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# INFLUENCE OF DAIDZEIN ON MEIOTIC MATURATION OF PIG OOCYTES

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## Abstract

Daidzein is ranked among phytoestrogens, natural substances which influence an organism due to their binding to estrogen receptors, similarly to endocrine disruptors. For that reason, their effects are manifested especially in the field of reproduction. One of the important presumptions of female reproduction is a physiological course of meotic maturation of oocytes. The high portion of soya in a pig's diet predetermines this species for the possible influence of daidzein on reproductive functions. The aim of this study was to determine the effects of an increasing concentrations of daidzein on the meiotic maturation progress of porcine oocytes under *in vitro* conditions. The oocytes were exposed to different concentrations of daidzein (10, 20, 40, 50µg/ml). After 24 hours cultivation the stage of nuclear maturation and the area of cumulus – oocyte complexes reached as a detector of cumular cell expansion were evaluated. The high level of daidzein's solubility was determinated by the HPLC method.

The effects of daidzein on oocytes were already manifested at the lowest concentration used. Nuclear maturation was inhibited in a dose dependent manner. The maximum effects were observed in the concentration of 20µg/ml, and inhibitory effects of higher concentrations were determinate at the same intensity. Daidzein suppressed the expansion of cumular cells as well. The lowest (10µg/ml) and highest (50µg/ml) concentrations had the strongest effects on cumular expansion. In conclusion, daidzein negatively influences the meiotic maturation of porcine oocytes, whereas the expansion of cumular cells reacts more sensitively than nuclear maturation of oocytes to this soya phytoestrogen.

**Keywords:** phytoestrogen, daidzein, pig, meiosis, oocyte

## Introduction

Phytoestrogens are related to a number of other natural, but also synthetic, substances, classified among so - called endocrinous disruptors – substances with a similar chemical structure to estradiol. This enables them to bind to estrogen receptors and elicit estrogenic or anti - estrogenic activities (Holoubek and Čadová, 2000; Tham et al., 1998). Estrogenic activity of phytoestrogens is dramatically lower than 17 ß- estradiol. However, their concentration in an organism may be markedly higher compared to endogenous estrogens (Tham et al. 1998). Therefore, the effects of phytoestrogens can be manifested in an organism very intensively.

Many livestock are exposed to the influence of phytoestrogens, because they especially appear in clover and legumes. This was confirmed in grazing ruminants, but also in pigs fed with a soya diet. The effects of phytoestrogens on animals are mainly unfavorable, in contrast to the human organism, and they especially affect the area of reproduction (Adams 1995). In many livestock, the effects of phytoestrogens on sexual differentiation and gonad development, as wel as on abortions, were documented (Romero et al., 2008). Pathological changes in the reproductive system (Adams, 1996; Burton and Wells, 2002), disturbances of

the estrous cycle (Adams 1995), infertility and a number of reproductive problems, including negative effects on gametes, were also observed (Burton and Wells, 2002; Kalač and Míka 1997; Kurzer and Xu, 1997; Rosselli et al. 2000; Dusza and Ciereszko, 2006).

The meiotic maturation of oocytes is one of the important moments of reproduction. It can be influenced by many internal and external factors, and its physiological course is fundamental for successful fertilization and resulting embryonic development. Experimentally, under *in vitro* conditions, it was found, that the addition of the naturally occurring endogenous hormone 17 ß- estradiol inhibits the meiotic maturation of porcine (Li et al., 2004) and bovine oocytes (Beker et. al., 2002). Various phytoestrogens affect oocytes similarly, but the intensity of their effects and their negative impact on the course of meiotic maturation are different.

Meiotic maturation was inhibited after the addition of another phytoestrogen – genistein – to the cultivation medium. This was confirmed in several animal species (Jung et al., 1993; Makarevich et al., 1997; Vodková et al., 2008 – pig, Van Cauwenberge and Alexander, 2000; Yoshida et Mizuno, 2012 – mouse). On the other hand, when low doses of genistein were added, the meiotic maturation of porcine oocytes was stimulated (Makarevich et al., 1997). Daidzein, similarly to genistein, has an inhibitory effect on the meiotic maturation of mouse oocytes. Nevertheless, the inhibitory effect of daidzein is markedly lower in comparison with genistein (Van Cauwenberge and Alexander, 2000). From the papers by Van Cauwenberge and Alexander (2000) and Yoshida et Mizuno (2012) it seems that it is difficult to prove the daidzein effects on mouse oocyte nuclear maturation at doses lower than 50 µM. The daidzein effects on pig oocyte maturation were not found (Galeati et al., 2010) but very low doses were tested in this experiment (1 a 10µM).

In general, the cumular cells also play an important role in the effect of estrogenic substances on oocytes, including their meiotic maturation. These cells enclose an oocyte in the antral follicle and harbor an estrogen receptor, similarly to oocytes (Wassarman and Albertini, 1994). The importance of cumular cells consists in the regulation of interruption and resumption of meiosis during oocyte development and in the support of the subsequent cytoplasmatic maturation of the oocyte (Tanghe, 2002). During meiotic maturation the cumular cells synthesize the structural components of the extracellular matrix, whereby their expansion and increase of the total cumulus-oocyte complex (COC) takes place (Eppig, 1979).

