when administered alone, promoted % of cells containing PCNA, suppressed bax accumulation, and stimulated progesterone release (only at dose 100 ng/ml). Testosterone release increased after the addition of IGF-I at 1 ng/ml, but decreased after IGF-I addition at 10 or 100 ng/ml. Both GTPP and EGCG suppressed or even reversed the effects of IGF-I on percentage of PCNA-positive cells, bax, testosterone output, and promoted IGF-I action on progesterone release. These observations suggested the inhibitory actions of green tea constituents on porcine ovarian granulosa cell functions were mediated through various regulatory mechanisms: suppression of the ovarian cell cycle in the S and G2 phases; promotion of cytoplasmic apoptosis; alteration of steroid hormone release; and, predominantly, prevention of the action of the hormonal stimulator IGF-I on ovarian cells. It can be also suggested that the major GTPP effects may result from the presence of EGCG.

Introduction

In China the medicinal use of green tea (*Camellia sinensis* L., Theaceae) is dated 4700 years back. Even now drinking green tea is regarded to be generally healthful practice (Cooper, 2012).

Numerous biological, clinical, and epidemiological studies have now reported health and therapeutic benefits of this tea, which are mainly provided by green tea polyphenols (GTPP), especially the polyphenol epigallocatechin-3-gallate (EGCG). Owing to the anti-microbial, anti-oxidative, pro-apoptotic,

metabolic, endocrine, and angiogenic properties of these molecules, green tea consumption can reduce the incidence of cardiovascular and inflammatory diseases, obesity, diabetes, and cancer (Kao et al., 2000; Cooper, 2012; Niedzwiecki et al., 2016; Saeed et al., 2017). In addition, the use of herbs, including anti-microbial green tea, as additives in livestock nutrition has been suggested as a novel alternative to antibiotics. Nevertheless, despite many potential benefits, the consumption of green tea and its constituent molecules should be evaluated in the context of possible reproduction-related consequences (Basini et al., 2005).

Unfortunately, the available data concerning effects of green tea and its components on reproduction are highly contradictory. There have been reported some concerns about the influence of green tea on male reproduction, including: the stimulatory action of green tea on rat testis weight (Hijazi et al., 2015) and EGCG on pig sperm quality (Spinaci et al., 2008); the protective action of green tea and EGCG against the degenerative changes in rat testis and steroidogenesis induced by diabetes (Kaplanoglu et al., 2013; Oliveira et al., 2015; Dias et al., 2016); ionizing radiation (Ding et al., 2015); and cadmium toxicity (Sharma and Goyal, 2015; Abdelrazek et al., 2016). In contrast, Bucci et al. (2017) did not confirm the influence of green tea extract or EGCG on porcine sperm quality. Moreover, in some studies the castration-like effects of either EGCG (Figueiro et al., 2009) or green tea extract (Chandra et al., 2011; Das et al., 2015) were shown in male rats. In these animals the administration of such substances reduced plasma gonadotropins and testosterone levels, testicular steroidogenic enzymes, testis weight, sperm count, and induced the degeneration of testicular tissues.

Similar contradictions were indicated in the studies on green tea and female reproductive processes. In some publications the applicability of green tea, GTPP or EGCG to increase either the bovine (Wang et al., 2007) or porcine (Spinaci et al., 2008) oocyte fertility rate and for the prevention and treatment of human and animal ovarian cell malignant transformation (Cooper, 2012; Niedzwiecki et al., 2016; Saeed et al., 2017), symptoms of polycystic ovarian syndrome (Ghafurniyan et al., 2015), and infertility (Roychoudhury et al., 2017) was demonstrated. Conversely, some authors reported no discernible effects of green tea on proliferation (the accumulation of PCNA and cyclin B1) and steroid hormones (progesterone and estradiol) in cultured porcine ovarian granulosa cells (Roychoudhury et al., 2018)

or the effects of EGCG on progesterone release in cultured porcine ovarian cells or on porcine oocyte maturation (Spinaci et al., 2008), or any influence of GTPP on bovine oocyte maturation (Wang et al., 2007). Finally, in several publications the suppressive action of green tea molecules on animal reproductive processes was described. Kao et al. (2000) reported the inhibitory action of EGCG injections on murine ovarian growth and plasma levels of reproductive hormones (LH, testosterone, estradiol, leptin and insulin-like growth factor I (IGF-I)). Other authors reported the ability of green tea to promote apoptosis (the accumulation of caspase 3 and p53) (Roychoudhury et al., 2018) and the potential for EGCG treatment to suppress cultured granulosa cell proliferation, progesterone, estradiol, VEGF release, and respiration (Basini et al., 2005) and to impair oocyte fertility and embryogenesis (Spinaci et al., 2008) in pigs.

The mechanisms of green tea and its constituents on the reproductive processes have not been sufficiently clarified yet; moreover, the available information is discrepant. Female reproduction and fecundity are defined by the upstream hormonal regulators and the response of basic ovarian cell functions, such as proliferation, apoptosis, differentiation. One of these key regulators is IGF-I, which can be a mediator of gonadotropin action, a suppressor of ovarian cell apoptosis, and a promoter of proliferation, which leads to ovarian follicular growth and development (Sirotkin, 2014). The publications listed below reported either no action of green tea (Roychoudhury et al., 2018) or EGCG (Spinaci et al., 2008; Roychoudhury et al., 2018) on these processes or their ability to promote apoptosis (Roychoudhury et al., 2018) and inhibit ovarian cell proliferation and steroidogenesis (Basini et al., 2005). Therefore, the available information on the action of green tea and its constituents on ovarian cells, even within a single species, is inconsistent and requires further investigation. The variations in green tea composition can be the cause of such discrepancies; therefore, pure green tea constituents (for example, GTPP or EGCG) would be preferable for such studies. Furthermore, although the influence of green tea on FSH-induced ovarian steroidogenesis has been studied (Roychoudhury et al., 2018), no influence of green tea or its constituents on ovarian response to upstream hormonal regulators has been detected yet.

