Mechanism of phytoestrogen action in reproductive processes of mammals and birds

Luiza Dusza¹,², Renata Ciereszko³, Dariusz J. Skarzyński³, Leszek Nogowski⁴, Marek Opalka², Barbara Kamińska², Anna Nynca², Olga Kraszewska², Maria Słomczyńska³, Izabela Woclawek-Potocka³, Anna Korzekwa², Ewa Pruszyńska-Oszmałek⁴, Katarzyna Szkudelska⁴

¹Department of Animal Physiology, University of Warmia and Mazury in Olsztyn; ²Department of Reproductive Immunology, Institute of Animal Reproduction and Food Research, Polish Academy of Sciences, Olsztyn; ³Department of Reproductive Immunology, Institute of Animal Reproduction and Food Research, Polish Academy of Sciences, Olsztyn; ⁴Department of Animal Physiology and Biochemistry, A. Cieszkowski University of Agriculture, Poznań; ⁵Laboratory of Endocrinology and Tissue Culture, Department of Animal Physiology, Institute of Zoology, Jagiellonian University, Krakow, Poland

SUMMARY

Phytoestrogens are polyphenolic compounds that occur ubiquitously in food of plant origin and they have a variety of biological effects in numerous animal cell systems in vivo as well as in vitro. Results of studies conducted on animals have shown that effects of phytoestrogens vary depending on species, sex, routes of administration, dose and exposure time. This review summarizes the results of ours studies concerning: 1/ molecular mechanism of phytoestrogen action in porcine granulosa cells, 2/ the involvement of phytoestrogens in immunological regulations of bovine corpus luteum function during luteolysis, 3/ genistein action on

¹Corresponding author: Department of Animal Physiology, University of Warmia and Mazury in Olsztyn, Oczapowskiego 1A, 10-718 Olsztyn; e-mail: ldusza@uwm.edu.pl

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**INTRODUCTION**

Phytoestrogens are polyphenolic non-steroidal plant compounds with estrogen-like biological activities. Based on their chemical structure, phytoestrogens can be classified into four main classes: isoflavonoids, flavonoids, stilbenes and lignans [12]. The most extensively studied subclasses of isolavonoids are isoflavones and coumestans. These phytoestrogens are widespread among legumes and beans. Phytoestrogen plant content is affected by cultivar and local conditions such temperature, rainfall, timing of harvest, soil fertility, pest and disease damage. Post-harvest storage conditions and food processing techniques can also affect the plant concentrations of phytoestrogens [8]. Phytoestrogens are present in several plants consumed by humans and animals [35]. Unprocessed soybeans contain 1.2-4.2 mg of isoflavones/g dry weight, mainly genistein, daidzein and their conjugates. The most significant sources of coumestans in food are sprouts of clover and alfalfa, with coumestrol contents of 5.6 and 0.7 mg/g dry weight, respectively [1, 27]. The biological potencies of phytoestrogens vary considerable and are vastly less potent that those of estrogens ($10^{-3}$ to $10^{-5}$). Most studies show the relation between the chemical structure of phytoestrogens and their estrogen-like activities [73]. Results of studies conducted on laboratory animals as well as on large farm animals have shown that effects of phytoestrogens vary depending on species, sex, routes of administration, dose and exposure time [40].

The estrogenic activity of isoflavones was first observed in relation to a syndrome known as “clover disease” in sheep in western Australia [40]. Observations made from 1940 to 1970 reported that the ingestion
of high levels of phytoestrogens by animals led to relatively consistent adverse effects on reproduction which were more marked in females than males. The consumption of high levels of isoflavones and cumestanes led to infertility in sheep, cattle and rodents [1, 5, 8, 68]. In sheep and cattle, equol was found to be responsible for these effects. Equol is formed in the digestive tract by the bacterial metabolism of formononetin. Others dietary phytoestrogens, especially genistein, daidzein and coumestrol, were also implicated in reproductive failure in animals.

When ingested in relatively large amounts, phytoestrogens have been shown to exert significant estrogenic and/or antiestrogenic effects on animals and humans [8, 68]. Genistein and coumestrol are good ligands for estrogen receptors (ERs), while some other isoflavones or flavonoids bind to ER with much lower affinity [39]. Kuiper et al. [26] showed that phytoestrogens preferentially bind ERβ.

