Effect of genistein on steroidogenic response of granulosa cell populations from porcine preovulatory follicles

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SUMMARY

Genistein affects reproductive processes in animals. However, the mechanism of its action is not fully elucidated and differs among species. The objectives of the current study were: 1/ to establish an in vitro model of granulosa cell culture for studying the intracellular mechanism of phytoestrogen action in porcine ovary; 2/ to determine an in vitro effect of genistein on basal and FSH-stimulated P_4 and E_2 production by porcine granulosa cell populations (antral, mural, total) isolated from large, preovulatory follicles. Granulosa cells were isolated from large (≥8 mm), preovulatory follicles and separated into antral and mural cell subpopulations. Cells were allowed to attach for 72 h (37°C, 10% serum, 95% air/5% CO_2) and then cultured for next 48 hours with or without serum (0, 5 and 10%), FSH (0, 10 or 100 ng/ml) and genistein (0, 0.5, 5 or 50 µM). Basal P_4 and E_2 production did not differ among antral, mural and unseparated granulosa cells isolated form porcine preovulatory follicles.
follicles. Only mural cells tended to secrete less \( P_4 \) and \( E_2 \) than other cell populations. FSH stimulated \( P_4 \) production in a dose dependent manner in all cell populations and culture systems. Genistein inhibited in a dose dependent manner basal and FSH-stimulated \( P_4 \) production by antral, mural and unseparated granulosa cells. However, genistein did not affect \( E_2 \) production by granulosa cells. In addition, viability of porcine granulosa cells was not affected by the phytoestrogen except the highest dose of genistein. It appears that genistein may be involved in the regulation of follicular function in pigs. Moreover, unseparated porcine granulosa cells may provide a suitable \textit{in vitro} model for studying the intracellular mechanism of phytoestrogen action in porcine ovary. Reproductive Biology 2006 6 (1): 31–50.

\textbf{Key words}: pigs, follicles, antral cells, mural cells, progesterone, estradiol, phytoestrogens

\section*{INTRODUCTION}

Phytoestrogens (isoflavones, lignans and coumestans) are naturally occurring plant compounds that resemble estrogens in structure and function. The most intensely studied phytoestrogen is genistein, an isoflavone believed to have beneficial effects in humans. These effects include reducing susceptibility to breast and prostate cancers, reducing serum cholesterol concentrations and ameliorating symptoms of menopause. Genistein was also found to affect reproduction of farm animals including pigs [2, 25, 38].

Isoflavones are abundant in whole grains, fruits, vegetables and legumes, especially in soy products. Soybean-derived feedstuffs make up a significant part of most swine diets. Genistein and daidzein were found in plasma and urine of humans, rats, ruminants and pigs indicating the possibility of indirect or direct action on ovarian cells. Moreover, it was suggested that isoflavones are metabolized differently in pigs than in other animals, with more active forms remaining in the bloodstream [24, 32, 39].

Isoflavones were reported to bind estrogen receptors and have rather weak estrogenic activities compared to endogenous estrogens. However,
high isoflavone concentrations in diet could account for biological effects attributed to these phytoestrogens [2]. This seems to be especially true in view of the facts that genistein was demonstrated to exhibit higher affinity to ER$\beta$ than to ER$\alpha$ [22] and immunohistochemical studies revealed that, in contrast to ER$\alpha$, ER$\beta$ is abundant in porcine granulosa cells [33].

Gonadotropins and estrogens are the main endogenous factors involved in the regulation of follicular cell functions [14, 18]. Intracellular action of FSH may be affected by an array of different environmental estrogens including genistein. Moreover, genistein may act to mimic, amplify or antagonize estrogen action [38]. Studies on the intracellular mechanism of phytoestrogen action require creating an appropriate *in vitro* model. Granulosa cells appear to be a good choice because of their availability, relatively easy isolation and culture methods and fairly well recognized regulatory system.

Porcine granulosa cells are not a homogenous population. To examine the relationships between oocyte and follicular cells, granulosa cells are usually divided into cumulus oophorus cells and cells present in antrum and/or follicular wall [31]. When some steroidogenic properties are the focus of research, granulosa cells are separated into antral and mural subpopulations [10, 11, 17].

