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# The Effects of Phytoestrogen Genistein on Steroidogenesis and Estrogen Receptor Expression in Porcine Granulosa Cells of Large Follicles*

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Genistein is a biologically active isoflavone with estrogenic or antiestrogenic activity which can be found in a variety of soy products. Since in pigs' diet soy is the main source of protein, genistein may affect the reproductive/endocrine systems in these animals. Genistein has been shown to alter porcine ovarian and adrenal steroidogenesis but the mechanism of this action is still not clear. It is known that genistein binds to both estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ), although it has a higher affinity to ERβ. Moreover, this phytoestrogen was demonstrated to posses the activity of protein tyrosine kinase (PTK) inhibitor. The aim of the study was to examine the *in vitro* effects of genistein on: (1) progesterone ($P_4$) and estradiol ($E_2$) secretion by porcine luteinized granulosa cells harvested from large follicles, and (2) the mRNA and protein expression of ERα and ERβ in these cells. In addition, to verify the role of PTK–dependent mechanisms possibly involved in genistein biological action, we tested the effects of lavendustin C, the nonsteroidal PTK inhibitor, on granulosa cell steroidogenesis. Genistein significantly inhibited $P_4$ and did not affect $E_2$ secretion by porcine luteinized granulosa cells isolated from large follicles. Lavendustin C did not affect basal steroids secretion by examined cells. Genistein did not alter ERα but increased ERβ mRNA levels in the cultured porcine granulosa cells. In contrast to medium follicles, the expression of ERβ protein was unaffected by genistein in granulosa cells of large follicles. To conclude, the soy phytoestrogen genistein acts directly on the porcine ovary to decrease progesterone production and to increase the expression of ERβ mRNA. Moreover, genistein-induced changes in follicular steroidogenesis and granulosal sensitivity to estrogens in pigs may depend on maturity of the follicles.

Key words: Phytoestrogen, genistein, lavendustin, estrogen receptors, steroidogenesis, pig.

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Genistein is one of the most abundant estrogenic compounds present in soy and soy-derived food which are often consumed as an alternative to hormonal therapy by menopausal women (HAJIRAHIMKHAN *et al.* 2013). Soy is also used in pig diets as a main protein source and it is known to contain not only genistein but also other phytoestrogens (PE) (KRASZEWSKA *et al*. 2007). Phytoestrogens were found to affect the functions of female and male reproductive systems in many species (for review see: CIERESZKO *et al.* 2007; KURZER *et al*. 1997). Specifically, they were reported to cause reproductive disorders in humans, especially during the period of reproductive activity, and in farm animals including pigs (CIERESZKO *et al*. 2007; DUSZA *et al*. 2006). It was found that PE inhibit progesterone ($P_4$) secretion by porcine (NYNCA *et al.* 2009; NYNCA *et al.* 2013a,b,c; TIEMANN *et al.* 2007) as well as human (WHITEHEAD *et al.* 2002) granulosa cells which may influence ovarian function.

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Genistein affects cellular function *via* estrogen receptor (ER)-dependent and ER-independent mechanisms. The influence of estrogens is usually mediated by ER (ERα and ERβ), intracellular levels of which are tightly regulated by complex mechanisms (MARINO *et al.* 2012; BYERS *et al.* 1997). Genistein may directly bind to the ER already present in the cell and activate or inactivate the ER-dependent pathways, and they may modulate the intracellular ER levels (mRNA and protein). The ER-independent mechanisms may, in turn, involve changes in the activity of tyrosine kinases (PTK) and mitogen-activated protein kinases. In addition, genistein action may result from its antiproliferative and anti-angiogenic properties (BENASSAYAG *et al*. 2002). Genistein was also reported to modulate the activity of steroidogenic enzymes such as aromatase, 3β-hydroxysteroid dehydrogenase (3β-HSD) and 17β-hydroxysteroid dehydrogenase (17β-HSD) (WHITEHEAD *et al*. 2006). Therefore, the overall biological action of genistein and other PE in the target cells is probably the result of a very complex interplay of various mechanisms, which are determined by the cell type, balance between the ER subtypes and genistein administration route and dose.

Phytoestrogens have been shown to affect ovarian and adrenal steroid production. Previously we demonstrated the inhibitory effects of PE on $P_4$ secretion by porcine granulosa cells (NYNCA *et al.* 2009; NYNCA *et al.* 2013a,b,c). Moreover, genistein and daidzein suppressed cortisol production and stimulated androstenedione production by porcine adrenocortical cells isolated during the follicular and luteal phase of the estrous cycle (KAMIŃSKA *et al*. 2012). It was also found that genistein mechanism of action did not involve the suppression of PTK since non-steroidal PTK inhibitors did not affect steroidogenesis in adrenocortical cells (KAMIŃSKA *et al*. 2012) or granulosa cells harvested from medium follicles (NYNCA *et al.* 2013b). Since granulosa cell function may depend on biological/endocrine maturity (medium *vs*. large follicles), we decided to study the influence of genistein on granulosa cells harvested from large, preovulatory follicles and compare the results of the current study with those obtained previously on medium, growing follicles. Therefore, the objectives of the study were to evaluate the *in vitro* effects of genistein on $P_4$ and estradiol ($E_2$) secretion by porcine luteinized granulosa cells isolated from large (≥8 mm) follicles. In the present study we also examined the effects of genistein on mRNA and protein expression levels of ERα and ERβ in these cells. To verify the role of tyrosine kinase-dependent mechanism in genistein biological action, we additionally tested the effects of the well known tyrosine kinase inhibitor, lavendustin C, on granulosa cell steroidogenesis.

