BIOCHANIN A AND DAIDZEIN INFLUENCE MEIOTIC MATURATION OF PIG OOCYTES IN A DIFFERENT MANNER*

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The aim of the study was to determine the influence of different concentrations of phytoestrogens biochanin A (BIO A; 20, 40, 50µg ml^{-1}) and daidzein (DAI; 10, 20, 40, 50µg ml^{-1}) on the course of meiotic maturation of pig oocytes. After a 24-hour cultivation, a stage of nuclear maturation was achieved and the areas of cumulus-oocyte complexes (COCs), as an indicator of cumulus expansion, were evaluated. The effects of both contaminants on oocytes were manifested from the lowest concentration used. Nuclear maturation was inhibited in a dose-dependent manner in the case of BIO A. Effects of DAI reached a plateau at a concentration of 20µg ml^{-1}.Possible relationship to limited solubility of DAI was excluded because limits of DAI solubility in culture medium were confirmed at 50 µg ml^{-1}.The cumulus expansion was also influenced in a different manner – reduction of the COC's area by BIO A was dose-dependent, whereas DAI had the strongest effect on CCs in the lowest and highest concentrations used. Both phytoestrogens negatively influence the meiotic maturation of porcine oocytes but there are significant differences in their concrete effects which could relate to the diverse mechanisms of their acting on target cells.

reproduction; sow; soya; phytoestrogens; meiosis

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INTRODUCTION

Phytoestrogens are substances of plant origin, which appear in many food and feed sources. Among them clover and soya are significant sources of the isoflavones biochanin A (BIO A), daidzein (DAI), and genistein (GEN) (Kalač, Míka, 1997).The phytoestrogens act similarly to estradiol through the binding to estrogen receptors (ERs) of cells (Tapiero et al., 2002; Dusza, Ciereszko, 2006). Therefore, reproductive functions are the primary target. (Benassayag et al., 2002; Vrzáňová, Heresová, 2003).Although the phytoestrogens are perceived in human nutrition rather positively, their effects on animals, including livestock, are mostly unfavourable (Adams, 1995). They can affect gonad development and/or cause abortions (Romero et al., 2008), disturbances of the estrous cycle (Adams, 1995), infertility, and a number of other reproductive problems, including disturbances of gametes (Kurzer, Xu, 1997; Rosselli et al. 2000; Burton, Wells, 2002; Dusza, Ciereszko, 2006).

Although the binding activity to ERs is a common character of the phytoestrogens, some differences in their action were described.Their binding affinity to ERs is not equal and estrogenic activity decreases in the sequence GEN > DAI > BIO (Benassayag et al., 2002 Gruber et al., 2002). Moreover, inhibition of tyrosin protein kinases (TPK),correlating to certain effects of phytoestrogens (Jung et al., 1993),may not necessarily be due to binding to ERs (Hubbard, Miller, 2007).GEN and BIO A, but not DAI, are traditionally ranked among the phytoestrogens with the capability of TPK inhibition. Although DAI has been considered as an inhibitor of TPK quite recently–based on software analysis of chemical structures (Chemschetch, Chemaxon, version 5.4) (Hashemi et al., 2012), its potential is

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possibly lower in this respect. It is obvious, that such differences do not allow extrapolating the effects of a particular phytoestrogen from any other representative of this group automatically.

Regarding the phytoestrogen influence on mammalian oocytes, GEN was repeatedly studied. There were discovered negative effects on nuclear maturation of oocytes as well as on the compartment of cumular cells manifested as inhibition of the germinal vesicle breakdown (GVBD),the extrusion of the polar body in several animal species, as well as the expansion of cumular cells (Jung et al., 1993; Makarevich et al., 1997; Vodková et al., 2008 – pig; Van Cauwenberge, Alexandre, 2000; Yoshida, Mizuno, 2012 – mouse). Interestingly, some stimulatory effects on the meiotic maturation of porcine oocytes were reported with low doses of GEN (Makarevich et al., 1997).

Although these effects on oocytes were primarily applied to inhibition of TPK, there is a surprising agreement with the influence of 17ß-estradiol acting through the ERs binding (Beker et al., 2002; Li et al., 2004).Vodková et al. (2008) confronted GEN effects with those of genistin (GIN), a glycosidic form of GEN without inhibitory action on TPK. Although less pronounced if compared to GEN, some effects of GIN were documented.