The objectives of the current study were: 1/ to establish an *in vitro* model of granulosa cell culture for studying the intracellular mechanism of phytoestrogen action in porcine ovary; 2/ to determine an *in vitro* effect of genistein on basal and FSH-stimulated $P_4$ and $E_2$ production by porcine granulosa cell populations (antral, mural, total) isolated from large, preovulatory follicles.

**MATERIAL AND METHODS**

**Isolation of granulosa cells**

Pig ovaries collected in a local slaughterhouse (Grupa Animex SA, Morliny, Ostróda) were kept in cold buffered physiological saline (PBS) supplemented with gentamycin and nystatin. Time from ovary excision to follicle processing
ranged from 60 to 90 min. Ovaries were placed in Petri dishes containing M199 medium with 5% bovine serum albumin (BSA). Granulosa cells were isolated from large (≥8 mm), preovulatory, non-atretic follicles according to modified method described by Stoklosowa et al 1978 [34]. Follicles were classified as non-atretic if they had extensive vascularization and were transparent with no free-floating particles in the follicular fluid. The entire follicles were dissected from ovaries and theca interna/granulosa layers were separated from external layers of follicular wall with a help of a pair of fine forceps. The remaining follicle was opened with forceps over a small flask collecting follicular fluid (FF) flowing out of the follicle. The follicular wall was placed in the flask together with FF, and granulosa cells attached to the theca layer were rinsed off by intensive pipetting (10 s). Supernatant containing granulosa cells was decanted. The cells were rinsed (medium M199) and decanted several times until supernatant became transparent. Then, the pellet (with theca layer) was discarded and granulosa cells were washed in M199 medium with 5% BSA, treated with red blood cell lysis buffer and again washed three times in M199 medium (10 min, 180×g).

The cells were filtered through a 70 µm nylon filter (Becton Dickinson Labware Europe, France) to yield two subpopulations of granulosa cells [12]. Cells that passed through the filter were designated as antral since they constituted of weakly associated cells present in antral granulosa layers. Cells remaining on the filter were designated as mural cells and constituted of tightly bound granulosa cells originating from the mural layers of the follicles [17]. Unseparated granulosa cells (total cells; TGC) as well as antral (AGC) and mural (MGC) cells were counted by haemocytometry. Cell viability was determined by 0.4% trypan blue dye exclusion (at the beginning of the culture; always higher than 97%) or by Alamar Blue test (after the end of the culture; fig. 8). All reagents were purchased in Sigma Chemical Co. (St. Louis, MO, USA) unless stated otherwise.

**Culture of granulosa cells**

In a series of preliminary experiments, conditions of cell culture were established. Granulosa cells (TGC, AGC, MGC) were resuspended (150
000 cells/ml) in incubation medium (Eagle’s + 10% calf serum, CS) containing gentamycin (0.05 mg/ml) and nystatin (120 U/ml). One milliliter of cell suspension was seeded into each individual well of a 24-well culture plate (Becton Dickinson Labware Europe, France). Cells were allowed to attach for 72 h (37°C, 10% CS, 95% air/5% CO₂). Following 48 h, when cells where cultured with treatments, the experiments were terminated and media were collected and stored at -20°C until radioimmunoassays (RIAs) of hormones were completed. The effect of cell type (TGC, AGC, MGC), serum concentration during the 48 h culture period (0, 5 or 10% CS; Biomed, Lublin, Poland) and FSH dose (0, 10 or 100 ng/ml) was determined. Genistein effect (0.5, 5, 50 µM) on basal and FSH (100 ng/ml)-stimulated P₄ and E₂ secretion were examined in all three granulosa cell preparations in the presence of 5% CS with other conditions as described above. Each treatment was examined in 4-5 independent experiments and particular treatments in each experiment were run in triplicates.