## Material and Methods

### Chemicals

Cell culture supplies, genistein, medium M199, nystatin, red blood cells lysing buffer and trypan blue solution were obtained from Sigma (St. Louis, MO, USA). Labeled (2,4,6,7-$^3$H) 17-estradiol and (1,2,6,7-$^3$H) progesterone were purchased from Amersham Pharmacia Biotech (Little Chalfont, Great Britain). Eagle's medium and calf serum (CS) were used from Biomed (Lublin, Poland), bovine serum albumin (BSA) from ICN Biomedicals (Irvine, CA, USA), gentamycin from KRKA (Novo Mesto, Slovenia) and culture plates from Becton Dickinson Labware Europe (Le Pont de Claix Cedex, France). The cell viability test was performed with alamarBlue™ dye (BioSource International, Cammarillo, CA, USA). For total RNA isolation from cells TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) was used and for RT-PCR Omniscript Reverse Transcription Kit and HotStarTaq Master Mix Kit (Hilden, Germany). The VECTASTAIN ABC System were obtained from Vector Laboratories (Burlingame, CA, USA) and 3,3'-diaminobenzidine from Dako (Glostrup, Denmark).

### Cell cultures and experimental design

All experiments were performed according to the ethical standards of the Animal Ethics Committee at the University of Warmia and Mazury in Olsztyn (permission no. 11/2010/DTN). Porcine ovaries with large, preovulatory (≥8 mm in diameter) follicles were collected in a local slaughterhouse (Krokowo/Jeziorany, Poland) and transported promptly in cold buffered physiological saline (PBS) supplemented with gentamycin (0.05 mg/ml) and nystatin (120 U/ml). Ovarian and follicular morphology were evaluated (AKINS & MORRISSETTE 1968) and granulosa cells were isolated from the ovaries as described previously (NYNCA *et al.* 2009). All stages of experiments were performed in sterile conditions. Cell viability (≥97%) was determined by 0.4% trypan blue dye exclusion. Cells were incubated in Eagle's medium containing 5% calf serum (CS), gentamycin (0.05 mg/ml) and nystatin (120 U/ml). Aliquots of granulosa cells were cultured in: 1/ 96-well plates ($0.2\times10^5$ cells/0.1 ml/well) to measure cell viability; 2/ 24-well plates ($1.5\times10^5$ cells/1 ml/well) to measure steroid secretion and to assess the level of ER proteins; and 3/ 6-well plates ($2\times10^6$ cells/3 ml/well) to analyze the level of ER mRNA. Following 48-72 h of preculture (37°C, 10% CS, 95% air/5% $CO_2$), cells were cultured with treatments (5% CS) for subsequent 48 h. When the experiments were terminated, the media (-20°C) and/or the cells (-80°C)

were collected and stored until all assays were completed. Genistein and lavendustin C were dissolved in ethanol. The highest concentration of ethanol (0.5%) did not affect the examined parameters (granulosa cell viability, steroid hormone secretion, ERs mRNA and protein expression).

### The effect of genistein on the viability of granulosa cells

Cell viability was determined after treatment of genistein with alamarBlue™ reagent (BANNERMAN *et al*. 2001). After preincubation (48 h), monolayers of granulosa cells were cultured for 48 h with or without genistein (0.05, 0.5, 5 or 50 $\mu$M) or staurosporin (STS; a positive control; 5 $\mu$M). Twenty four hours before the end of cell culture, alamarBlue™ dye was added to all wells. The medium was collected 24 h later and alamarBlue™ reduction was measured spectrophotometrically at 565 and 595 nm and expressed as a percentage according to the manufacturer's calculations. All analyses were performed in quadruplicate.

### The effects of genistein and lavendustin C on steroid hormone secretion

For estimation of $P_4$ and $E_2$ secretion, granulosa cell monolayers were preincubated for 72 h and then cultured for 48 h with or without genistein (0.05, 0.5, 5 or 50 $\mu$M) in the absence or presence of luteinizing hormone (LH; 100 ng/ml). To compare the effects of genistein and lavendustin C (a protein tyrosine kinase inhibitor that is not a phytoestrogen) on steroid hormone production, cells were also incubated with lavendustin C (0.05, 0.5, 5 or 50 $\mu$M) and/or LH (100 ng/ml). In all experiments, medium without treatments served as a control. Medium concentrations of steroid hormones were measured by a previously validated $^3$H-radioimmunoassays (CIERESZKO *et al*. 1998, 2001; SZAFRAŃSKA *et al*. 2002) Intra- and inter-assay coefficients of variation for $P_4$ were 3.75 and 2.45%, respectively. Intra- and inter-assay coefficients of variation for $E_2$ were 3.1 and 2.25%, respectively. Sensitivities of the $P_4$ and $E_2$ assays were 6 and 1 pg/tube, respectively, and were not altered by treatments. Serial dilutions of medium samples showed parallelism with the standard curves of examined steroids. All analyses were performed in triplicate.