In contrast to GEN, the number of studies focused on DAI or BIO A effects on meiotic maturation is limited. Van Cauwenberge, Alexandre (2000) reported inhibitory effects of DAI on mouse oocyte transition from germinal vesicle (GV) stage to metaphase I (MI) at concentrations of 50, 100, and 200µM, although the general effects on mouse oocytes were weaker than those of GEN. Yoshida, Mizuno (2012) reported inhibitory effects on mouse oocytes at a concentration of 100µM, but they failed at 5 and 25µM concentrations. In pig, Galeati et al. (2010) did not observe any effects on porcine oocyte nuclear or cytoplasmic maturation but they noticed a decrease of progesterone production by cumular cells at concentrations of 1 or 10µM of DAI. The cumular cell expansion was not evaluated. How is the DAI concentration affecting nuclear maturation of porcine oocytes is not clear. Concerning BIO A, we did not find any data on its effects on oocyte meiotic maturation.

The aim of this study was to determine and compare the effects of BIO A and DAI on the nuclear maturation and cumulus expansion at porcine oocytes after 24-hour *in vitro* maturation, and to determine their effective concentrations.

MATERIAL AND METHODS

Ovaries collection

Porcine ovaries were obtained from non-cycling gilts at a slaughterhouse. The ovaries were transported to the laboratory in a saline solution (0.9% NaCl) at 39°C.

Oocyte isolation and culture

Oocytes were collected from the ovarian follicles (3–5 mm) with a 20-gauge aspirating needle. The oocytes were maturing in 4-well dishes (Nunc, Roskilde, Denmark) in 1 ml of modified M199 medium (Sigma-Aldrich, St. Louis, USA), in a thermo box at 39°C in an air mixture with 5.0% CO_2. In 1 ml of culture medium there were 50 oocytes at the most. Before oocyte maturation the medium M199 was enriched with 13.5 IU eCG : 6.6 IU hCG ml^{-1}(P.G.600; Intervet International B.V., Boxmeer, the Netherlands) and with calf serum growth proteins (8.14g l^{-1}) in the amount of 20µl ml^{-1} of maturating medium (BIOPHARM, Research Institute of Biopharmacy and Veterinary Drugs, a.s., Jílové u Prahy, Czech Republic). Only fully-grown oocytes with intact cytoplasm and compact cumuli were used in the experiments. The oocytes were cultured for 24 h to metaphase I (MI) with the addition of the relevant dose of BIO A in concentrations of 0, 20, 40, or 50 µg ml^{-1}or DAI (both Sigma-Aldrich) in concentrations of 0, 10, 20, 40, or 50 µg ml^{-1}. As the HPLC analysis revealed significant limitation of DAI solubility at the highest concentration tested, the concentration of 50 µg ml^{-1} in culture medium in fact corresponds to the addition of 80 µg ml^{-1}.

Oocyte evaluation

After 24h of maturation a digital image of the oocytes and their cumular cells (cumulus-oocytes complexes; COCs) was recorded. The cumular cells were then removed by pipetting through a narrow glass pipette. The oocytes were mounted on microscope slides and fixed in acetic acid/ethanol (1:3) for 24 h. The area of the COC was measured and evaluated by digital image analysis using NIS-Elements AR software (Version 3.10, 2009). Nuclear maturation was evaluated under light microscope after staining the oocytes with 1% orcein.

Statistical analysis

The oocytes were subjected to further evaluation only if at least 85% of the control group oocytes reached the MI stage after a 24-hour cultivation. All the experiments were repeated four times at least. For statistical evaluation of COC expansion, the Kruskal-Wallis ANOVA test was used, and for nuclear maturation, the chi-square test.

