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IN VITRO EFFECTS OF GENISTEIN AND DAIDZEIN ON THE ACTIVITY OF ADRENOCORTICAL STEROIDOGENIC ENZYMES IN MATURE FEMALE PIGS

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Soy products, commonly used as a protein source in farm animals' diets, contain considerable quantities of non-nutrient constituents such as phytoestrogens. Genistein and daidzein are known to affect the reproductive processes in humans and animals. However, reports concerning phytoestrogens and porcine adrenal steroidogenesis are scarce, and the adrenal mechanism of phytoestrogen action in species other than humans and rodents is poorly recognized. The goal of the present paper was to examine the *in vitro* effects of genistein and daidzein on the activity of key enzymes for cortisol and corticosterone synthesis in porcine adrenocortical cells harvested during the luteal or follicular phase of the porcine estrous cycle. The cells were treated with genistein or daidzein (10 μM), with or without ACTH (5 nM), in the presence or absence of precursors (1 μM) of cortisol (pregnenolone, P_5; progesterone, P_4; 17-hydroxyprogesterone, 17OH-P_4; or 11-deoxycortisol, 11d-cortisol) or corticosterone: (P_5 or P_4) synthesis. The supplementation of a medium with P_5, P_4, 17OH-P_4 or 11d-cortisol enabled us to measure the activity of cholesterol side-chain cleavage enzyme (P450scc), 3β-hydroxysteroid dehydrogenase (3β-HSD), 17α-hydroxylase/C17-20 lyase (P450c17) or 21-hydroxylase (P450c21) and 11β-hydroxylase (P45011β), respectively. We demonstrated that in sexually mature, cyclic pigs, regardless of the phase of the estrous cycle, phytoestrogens genistein and daidzein suppressed basal and ACTH-stimulated *in vitro* secretion of cortisol and corticosterone *via* progesterone synthesis inhibition. This indicates that phytoestrogens specifically inhibit the 3β-HSD activity in porcine adrenocortical cells. We suggest that genistein and daidzein present in soy products may negatively affect glucocorticoid synthesis of mature gilts by disrupting adrenal steroidogenesis at the 3β-HSD level.

Key words: *adrenocortical cells, daidzein, genistein, phytoestrogens, steroidogenesis, estrous cycle, isoflavones*

INTRODUCTION

Phytoestrogens are plant-derived and chemically differentiated substances that exhibit a weak estrogen-like activity. Soy products, commonly used as a protein source in farm animals' diets, contain considerable quantities of non-nutrient substances including phytoestrogens *i.e.*, isoflavones such as genistein and daidzein (1). Micromolar amounts of these isoflavones were found in plasma of pigs fed diets rich in soy bean meal or soy protein isolate (2, 3). The endogenous estrogen plasma concentration in sexually mature cyclic gilts is more than 1000 times lower (4). Thus, despite the differences in the binding affinity to estrogen receptors (ER), an effective competition between estrogens and phytoestrogens is possible and enables the latter hormones to affect intracellular biological processes *via* estrogen-specific mechanisms.

Phytoestrogens were reported to mimic or antagonize estrogen action in some target cells and ER were suggested to mediate the intracellular action of phytoestrogens (5, 6). In addition to binding to ER, genistein is known to act as a relatively specific inhibitor of protein tyrosine kinases (PTK) (7). Moreover, the results of some studies indicate that phytoestrogens may affect the activity of steroidogenic enzymes as well as act as modulators of sex steroid synthesis (8, 9).

Until now, most studies have focused on phytoestrogen effects on the reproduction of humans and rats. Recently, more and more attention has been paid to the phytoestrogen significance for the etiology of reproductive disorders in farm animals including those in pigs (10-12). However, reports concerning phytoestrogens and adrenal steroidogenesis in species other than humans and rodents are scarce, and the adrenal mechanism of phytoestrogen action in pigs is poorly recognized. In our previous study, genistein, daidzein and biochanin A inhibited the secretion of cortisol and stimulated the secretion of androstenedione by porcine adrenocortical cells (13). Results of our other study suggested that ER are not involved in the influence of genistein and daidzein on adrenal steroidogenesis and the action of genistein in porcine adrenocortical cells was not mediated by the inhibition of PTK (14). In view of these findings it is possible that in pigs phytoestrogens may directly and specifically affect the activity of some adrenal steroidogenesis enzymes.