During the last decade, multiple mechanisms of phytoestrogen action have been reported [5, 12, 68]. Isoflavones were found to compete with endogenous substrates for active sites of enzymes involved in estrogen synthesis and metabolism. These phytoestrogens were also demonstrated to stimulate in vitro production of sex hormone binding globulin and to inhibit angiogenesis. Some phytoestrogens may exert antiproliferative or antioxidant activities, while others may inhibit tyrosine kinase, protein kinase C or topoisomerase II. The effects of individual phytoestrogens can vary considerably, being both species- and tissue-dependent [57]. In order to determine the details concerning phytoestrogen action on reproductive system, one would have to study each environmental estrogen individually in different organs and species.

MOLECULAR MECHANISM OF PHYTOESTROGEN ACTION IN PORCINE GRANULOSA CELLS

Phytoestrogens are known to affect reproductive functions in many animal species including humans. There is a growing body of evidence that these plant-derived substances may alter synthesis, secretion and metabolism
of hormones involved in the regulation of reproductive processes. Phytoestrogens have also been shown to affect ovarian steroidogenesis in females [4, 33, 76].

The phytoestrogen effects on ovarian steroidogenesis

Results of epidemiological studies on pre- and post-menopausal women and in vivo experiments conducted on animals show that multiple components of soya diet, including isoflavones, may modulate ovarian hormone plasma levels. This modulation may occur either directly or indirectly via gonadotropins. Dietary isoflavones were demonstrated to cause a decrease, an increase or a lack of changes in serum progesterone (P₄) and estradiol (E₂) concentrations [5, 31, 33]. Such inconsistency may likely be explained by differences in length of the experiment, form of the applied soy product, type of isoflavone and its concentration in the diet. In addition, direction and degree of genistein or daidzein in vivo effects appear to be dependent on species, reproductive status of a subject, individual phytoestrogen metabolism and even a blood sampling schedule.

In vitro studies concerning phytoestrogen action on steroidogenesis were performed mostly on granulosa cells. Genistein was found to inhibit basal and stimulated P₄ production by human, rat and bovine granulosa cells as well as porcine theca and luteal cells [44]. The P₄ inhibition was demonstrated for genistein concentration ranging from 0.5 to 100 µM. A biphasic mode of genistein action was observed in bovine (185-1850 nmol/l; [20]) and rat (0.3 µM-100 µM; [16]) granulosa cells. These authors found that low concentrations of genistein stimulated and high concentrations inhibited P₄ production. In contrast, Makarevich et al. [34] demonstrated that genistein (37 nM-37 µM) stimulated P₄ production by bovine and rabbit granulosa cells. Estradiol production was either stimulated (rabbit granulosa cells; [34]) or inhibited (rat [17] and human [75] granulosa cells) by genistein. In pigs, genistein was found to stimulate E₂ production by whole follicles [34] and inhibited P₄ production by theca [11] and luteal [10] cells.

Our recent in vitro studies revealed that isoflavones: genistein, daidzein and biochanin A, affected steroidogenesis of luteinized granulosa
cells originated from growing (GRO), antral (3-6 mm) as well as large, preovulatory (PO) porcine follicles [44, 45, 46; Ciereszko et al., unpublished]. The phytoestrogens inhibited basal and gonadotropin-stimulated $P_4$ production by granulosa cells from PO follicles in a dose dependent manner (fig. 1). Similar to PO follicles, all examined isoflavones significantly reduced basal and FSH-stimulated $P_4$ production by cultured granulosa cells isolated from GRO follicles.

The effect of phytoestrogens on $E_2$ production differed from their effect on $P_4$. In PO follicles, genistein and daidzein did not affect basal and LH-stimulated $E_2$ production. However, $E_2$ production was inhibited by biochanin A. In contrast to PO follicles, genistein-stimulated basal production of $E_2$ by granulosa cells originated from GRO follicles [45]. Such effects of daidzein and biochanin A were not observed. The

Figure 1. Effect of increasing concentrations of genistein on basal and FSH-stimulated progesterone production (mean±SEM) by granulosa cells originated from porcine preovulatory ($\geq$8 mm) follicles (n=4 independent experiments). The cells (150 000 cells/ml) were allowed to attach for 72 h (37°C, 95% air/5% CO$_2$) in Eagle’s medium supplemented with 10% calf serum and then were cultured (5% serum) in the absence (C) or presence of FSH (FSH; 100 ng/ml) for subsequent 48 h. Bars without common superscripts are significantly different (p<0.05).
appearance of granulosa cells following culture with genistein, daidzein or biochanin A as well as the stimulating effect of genistein on E₂ production by the cells from GRO follicles, do not support the concept that inhibition of P₄ production exerted by phytoestrogens might be of a cytotoxic nature. This notion was confirmed by results of the cell viability Alamar Blue test. It was demonstrated that all doses of genistein, except the highest dose (50 μM), did not affect the viability of granulosa cells harvested from PO follicles (fig. 2; [44, 45]).