### The effects of genistein on ERα and ERβ mRNA expression

The levels of ERα and ERβ mRNA expression were measured in luteinized granulosa cells preincubated for 48 h and then cultured for subsequent 48 h with or without genistein (0.5 or 5 $\mu$M). Total RNA was extracted from luteinized granulosa cells after the culture using TRIzol Reagent. For cDNA synthesis, 1 $\mu$g of RNA was reverse transcribed in a 20 $\mu$l reaction volume with 0.5 $\mu$g oligo(dT)$_{15}$ primer (Roche, Penzberg, Germany) using the Omniscript RT Kit (Hilden, Germany). Complementary DNA was amplified by polymerase chain reaction (PCR; GeneAmp PCR System 2400, Perkin Elmer, Waltham, MA, USA) in a total volume of 50 $\mu$l using 40 pmol of porcine ERα (MUTEMBEI *et al*. 2005) or ERRβ (PFAFFL *et al*. 2001) sense and antisense primer pairs: ERα sense 5'AGGGAGAGGAGTTTGTGTG 3' and antisense 5'TCTCCAGCAGCAGGTCATAG 3'; ERβ sense 5'GCTTCGTGGAGCTCAGCCTG 3' and antisense 5'AGGATCATGGCCTTGACACAGA 3'. To provide an appropriate internal control, coamplification of GAPDH was carried out in each sample using the GAPDH-sense (5'ATGGTGAAGGTCGGAGTGAA 3') and antisense (5'CTTGGCAGCGCCGGTAGAAGC 3') primer pair. Amplification tubes also contained 5 $\mu$l of the first strand cDNA, 25 $\mu$l of HotStarTaq Master Mix (2.5 U HotStarTaq DNA Polymerase, 1×PCR buffer containing 1.5 mM $MgCl_2$, 200 $\mu$M of each dNTP). The optimal number of cycles, ensuring the termination of amplification for the genes in the log phase was established by primer dropping method (WONG *et al*. 1994): 1) 40 and 30 cycles were employed for ERα and GAPDH, respectively, and 2) 38 and 26 cycles were employed for ERβ and GAPDH, respectively. PCR reactions were performed under the following cycling conditions: initial denaturation at 95°C for 15 min, an appropriate number of cycles including denaturation at 95ºC for 20 s, annealing at 59ºC (for ERα gene) or 58ºC (for ERβ gene) for 30 s and elongation at 72ºC for 1 min, followed by a final extension at 72ºC for 7 min. Negative controls were performed without reverse transcriptase and for each pair of primers the non-template controls were carried out.

Aliquots of PCR reaction products were electrophoresed on a 1.5% agarose gel stained with ethidium bromide and visualized under ultraviolet illumination. Gel images were saved by FOTO/Analist Achiever software (Fotodyne, Hartland, WI, USA) and product yield was determined using GelScan for Windows ver.1.45 software (Kucharczyk, Warszawa, Poland). Data were expressed as a ratio of ERα or ERβ mRNA relative to GAPDH mRNA in arbitrary optical density units (OD).

In addition, the PCR-amplified DNA was sequenced (by DNA Sequencing and Synthesis Laboratory, Institute of Biochemistry and Biophysics, Polish Academy of Science, Warsaw, Poland) in both directions to confirm the accuracy of amplification. Comparison of the PCR-amplified

DNA sequence to that in the database indicated 100 and 99% homology for ERα and ERβ, respectively, at the nucleotide level.

### The effects of genistein on ERα and ERβ protein expression

ERα and ERβ protein expression in luteinized granulosa cells preincubated for 72 h and then cultured for subsequent 48 h with or without genistein (0.05, 0.5, 5 or 50 $\mu$M) was assessed by immunocytochemistry. Estrogen receptor α and β proteins were detected in granulosa cells plated on Thermanox™ Coverslips (Nunc, Roskilde, Denmark) using primary mouse monoclonal antibodies against ERα (Dako, Glostrup, Denmark) or ERβ (Serotec, Kidlington, Great Britain) at a dilution of 1:100 (PETTERSSON *et al*. 1997; SLOMCZYNSKA *et al*. 2001) or 1:20 (SAUNDERS *et al*. 2000; SLOMCZYNSKA *et al*. 2001), respectively. After culture, luteinized granulosa cells were rinsed in PBS and fixed in 4% paraformaldehyde. Then, following washing in PBS, cells were incubated with 0.01% Triton X-100 in PBS for 1 min to permeabilize the cell membranes. Thereafter, to quench endogenous peroxidase activity, cells were incubated with 0.3% $H_2O_2$ in PBS for 20 min, and then in 5% normal horse serum in Tris-buffered saline plus Tween-20 (TBST) for 50 min to block the non-specific binding of the secondary antibody. Subsequently, cells were incubated overnight with primary antibodies, followed by incubation with biotinylated horse anti-mouse antibody (Vector Laboratories, Burlingame, CA, USA) at a 1:300 dilution in TBST for 60 min. Next, the cells were treated with streptavidin-horseradish peroxidase complex (ABC/HRP; Vector Laboratories, USA) at a 1:100 dilution in TBST for 50 min. The color reaction was developed for 5–10 min in diaminobenzidine (DAB) solution (Dako, Glostrup, Denmark). The specificity of immunostaining for ERα and ERβ was tested by omitting the primary antibodies (Fig. 7F). Since no expression of ERα protein was detected in granulosa cells, porcine uterine slices were examined immunohistochemically to additionally analyze the specificity of the used antibodies. Images were recorded for data analysis using a CH30/CH40 microscope and a C-5060 WZ digital camera (Olympus, Tokyo, Japan). The staining distribution (number of stained cells and staining intensity) was quantified using 5 Soft Imaging System (Olympus, Tokyo, Japan). All treatments were run in duplicate and repeated in four separate experiments. Six pictures of the stained cells were taken from each duplicate. The pictures were always taken from the same precisely defined six areas of the coverslip. Data from each duplicate were archived, analyzed and expressed as a number of stained cells (%) and as the intensity staining of luteinized granulosa cells nuclei (arbitrary units; range: 0-255).

### Statistical analysis

Analyses were performed using Statistica program (StatSoft Inc., Tulsa, OK, USA). The raw hormone concentrations data were log transformed and then statistically analyzed. Data expressed as a percentage of the number of stained cells were arcsine transformed before the statistical analyses. Amounts of $P_4$ secreted in the absence (control) or presence of LH were compared by the Student's *t*-test. All other data were analyzed by one-way ANOVA for repeated measurements followed by the least significant difference (LSD) post hoc test. The level of significance was set at $P<0.05$ for all analyses. RIA data are expressed as a percentage of the control culture (100%); all other data are presented as mean±SEM.