It was reported that isoflavones (genistein, daidzein, biochanin A and formononetin) inhibited the activity of 3β-hydroxysteroid dehydrogenase (3β-HSD) in microsomal preparations of bovine adrenocortical cells and H295R human adrenocortical carcinoma cells (15, 16). Moreover, microsomal 21-hydroxylase (P450c21) activity was found to

be inhibited by these isoflavones in H295R cells (16). Genistein and daidzein were also shown to specifically inhibit P450c21 in the H295R cell line and human fetal adrenocortical cells (17).

Since the mechanism of phytoestrogen action was mostly examined in cell lines and mitochondrial/microsomal preparations of adrenal cells (15-17) and there is no data on porcine adrenocortical cells, the goal of the present paper was to examine the effects of genistein and daidzein on the activity of key enzymes for cortisol and corticosterone synthesis in intact porcine adrenocortical cells. To meet this goal, the incubation medium was supplemented with specific steroid products of particular steroidogenic enzymes (*Fig. 1*), and the phytoestrogen-inhibited glucocorticoid production was compared in the presence and absence of steroid precursors. This approach enabled to infer on the genistein and daidzein-induced changes in respective enzyme activity. Moreover, since it is not known whether the hormonal milieu associated with reproductive status may influence the intracellular action of soy isoflavones, the *in vitro* effect of phytoestrogens on the enzyme activity was examined in adrenocortical cells harvested during the luteal or follicular phase of the porcine estrous cycle.

MATERIALS AND METHODS

Chemicals and animals

Ham's F-12 medium and bovine serum albumin (BSA) were obtained from AppliChem GmbH (Darmstadt, Germany) and Carl Roth GmbH (Karlsruhe, Germany), respectively. Radiochemicals were purchased from DuPont NEN (Boston, MA, USA). The phytoestrogens (genistein and daidzein), $ACTH_{1-24}$ (ACTH) and steroid enzyme products, pregnenolone (P_5), progesterone (P_4), 17-hydroxyprogesterone (17OH-P_4) and 11-deoxycortisol (11d-cortisol) as well as other reagents were purchased from Sigma (St. Louis, MO, USA).

Adrenal glands were harvested on days 6-10 (luteal phase) and 17–20 (follicular phase) of the estrous cycle from locally slaughtered mature crossbred gilts. The glands were transported within 60 min to the laboratory in ice-cold Ham's F-12 medium. The phase of the estrous cycle was assessed according to Akins and Morrissette (18). All experiments were performed in accordance with the principles and procedures of the Animal Ethics Committee of the University of Warmia and Mazury in Olsztyn, Poland.

Dispersion and incubation of adrenocortical cells

Porcine adrenocortical cells were isolated as previously reported (19). In brief, adrenals were decapsulated and medullae were discarded. The cells were dispersed by sequential treatment with collagenase type V (0.03%). Then, the cells were seeded into 24-well culture plates at a density of 3×10^5/mL. Ham's F-12 (pH 7.4) containing 2% BSA and supplemented with 25 mM Hepes buffer, 1.2 g/L $NaHCO_3$ and antibiotics was used as an incubation medium. All incubations were performed at 37°C under the water-saturated atmosphere of 95% air and 5% CO_2. Cell viability, determined by the trypan blue exclusion method, was >95%. None of the solvents at the concentrations used in this study affected the cell function (examined by radioimmunoassay) or cell viability (examined by trypan blue exclusion and microscopical observation).