So, the aim of our study was to examine the action of the green tea constituents, GTPP and EGCG, on the basic functions of cultured porcine ovarian granulosa cells: proliferation, apoptosis, steroidogenesis, and their response to IGF-I. For the markers of

proliferation, the accumulation of PCNA and cyclin B1, which are markers of the S phase and G2 phase of the cell cycle, respectively was used (Naryzhny and Lee, 2001; Liebermann and Hoffman, 2007). As markers of cytoplasmic apoptosis, we used the accumulation of bax and caspase 3 (Karran and Dyer, 2001; Zheng et al., 2016). Steroid hormones progesterone, testosterone and estradiol, which are the key regulators and markers of the ovarian follicle state, were also analysed (Sirotkin, 2014).

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 without previous artificial stimulation of ovarian functions. The cells were aspirated from ovarian follicles (0.3–0.6 mm in diameter) without visible signs of atresia (weak vascularization, thin follicular wall, pale follicular fluid). After the aspiration, these cells were then washed in sterile DMEM/F12 1:1 medium (BioWhittaker™, Verviers, Belgium), resuspended in the same medium supplemented with 10% foetal calf serum (BioWhittaker™, Verviers, Belgium) and 1% antibiotic-antimycotic solution (Sigma-Aldrich, St. Louis, MO, USA) at a final concentration of 10^6 cells/ml medium. Aliquots of the cell suspension were dispensed in 24-well culture plates (Nunc™, Roskilde, Denmark; 1 ml suspension/well for hormones assay) or 16-well chamber slides (Nunc Inc., Naperville, IL, USA; 200 µl/well for immunocytochemistry). Both the plate wells and chamber slides were incubated at 37 °C in an atmosphere of 5% CO₂ in humidified air until a 60–75% confluent monolayer was formed (3–5 days), then the medium was renewed. Further culture was performed in 2 ml culture medium in 24-well plates (for hormones enzyme immunoassay (EIA)) or 200 µl/medium in 16-well chamber slides (for examined proteins immunocytochemistry). In the first series of experiments, after medium replacement, the experimental cells were cultured in the presence of either GTPP or EGCG (Changsha Sunfull Bio-tech Co., Changsha, P.R. China) at concentrations of 0, 1, 10, and 100 µg/ml. These doses are comparable with green tea constituents level in animal and human blood after different applications of green tea extract (Wein et al., 2016; Kulandaivelu and Mandal, 2017; Law et al., 2017) and with doses used in similar previous *in vitro* studies (Basini et al., 2005; Wang et al., 2007; Spinaci

et al., 2008; Roychoudhury et al., 2018). Substances were dissolved in culture medium immediately before their addition to the cells. In the second series of experiments, the cells were cultured with 0, 1, 10 or 100 ng/ml recombinant IGF-I (Peptides International Inc., Louisville, KY, USA) with and without either GTPP or EGCG (both at dose of 10 µg/ml). After 2 days of culture, the medium from the 24-well plates was gently aspirated and frozen at –24 °C prior to hormones assay. After removal of the medium from chamber slides, the cells were washed in ice-cold phosphate-buffered saline (PBS; pH 7.5), fixed in paraformaldehyde (4% in PBS, pH 7.2–7.4) for 60 min and stored at 4 °C prior to immunocytochemistry.

The data were presented as the mean of values obtained from three separate experiments performed on separate days using separate pools of ovaries obtained from 10–12 animals in each. Taken together, granulosa cells isolated from 60–70 ovaries were used in experiments. For each experimental group, six culture wells or four slide chambers were analysed. The proportions of cells containing specific immunoreactivity were calculated from the inspection of a minimum of 1000 cells per chamber. The assays of hormones in the incubation medium were performed in duplicate. The values of the blank control were subtracted from the value determined by EIA in cell-conditioned medium to exclude any non-specific background (less than 7% of total values). The rates of substance secretion were calculated per 10^6 cells/day.

Immunocytochemical analysis

After washing and fixation, the cells were incubated in the blocking solution (1% goat serum in PBS at +20 °C for 1 h to block nonspecific binding of the antiserum). Subsequently, the cells were incubated in the presence of monoclonal antibodies against either PCNA, cyclin B1, bax, and caspase 3 (all from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA; dilution 1:500 in PBS) for 2 h at room temperature or overnight at 4 °C. The specificity and suitability of these antibodies for analysis of the corresponding antigens in cultured porcine granulosa cells were validated previously by Western immunoblotting (Mészárosová et al., 2008; Sirotkin, 2010) and RT-PCR (Kádasi et al., 2017; Štochmařová et al., 2018). The results obtained by Western immunoblotting and RT-PCR corresponded to the results obtained by using quantitative immunocytochemistry. To detect the binding sites of the primary antibody, the cells were incubated in secondary swine anti-mouse IgG labelled with FITC

(Servac, Prague, Czech Republic; dilution 1:1000) for 1 h. The negative controls were composed of cells stained without the primary antibody. Cells containing FITC were detected using a fluorescence microscope (Leica GmbH, Wetzlar, Germany). The ratio of stained cells to the total cell number was calculated.