## Results

### The effect of genistein on the viability of granulosa cells

Genistein at concentrations ranging from 0.05 to 5 $\mu$M did not affect the viability of porcine luteinized granulosa cells harvested from large, preovulatory follicles (Fig. 1). A significant decrease ($P<0.05$) in cell viability was, however, seen when the highest dose of genistein (50 $\mu$M) was used. Moreover, a positive control (5 $\mu$M, staurosporin) caused a prominent decrease in the viability of the granulosa cells.

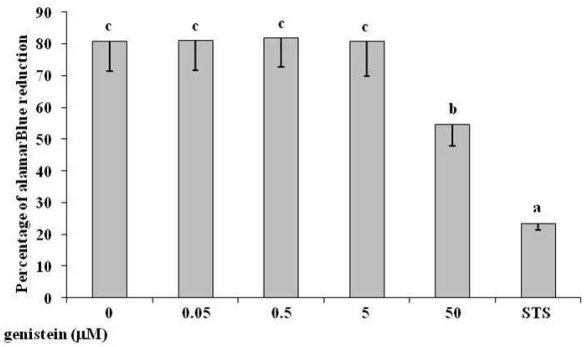


Fig. 1. The effect of genistein on porcine granulosa cell viability (mean±SEM) determined by alamarBlue test (n=4) and expressed as a percentage of alamarBlue dye reduction. Following preincubation (48 h), the cells were cultured for 48 h ($0.2\times10^5$ cells/100 $\mu$l Eagle's medium) with genistein (0.05-50 $\mu$M) and staurosporin (STS; 5 $\mu$M; a positive control). Bars with different superscripts depict significant differences ($P<0.05$).

### The effects of genistein and lavendustin C on steroid hormone secretion

Basal $P_4$ level (mean ± SEM) in the control granulosa cell medium was 30.8 ± 21.1 ng/mL. LH stimulated ($P<0.05$; 398.5 ± 201.8 ng/ml) $P_4$ secretion by porcine luteinized granulosa cells (data not shown). Genistein inhibited ($P<0.05$) basal $P_4$ production by luteinized granulosa cells cultured with the phytoestrogen for 48 hours (0.5-50 $\mu$M; Fig. 2A). In the presence of LH in the medium, the highest concentration of genistein (50 $\mu$M) also decreased ($P<0.05$) $P_4$ secretion (Fig. 2B). The inhibition caused by the remaining concentrations of genistein were not significant. In contrast, lavendustin C (0.05-50 $\mu$M) did not affect basal $P_4$ secretion by granulosa cells (Fig. 3A). Only two doses of lavendustin C (0.05 and 0.5 $\mu$M) inhibited the LH-stimulated $P_4$ secretion by luteinized granulosa cells originated from large follicles (Fig. 3B).

Basal $E_2$ level (mean ± SEM) in control granulosa cell medium was 45.2 ± 11 pg/ml. Genistein did not affect $E_2$ secretion by granulosa cells cultured for 48 h (Fig. 4A and 4B). In contrast to genistein, the highest dose of lavendustin (50 $\mu$M) inhibited basal $E_2$ secretion (Fig. 5A).

### The effects of genistein on ERα and ERβ mRNA expression

Genistein did not affect ERα mRNA expression in cultured porcine granulosa cells (Fig. 6A). In contrast, transcript concentration of ERβ gene was significantly higher ($P<0.05$) in the cells cultured with 5 $\mu$M of genistein comparing to controls (Fig. 6B). The lower dose of genistein (0.5 $\mu$M) had a tendency ($P=0.07$) to increase the ERβ mRNA level.

### The effects of genistein on ERα and ERβ protein expression

Using immunocytochemistry we found that ERβ protein was present in luteinized granulosa cells isolated from large porcine follicles. The staining was located predominantly in the nuclei of the cells (Fig. 7). In contrast, the immunoreaction of ER was not observed in these cells. Due to the absence

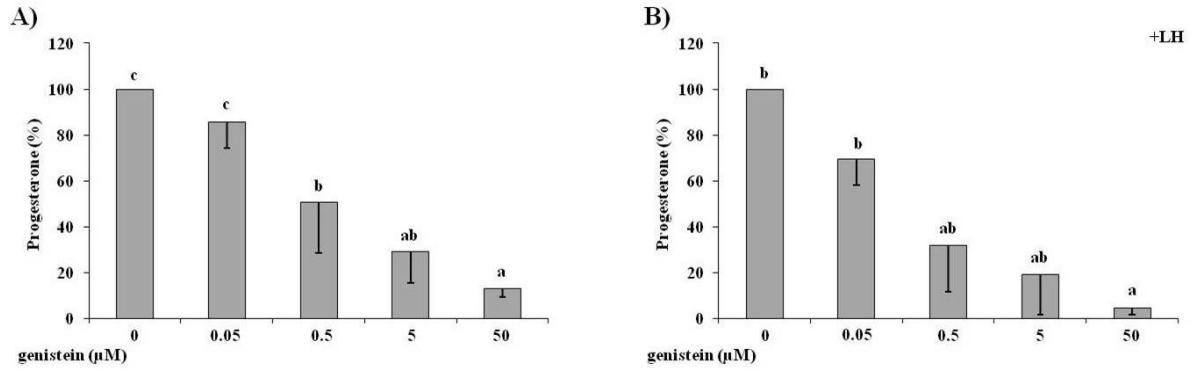


Fig. 2. The effects of genistein on basal (A) and LH-stimulated (B) $P_4$ secretion (mean±SEM) by porcine luteinized granulosa cells originated from large follicles (n=4). Following preincubation (72 h), the cells were cultured for 48 h ($1.5\times10^5$ cells/ml; 5% calf serum) with genistein (0.05-50 $\mu$M) or genistein and LH (100 ng/ml). Data are expressed as a percentage of control culture (100%). One-way ANOVA for repeated measurements was performed on log transformed data. Bars with different superscripts depict significant differences ($P<0.05$).