Experimental design

After pre-incubation (30 min), adrenocortical cells were exposed for eight hours (95% air and 5% CO_2, 37°C) to genistein or daidzein (10 μM), with or without ACTH (5 nM), in the presence or absence of precursors of cortisol (P_5; P_4; 17OH-P_4 or 11d-cortisol; 1 μM) or corticosterone (P_5 or P_4; 1 μM) synthesis. Supplementation of a medium with P_5, P_4, 17OH-P_4 or 11d-cortisol enabled us to measure the activity of cholesterol side-chain cleavage enzyme (P450scc), 3β-HSD, 17α-hydroxylase/C17-20 lyase (P450c17) or 21-hydroxylase (P450c21) and 11β-hydroxylase (P45011β), respectively (*Fig. 1*). Control cells were incubated: 1 - without treatments, or 2 - with ACTH only (5 nM, a positive control) or 3 - with a respective steroid precursor (1 μM). Each experiment was repeated 5–6 times. The applied concentrations of phytoestrogens and steroid precursors were either established during our dose-response preliminary experiments or taken from previously published studies (13, 17, 20). At the end of incubation, medium was collected and stored at -20°C. Concentrations of cortisol and corticosterone were determined by radioimmunoassay (RIA).

Hormone assays

Cortisol and corticosterone concentrations were measured in a medium using specific RIAs (19-21). The specificities of cortisol (22) and corticosterone (23) antibodies have been previously reported. The sensitivity of both assays was 15

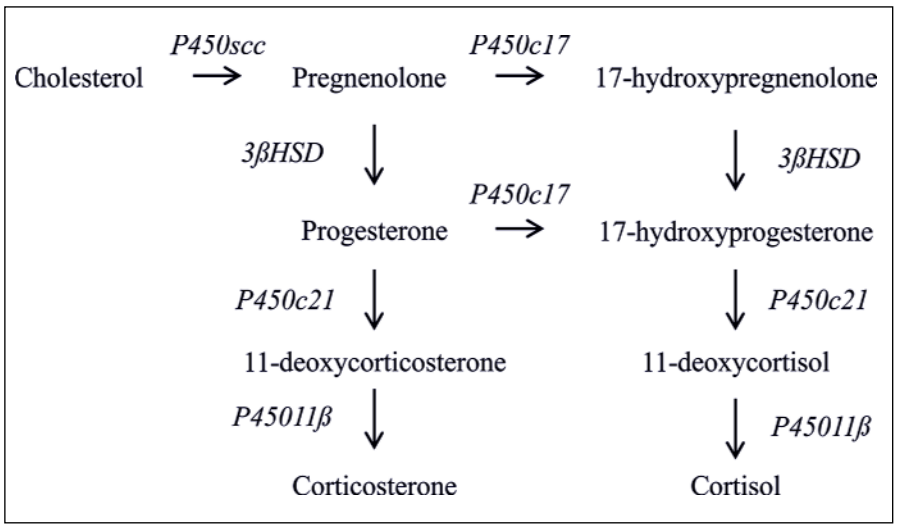

Fig. 1. The simplified pathways of glucocorticoid synthesis. Cholesterol side-chain cleavage enzyme (*P450scc*); 3β-hydroxysteroid dehydrogenase (*3β-HSD*); 17α-hydroxylase/C17-20 lyase (*P450c17*); 21-hydroxylase (*P450c21*); 11β-hydroxylase (*P45011β*).

pg/tube. The intra- and inter-assay coefficients of variation for cortisol and corticosterone assays were less than 7%.

Statistical analysis

One-way analysis of variance for repeated measurements followed by LSD-test was used to compare the effects of different treatments on steroid secretion *in vitro* (Statistica, StatSoft Inc., Tulsa, OK., USA). Statistical analysis of corticosterone was performed on raw data (ng/mL). Due to the high heterogeneity in basal cortisol secretion among pigs, cortisol data (ng/mL) were log transformed (19, 24) before statistical analysis. Differences were considered to be significant at $p<0.05$. Cortisol and corticosterone medium concentrations were presented as percentage change from respective control values (mean±S.E.M.).