Immunoassay

The concentrations of progesterone, testosterone and estradiol were determined in 25–100 μ l of incubation medium by using enzyme immunoassay (EIA). These antisera against steroids were produced in the Institute of Animal Science (Neustadt, Germany). The assays were previously validated for use in culture medium. The progesterone concentrations were measured by using EIA, as described previously (Prakash et al., 1987). Rabbit antiserum against progesterone was obtained from Research Institute for Animal Production (Schoonoord, The Netherlands). Its cross-reactivity was $\leq 0.1\%$ to estradiol, dihydrotestosterone, testosterone and 17- β -hydroxyprogesterone; the sensitivity was 12.5 pg/ml and the inter- and intra-assay coefficients of variation did not exceed 3.3 and 3.0%, respectively. Testosterone was assayed by using EIA in accordance with the method of Münster (1989). Sensitivity was 10 pg/ml. Cross-reactivity of antiserum was $\leq 96\%$ to dihydrotestosterone, $\leq 3\%$ to androstenedione, and $\leq 0.01\%$ to progesterone and estradiol, $\leq 0.02\%$ to cortisol and $\leq 0.001\%$ to corticosterone. The inter- and intra-assay coefficients of variation were 12.3 and 6.8%, respectively. The estradiol concentrations were evaluated by using EIA in accordance with the method of Münster (1989); the sensitivity was 5 pg/ml and the cross-reactivity of the estradiol antiserum was $< 2\%$ to estrone, $\leq 0.3\%$ to estriol, $\leq 0.004\%$ to testosterone and $\leq 0.0001\%$ to progesterone and cortisol. The inter- and intra-assay coefficients of variation did not exceed 16.6 and 11.7%, respectively.

Statistical analysis

Significant differences between the experiments and groups were evaluated by using paired Wilcoxon-Mann-Whitney test (Sigma Plot 11.0 software; Systat Software, GmbH, Erkhart, Germany). Differences compared with the control at $P < 0.05$ were considered to be significant.

Results

During culture, the cells produced substantial amounts of both progesterone and testosterone, but the amount of released estradiol was below the limit of the assay sensitivity. The cells collected after cul-

ture contained visible staining for PCNA, cyclin B1, bax, and caspase 3. PCNA was located exclusively in the cell nuclei, whilst cyclin B1, bax and caspase 3 were present mainly in cytoplasm (Figure 1A–D, respectively). The measured parameters were affected by the addition of GTPP, EGCG, and IGF-I alone or in combination with GTPP or EGCG.

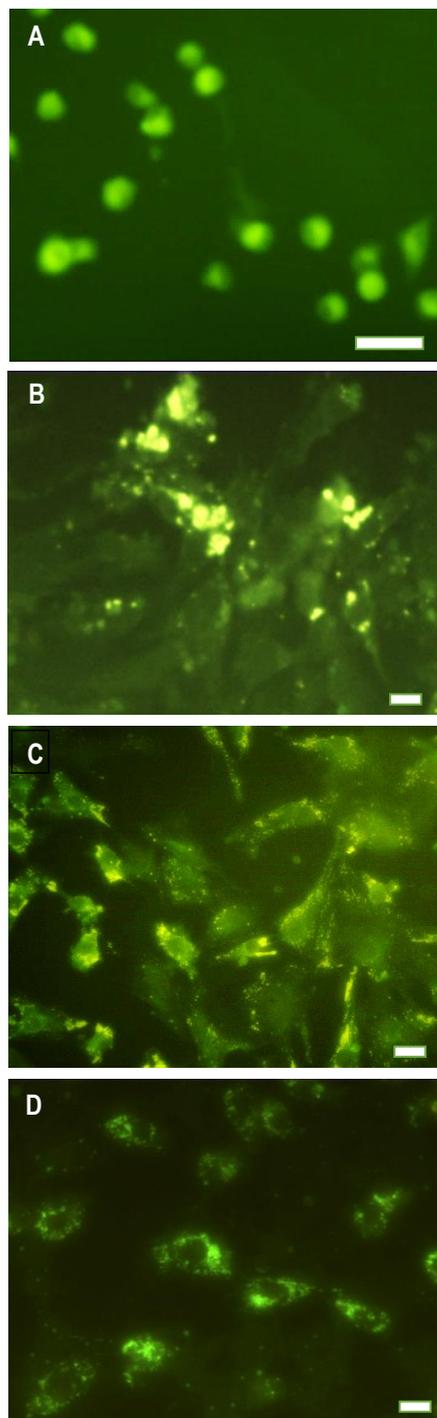


Figure 1. Fluorescent images of porcine ovarian granulosa cells containing PCNA (A), cyclin B1 (B), bax (C) and caspase 3 (D). Cells were stained with fluorescein isothiocyanate (FITC; green); scale bars: 1 cm = 20 μ m

The proportion of cells containing PCNA was reduced after the addition of both GTPP and EGCG at all treatment doses (Figure 2A,B). The percentage of cells containing cyclin B1 was also reduced after addition of GTPP (all doses) or EGCG (10 or 100 $\mu\text{g/ml}$) (Figure 2C,D). At all tested doses, both GTPP and EGCG increased the percentage of bax-positive (Figure 2E,F) and caspase 3-positive (Figure 2G,H) cells. The progesterone release was increased after addition of GTPP regardless used dose (Figure 2I), after EGCG addition the increase was stated only after the highest dose (Figure 2J). The testosterone release was either increased (at a dose of 1 $\mu\text{g/ml}$ medium) or decreased (at a dose

of 10 and 100 $\mu\text{g/ml}$ medium) after GTPP addition (Figure 2K). There was no influence of EGCG on testosterone release (Figure 2L).