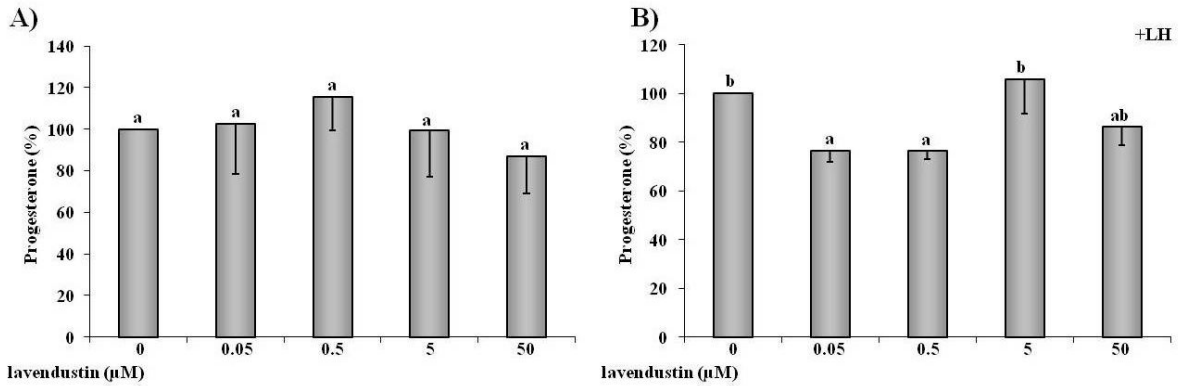


Fig. 3. The effects of lavendustin C on basal (A) and LH-stimulated (B) $P_4$ secretion (mean±SEM) by porcine luteinized granulosa cells originated from large follicles (n=4). Following preincubation (72 h), the cells were cultured for 48 h ($1.5\times10^5$ cells/ml; 5% calf serum) with lavendustin C (0.05-50 $\mu$M) or lavendustin C and LH (100 ng/ml). Data are expressed as a percentage of control culture (100%). One-way ANOVA for repeated measurements was performed on log transformed data. Bars with different superscripts depict significant differences ($P<0.05$).

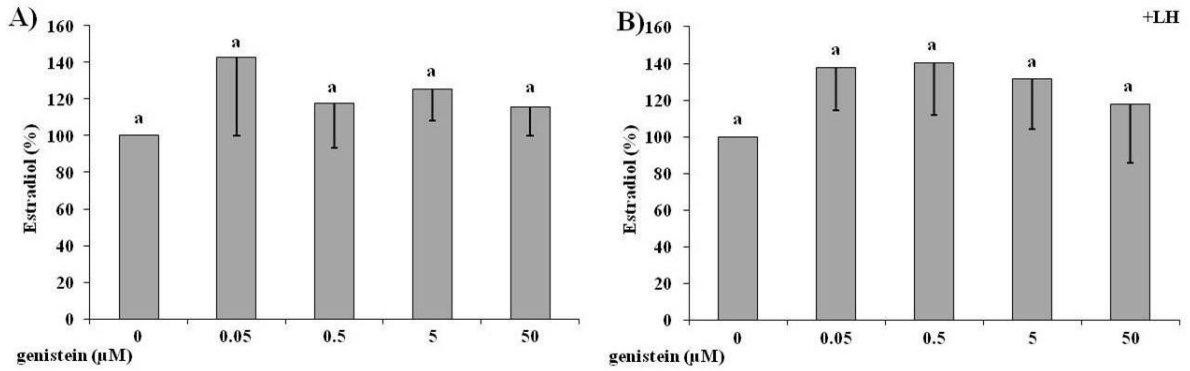


Fig. 4. The effects of genistein on basal (A) and LH-stimulated (B) $E_2$ secretion (mean±SEM) by porcine luteinized granulosa cells originated from large follicles (n=4). Following preincubation (72 h), the cells were cultured for 48 h ($1.5\times10^5$ cells/ml; 5% calf serum) with genistein (0.05-50 $\mu$M) or genistein and LH (100 ng/ml). Data are expressed as a percentage of control culture (100%). One-way ANOVA for repeated measurements was performed on log transformed data. Bars with different superscripts depict significant differences ($P<0.05$).