**Cell viability test**

Granulosa cell viability was measured with Alamar Blue vital dye [1]. The Alamar Blue (BioSource International, Nivelles, Belgium) test enables to monitor the innate metabolic activity of cells, which ceases in the presence of toxic compounds. To assess viability, granulosa cells resuspended in Eagle’s medium (Biomed, Lublin, Poland) supplemented with 10% CS, gentamycin (0.05 mg/ml) and nystatin (120 U/ml) were seeded into 96-well plates at a density of 20 000 cells/well. After 48 h in culture (37°C, 95% air/5% CO₂) cells were treated with genistein (0, 0.5, 5, 50 µM) or staurosporin (5 µM; a positive control) for subsequent 48 h. Twenty four hours prior to the end of treatment, Alamar Blue reagent was added at 10% of the culture volume (10 µl). Monolayers were assayed in a spectrophotometric microtiter well plate reader (Labsystem Multiskan EX, Finland) at two wavelengths (570 and 595 nm). Cell viability was expressed as percentage of Alamar Blue dye reduction. Each treatment was examined in 6 independent experiments and particular treatments in each experiment were run in triplicates.
Hormone assays

Medium P₄ and E₂ levels were determined by RIA. The validations of this assay for use in experiments with porcine ovarian cells have been reported previously [5]. Specificity of the anti-progesterone (SO/91/4) and anti-estradiol antibodies (BS/88/733) is described elsewhere [6, 35]. Exogenous treatments did not interfere with the RIA. Intra- and inter-assay coefficients of variation for P₄ were 3.75 and 2.45%, respectively. Intra- and inter-assay coefficients of variation for E₂ were 3.1 and 2.25%, respectively. Sensitivities of the P₄ and E₂ assays were 6 and 1 pg/tube, respectively. Serial dilutions of medium samples showed parallelism with the standard curves of examined steroids. All analyses were performed in triplicates.

Statistical analysis

Data are presented as mean±SEM. Analyses were performed using Statistica programs (StatSoft Inc., Tulsa, OK, USA). Due to variability among basal medium hormones levels all data were log transformed. One-way analysis of variance for repeated measurements was used to test the effects of genistein on basal (P₄, E₂) or FSH-stimulated (P₄) hormone concentrations in medium as well as the cell viability. Mean values of basal P₄ and E₂ concentrations in different granulosa cells (TGC, AGC and MGC) were compared by one-way ANOVA. The effect of FSH and granulosa cell type on P₄ level was examined by two-way ANOVA. Where significant effects were seen, post hoc test was used for multiple comparisons. Student’s t test was used to test the effect of FSH on P₄ level in media supplemented with 0, 5 or 10% of CS. Differences with a probability of p<0.05 were considered significant.

RESULTS

Basal P₄ production by antral, mural and unseparated granulosa cells isolated from porcine preovulatory follicles did not differ following 5-day culture in medium supplemented with 0, 10 (fig. 1) or 5% of CS (tab. 1). However,
Figure 1. Progesterone concentrations (mean±SEM) in medium collected following 5-day culture of antral (AGC), mural (MGC) and total (TGC) granulosa cells originated from porcine preovulatory (≥8 mm) follicles (n=4 independent experiments). The cells (150 000 cells/ml) were allowed to attach for 72 h (37°C, 95% air/5% CO₂) in Eagle’s medium supplemented with 10% calf serum and then treated with FSH (0, 10, 100 ng/ml) and cultured in the absence (A) or presence (B; 10%) of serum for subsequent 48 h. Bars without common superscripts are significantly different (p<0.05).
Table 1. Basal progesterone and estradiol concentrations (mean±SEM) in granulosa cell (150 000/well) populations cultured for 5 days in Eagle’s medium supplemented with 5% serum (n=4 independent experiments)

<table>
<thead>
<tr>
<th>Steroid hormone</th>
<th>Cell population</th>
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<tbody>
<tr>
<td></td>
<td>AGC</td>
</tr>
<tr>
<td>Progesterone (ng/ml)</td>
<td>85.6±44.7(^{a})</td>
</tr>
<tr>
<td>Estradiol (pg/ml)</td>
<td>32.0±5.0(^{ab})</td>
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Different superscripts designate significant differences at p<0.05

MGC cultured in the presence of 5% CS tended to secrete less P\(_{4}\) than other cell populations. FSH stimulated P\(_{4}\) production in a dose-dependent manner in all cell populations and culture systems, and the stimulation level was similar for AGC, MGC and TGC. Basal E\(_{2}\) production was similar in all granulosa cell types except MGC that secreted significantly less E\(_{2}\) than TGC.