Primary cultures of porcine granulosa cells appear to provide a good model for studying the mechanism of phytoestrogen action in reproductive tissues. The cells are relatively easy to isolate and culture as well as responsive to phytoestrogens with a very high degree of consistency.

Figure 2. Effect of genistein on viability of granulosa cells (mean±SEM) originated from porcine preovulatory (≥8 mm) follicles (n=6 independent experiments). Data are expressed as percentage of Alamar Blue reduction. The cells (20 000/100 μl) were allowed to attach for 48 h (37°C, 95% air/ 5%CO₂) in Eagle’s medium supplemented with 10% calf serum and then cultured (5% serum) in the absence or presence of genistein or staurosporine (STS; 5 μM; a positive control) for subsequent 48 h. Alamar Blue reagent was added 24 h prior to the end of treatment. Bars without common superscripts are significantly different (p<0.05). Reproduced with permission from [44].
Mechanism of phytoestrogen action

The intracellular mechanism of phytoestrogen action may include: a/ classical genomic effects on ERα, ERβ and other nuclear receptors (progesterone, androgen or aryl hydrocarbon receptor); b/ inhibition of enzymes involved in steroidogenesis (3β- and 17β-hydroxysteroid dehydrogenase, aromatase); c/ stimulation of sex hormone-binding globulin; d/ inhibition of protein tyrosine kinase, important for signal transduction; e/ inhibition of DNA topoisomerases I and II, essential for DNA replication; and f/ antioxidant activity [4, 33, 76]. Estrogen binding is the most thoroughly investigated mechanism of phytoestrogen action. Many studies have shown that phytoestrogens bind both ER (α and β) and can mimic or block the actions of endogenous E₂. Competition binding assays demonstrate that phytoestrogens have a significantly higher affinity for ERβ protein than ERα. Recombinant assays, performed mostly in yeast model system and breast cancer cell lines, provide evidence that phytoestrogens not only bind to ERs but also initiate gene transcription. Phytoestrogens were found to either stimulate or inhibit the expression of ERα and/or ERβ protein and mRNA in nervous and reproductive tissues of rodents. Review of literature data makes it evident that the effects of individual phytoestrogens vary considerable and that the effects are species and tissue dependent [4, 5, 57].

Estrogen receptor β is a predominant ER in granulosa cells of humans, rats and pigs [66]. We also have not been able to demonstrate the immunoreactivity of ERα in porcine granulosa cells from GRO and PO follicles. In contrast to ERα, the immunostaining of ERβ was found to be present in these cells, mostly located in the nuclei. The isoflavones tended to increase the intensity of ERβ staining of granulosa cell nuclei in PO follicles as was measured by means of Olympus software (analySIS). Daidzein and biochanin A significantly increased the number of stained cells, while the effect of genistein was visible but not always significant. The effect of phytoestrogens on ERβ immunostaining in GRO follicles appeared to be stronger in comparison to that exerted in PO follicles. Genistein, daidzein and biochanin A (fig. 3) significantly stimulated
immunoexpression of ERβ measured both, as the intensity of staining and the percentage of stained granulosa cells originated from GRO follicles [45, 46; Ciereszko et al., unpublished]. In addition, our preliminary data indicated that genistein decreased the amount of ERβ mRNA in granulosa cells from GRO porcine follicles.

In conclusion, genistein, daidzein and biochanin A were found to affect steroidogenesis in porcine granulosa cells. It is possible that the phytoestrogen action is mediated via ERβ. Further studies are required to determine the effect of very low doses (<50 nM) of phytoestrogens on porcine ovarian steroidogenesis and ER activation as well as to establish whether other than ER signal transduction pathways are involved in phytoestrogen action in porcine ovary.
PHYTOESTROGENS MODULATE IMMUNOLOGICAL REGULATIONS IN THE BOVINE CORPUS LUTEUM DURING LUTEOLYSIS