Analysis of solubility by the HPLC method

The actual concentrations of DAI in the medium prepared were determined using the HPLC technique with

Table 1. Effect of BIO A on nuclear maturation of pig oocytes cultured for 24h

Meiotic maturation stage (%)	Concentration of BIO A (μg/ml)			
	0	20	40	50
GV	3^{A}	4^{A}	54^{B}	72^{C}
LD+PM	5^{AC}	12^{AB}	20^{B}	3^{C}
MI	88^{A}	61^{B}	17^{C}	5^{D}
MII	2^{A}	19^{B}	2^{A}	0^{A}
Ab+deg	2^{A}	4^{AB}	7^{C}	20^{D}
Number of oocytes	143	96	98	101

GV – germinal vesicle, LD + PM – late diakinesis + premetaphase, MI – metaphase I, MII – metaphase II, Ab. + deg. – abnormal and degenerate oocytes.
^{A,B,C,D} – values with different superscripts in the row differ on the level ($P < 0,05$)

Table 2. Effect of DAI on nuclear maturation of pig oocytes cultured for 24h.

Meiotic maturation stage (%)	Concentration of DAI (μg/ml)		
	0	10	20
GV	4^{A}	4^{A}	14^{B}
LD+PM	2^{A}	10^{B}	4^{AB}
MI	88^{A}	85^{A}	72^{B}
MII	5^{A}	0^{B}	5^{A}
Ab+deg	1^{A}	1^{A}	5^{B}
Number of oocytes	159	97	125

GV – germinal vesicle, LD + PM – late diakinesis + premetaphase, MI – metaphase I, AI – TI – anaphase to telophase, MII – metaphase II, Ab. + deg. – abnormal and degenerate oocytes.
^{A,B} – values with different superscripts in the row differ on the level ($P < 0,05$)

UV detection. The Summit®HPLC Analytical System (Dionex Corp., Sunnyvale, USA) was equipped by a Gemini C18 column (5 μm, 110A, 4.6×250 mm) with a HPLC Guard Column (Phenomenex Inc., Torrance, USA). Binary gradient elution was used by means of acetonitrile (ACN) (LAB-SCAN, Gliwice, Poland) and trifluoroacetic acid (TFA) (Sigma-Aldrich). The UV detection was performed at a wavelength of 260 nm (L e u n e r et al., 2013).

RESULTS

The pig oocytes were exposed to DAI and BIO A for 24 h and their influence on meiotic maturation – nuclear maturation and cumulus expansion – was evaluated under *in vitro* conditions. The resulting levels of nuclear maturation of oocytes exposed to BIO A or DAI are presented in Tables 1 and 2.

Influence of BIO A and DAI on nuclear maturation of pig oocytes

BIO A, as well as DAI, blocked nuclear maturation of porcine oocytes. Both phytoestrogens manifested their effects from the lowest concentrations used. However, they differed in the strength and trend of their influence. BIO A decreased the number of oocytes achieving the MI stage after a 24-hour cultivation in a dose-dependent manner. This effect was significant ($P< 0.05$) already at the lowest concentration of BIO A (20μg ml^{-1}) in the cultivation medium used. However, this concentration accelerated a nuclear maturation to metaphase II (MII) stage in 19% of oocytes. This effect was significant and did not repeat at higher concentrations. With the further increasing of BIO A concentrations in the culture medium the percentage of oocytes, which did not commence meiotic maturation and were arrested in the germinal vesicle (GV) stage, significantly increased. The occurrence of altered oocytes, particularly with abnormalities in the metaphase plate, with the absence of chromosomes or degenerated oocytes, also rose with the increasing concentrations (Table 1).

DAI slowed down nuclear maturation at all the concentrations tested. Whereas the lowest concentration of DAI (10μg ml^{-1}) in the culture medium significantly ($P< 0.05$) increased a portion of oocytes on the level of late diakinesis (LD) or premetaphase (PM), when the higher concentrations of DAI were used, significantly more oocytes ($P< 0.05$) did not begin nuclear maturation and remained at the GV stage. There was no difference in the intensity of this DAI effect among the concentrations of 20, 40, or 50 μg ml^{-1}. Therefore, we do not present the data on the two highest concentrations in the table (Table 2).

Table 3. Influence of BIO A on area of COCs after 24-hour cultivation

Concentration of BIO A (µg/ml)	Mean of area of COCs (%)
0	100^{A}
20	69^{B}
40	49^{C}
50	36^{D}

^{A,B,C,D} – values with different superscripts in the column differ on the level ($P < 0,05$)

Table 4. Influence of DAI on area of COCs after 24-hour cultivation

Concentration of DAI (µg/ml)	Mean of area of COCs (%)
0	100^{A}
10	52^{B}
20	70^{C}
40	64^{C}
50	37^{D}

^{A,B,C,D} – values with different superscripts in the column differ on the level ($P < 0,05$)

Influence of BIO A and DAI on cumulus expansion

The degree of cumulus expansion is expressed as percentage of the average area of the COCs in the experimental medium to the corresponding control. Data for BIO A are illustrated in Table 3 and for DAI in Table 4 as means of partial experiments. Both the tested phytoestrogens reduced the area of COCs during meiotic maturation at all the concentrations used ($P< 0.05$).