IGF-I increased the proportion of cells containing PCNA regardless the used dose (Figure 3A,B). On the other hand the addition of GTPP at a dose of 10 $\mu\text{g/ml}$ into cell culture without IGF-I caused a significant decrease in the percentage of PCNA-positive cells, whereas for EGCG (at a dose of 10 $\mu\text{g/ml}$) no such effect was stated. In the presence of GTPP and EGCG, IGF-I also increased PCNA accumulation but only at the highest dose (100 ng/ml). However in case of GTPP the increase was not so pronounced as when IGF-I was added alone (Figure 3A), and in case of

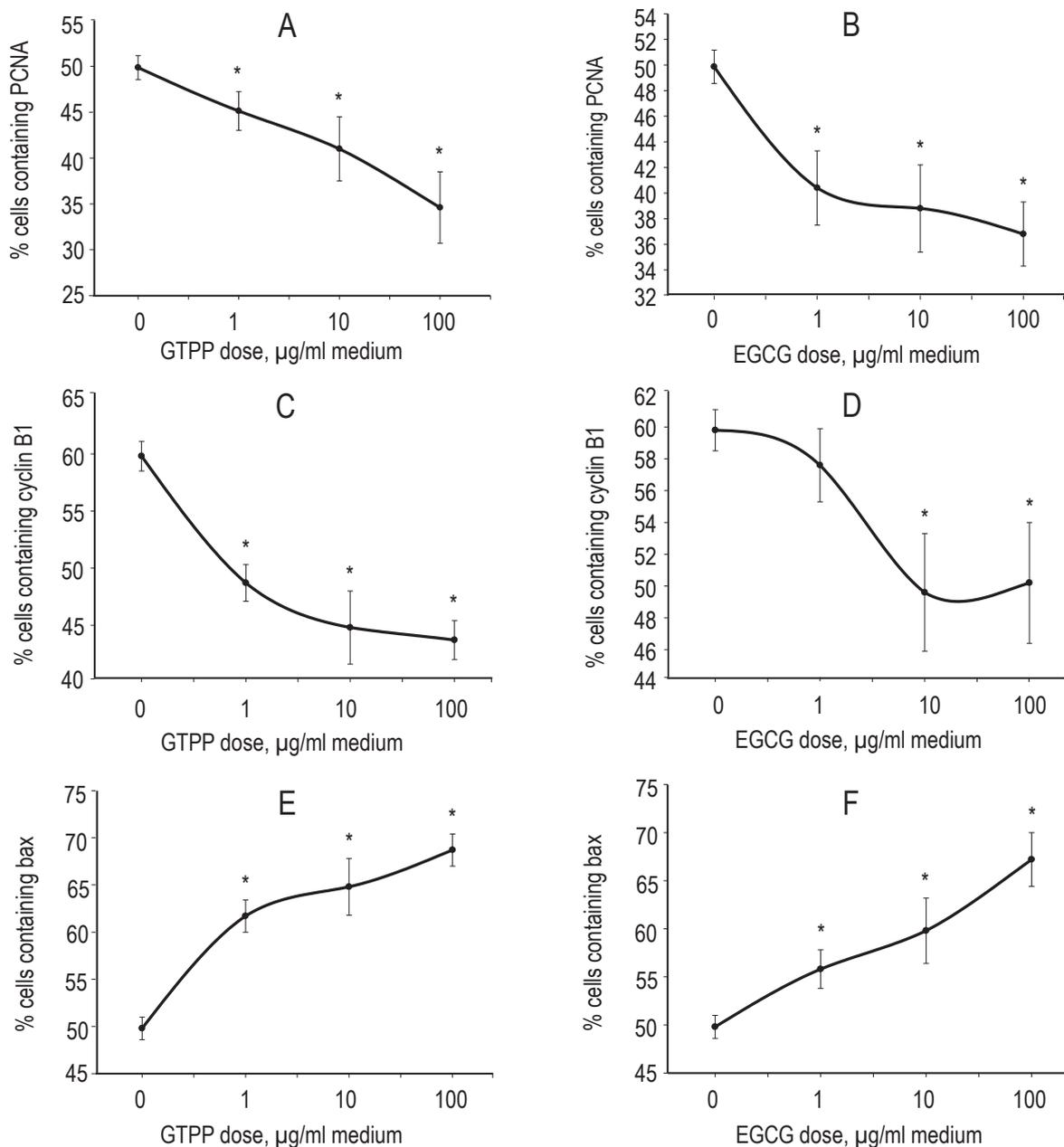


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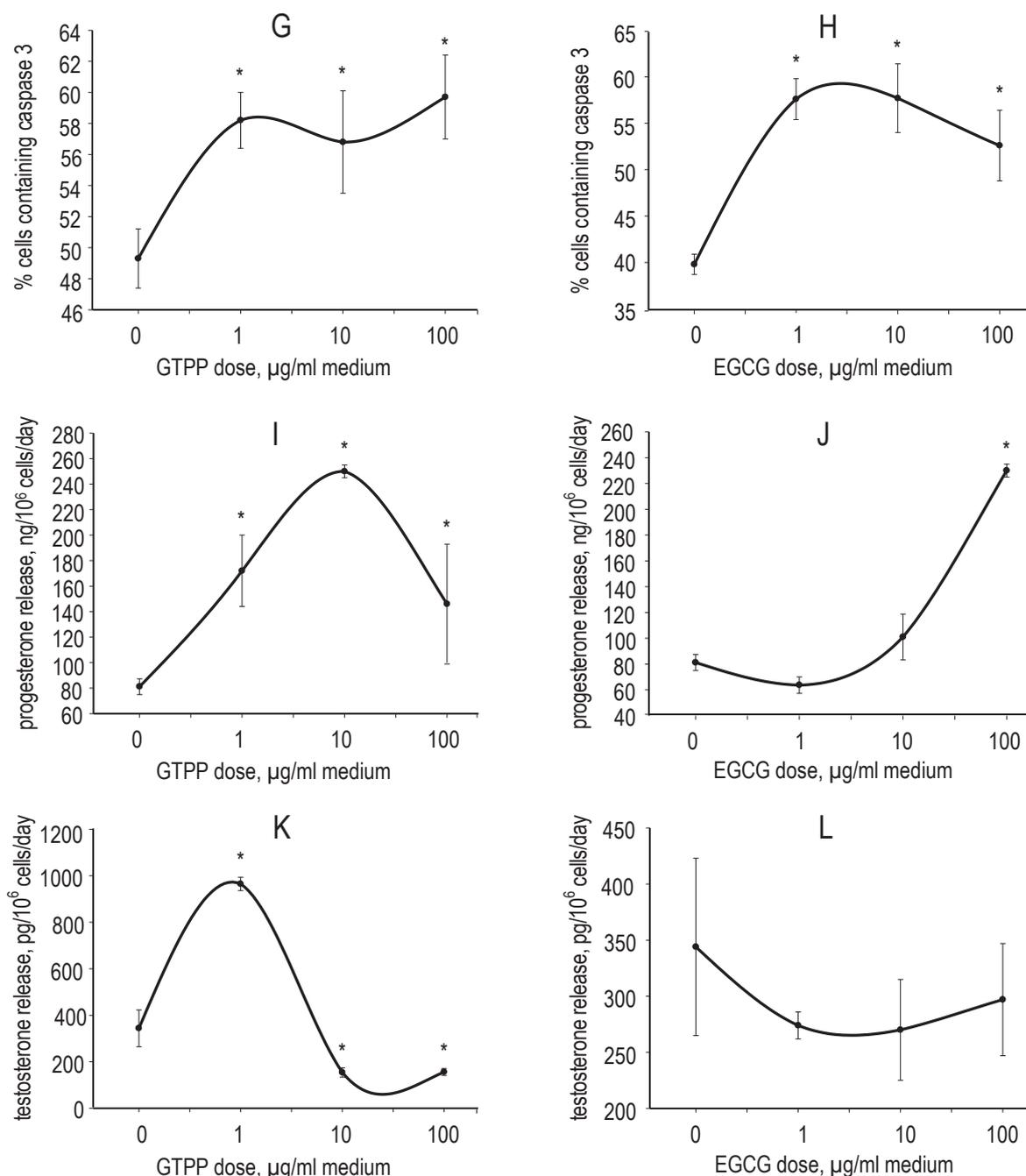


Figure 2. Effect of green tea polyphenols (GTPP) (left) and the polyphenol epigallocatechin-3-gallate (EGCG) (right) on the accumulation of proliferation markers PCNA (A,B) and cyclin B1 (C,D), the apoptosis markers bax (E,F) and caspase 3 (G,H), and the release of progesterone (I,J) and testosterone (K,L) by cultured porcine ovarian granulosa cells, as determined by immunocytochemistry (A–H) and enzyme immunoassay EIA (I–L)

data are the mean + S.E.M.; * – effect of green tea constituent, significant ($P < 0.05$) differences between the cells cultured with and without green tea constituent (0 µg/ml)

EGCG increase the value observed for IGF-I given alone (Figure 3B).

IGF-I reduced the accumulation of bax at all doses, whereas the addition of GTPP and EGCG without IGF-I caused a significant increase in the percentage of cells containing bax (Figure 3C,D). In the presence of GTPP the IGF-I reduced bax accumulation only

at two higher doses (10 or 100 ng/ml) but still for the highest dose the percentage of cells containing bax was higher in comparison to values for IGF-I given alone (Figure 3D). In the presence of EGCG the IGF-I reducing effect was stated only at the highest dose (100 ng/ml) and it was the same as the value observed for IGF-I given alone (Figure 3D).

IGF-I alone promoted progesterone output but only at the highest dose (100 ng/ml) (Figure 3E,F). When GTPP and EGCG were added without IGF-I, there was a significant increase in progesterone release. In presence of GTPP, two doses of IGF-I, 1 and 10 ng/ml, promoted progesterone release, but the highest used dose of IGF-I, 100 ng/ml, reduced progesterone release (Figure 3E). In the presence of EGCG, IGF-I at doses 10 or 100 ng/ml progesterone

output and this effect was more pronounced in comparison to the situation when IGF-I was added alone (Figure 3F).