Role of cytokines and nitric oxide in the regulation of luteolysis in cattle

The main hormone produced by corpus luteum (CL) is P₄, whereas prostaglandin (PG)F₂α, leukotriene (LT)C₄, nitric oxide (NO), cytokines (tumor necrosis factor: TNFα, interferon: IFNγ), oxytocin (OT), estrogens (E₂) and testosterone (T) are factors involved in the termination of CL lifespan [36, 53, 58, 62, 63]. PGF₂α acts as a luteolytic agent when administered parenterally in ruminants [36]. Nevertheless, in the first steps of our reported studies we showed that PGF₂α did not have a direct, luteolytic influence on steroidogenic cells of the bovine CL [3, 21, 59]. Unexpectedly, PGF₂α was found to act within the CL as a local factor stimulating P₄ production in steroidogenic cells [21, 59] and modulating frequency of episodic P₄ output [3]. Therefore, it is suggested that different subpopulations of steroidogenic and non-steroidogenic cells interact via auto- and paracrine pathways and the number of substances produced by endothelial and immune cells of bovine CL, locally mediate luteolytic action of PGF₂ and modulate the function and life-span of the CL [21, 61, 64].

One of the main PGF₂α mediators during luteolysis is endothelin-1 (ET-1) – the product of endothelial cells [38]. Moreover, the number of leukocytes increases in the CL at the time of luteolysis [53, 58]. The subluteolytic release of PGF₂α from the uterus acts on T lymphocyte and macrophages to promote cytokine production during luteolysis [53, 58, 61, 64]. As shown in our studies, TNFα in combination with IFNγ reduces P₄ production and induces the apoptotic events in the bovine CL that finally leads to functional and structural luteolysis [23, 48, 62, 64]. Moreover, we recommended that NO serve as a universal mediator of PGF₂α action in the functional regression of the bovine CL [63]. NO is produced by three main types of CL cells: steroidogenic, endothelial and immune cells. The infusion of L-NAME (a NO synthase inhibitor) stimulated P₄ secretion and concomitantly inhibited PGF₂α-induced luteolysis [63]. Additionally,
we showed that NO donor (NONOate Spermine) inhibited P$_4$ secretion from the CL cells [21] and induced intracellular, enzymatic and molecular mechanisms involved in apoptosis [23, 64]. NO induces apoptosis in the bovine CL by stimulation of intracellular mobilization of calcium ions, increase of Fas, bax and caspase-3 gene expression, as well as caspase-3 activity [23].

Apart from cytokines, NO produced by endothelial cells as well as P$_4$ and E$_2$ produced inside and outside the CL influence the regression of the bovine CL [48, 58]. We showed that an antagonist of P$_4$ (Onapristone) increased Fas mRNA expression and enhanced both, gene expression and activity of caspase-3 in the cultured steroidogenic cells of the bovine CL [48]. This suggests that intra-luteal P$_4$ suppresses apoptosis in the bovine CL through the inhibition of Fas and caspase-3 mRNA expression and inhibition of caspase-3 activation [64]. Since estrogen receptors are present in the bovine CL, luteal and exogenous estrogens may play an important role in regulation of CL function [55]. Endogenous estrogens control the estrous cycle influencing PGF$_{2a}$ synthesis and action. Because the structure and functional action of E$_2$ is mimicked by phytoestrogens [32], the latter may act like antagonists or agonists of endogenous estrogens and influence the same reproductive processes as those regulated by E$_2$.