RESULTS

Genistein and daidzein (10 μM) significantly ($p<0.05$) decreased basal and ACTH-stimulated cortisol (*Fig. 2A*, n=5 and *Fig. 2B*, n=6) and corticosterone (*Fig. 2C*, n=6 and *Fig. 2D*, n=6)

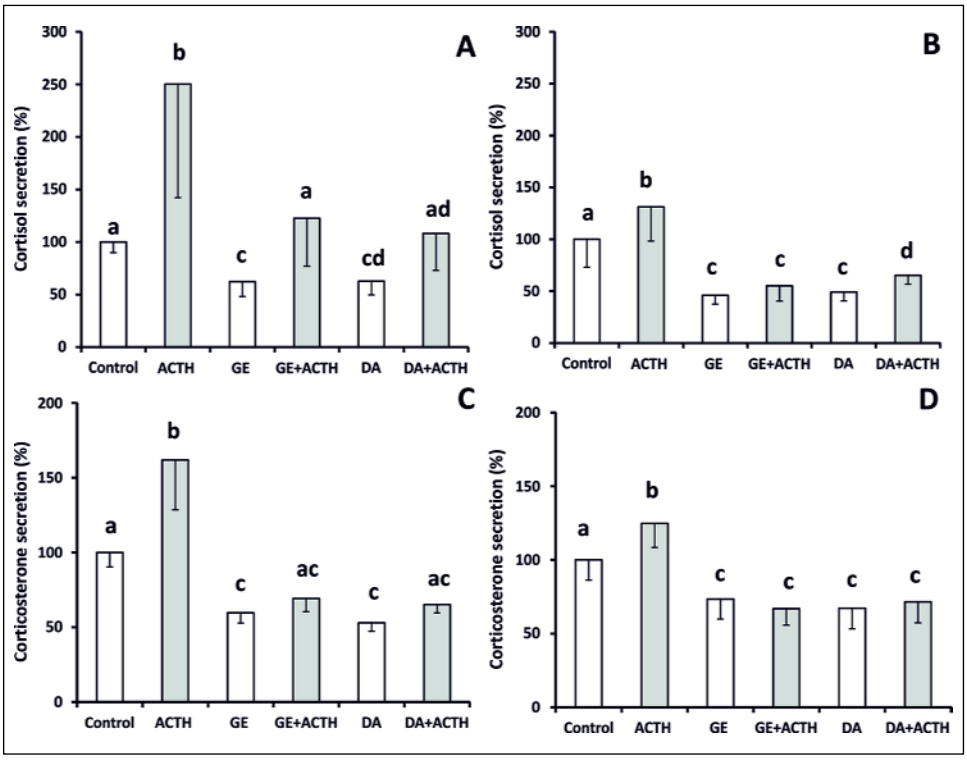

Fig. 2. Effects of genistein (GE; 10 μM) and daidzein (DA; 10 μM) on basal and ACTH (5 nM) -stimulated cortisol (*Figs. A*, n=5; B, n=6) and corticosterone (*Figs. C* and *D*; n=6) secretion by adrenocortical cells (3×10^5/mL, 8 h incubation) obtained during the luteal- (*Figs. A* and *C*) and follicular- (*Figs. B* and *D*) phases of the porcine estrous cycle. Data (mean±S.E.M.) are expressed as a percentage of control (no treatment) cultures. Bars without common superscripts are significantly different ($p<0.05$). White bars: samples without ACTH; grey bars: samples with ACTH.