Testosterone release was increased by the addition of 1 ng/ml IGF-I alone, but decreased below the control value when 10 or 100 ng/ml IGF-I was added (Figure 3G,H). The addition of both GTPP and EGCG without IGF-I presence caused significant decrease in the testosterone release. Moreover

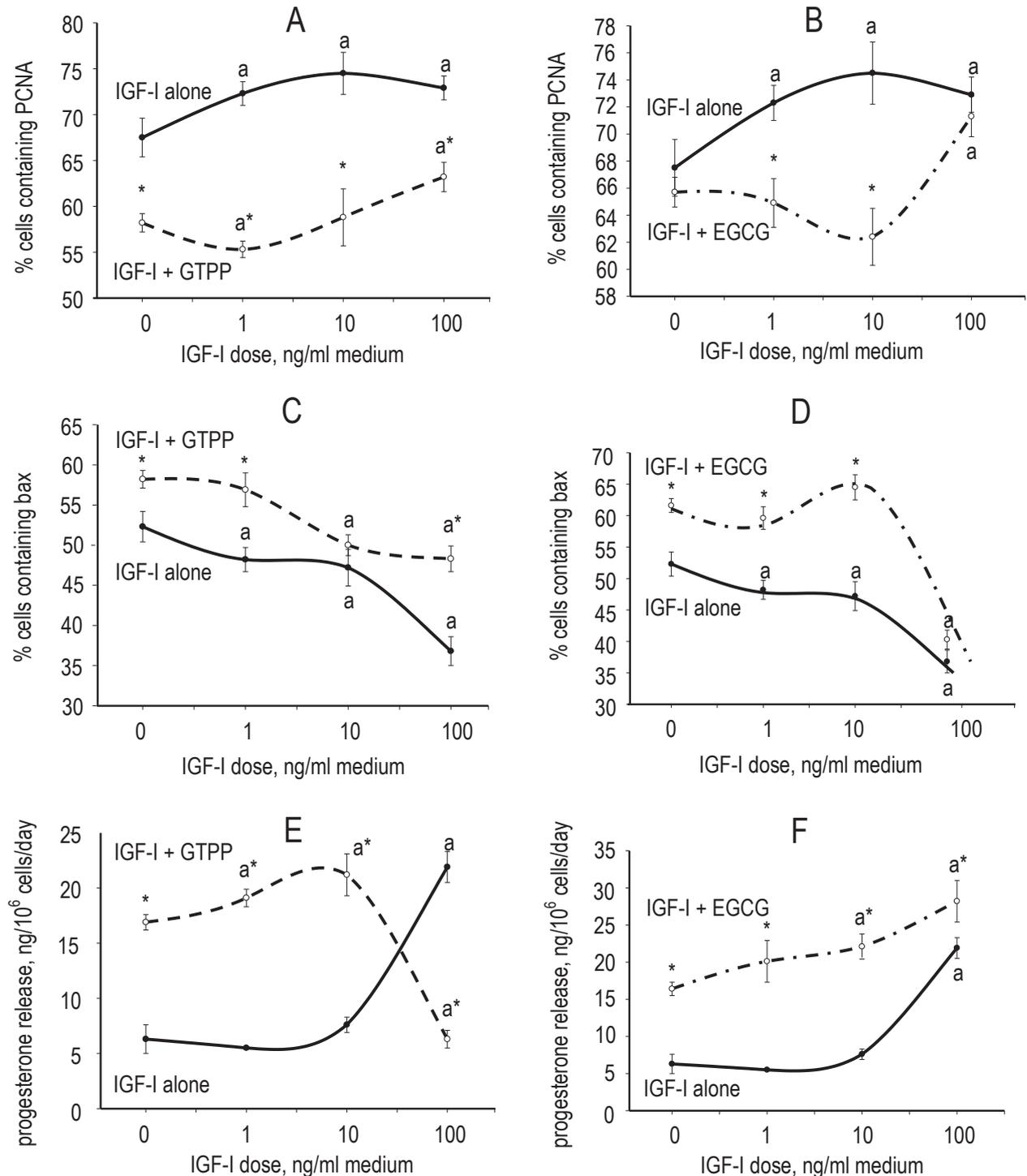


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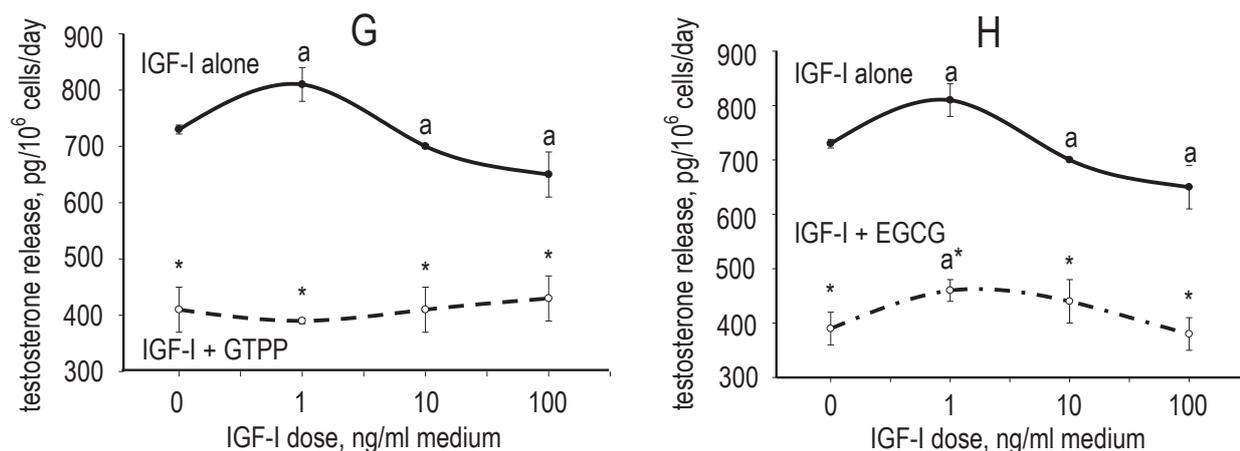