**Phytoestrogens and their metabolites modulate secretory function of bovine steroidogenic luteal cells**

Ingestion of clover rich in plant estrogens was shown to cause several reproductive disorders in various species [6, 18, 37, 57, 78]. Daidzein and genistein are two major phytoestrogens present in soy [54, 60]. Rumen microorganisms convert daidzein and genistein into two active metabolites: equol and para-ethyl-phenol, respectively [32]. We showed that phytoestrogens and their active metabolites did not affect basal P$_4$ secretion in steroidogenic cells of the bovine CL [65]. However, in cows fed with the soy-bean diet, P$_4$ concentrations in luteal tissue and in the blood samples were lower than in control animals [55]. It seems that
the phytoestrogen influence on \( P_4 \) secretion is indirect and may depend, either on their effect on luteal production of a luteolytic substance (PGF\(_{2\alpha}\), cytokines, NO, LTC\(_4\), [64]), or on their ability to inhibit LH- and PGE\(_2\)-stimulated \( P_4 \) secretion [55]. We showed that phytoestrogens and their active metabolites may disturb CL secretory functions through stimulation of T, PGF\(_{2\alpha}\), NO and LTC\(_4\) secretion [22, 64]. This is in agreement with our previous in vivo study, which proved that high soy diet significantly increased PGF\(_{2\alpha}\) secretion in soy-fed cows, causing the decrease of the rate of successful pregnancies and the increase in the mean insemination rate [79]. In addition, we previously showed that phytoestrogens and their metabolites may disturb CL secretory functions through stimulation of T, PGF\(_{2\alpha}\), NO and LTC\(_4\) secretion [22, 64]. This is in agreement with our previous in vivo study, which proved that high soy diet significantly increased PGF\(_{2\alpha}\) secretion in soy-fed cows, causing the decrease of the rate of successful pregnancies and the increase in the mean insemination rate [79]. In addition, we previously showed that phytoestrogens and their metabolites may disturb CL secretory functions through stimulation of T, PGF\(_{2\alpha}\), NO and LTC\(_4\) secretion [22, 64]. This is in agreement with our previous in vivo study, which proved that high soy diet significantly increased PGF\(_{2\alpha}\) secretion in soy-fed cows, causing the decrease of the rate of successful pregnancies and the increase in the mean insemination rate [79]. In addition, we previously showed that phytoestrogens and their metabolites may disturb CL secretory functions through stimulation of T, PGF\(_{2\alpha}\), NO and LTC\(_4\) secretion [22, 64]. This is in agreement with our previous in vivo study, which proved that high soy diet significantly increased PGF\(_{2\alpha}\) secretion in soy-fed cows, causing the decrease of the rate of successful pregnancies and the increase in the mean insemination rate [79]. In addition, we previously showed that phytoestrogens and their metabolites may disturb CL secretory functions through stimulation of T, PGF\(_{2\alpha}\), NO and LTC\(_4\) secretion [22, 64].

**Phytoestrogens augment the cytokine actions in bovine CL during luteolysis**

Immune cells are present in the CL during the whole estrous cycle and their number increases during luteolysis [53]. Concentrations and distributions of immune cells depend on ovarian steroids including \( E_2 \) [58]. Phytoestrogens may affect \( E_2 \) action [32]. We investigated the involvement of selected isoflavones in mechanisms of CL regression by determining the influence of p-ethyl phenol and equol on luteolytic action of cytokines [22]. Steroidogenic CL cells were preincubated with phytoestrogenes and then treated with cytokines (TNF\( \alpha \), IL-1\( \beta \), Fas-Ligand and IFN\( \gamma \)). Isoflavones and \( E_2 \) augmented the stimulatory effect of cytokines on PGF\(_{2\alpha}\), LTC\(_4\) and NO secretion. Moreover, the influence of p-ethyl phenol and equol on the cytokine-induced cell death
was determined in the steroidogenic CL cells [22]. Although, cytokines alone did not influence cell viability, in the cells preincubated with isoflavones all cytokines revealed the cytotoxic effect. This suggests that the steroidogenic luteal cell exposure to phytoestrogens or E₂ rendered the cells more sensitive to luteolytic-cytotoxic action of cytokines. Thus, para-ethyl phenol and equol act as ER agonists modulating CL regression dependent on the presence of immune cells. Moreover, we examined the involvement of phytoestrogen metabolites (para-ethyl phenol, equol) and E₂ in the regulatory mechanisms functioning in different CL cell types (steroidogenic cells, endothelial cells and lymphocytes; [22]). Para-ethyl-phenol, equol and E₂ stimulated secretion of the main luteolytic factors, such as PGF₂α, LTC₄ and NO in steroidogenic cells alone and in cocultures with other types of CL cells [22].

In conclusion, at the time of luteolysis, structural and functional regression of bovine CL is activated by a cascade of products/factors of the CL cells (PGF₂α, cytokines, NO, LTC₄). Progesterone is the major auto- and/or paracrine factor preventing the induction of structural luteolysis (apoptosis) in the bovine CL. Endogenous and exogenous estrogenic compounds (phytoestrogens-isoflavones) play an opposite role to P₄ in the regulation of cell death and in the induction of luteolysis. We suggest that phytoestrogen-dependent stimulation of luteolytic substance production in the CL affects different regulatory levels and cause premature luteolysis, leading to embryonic loss during early pregnancy in cattle [55, 57]. Phytoestrogens sensitize steroidogenic cells of the bovine CL to cytotoxic cytokine action. Although phytoestrogens and their active metabolites do not influence basic and pulsatile P₄ secretion, they suppress the sensitivity of the CL to luteotropic factors (LH and PGE₂). Moreover, active phytoestrogen metabolites: para-ethyl-phenol and equol, stimulate production of local mediators of luteolysis in the bovine CL (PGF₂α, cytokines, NO, LTC₄). Finally, phytoestrogens, in contrast to E₂, exert only genomic, estrogen/androgen nuclear receptor dependent mechanism of action in the bovine CL cells.
GENISTEIN ACTION ON METABOTROPIC HORMONES AND LIPID-CARBOHYDRATE METABOLISM IN RATS DURING PREGNANCY