*Figure 2. Estradiol concentrations (mean±SEM) in medium collected following 5-day culture of mural granulosa cells (MGC) originated from porcine preovulatory (≥8 mm) follicles (n=3 independent experiments). The cells (150 000 cells/ml) were allowed to attach for 72 h (37°C, 95% air/5% CO\(_{2}\)) in Eagle’s medium supplemented with 10% calf serum and then cultured in the absence or presence of FSH (10 or 100 ng/ml) and serum (10%) for subsequent 48 h. Asterisks depict a tendency (p<0.07) for the FSH doses stimulating estradiol production.
FSH did not affect E₂ production in any of AGC and TGC cultures, but tended (p=0.07) to stimulate E₂ in MGC cultures supplemented with 10% serum (fig. 2). Steroid concentration was always higher in medium containing serum than in serum-free medium. Reducing serum content in medium from 10 to 5% did not affect FSH ability to stimulate P₄ production in TGC cultures (fig. 3).

The effect of genistein on steroidogenic response of porcine granulosa cells was investigated in AGC, MGC and TGC cultures supplemented with 5% serum and 100 ng of FSH. Genistein inhibited in a dose dependent manner basal and FSH-stimulated P₄ production by all granulosa cell populations (figs. 4, 5 and 6). The lowest dose of genistein significantly affected only the FSH-stimulated P₄ production in TGC cultures.

The inhibition rate was similar in cultures of AGC and TGC. The mean medium concentration of P₄ in basal and FSH-stimulated AGC cultures...
Figure 4. Effect of increasing concentrations of genistein on basal and FSH-stimulated progesterone production (mean±SEM) by antral granulosa cells (AGC) originated from porcine preovulatory (≥8 mm) follicles (n=4 independent experiments). The cells (150 000 cells/ml) were allowed to attach for 72 h (37°C, 95% air/5% CO₂) in Eagle’s medium supplemented with 10% calf serum and then were cultured (5% serum) in the absence (-FSH) or presence of FSH (+FSH; 100 ng/ml) for subsequent 48 h. Bars without common superscripts are significantly different (p<0.05).
Figure 5. Effect of increasing concentrations of genistein on basal and FSH-stimulated progesterone production (mean±SEM) by mural granulosa cells (MGC) originated from porcine preovulatory (≥8 mm) follicles (n=4 independent experiments). The cells (150 000 cells/ml) were allowed to attach for 72 h (37°C, 95% air/5% CO₂) in Eagle’s medium supplemented with 10% calf serum and then were cultured (5% serum) in the absence (-FSH) or presence of FSH (+FSH; 100 ng/ml) for subsequent 48 h. Bars without common superscripts are significantly different (p<0.05).
Figure 6. Effect of increasing concentrations of genistein on basal and FSH-stimulated progesterone production (mean±SEM) by total granulosa cells (TGC) originated from porcine preovulatory (≥8 mm) follicles (n=4 independent experiments). The cells (150 000 cells/ml) were allowed to attach for 72 h (37°C, 95% air/5% CO₂) in Eagle’s medium supplemented with 10% calf serum and then were cultured (5% serum) in the absence (-FSH) or presence of FSH (+FSH; 100 ng/ml) for subsequent 48 h. Bars without common superscripts are significantly different (p<0.05).
treated with 5 and 50 µM of genistein represented, respectively, 28.5±15% and 9.8±5% of those found in the absence of genistein. The corresponding numbers for TGC cultures were 32.6±15% and 17.6±10.5%. In contrast, in culture medium of MGC treated with 5 and 50 µM of genistein, the mean P<sub>4</sub> concentrations were, respectively, 56±16% and 49.1±14% of those found in medium cultures without genistein.

Genistein did not affect E<sub>2</sub> production by granulosa cells under any studied conditions (fig. 7). In addition, viability of granulosa cells was not affected by 0.5 and 5 µM of genistein (fig. 8). Conversely, the highest dose of genistein (50 µM) as well as 5 µM of staurosporine (a positive control) decreased cell viability measured by Alamar Blue test.