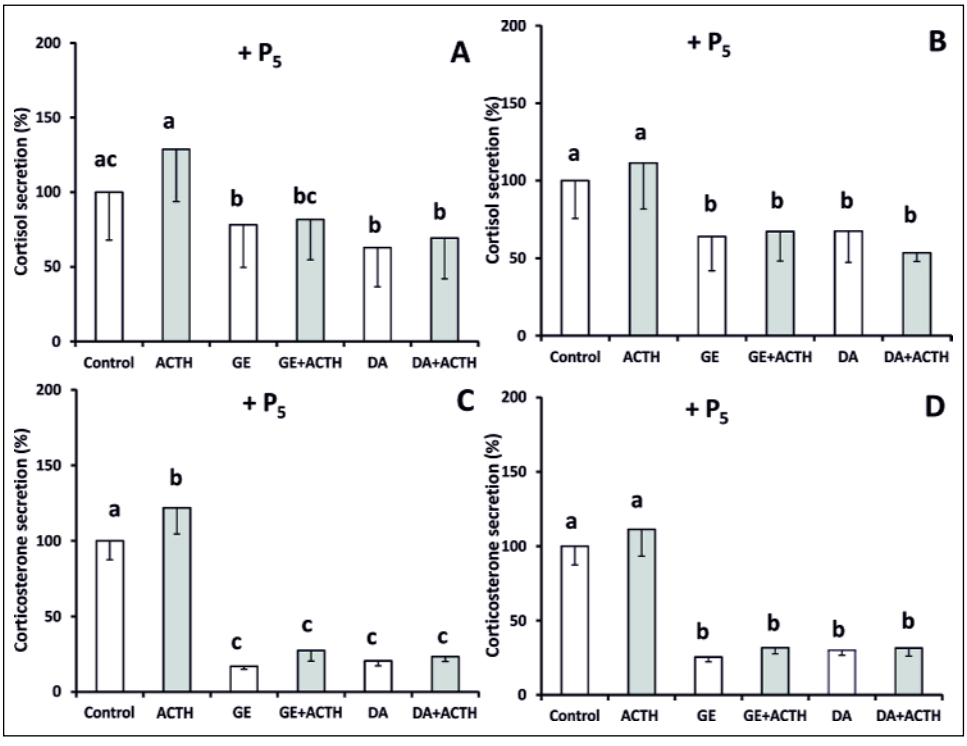

Fig. 3. Effects of genistein (GE; 10 μM) and daidzein (DA; 10 μM) on basal and ACTH (5 nM) -stimulated cortisol (*Figs. A*, n=6; *B*, n=6) and corticosterone (*Figs. C* and *D*; n=6) secretion by porcine adrenocortical cells (3×10^5/mL, 8 h incubation) incubated in the presence of pregnenolone (P_5; 1 μM). The cells were obtained during the luteal- (*Figs. A* and *C*) and follicular- (*Figs. B* and *D*) phases of the porcine estrous cycle. Data (mean±S.E.M.) are expressed as a percentage of control (P_5 only) cultures. Bars without common superscripts are significantly different ($p<0.05$). White bars: samples without ACTH; grey bars: samples with ACTH.

secretion by porcine adrenocortical cells harvested during the luteal (*Figs. 2A* and *2C*) as well as follicular phase (*Figs. 2B* and *2D*) of the estrous cycle. ACTH (5 nM, a positive control) significantly ($p<0.05$) increased adrenocortical secretion of both steroids (*Fig. 2*).

The addition of P_5 (1 μM) to the medium did not alter the inhibitory effect of phytoestrogens on cortisol (*Figs. 3A* and *3B*; n=6) and corticosterone (*Figs. 3C* and *3D*; n=6) secretion in both luteal- (*Figs. 3A* and *3C*) and follicular- phase gilts (*Figs. 3B* and *3D*). In contrast, the addition of P_4 (1 μM) abolished the inhibitory effects of 1/ genistein and daidzein on basal steroid secretion and 2/ daidzein on ACTH-stimulated steroid secretion during the luteal- (*Figs. 4A* and *4C*; n=6) and follicular- phase (*Fig. 4B*, n=6; *Fig. 4D*, n=6). However, P_4 added to the medium did not affect the inhibitory effects of genistein on the ACTH-stimulated cortisol (luteal phase; *Fig. 4A*) and corticosterone (follicular phase; *Fig. 4D*) secretion.

Medium supplementation with precursors (1 μM) of cortisol synthesis *i.e.* 17OH-P_4 (*Fig. 5A*, n=5; *5B*, n=6) or 11d-cortisol (*Fig. 5C*, n=5; *5D*, n=6) abolished the inhibitory effect of both phytoestrogens on basal cortisol secretion by porcine adrenocortical cells harvested during the luteal (*Figs. 5A* and *5C*) as well as follicular phase (*Figs. 5B* and *5D*) of the estrous cycle. In addition, such abolition was usually demonstrated for the