The influence of genistein on metabolism in pregnant rats

Studies concerning the influence of phytoestrogens on pregnant animals concentrate mostly on the effects exerted on the fetus or neonate. Data pertaining to phytoestrogens action on pregnant animal itself are scant. It was found recently that daidzein significantly diminished the blastocyst implantation rate and serum levels of gonadotropin-releasing hormone (GnRH), gonadotropins (FSH and LH) and P₄ in pregnant rats [80]. Harrison et al. [15] demonstrated that dietary genistein increased E₂ level during advanced pregnancy and delivery in pregnant rhesus monkeys without altering the P₄ level. Dietary genistein can also modify the expression of the estrogen-regulated P₄ receptor in the uterus during pregnancy and lactation in rats and, consequently, may have long-term reproductive health consequences [19].

Pregnancy demands motherly adaptation of metabolism and endocrine system suitably for the developing fetus. This adaptation may be disturbed by dietary factors which influence hormone and metabolic status. The effect of dietary genistein on this status in non-pregnant animals is relatively well recognized, but there are no data concerning pregnant animals. In our experiment [43] genistein was administered in the diet of Wistar rats throughout the entire pregnancy. Females were divided into two groups: 1/ genistein-fed rats (100 mg/kg of feed), and 2/ controls with no genistein in diet. Genistein did not change the serum insulin concentration in pregnant rats [43]. During pregnancy in the rat, higher concentrations of endogenous estrogens and P₄ are responsible for hypertrophy of pancreatic islets and enhanced insulin secretion. Probably, genistein as a compound with a lower affinity to ER than estrogens was not able, under examined conditions, to manifest its effect on plasma insulin. The results of semiquantitative RT-PCR analysis and western blotting also indicated a lack of significant effects of dietary genistein
action on insulin receptor expression in liver, adrenal and thyroid gland during pregnancy (Kaczmarek, unpublished).

Leptin, a fat tissue hormone known as a regulator of food intake and energy expenditure, is also involved in the regulation of reproductive processes. Both, leptin synthesis in fat tissue and serum concentration are enhanced during pregnancy [9]. During late pregnancy and on the first day after delivery, serum leptin concentration was reduced in genistein-treated rats [43] in spite of non-altered serum insulin level. The inhibitory effect of genistein on leptin was probably insulin-independent. It should be emphasized that this isoflavone was shown previously to reduce leptin secretion acting directly on isolated rat adipocytes [72]. Some other examples of the direct genistein action on rat adipocytes were also reported e.g. the restriction of insulin-stimulated glucose transport [67] and inhibition of lipogenesis [72].

Progesterone, an important gestation hormone is also an endogenous substrate for 21-hydroxylase in corticosterone synthesis. Genistein as a competitive inhibitor of 21-hydroxylase in vitro [47] is known to decrease adrenal steroidogenesis. During pregnancy, however, genistein did not manifest its inhibitory action on serum corticosterone concentration [43] probably due to the higher concentration of $P_4$. ACTH level in pregnant rats was also not affected by genistein. In contrast, during the post-parturition period a significant decrease in ACTH was observed in genistein-treated females in comparison to controls [43].

Glucose concentration (an important parameter of carbohydrate metabolism) in pregnant rats was not altered by genistein [43]. Available data concerning the effect of genistein on glucose concentration are unclear since the isoflavone was reported to evoke contradictory effects [42, 71]. In addition, lack of genistein effects on glycogen content was observed in liver and muscles of pregnant rats [43], although there are some reports indicating that genistein alters the activity of glycogen phosphorylase and modifies glycogen stores.

Plasma lipid (triglycerides, cholesterol and free fatty acids) concentrations and liver synthesis of triglycerides are elevated during gestation [17]. Activities of enzymes engaged in lipid metabolism (e.g. lipoprotein lipase, hormone sensitive lipase) are changed in pregnant females. Genistein can modify serum lipids in non-pregnant animals:
concentrations of triglycerides and free cholesterol were decreased and free fatty acids were increased in ovariectomized rats [42]. However, serum triglycerides, free fatty acids, cholesterol (total, free and HDL) as well as triglycerides and cholesterol in liver and muscle during pregnancy remained unchanged in genistein-treated females [43].