BIO A inhibited the cumular cell expansion area in a dose-dependent manner, whereas DAI manifested the most effective influence when the lowest and the highest concentrations were used. The both substances exhibited similar effects, i.e. an equal rate of COC area inhibition at a concentration of 20 or 50 µg ml^{-1}.

HPLC analysis of DAI solubility

In contrast to BIO A, the DAI effects to nuclear maturation reached a plateau at a concentration of 20 µg ml^{-1}. For this reason, the solubility of DAI in the culture medium was verified. HPLC analyses showed standard solubility up to 40 µg ml^{-1} with its subsequent sharp decrease. Maximum concentration of DAI reached in the culture medium was 50 µg ml^{-1} after a dose of 80 µg ml^{-1} and a higher dose of 100 µg ml^{-1}did not increase the amount of dissolved DAI in the culture medium.

DISCUSSION

Our data demonstrate the negative influence of BIO A and DAI on the initiation and subsequent course of meiotic maturation of porcine oocytes under *in vitro* conditions. Both phytoestrogens inhibited the nuclear maturation of oocytes. However, there are differences in the intensity of their inhibitory effects and the level of negative influence on chromosomal formation.

In this study, BIO A mostly inhibited the nuclear maturation of oocytes. This is not a surprising result, if we consider that negative effects of BIO A are also described in somatic cells. This phytoestrogen inhibits the cell cycle and suppresses the synthesis of cyclin B (Rice et al., 2002), which is a protein necessary for the resumption of meiosis of mammalian oocytes (Levesque, Sirard, 1996; Reis et al., 2006). Both these facts are probably reflected in BIO A effects on oocytes. Acceleration of oocyte maturation observed at the lowest concentration used seems to be surprising. But we can find a parallel in the paper by Makarevich et al. (1997) who presented some stimulatory effects of low doses of GEN, too.

In our experiment, the number of oocytes with the abnormal configuration of the metaphase plate did increase (up to 20%) with an increasing dose of BIO A. The abnormalities were manifested especially in the unequal distribution of chromosomes, duplication of the chromosomal set or a total absence of chromosomes. We may observe analogical situations in somatic cells exposed to the influence of BIO A (Rice et al., 2002). This phytoestrogen is known for its inhibitory effect on DNA topoisomerase II, the enzyme which participates in DNA replication, particularly in the condensation and segregation of chromosomes (Tsutsui et al., 2003). This was proved by an increased occurrence of aneuploidies, DNA adducts, and morphological changes of embryonic cells after cultivation with doses of 50 and 100µM of BIO A. This phytoestrogen changed the expression of the number of genes which ensure the physiological formation of microtubules (Rice et al., 2002). Regarding the fact that a mature oocyte is transcriptionally inactive (Fuente, Eppig, 2001), the influence of BIO A on the aneuploidy of maturing mammalian oocytes must be explained in a different way.

The BIO A effects observed in this study were of a very similar pattern to GEN tested in the same culture system by Vodková et al. (2008). The effects of BIO A could be related to its similarity to GEN but also to its ability to be metabolized to the GEN in the organism (King, 2002). In general, GEN is considered as a phytoestrogen with a higher binding affinity to ERs and estrogenic activity compared to BIO A (Benassayag et al., 2002). It is interesting that BIO A in this study inhibited nuclear maturation more intensively than GEN did in comparable concentrations in the study by Vodková et al. (2008). It is well known that GEN inhibits somatic cells in G2/M transition of the cell cycle (Mukherjee et al., 2010), as well as the meiotic maturation of oocytes