Figure 7. Effect of increasing concentrations of genistein on estradiol production (mean±SEM) by total granulosa cells (TGC) originated from porcine preovulatory (≥8 mm) follicles (n=4 independent experiments). The cells (150 000 cells/ml) were allowed to attach for 72 h (37°C, 95% air/5% CO<sub>2</sub>) in Eagle’s medium supplemented with 10% calf serum and then cultured in the presence of 5% serum for subsequent 48 h. Bars with common superscripts are not significantly different (p>0.05).
DISCUSSION

Porcine granulosa cells cultured in the presence of serum differentiate and undergo luteinization. The cells within two days of culture cease to produce significant amounts of E₂ and start to produce more P₄. Moreover, they change morphologically and, with respect to E₂ secretion, become less responsive to FSH [4, 28]. In our study, granulosa cells from porcine preovulatory follicles, either separated into two subpopulations (AGC, MGC) or unseparated (TGC), after a five-day culture period produced marked and equal amounts of P₄. In addition, FSH significantly stimulated production of this steroid. This was true for all examined medium concentrations of serum. Similar results concerning basal and FSH-stimulated P₄ production by granulosa cell subpopulations isolated from porcine follicles were reported previously [12, 13, 17].
In the current study, the basal medium concentration of E\(_2\) did not differ among studied cell types (0 and 10% serum) or was lower in MGC than in TGC cultures (5% serum). FSH tended to stimulate E\(_2\) production by MGC, but not by the other cell populations. When Ford [12] and Ford and Howard [13] cultured granulosa cells from small follicles for 3-8 days in the presence of 10% serum and androstenedione (A\(_4\)), they found that FSH stimulated E\(_2\) production. This stimulation was higher in mural than antral cells. The authors also investigated granulosa cells from medium follicles which were cultured with A\(_4\) for 3-5 days [13, 17]. They demonstrated that, similar to small follicles, E\(_2\) production in mural cells was higher than that in antral cells. Moreover, FSH stimulated E\(_2\) production either exclusively in mural cells (10% serum) or this stimulation was higher in mural than in antral cells (0% serum). Both granulosa cell subpopulations isolated from large follicles [10] did not respond to FSH with elevated E\(_2\) secretion when they were incubated (24 h, 10% serum) in the absence of androgen substrate. Conversely, in the presence of testosterone (T), the E\(_2\) secretion was stimulated by FSH. In general, follicle size, cell type and culture conditions, including serum concentrations in medium, affect steroidogenic features of porcine granulosa cells. It appears that 1/ granulosa responsiveness to FSH with respect to E\(_2\) secretion was affected by presence of androgen substrate in medium and cell type; 2/ independently on culture conditions (serum content, days of culture) and follicular size, FSH stimulated P\(_4\) secretion in all granulosa cell populations in pigs.

In view of data presented above we have decided to examine the effect of genistein on steroidogenic response of AGC, MGC and TGC cultured in medium supplemented with 5% serum and 100 ng of FSH. In addition, we believe that the luteinized granulosa cell cultures provide a suitable in vitro model for our future research pertaining with elucidation of the intracellular mechanism of phytoestrogen action in porcine ovarian cells.

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In the current paper, genistein inhibited, in a dose dependent manner, basal and FSH-stimulated P\(_4\) production by luteinized granulosa cells harvested from large, preovulatory porcine follicles. The cells were cultured (for five days) in the presence of 0.5-50 µM genistein and 5% serum (for two days). Similar doses of genistein were found to inhibit
basal, forskolin-[36] and FSH-[29] stimulated $P_4$ production by luteinized rat granulosa cells cultured (for three days) in the absence of serum (for two days). These data are supported by the results of in vitro studies which were aimed to investigate the involvement of genistein, a protein tyrosine kinase inhibitor, in the regulation of steroidogenic function of several cell types. The short term incubations (8-24 h) demonstrated that genistein (1-50 µM) inhibited prolactin-stimulated $P_4$ production by porcine theca, luteal [6, 7, 15] and adrenocortical [20] cells as well as testicular cells of rooster [30]. These results support the notion that genistein-related in vitro inhibition of $P_4$ secretion is not dependent on species, cell type, and presence of serum or incubation/culture time.