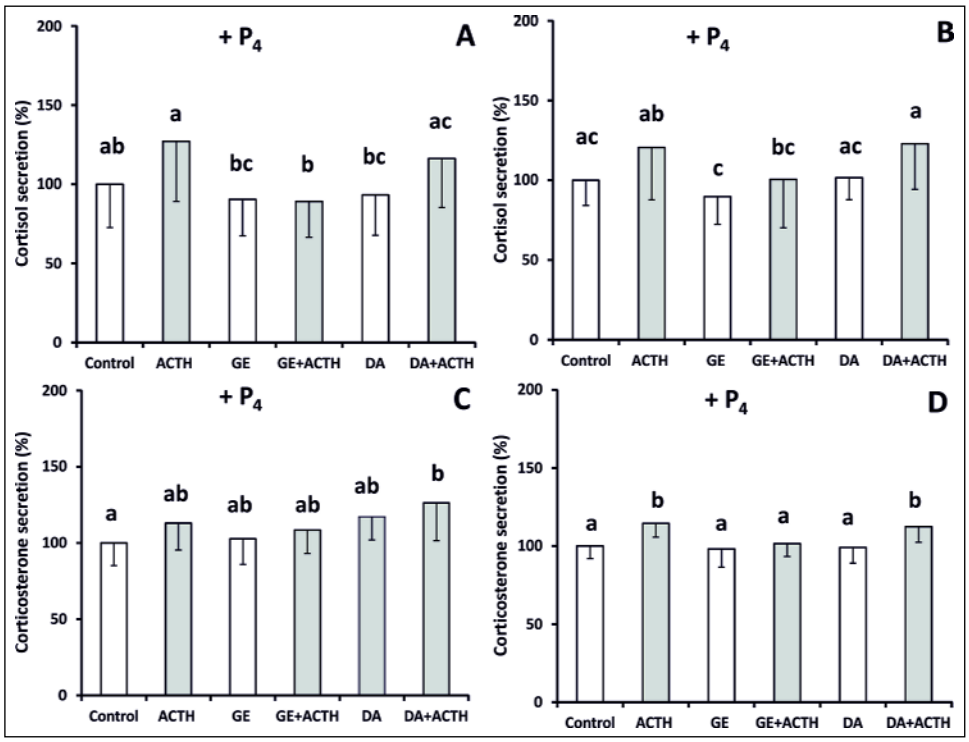

Fig. 4. Effects of genistein (GE; 10 μM) and daidzein (DA; 10 μM) on basal and ACTH (5 nM) -stimulated cortisol (*Figs. A*, n=6; *B*, n=6) and corticosterone (*Figs. C*, n=6; *D*, n=6) secretion by porcine adrenocortical cells (3×10^5/mL, 8 h incubation) incubated in the presence of progesterone (P_4; 1 μM). The cells were obtained during the luteal- (*Figs. A* and *C*) and follicular- (*Figs. B* and *D*) phases of the estrous cycle. Data (mean±S.E.M.) are expressed as a percentage of control (P_4 only) cultures. Bars without common superscripts are significantly different ($p<0.05$). White bars: samples without ACTH; grey bars: samples with ACTH.