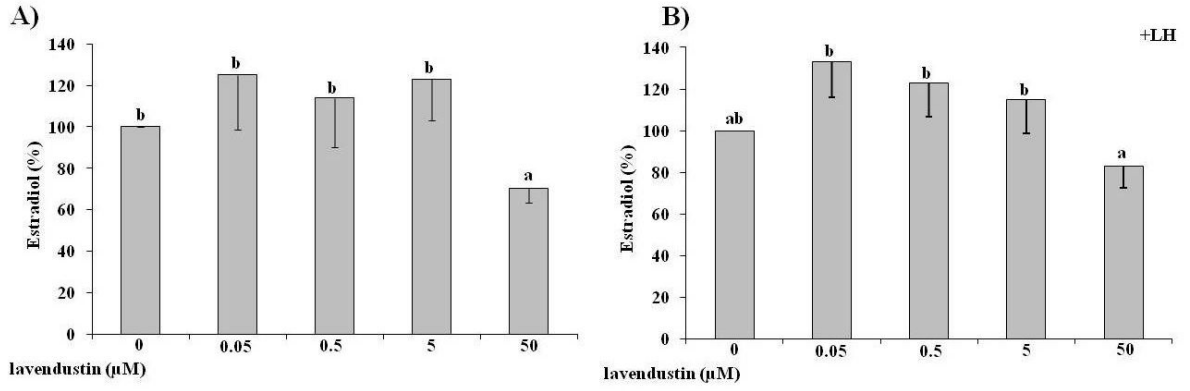


Fig. 5. The effects of lavendustin C on basal (A) and LH-stimulated (B) $E_2$ secretion (mean±SEM) by porcine luteinized granulosa cells originated from large follicles (n=4). Following preincubation (72 h), the cells were cultured for 48 h ($1.5\times10^5$ cells/ml; 5% calf serum) with lavendustin C (0.05-50 $\mu$M) or lavendustin C and LH (100 ng/ml). Data are expressed as a percentage of control culture (100%). One-way ANOVA for repeated measurements was performed on log transformed data. Bars with different superscripts depict significant differences ($P<0.05$).

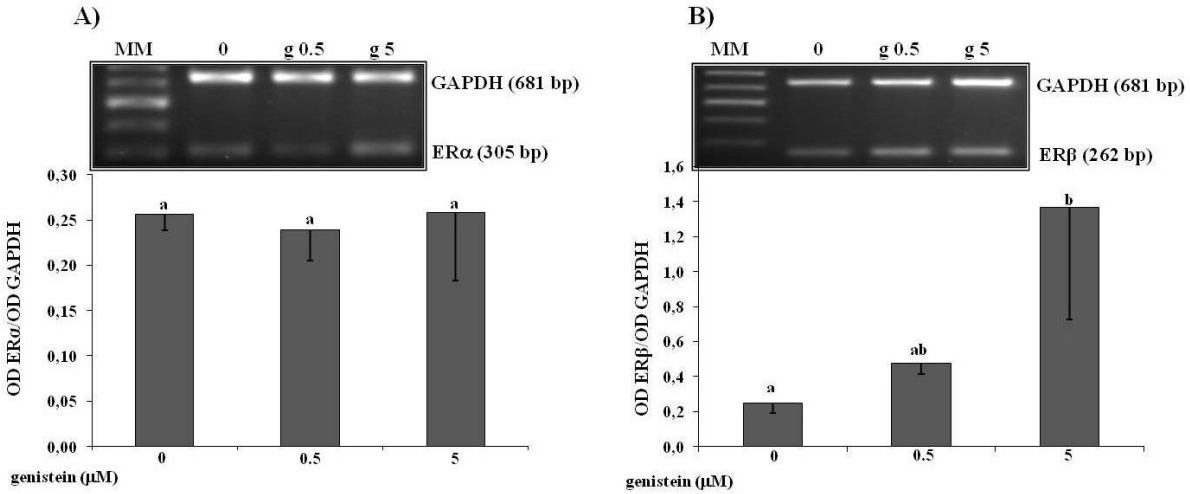


Fig. 6. The effects of genistein on estrogen receptor α (ERα; A) and estrogen receptor β (ERβ; B) mRNA expression determined by semiquantitative RT-PCR in porcine luteinized granulosa cells originated from large follicles (n=4). Upper panels show representative images of agarose gels; MM: molecular marker, g: genistein, bp: base pairs, GAPDH: housekeeping gene. Lower panels show the results of densitometric analysis of ERα mRNA and ERβ mRNA relative to GAPDH mRNA. Values are expressed as means±SEM of arbitrary optical density units (OD). Bars with different superscripts depict significant differences ($P<0.05$).