In contrast, Haynes-Johnson et al [16] reported that only high doses of genistein inhibited FSH-stimulated $P_4$ production by granulosa cells isolated from follicles of immature, diethylstilbestrol-treated rats. The stimulatory effect of genistein observed in this study, in both the presence or absence of serum, was demonstrated for doses ranging from 0.3 to 3 µM. Similar doses, however, were found to inhibit $P_4$ production in another study performed on rat granulosa cells [36]. It is possible that this inconsistency was caused by the presence of $A_4$ in the culture medium in the former experiment. Androgens are known to stimulate granulosal $P_4$ secretion in rats [8, 19]. It cannot be excluded that the addition of $A_4$ to the medium masked the inhibition caused by low doses of genistein.

Biphasic mode of genistein action was also observed by Kaplanski et al [21] who examined the effect of genistein and biochanin A on basal $P_4$ production by bovine granulosa cells (24 h, 5% serum) from large follicles. They found that genistein doses of 185 pM stimulated, whilst those higher than 1850 pM inhibited $P_4$ synthesis. The phytoestrogen doses used in this experiment were extremely low. In one of our recent experiments, 50 nM of genistein did not affect steroid production by granulosa cells from growing or large porcine follicles (Ciereszko et al, unpublished). It appears that the effect of such low concentrations of genistein (pM) should be tested additionally in follicular cells other than bovine.

Concentrations of genistein ranging from 37 nM to 37 µM were reported to stimulate basal $P_4$ production by bovine and rabbit granulosa
cells [26]. Unfortunately, many details concerning cell culture conditions were omitted in this report. In addition, no effect of genistein on $P_4$ secretion by whole porcine ovarian follicles (5 mm) was found in this study. Taken together, despite the data presented by Makarevich et al [26] and Haynes-Johnson et al [16], the majority of authors demonstrated the inhibitory *in vitro* action of genistein on steroidogenic cells. The fact that genistein and other phytoestrogens may inhibit the activity of 3$\beta$-hydroxysteroid dehydrogenase, an enzyme involved in $P_4$ synthesis, in luteinized human granulosa cells [23, 37] additionally strengthens such conjecture.

The inhibitory effect of genistein could not be attributed to its cytotoxic properties since viability of granulosa cells was not affected by 0.5 and 5 $\mu$M of the phytoestrogen. The latter dose of genistein was found to be effective in reducing $P_4$ production. Only the highest dose lowered the cell viability, although in a degree markedly less than that caused by 5 $\mu$M of staurosporine.

Basal estradiol production by luteinized granulosa cells isolated from large, preovulatory porcine follicles was not affected by genistein in this study. Genistein, however, inhibited basal and gonadotropin-stimulated $E_2$ production by human and rat granulosa cells [16, 37]. In contrast, Makarevich et al [26] demonstrated the stimulatory effect of genistein on $E_2$ production by rabbit granulosa cells and whole porcine follicles. This stimulation as well as the observed lack of genistein effect on $E_2$ in our study are difficult to explain in a view of several studies demonstrating the genistein-induced inhibition of 17$\beta$-hydroxysteroid dehydrogenase and aromatase [3, 9, 23, 27], enzymes involved in synthesis of $E_2$.

The similar pattern of genistein action on steroid production was observed in all examined granulosa cell populations. The inhibitory effect on basal and FSH-stimulated $P_4$ production as well as the lack of effect on $E_2$ was demonstrated for AGC, MGC and TGC. The only difference observed was a less pronounced reduction in $P_4$ secretion found in genistein-treated MGC in comparison to AGC and TGC.

In summary, basal $P_4$ and $E_2$ production did not differ among antral, mural and unseparated granulosa cells isolated from porcine preovulatory follicles. Only mural cells tended to secrete less $P_4$ and $E_2$ than other cell populations. FSH stimulated $P_4$ production in a dose-dependent manner.
in all cell populations and culture systems. Genistein inhibited in a dose-dependent manner basal and FSH-stimulated \( P_4 \) production by antral, mural and unseparated granulosa cells. However, genistein did not affect \( E_2 \) production by granulosa cells. In addition, viability of porcine granulosa cells was not affected by the phytoestrogen except the highest dose of genistein. It appears that genistein may be involved in the regulation of follicular function in pigs. Moreover, unseparated porcine granulosa cells may provide a suitable in vitro model for studying the intracellular mechanism of phytoestrogen action in porcine ovary.

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