The effect of genistein on hormonal status and some metabolic parameters observed in pregnant rats is probably due to affinity of genistein to ER. The influence of genistein is clear in ovariectomized or male rats. However, during pregnancy – associated with a higher estrogen level – such an effect of genistein cannot be manifested. The reduced serum leptin concentration in genistein-treated pregnant animals and the decreased ACTH concentration after parturition are rather the result of ER-independent activity of genistein.

### The effect of genistein on metabolism in the immature female rats

Very young organisms i.e. fetus and neonate are sensitive to estrogen exposure. Genistein as a diet component possessing estrogen activity may affect the development of these organisms. Some genistein effects include disturbances of sexual differentiation, smaller anogenital distance, decreased birth body weight, delayed puberty and disrupted fertility of female rats [29]. In our study the effects of genistein on selected plasma hormones and metabolic parameters were investigated in the immature, female rats (Nowicka, unpublished). Genistein was administered intragastrically in two doses (1 and 5mg/kg b.w.) for seven days (once a day). Both doses of genistein significantly decreased serum insulin concentration and the higher dose reduced serum leptin concentration. The latter effect could be partially the consequence of a reduced serum insulin level or a direct influence of genistein on leptin secretion from adipocytes.

Genistein is known to exert a goitrogenic action because it inhibits thyroid peroxidase activity usually without affecting serum concentration of thyroid hormones. In young female rats receiving genistein no changes were found in total and free $T_3$ and $T_4$ as well as TSH blood levels (Nowicka, unpublished).
Among metabolic parameters determined in serum, liver and skeletal muscles, only the skeletal muscle content of triglycerides was significantly reduced in immature female rats after genistein treatment (5 mg/kg b.w.; Nowicka, unpublished). Some previous reports described this effect in mature male rats and in ovariectomized rats [42, 71]. Moreover, E$_2$ administered to male rats at a dose much lower than that of genistein also reduced muscle triglyceride content [71]. This suggests that the ER pathway might be involved in the genistein action. It can be concluded that genistein influence on hormonal and metabolic status is more pronounced in immature female than in pregnant rats.

THE EFFECTS OF PHYTOESTROGENS ON REPRODUCTIVE PROCESSES IN MALES OF BIŁGORAJ GOOSE

Up to date studies concerning phytoestrogens and male reproduction in mammals were focused on: 1/ the interactions between high dietary levels of phytoestrogens and processes during particular reproductive periods, and 2/ mechanism of phytoestrogen action in the prostate cancer protection. Available data [14] indicate that phytoestrogens may affect reproduction in males especially when consumed at high doses.

The effects of dietary phytoestrogens on avian reproduction have not been extensively studied. The California quail had poor breeding success after consuming foods with high phytoestrogen concentration (genistein, formononetin; [28]). On the other hand, no negative effects on reproductive performance were observed in bobwhites fed biochanin A [30]. Moreover, the increased weight of oviduct was noted in 12-day old chickens injected with genistein [7]. Dietary daidzein also exerted dose-dependent, divergent (negative or positive) effects on the egg-laying performance of Shaoxing ducks [81]. There is no data concerning the effect of phytoestrogens on male reproduction in geese. The Biłgoraj goose, a local breed in Poland, which is a domestic form of the Greylag goose (Anser anser) is characterized by short reproductive season and relatively low reproduction compared to other poultry species. Geese are fed diets containing plant-derived phytoestrogens in mammalian and avian reproduction
components rich in phytoestrogens (soy, alfalfa). Thus, research concerning the effects of phytoestrogens on reproductive processes in ganders seems to be vital. The aim of our studies was to examine: 1/ the influence of dietary phytoestrogens on plasma androgen levels, semen characteristics, fertility parameters and T secretion by testicular cells in ganders, 2/ an in vitro production of T by gander Leydig cells treated with phytoestrogens, and 3/ mechanism of phytoestrogen action in these cells.

The effects of dietary phytoestrogens on reproductive parameters in ganders

The influence of long-term dietary phytoestrogen exposure on reproduction of Biłgoraj ganders was investigated for two years (from August 2002 to July 2004). In 2002 year, 15 week-old ganders were divided into two groups: 1/ control group (C) fed diets containing grass meal (with low level of phytoestrogens), and 2/ experimental group (P) fed diets containing soy and alfalfa meal (with high level of phytoestrogens). Birds were fed the C and P diets during the photorefractoriness and laying periods. In the next year (2003), male goslings, derived from parents fed the C or P diets, were respectively divided (immediately after hatching) into the C or P group. Ganders were fed the C or P diets during the periods of growth, photorefractoriness and laying. All diets were balanced (crude protein, metabolizable energy, exogenous amino acids and minerals) according to nutrient requirements of domestic fowl. In all experiments, dietary phytoestrogen levels were determined and they were higher in the P group than in the C group. During the laying period, the concentration of phytoestrogens in the C and P groups amounted to 15.40 µg and 140.83 µg of total phytoestrogens per g of diet, respectively [50].