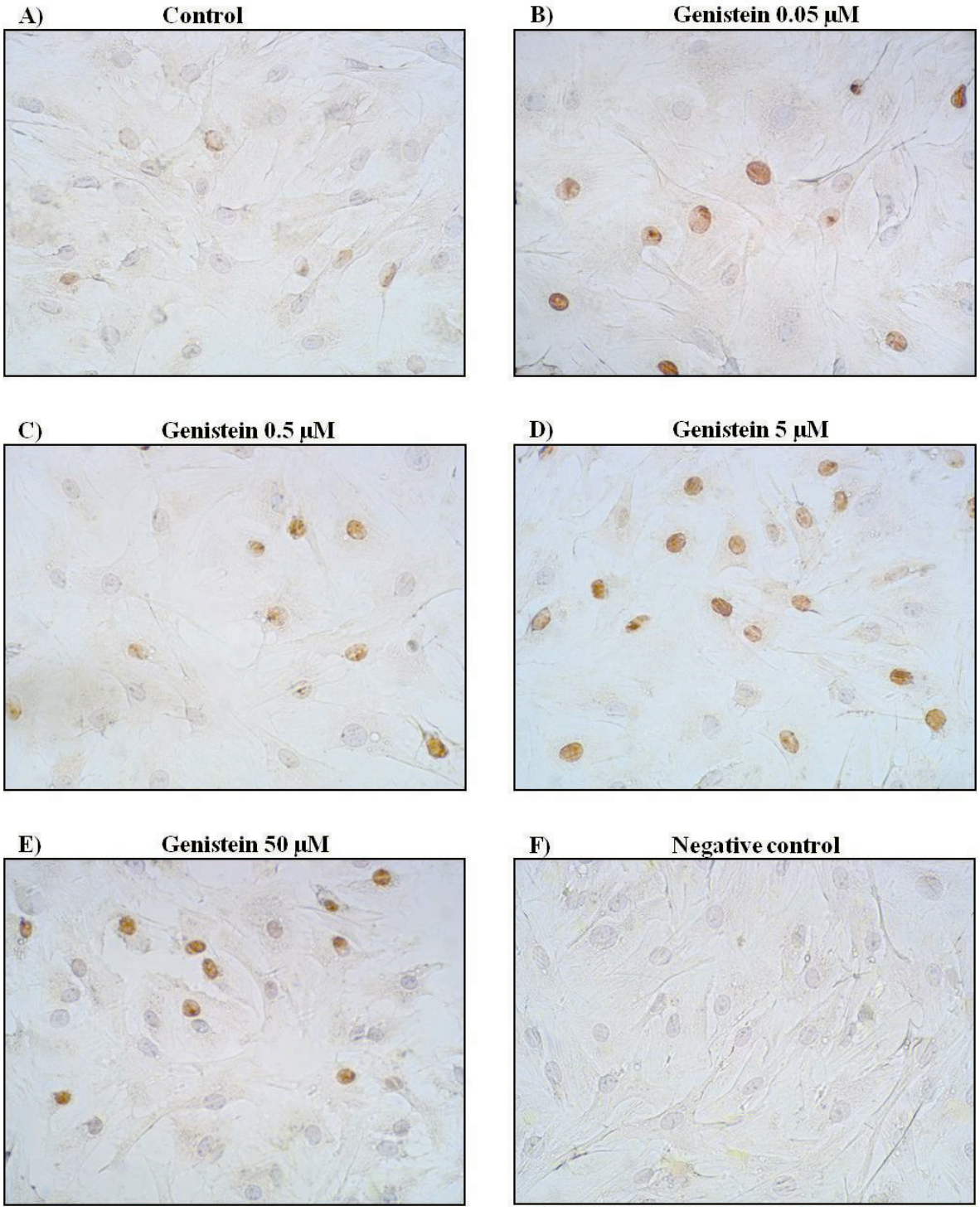


Fig. 7. Exemplary images of immunoexpression of ERβ determined by immunocytochemistry in porcine luteinized granulosa cells originated from large follicles (n=4). A – cells without treatments, B-E – cells treated with genistein (0.05-50 $\mu$M), F – a negative control. Bar = 5 $\mu$m.

of the ER protein in the examined granulosa cells, the effects of genistein on ER protein expression could be examined only with respect to ERβ. No effect of genistein on the percentage of the ERβ positively stained granulosa cells and the intensity of cell nuclei immunostaining was observed (Table 1).

## Discussion

The current study demonstrated that genistein (0.5-50 $\mu$M) inhibited $P_4$ secretion by porcine luteinized granulosa cells isolated from large, preovulatory follicles. Genistein (0.5-50 $\mu$M) also

Table 1

The effect of genistein on the percentage of the stained granulosa cells and on the intensity of the ERβ immunostaining (mean±SEM) in the granulosa cells harvested from the large porcine ovarian follicles (n=4)

| Genistein ($\mu$M) | Percentage of the stained cells (%) | | Intensity of the immunostaining (arbitrary units) | |
|---|---|---|---|---|
| | Mean | SEM | Mean | SEM |
| 0 | 38.2 | 20.8 | 140.5 | 23.3 |
| 0.05 | 47.1 | 6.6 | 153.0 | 19.9 |
| 0.5 | 43.5 | 9.9 | 201.1 | 50.4 |
| 5 | 49.1 | 8.9 | 152.9 | 20.5 |
| 50 | 34.3 | 7.0 | 158.4 | 18.1 |

decreased production of $P_4$ in granulosa cells from pig medium follicles (NYNCA *et al.* 2013b). This is consistent with our previous reports describing the inhibitory effects of daidzein (NYNCA *et al.* 2009; NYNCA *et al.* 2013a) and biochanin A (NYNCA *et al.* 2013c) on $P_4$ secretion by cultured granulosa cells isolated from either medium or large follicles of pigs. Similar inhibitory effects of phytoestrogens were shown in other reports, including one describing genistein-induced inhibition of steroidogenesis in human granulosa cells (WHITEHEAD *et al.* 2002). In contrast to $P_4$, the effect of genistein on $E_2$ secretion appears to depend on the developmental stage of ovarian follicles. Genistein (0.05-50 $\mu$M) did not affect $E_2$ secretion in the current study performed on luteinized granulosa cells from large follicles, while it increased $E_2$ secretion by granulosa cells from medium follicles (NYNCA *et al.* 2013b). Interestingly, BASINI *et al*. (2010) reported a biphasic genistein action on $E_2$ production by porcine granulosa cells; a low dose (1 $\mu$M) stimulated, whereas a high dose (185 $\mu$M) inhibited $E_2$ levels. It should be emphasized that our radioimmunological data (i.e., genistein-induced decrease in $P_4$ secretion accompanied by a lack of effect on $E_2$ secretion) together with the viability results (only 50 $\mu$M of genistein reduced granulosa cell viability) confirmed that genistein at concentrations 0.05, 0.5 and 5 $\mu$M was not cytotoxic. Summarizing, in most experiments performed on ovarian cells, phytoestrogens decreased $P_4$, while the effect on $E_2$ secretion was related to treatment doses and/or the maturational status of follicles used as a granulosa cell source (NYNCA *et al.* 2009; NYNCA *et al.* 2013a,b).