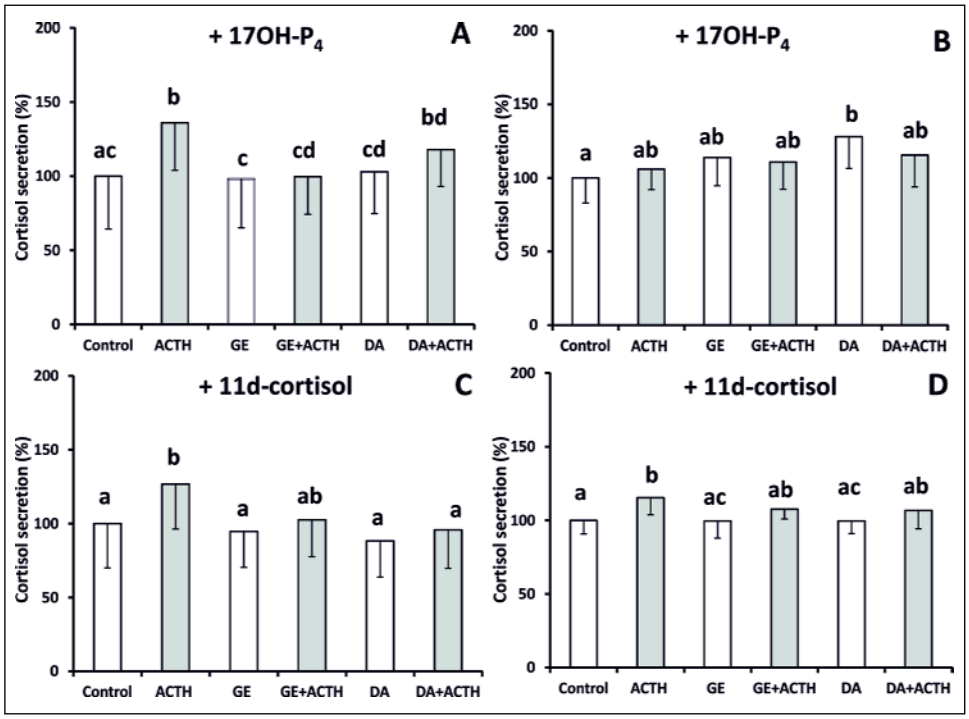

Fig. 5. Effects of genistein (GE; 10 μM) and daidzein (DA; 10 μM) on basal and ACTH (5 nM) -stimulated cortisol secretion by porcine adrenocortical cells (3×10^5/mL) incubated in the presence of 17-hydroxyprogesterone (17OH-P_4; *Figs. A* and *B*; 1 μM) or 11-deoxycortisol (11d-cortisol; *Figs. C* and *D*; 1 μM). The cells were obtained during the luteal- (*Figs. A* and *C*; n=5) and follicular- (*Figs. B* and *D*; n=6) phases of the estrous cycle. Data (mean±S.E.M.) are expressed as a percentage of control (17OH-P_4 or 11d-cortisol only) cultures. Bars without common superscripts are significantly different ($p<0.05$). White bars: samples without ACTH; grey bars: samples with ACTH.

inhibitory effects of phytoestrogens on ACTH-stimulated cortisol secretion. Genistein or daidzein effects on ACTH-stimulated cortisol secretion in the presence of 17OH-P_4 or 11d-cortisol, respectively, remained inhibitory only in adrenocortical cells obtained during the luteal phase of the porcine estrous cycle.

DISCUSSION

In the current *in vitro* study, phytoestrogens, genistein and daidzein inhibited basal and ACTH-stimulated adrenocortical secretion of cortisol and corticosterone. The examined cells were isolated from adrenals of mature gilts, and the inhibition was demonstrated in both luteal- and follicular-phase gilts. Previously, we had reported that genistein, daidzein and biochanin A suppressed basal cortisol secretion by adrenocortical cells harvested from luteal-phase gilts (13). Genistein and daidzein were also found to decrease basal or ACTH-stimulated cortisol production in human fetal and postnatal adrenocortical cells (17). Moreover, cAMP-stimulated secretion of cortisol was reduced by genistein and daidzein in H295R cells (16, 17). Recently, Ohlsson *et al.* (25) demonstrated an inhibitory effect of genistein, daidzein and/or apigenin (a flavone) on basal secretion of cortisol, aldosterone and testosterone in H295R cells. The thesis that phytoestrogens inhibit adrenal steroidogenesis in humans and animals appears to be strongly supported by results of *in vitro* studies.

Since the mechanism of this inhibition in domestic animals including pigs is unclear, we have examined whether genistein or daidzein affected the activity of enzymes involved in adrenal steroidogenesis. To meet this goal, porcine adrenocortical cells were treated with genistein or daidzein in the presence of precursors of cortisol (P_5; P_4; 17OH-P_4 or 11d-cortisol) or corticosterone (P_5 or P_4) synthesis. Pregnenolone, P_4, 17OH-P_4 and 11d-cortisol synthesis is the result of the enzymatic activity of P450scc, 3β-HSD, P450c17 and P450c21, respectively (*Fig. 1*). If an additional particular steroid synthesis precursor eliminates the effect of phytoestrogens on glucocorticoid secretion, the enzyme responsible for the precursor synthesis has to be inhibited.