Blood for T and androstenedione (A₄) determination was collected monthly from December to June. No differences in T concentrations among the C and P groups were observed. In April, plasma A₄ level in ganders from the P group was higher than that of the C group [24]. Semen was sampled from February to May. Feeding of diets with high phytoestrogen levels decreased the volume of ejaculates and increased the number of abnormal spermatozoa [25].
Reproductive parameters (number and average weight of eggs, fertility of eggs, embryo mortality, percentage of normal hatching) were not different in herds (males and females) fed the C and P diets during the breeding season (Kugla-Owczarska, unpublished). These results are similar to those previously reported for Biłgoraj goose [56]. In male rats, the phytoestrogen-rich diet (600 µg isoflavones/g of diet) decreased plasma T and A4 concentrations [74]. Exposure to dietary genistein during gestation and lactation of female rats resulted in a lower plasma T concentration in their adult male offspring [77]. The aberrant or delayed spermatogenesis was observed in male rats exposed to dietary genistein during their development [13]. On the other hand, neither serum T concentration nor sperm counts in male rats were changed by neonatal oral administration of genistein [41]. Similarly, neonatal coumestrol injected into male rats did not alter the weight of testes, sex accessory organs, sperm count, as well as serum concentrations of T, LHβ and FSH [2]. These results indicate that phytoestrogens may affect reproduction in males, especially when they are consumed at high doses.

The phytoestrogens effects on testosterone secretion by gander Leydig cells

Testes used in the in vitro study were obtained from both groups of ganders at three different times of the breeding season: the peak of reproductive activity (March), the second half of reproductive activity (May) and the beginning of photorefractoriness (July). The isolated Leydig cells (1×10⁵/ml) were incubated (37°C) for 20 hours. No differences in the mean weight of testes from ganders were noted between experimental groups. Feeding of diets with high levels of phytoestrogens did not change the basal and LH-stimulated T secretion by gander Leydig cells. In vitro treatment of genistein, daidzein and equol inhibited basal and LH-stimulated T production by Leydig cells from both, the C and P groups. Coumestrol inhibited basal in vitro T secretion only in March in the C group. Genistein showed the strongest and coumestrol the weakest effect on testicular secretion [50]. Similarly, in our previous study [49], genistein decreased T secretion...
by Leydig cell in roosters (*Gallus gallus domesticus*). Additionally, hollyhock extract containing other phytoestrogens, quercetin and kaempferol, reduced Leydig cells production of T in rats [52]. Moreover, long-term dietary administration of genistein inhibited Leydig cell steroidogenesis *ex vivo* [69], but without altering the serum level of either LH or T.

Since the mechanism of phytoestrogen action in gonadal cells of ganders has not been investigated, the possibility of phytoestrogen action via estrogen/androgen receptors or protein tyrosine kinases pathway was examined in incubated gander Leydig cells (1×10^5/ml; 20 h; 37°C). The effects of hydroxytamoxifen (ER inhibitor) and cyproterone acetate (androgen receptor antagonist) on phytoestrogen inhibition of T release by Leydig cells were not observed. Lavendustin A (protein tyrosine kinases inhibitor) did not affect T production. These results suggest that phytoestrogen action in Leydig cells of ganders did not depend on androgen receptor or inhibition of protein tyrosine kinases. In addition, the influence of phytoestrogens seems not to be conducted by ERs [51]. Further studies are required to reveal the mechanism of phytoestrogen action in gander testis.

In conclusion, we showed that in ganders, dietary exposure to phytoestrogens affected plasma A_4 concentration and semen characteristics, but did not change plasma T concentration and reproductive parameters. In general, phytoestrogens exerted an inhibitory effect on *in vitro* secretion of T by gander Leydig cells. This inhibitory effect was demonstrated in both examined groups. The influence of phytoestrogens seems not to be conducted via estrogen and androgen receptors or protein tyrosine kinases system in these cells. However, further studies are required to elucidate the mechanism of phytoestrogen action in testis of ganders.

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