Figure 3. Effect of different doses of insulin-like growth factor (IGF-I) given alone and in combination with green tea polyphenols (GTPP; at dose of 10 µg/ml) (left) or the polyphenol epigallocatechin-3-gallate (EGCG; at dose of 10 µg/ml) (right) on the accumulation of proliferation marker PCNA (A,B), apoptosis marker bax (C,D), and the release of progesterone (E,F) and testosterone (G,H) in cultured porcine ovarian granulosa cells, as determined by immunocytochemistry (A–D) and EIA (E–H)

data are the mean \pm S.E.M.; * – effect of green tea constituent, significant ($P < 0.05$) differences between the corresponding groups of cells cultured with and without green tea constituent; a – effect of IGF-I, significant ($P < 0.05$) differences between the cells cultured with and without IGF-I (0 ng/ml)

the presence of GTPP prevented both the stimulatory and inhibitory action of IGF-I on testosterone release (Figure 3G). Conversely, EGCG prevented the inhibitory, but not the stimulatory, action of IGF-I on testosterone release (Figure 3F).

Discussion

The creation of a cell monolayer, the presence of proliferation-related molecules within the cells, and the release of substantial amounts of progesterone and testosterone by cultured cells suggested that the cells were viable and presented a suitable system to test the treatments. Furthermore, the high production of progesterone and low estradiol output indicated cell luteinisation during culture (Sirotkin, 2014).

GTPP and EGCG action on basic ovarian granulosa cell functions. It was observed a decrease in the proportion of cells containing both PCNA and cyclin B1 after the addition of both GTPP and EGCG, which suggested that these green tea constituents inhibit ovarian granulosa cell proliferation. These observations were in agreement with those of Basini et al. (2005), who observed that EGCG suppressed the proliferation of cultured porcine granulosa cells, but not with the report of Roudhury et al. (2018), who did not find any effect of whole green tea extract on the same markers of proliferation as used in our study. This failure could result from the testing of whole green tea extract, which may contain a variety of molecules characterised by opposing actions on the cell cy-

cle, and may mask the effect of particular tea constituents. As PCNA is considered to be a promoter and marker of DNA replication and repair during the S-phase of the cell cycle, cyclin B1 is considered to be a marker and promoter of the transition through the G2 phase to mitosis (Naryzhny and Lee, 2001; Liebermann and Hoffman, 2007), our observations suggested that green tea constituents were able to promote both S and G2 phases of the cell cycle.

In contrast with the proliferation markers, in our experiments it was shown that the expression of two cytoplasmic apoptosis markers, bax and caspase 3 (Karran and Dyer, 2001; Zheng et al., 2016), was increased after the addition of GTPP or EGCG. These observations were in line with the previous report (Roychoudhury et al., 2018) on the promotion of apoptosis in cultured porcine granulosa cells, as shown by the percentage of cells containing caspase 3 and p53.

In our study, both GTPP and EGCG promoted progesterone release and inhibited testosterone output. These observations corresponded to the report of Kao et al. (2000), in which the ability of EGCG to suppress testosterone release through murine ovaries *in vivo* was demonstrated. However, they are in contrast to previous reports of Spinaci et al. (2008) and Roychoudhury et al. (2018), in which the lack of GTPP and the whole green tea effect on progesterone release from cultured porcine ovarian cells was revealed or the ability of EGCG to suppress progesterone output from cultured porcine

granulosa cells was studied (Basini et al., 2005). The differences between effects of whole green tea extract, GTPP and EGCG on ovarian steroidogenesis could be explained by differences in phytoestrogen activity of these substances. GTPP contain different kinds and amounts of phytoestrogens which can exert different action on steroid hormone receptors in turn influencing ovarian cell proliferation, apoptosis, steroidogenic enzymes and luteinisation (Sirotkin, 2014; Sirotkin and Harrath, 2014). We could not exclude the possibility that the effects of green tea extract and its constituents were dependent on the species and state of the target cells used, but our observations for the first time demonstrate the ability of green tea constituents to promote ovarian steroidogenesis. The ability of progesterone to inhibit ovarian follicular growth was associated with the reduction of granulosa cell proliferation, and the ability of testosterone to promote ovarian atresia associated with apoptosis (Sirotkin, 2014) suggested that the constituents of green tea can affect ovarian cell proliferation and apoptosis *via* changes in steroidal hormone output. Furthermore, the action of green tea molecules on ovarian cell steroidogenesis and other functions could result from their ability to affect ovarian cell luteinisation (Sirotkin, 2014), steroid hormone receptors (Sirotkin and Harrath, 2014), the sirtuin/mTOR signalling pathway (Sirotkin, 2016), or to their antioxidant properties (Basini et al., 2005).