The addition of P_5 to the medium did not alter the inhibitory effect of phytoestrogens on basal and ACTH-stimulated cortisol and corticosterone secretion by adrenocortical cells in both luteal- and follicular-phase gilts. This suggests that genistein and daidzein did not affect the P450scc activity in porcine adrenals. Similarly, genistein and daidzein did not alter the activity of P450scc in human fetal cells and H295R cells (16, 17). This indicates that P450scc is not involved in the inhibitory action of phytoestrogens on the adrenal synthesis of glucocorticoids, and that the inhibition probably occurs in later steps of steroidogenesis.

In contrast to P_5, the addition of P_4 to the medium abolished the inhibitory effects of genistein and daidzein on basal adrenal cortisol and corticosterone secretion. Moreover, this effect of P_4 was usually demonstrated in ACTH-stimulated cell cultures. This suggests that genistein and daidzein inhibited the 3β-HSD activity in porcine adrenocortical cells. Genistein- or daidzein-induced suppression of 3β-HSD activity was previously observed in ovarian granulosa cells in pigs (26). To date majority of experiments were performed on cell lines (H295R) or mitochondria/microsomal preparations. It was found that isoflavones - genistein, daidzein, formononetin and biochanin A - inhibited, in a dose dependent manner, microsomal activity of bovine adrenal 3β-HSD (15). The isoflavones also significantly inhibited microsomal 3β-HSD type II in H295R cells and Sf9 insect cells (16, 27). Since, in our experiment, the conversion of P_4 to cortisol and corticosterone was not inhibited by phytoestrogens, we suggest that phytoestrogens suppress 3β-HSD activity in porcine adrenal glands.

The addition of 17OH-P_4 and 11d-cortisol also abolished the inhibitory effects of genistein and daidzein on cortisol secretion. In the steroidogenesis pathways, 17OH-P_4 and 11d-cortisol follow progesterone (*Fig. 1*), so their similarity to the effect of P_4 on phytoestrogen-inhibited glucocorticoid synthesis is not surprising. These data confirm that 3β-HSD, but not P450c17, P450c21 and P45011β, is affected by genistein and daidzein in porcine adrenocortical cells. Genistein and daidzein did not affect P450c17 and P45011β activity in human fetal adrenocortical cells and H295R cells (16, 17). These authors, however, demonstrated that phytoestrogens decreased P450c21 activity in the examined cells. Although genistein and daidzein did not affect the activity of this enzyme in our study, P450c21 as well as 3β-HSD were reported to be affected by phytoestrogens (16, 17, 27). Moreover, inhibition of src tyrosine kinases was found to increase P450c17 activity in H295R cells (28) and genistein is known as a tyrosine kinase inhibitor (7). Nevertheless, results of the current study are consistent with a notion that 3β-HSD is the main target for phytoestrogens in porcine adrenals.

In the current study, the *in vitro* effects of genistein and daidzein on glucocorticoid secretion and the activity of adrenocortical steroidogenic enzymes were similar during the follicular and luteal phases of the porcine estrous cycle. This is consistent with the results of *in vivo* studies in which no differences in peripheral blood plasma glucocorticoid concentration were found between the two phases of the porcine estrous cycle (29, 30). In contrast, earlier studies by McGuire *et al.* (31) demonstrated a positive correlation between corticoid and estrogen plasma concentrations during the estrous cycle of pigs. It should also be noted that *in vitro* adrenocortical sensitivity to oxytocin differed between the follicular and luteal phase of the porcine oestrous cycle (21). It appears that the nature of the relationship between the sensitivity of adrenocortical cells to phytoestrogens and cycle-dependent changes in plasma hormone concentrations is still not elucidated.

In summary, we demonstrated that in sexually mature cyclic pigs, regardless of the phase of the porcine estrous cycle, phytoestrogens genistein and daidzein suppressed basal and ACTH-stimulated *in vitro* secretion of cortisol and corticosterone *via* progesterone synthesis inhibition. This suggests that phytoestrogens specifically inhibit the 3β-HSD activity in porcine adrenocortical cells. Therefore, genistein and daidzein present in soy products, may negatively affect glucocorticoid synthesis of mature gilts by disrupting adrenal steroidogenesis at 3β-HSD level.

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Conflict of interests: None declared.

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