Genistein may affect its target cells *via* several intracellular ways. Since genistein is commonly considered to be a specific PTK inhibitor (for review see KRASZEWSKA *et al*. 2007; MURKIES *et al*. 1998) we have compared the effects of genistein and lavendustin C (another PTK inhibitor) on granulosal steroidogenesis. As mentioned above, genistein significantly affected basal and gonadotropin-stimulated $P_4$ secretion by porcine luteinized granulosa cells harvested from large follicles. In contrast, lavendustin C (0.05-50 $\mu$M) did not affect basal secretion of $P_4$. Similar lack of effects on gonadotropin-stimulated $P_4$ production was observed when two highest doses of lavendustin C were applied. However, the two lowest doses of lavendustin C inhibited gonadotropin-stimulated $P_4$ secretion by the examined cells. In view of different patterns of genistein and lavendustin C actions, the effects of genistein in these cells are probably not mediated by PTK.

Interestingly, genistein was reported to affect the activity or mRNA/protein expression of steroidogenic enzymes in some cells (TIEMANN *et al.* 2007; RICE *et al*. 2006; KAMINSKA *et al*. 2013; WONG & KEUNG 1999). Moreover, we have demonstrated recently that genistein inhibited $P_4$ production and decreased the activity of cytochrome P450 cholesterol side chain-cleavage (P450scc) and 3β-hydroksysteroid dehydrogenase (3β-HSD) in granulosa cells isolated from both medium and large porcine follicles (PIASECKA-SRADER *et al*. 2014). It is not known, however, whether these genistein-induced changes in the activity of steroidogenic enzymes are functionally associated with the activation of ER-mediated pathways.

The presence of both mRNA and protein of ERβ was demonstrated in porcine luteinized granulosa cells isolated from large (current study) and medium follicles (NYNCA *et al.* 2013b). In contrast, we have not detected ERα protein in these cells. This finding is consistent with the note that ERβ is the primary ER subtype present in the ovary, particularly in granulosa cells (BYERS *et al*. 1997; SŁOMCZYŃSKA & WOŹNIAK 2001) as well as that it plays a direct role in follicular development. Transgenic mice lacking ERβ gene demonstrated changes in the expression of genes important for ovarian differentiation i.e., aromatase, LH receptor or prostaglandin synthase (DRUMMOND &

FULLER 2012). Since ERβ is of great importance for follicular development, any change in its expression (mRNA and protein) may influence ovarian functions. Moreover, the affinity of genistein for ERβ is very high (HWANG *et al*. 2006; KUIPER *et al*. 1998), and part of genistein's action is mediated *via* this receptor. Therefore, granulosa cells are an appropriate *in vitro* model for investigating the mechanism of genistein action in the ovary.

In the present study, genistein increased the mRNA level of ERβ, but not that of ERα, in luteinized granulosa cells isolated from large porcine follicles. Similar results were demonstrated in granulosa cells harvested from medium follicles (NYNCA *et al.* 2013a). Daidzein, another phytoestrogen, caused a significant increase in mRNA levels of both ERs in granulosa cells from large follicles (NYNCA *et al.* 2009). In medium follicles, daidzein decreased ERα mRNA and did not affect ERβ mRNA level (NYNCA *et al.* 2013b). Interestingly, biochanin A did not alter the ER mRNA expression regardless of the source of granulosa cells (medium *vs*. large follicles) (NYNCA *et al.* 2013c). It seems that responsiveness of granulosa cells to phytoestrogens, examined at the mRNA level, depends on the type of phytoestrogen and/or the developmental stage of follicles used for granulosa cell isolation.

Despite that the intensity of ERβ immunostaining or the percentage of stained granulosa cells harvested from large ovarian follicles of pigs increased after genistein (0.05, 0.5 and 5 $\mu$M) treatment, none of the genistein concentration significantly affected ERβ protein expression. It is of interest that genistein enhanced the intensity of ERβ immunostaining in granulosa cells harvested from medium follicles (NYNCA *et al.* 2013b). Moreover, we demonstrated previously that daidzein (NYNCA *et al.* 2009, NYNCA *et al.* 2013a) and biochanin A (NYNCA *et al.* 2013c) enhanced ERβ protein expression in granulosa cells from both medium and large follicles. It appears that genistein-induced changes in concentrations of steroid hormones and granulosal level of ERβ mRNA are not parallel to the changes in ERβ protein expression. The lack of unequivocal compatibility in the patterns of gene and protein expression occurs quite often (YANG *et al.* 2001; TIAN *et al*. 2004; KRETZSCHMAR *et al*. 2010). Frequently, RNA and protein levels are regulated independently, which may be due to several factors including a negative feedback i.e., the high level of gene expression may diminish the posttranscriptional processes of its protein product.

It should be emphasized that genistein inhibited $P_4$ production by granulosa cells of medium as well as large follicles. In contrast, genistein did not alter the expression of ERβ protein in granulosa cells from large follicles (the current paper) while increased it in cells harvested from medium follicles (NYNCA *et al.* 2013a). It appears that the mechanism of genistein action differs between the two follicle types. This difference depends probably on a hormonal status of the animal, which affects subsequent *in vitro* granulosa cell sensitivity to phytoestrogens.

In summary, we demonstrated that genistein markedly decreased $P_4$ secretion and increased mRNA ERβ expression in porcine luteinized granulosa cells isolated from large follicles. These findings were not, however, accompanied by changes in ERβ protein expression. Moreover, the intracellular mechanism of genistein action did not result from the inhibition of PTK but, according to our recent report (PIASECKA-SRADER *et al*. 2014), might be associated with a decrease in the activity of P450scc and 3β-HSD. Different response of granulosa cells isolated from large (lack of significant effect of genistein on ERβ protein: the current study) and medium (stimulatory effect of genistein on ERβ protein: (NYNCA *et al.* 2013a)) follicles suggests that mechanisms of intracellular action of genistein differ between the two types of porcine follicles.

## Conflict of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the article reported.

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