IGF-I action on basic ovarian granulosa cell functions. The IGF-I-induced increase in the proportion of PCNA-positive cells and the decrease in cellular bax accumulation confirmed the previous data on the ability of this growth factor to promote proliferation and to suppress the apoptosis of ovarian cells, and therefore to simulate growth and prevent apoptosis-related atresia of ovarian follicles (Quirk et al., 2004; Sirotkin, 2014). Furthermore, the increase in progesterone and testosterone release by cells after the addition of IGF-I supported previous reports on the ability of IGF-I to promote ovarian steroidogenesis (Sirotkin, 2014). On the other hand, in our study the biphasic effect of IGF-I on testosterone output is presented for the first time – testosterone release was promoted at low IGF-I concentration (1 ng/ml), but it was inhibited at higher concentrations (10 and 100 ng/ml). The functional interrelationships between the events induced by IGF-I remain unknown. Although the fact that progesterone can promote the production of its metabolite, testosterone, and that both hormones are involved in the proliferation and apoptosis (Sirotkin,

2014), no association between the IGF-I concentrations affecting progesterone, testosterone, proliferation, and apoptosis were found. This indicated that the IGF-I actions on ovarian functions were not mediated by steroidal hormones.

GTPP and EGCG modify effects of IGF-I on basic ovarian granulosa cell functions. The effects of IGF-I on all measured parameters were quantitatively and qualitatively modified by both GTPP and EGCG. Both treatments suppressed the response of cells to IGF-I. In their presence, IGF-I affected proliferation and apoptosis when added only at highest dose, whereas GTPP was able to not only prevent, but even reverse, the PCNA-promoting effect of IGF-I. The presence of GTPP and EGCG increased the IGF-I-induced stimulation of progesterone release; however, in the presence of GTPP, the highest dose of IGF-I did not promote, but instead inhibited, progesterone output. GTPP prevented both the stimulatory and inhibitory action of IGF-I on testosterone release, whereas EGCG prevented the inhibitory action of IGF-I on this parameter. Although green tea constituents were able to support the stimulatory action of IGF-I on progesterone output, they prevented and even reversed all other stimulatory and inhibitory effects of IGF-I on proliferation, apoptosis, and testosterone. Moreover, GTPP changed the stimulatory action of IGF-I at 100 ng/ml on progesterone release to the inhibitory one, i.e. it was able to reverse IGF-I action on progesterone output too. These observations suggested that the novel mechanism of green tea constituents on ovarian functions acted to modify the response of ovarian cells to the upstream hormonal regulators.

Comparison of effects of GTPP and EGCG. GTPP and EGCG showed similar dose-dependent effects on all markers of proliferation (PCNA and cyclin B1), apoptosis (bax and caspase 3) and steroidogenesis (progesterone and testosterone release); however, in the second series of experiments, the tendency of EGCG to inhibit ovarian PCNA accumulation was not statistically significant.

In contrast, some differences in GTPP and EGCG action on IGF-I were observed. In the presence of GTPP, IGF-I was able to inhibit PCNA accumulation and progesterone release, but EGCG did not have this effect. Furthermore, GTPP prevented both the stimulatory and inhibitory actions of IGF-I on testosterone release, but EGCG prevented only the inhibitory action.

The similarity of the effects of GTPP and EGCG on proliferation, apoptosis, and steroidogenesis suggested that the action of GTPP on these pro-

cesses can be attributed to the presence of EGCG. In contrast, the difference in the ability of GTPP and EGCG to influence IGF-I effects on progesterone and testosterone indicated that GTPP may contain molecules in addition to EGCG, which can modify the action of IGF-I on ovarian steroidogenesis and maybe luteinisation.

Conclusions

The inhibitory action of green tea constituents on porcine luteinized ovarian granulosa cell functions was mediated through various regulatory mechanisms: the suppression of the ovarian cell cycle; the promotion of cytoplasmic apoptosis; the alteration of steroid hormone release; and the prevention of the stimulatory action of IGF-I on ovarian granulosa cells. The major effects of green tea polyphenols (GTPP) are probably a result of the presence of epigallocatechin-3-gallate (EGCG), although the influence of GTPP on the effects of IGF-I on steroidogenesis could be mediated by other, unknown GTPP components. The ability of green tea constituents to suppress ovarian cell functions suggested not only their applicability for the prevention and treatment of ovarian cancer, which has been previously demonstrated (Cooper, 2012; Niedzwiecki et al., 2016; Saeed et al., 2017), but explain the previous observations of their adverse action on ovarian growth (Kao et al., 2000) and fecundity (Spinaci et al., 2006). Similar negative effects on green tea on male reproductive processes have also been reported. This anti-reproductive action of green tea constituents could be potentially applicable to synchronize ovarian cycles in pig production. In our experiments the anti-reproductive action of green tea constituents was demonstrated not only by changes in steroidogenesis, but also by inhibition of proliferation (at both S- and G-stage), promotion of apoptosis and reduction in response to the upstream stimulator. Furthermore, as such adverse action may not only occur in animals, but also in humans, this anti-reproductive effect of green tea constituents should be taken into account in terms of green tea consumption by humans.

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