in pigs (Jung et al., 1993), cattle (Borzym et al., 2008), and mice (Yoshida, Mizuno, 2012). The effects of GEN are often primarily attributed to its inhibitory effect on protein kinases (Jung et al., 1993), although Vodková et al. (2008) observed some inhibitory effects also at GIN which does not inhibit protein kinases, nevertheless it has a similar affinity to ERs as GEN (Li et al., 2004 in Vodková et al., 2008). Also, 17ß-estradiol acting through the ERs binding revealed similar effects on oocyte maturation (Beker et al., 2002; Li et al., 2004). Thus, the observed effects of BIO A as well as GEN might be – at least partially– a result of interaction with ERs. The effect of GEN could explain an observed abnormal configuration of chromatin, because GEN induces a depolymerization of microtubules in somatic cells by binding to a specific position on tubulin (Mukherjee et al., 2010). However, we cannot exclude the other mechanisms of action, because BIO A and GEN inhibit the expression of polo-like kinase 1, for example, in somatic cells (Seo et al., 2011), which participate in the regulation of meiosis of mammalian oocytes (Yao et al., 2003).

In our study, DAI also manifested negative effects on the nuclear maturation of oocytes. The lowest concentration – of 10µg ml^{-1} (i.e. 40µM) – only partially inhibited nuclear maturation, because a significant part of the oocytes (10%) achieved late diakinesis, not the MI stage. A higher concentration of DAI – 20µg ml^{-1} (i.e. 80µM) – already prevented the GVBD during meiotic maturation.

Van Cauwenberge, Alexandre (2000) used concentrations similar to ours (DAI 50, 100, 200µM) and also noted the inhibitory effects of DAI on nuclear maturation of mouse oocytes. Yoshida, Mizuno (2012) also noted this, at a dose of 100µM of DAI. However, the authors in the afore-mentioned experiments found a markedly higher percentage of inhibited oocytes compared to our data. This may indicate that porcine oocytes might be less sensitive to the effects of DAI than mouse oocytes. Nevertheless, the experiments of testing markedly lower doses of DAI (1 and 10µM, or 5 and 25µM, respectively) do not describe the effects on the course of nuclear maturation of either porcine (Galeati et al., 2010) or mouse (Yoshida, Mizuno, 2012) oocytes cultured to MII stage.

In contrast to BIO A, the negative effects of DAI were not manifested on the chromosomes of oocytes. Yoshida, Mizuno (2012) also did not notice any changes on the meiotic spindle in mice oocytes. This fact is also supported by the fact that DAI, in contrast to BIO A, does not influence the activity of the DNA topoisomerases (Cornwell et al., 2004) which partake in the general dynamics of chromosomes.

We demonstrated the effects of both phytoestrogens on cumular cell expansion, already from the lowest concentrations used. BIO A inhibited the expansion of cumular cells in a dose-dependent manner, similarly to nuclear maturation. The inhibitory effect of DAI was not dose-dependent and the maximum intensity was achieved when the lowest and the highest concentrations of supplement were used.

The results of the other studies focused on the influence of BIO A and DAI on the expansion of cumular cells are missing. Nevertheless, the study from our laboratory focused on GEN, the other flavonoid mentioned above, shows inhibition of the expansion of cumular cells in comparable concentrations to BIO A and DAI (Vodková et al., 2008), in a dose-dependent manner like BIO A. The effect of GIN, a glycosidic form of GEN, on the expansion of cumular cells was markedly weaker (Vodková et al., 2008) and did not suppress the expansion of cumular cells in a dose-dependent manner, similarly to DAI. The absence of a dose effect of DAI on the expansion of cumular cells could be due to the phenomenon referred to as low-dose effect, which is typical for endocrine disruptors (Vandenberg et al., 2012). Regarding similarities between DAI and GIN effects on cumulus expansion, it is noticeable that the both were considered not to inhibit TPK (Li et al., 2004; Van Cauwenberge, Alexandre, 2000) although according to software analysis of chemical structures by Hashemi et al. (2012), DAI should have an inhibitory potential.

CONCLUSION

Finally, BIO A as well as DAI manifested their inhibitory effects on nuclear maturation, as well as on the expansion of cumular cells of oocytes. There are differences between BIO A and DAI in the intensity and pattern of their negative impact on the course of meiotic maturation. Their specific effects on oocytes could be caused by the various mechanisms of action, different affinity to ERs or different target structures which partake in the final effect of these substances, because the exact mechanism of the action of phytoestrogen has been unclear as yet.

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