Similarly to nuclear maturation, expansion is influenced by estradiol (Li et al., 2004) as well as by some phytoestrogens. Whereas genistein inhibits the cumular cell expansion of oocytes in mice (Tirone et al., 1997) and in pigs (Jung et al., 1993; Vodková et al., 2008), in daidzein such data are missing. Nevertheless, the decrease of progesterone production during pig oocyte *in vitro* maturation noted by Galeati et al. (2010) indicates the effects on cumular cells activity, even at above mentioned very low daidzein concentrations.

The aim of this experiment was to determine the influence of various concentrations of daidzein on meiotic maturation of pig oocytes after 24 hour *in vitro* cultivation. The achieved stage of nuclear maturation reached and the area of expanded cumular cells or the entire cumulo-oocyte complex (COC) were evaluated.

## Materials and Methods

Acquisition of ovaries

Porcine ovaries were obtained from non-cycling gilts at an unknown phase of estrus cycle at a slaughterhouse. The ovaries were transported to the laboratory in a saline solution (0.9 % NaCl) at 39 °C. Oocytes were collected from the ovarian follicles (3 – 5 mm) with a 20-gauge aspirating needle. Only fully-grown oocytes with intact cytoplasm and compact cumuli were used in the experiments.

Selection, cultivation and evaluation of oocytes

The oocytes were cultivated in modified M199 medium (Sigma-Aldrich, USA), containing sodium bicarbonate (32.5 mM), calcium L-lactate (2.75 mM), gentamicin (0.025 mg/ml), HEPES (6.3 mM), 13.5 IU eCG: 6.6 IU hCG/ml (P.G.600; Intervet, Holland) and fetal calf serum (8,14g/l) in the amount of 20µl/ml cultivation medium (BIOPHARM, Research Institute of Biopharmacy and Veterinery Drugs, a.s.). The oocytes were matured in 4 – well dishes (Nunc, Roskilde, Denmark) containing 1 ml of culture medium at 39 °C in an air mixture of 5.0 % $CO_2$. The oocytes were cultivated for 24hours to metaphase I (MI) with the addition of relevant dose of daidzein (Sigma Aldrich) in concentrations 0, 10, 20, 40, 50µg/ml. After cultivation a digital image of the oocytes and their cumular cells (COC) was recorded. Then the cumular cells were removed by pipetting through a narrow glass pipette. The oocytes were fixed in a solution of acetic acid and ethanol (1:3).

The area of COC was measured and evaluated by digital image analysis by NIS-Elements software (AR, 3.10, Laboratory Imaging, CZ). 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 24hours cultivation. For statistical evaluation of COCs the Kruskal – Wallis ANOVA test was used, and for nuclear maturation, the chi-square test.

Analysis of solubility by HPLC method

The actual concentrations of daidzein in the solutions created were determined using the HPLC technique with UV detection. The HPLC SUMMIT instrumentation (automated sample injector ASI-100; thermostatted column compartment TCC-100; dual gradient pump P680 and photodiode array detector PDA-100) was manufactured by Dionex (Germany and USA) and operated by software Chromeleon (Dionex, Germany). A Gemini C18 column, 5 µm, 110A, 4.6×250 mm with a guard column (Phenomenex, Torrance, CA, USA) was used, and the column temperature was set to 45 °C. Binary gradient elution was used using 100% acetonitrile (ACN) (LAB-SCAN - Gliwice, Poland) and 0.1% trifluoroacetic acid (TFA) (Sigma-Aldrich) as the mobile phase at a ratio of 15 : 85 rising to 70 : 30 over 40 min; the flow rate was 0.8 mL $min^{-1}$ The UV detection was performed at a wavelength of 260 nm (Leuner et al.2013).

## Results and discussion

The pig oocytes were exposed to daidzein for 24 hours, and it's influence on meiotic maturation, nuclear maturation and expansion of cumular cells was evaluated *in vitro* (Table 1, Table 2.)

1. Influence of daidzein on nuclear maturation of oocytes

Daidzein blocked nuclear maturation in all concentrations tested. The lowest concentration of daidzein (10µg/ml) in the cultivation medium significantly ($P < 0.05$) affected the nuclear maturation of oocytes, when expressive retardation occurred and oocytes after 24 hour cultivation did not reach the MI stage, but only late diakinesis (LD) or premetaphase (PM). When higher concentrations of daidzein (20, 40, 50µg/ml) were used, significantly more oocytes ($P < 0.05$) did not begin nuclear maturation and remained in the germinal vesicle (GV) stage, where there was no difference in intensity of effect between individual concentrations (Table 1).

Table 1 – Nuclear maturation of oocytes after 24hours cultivation with daidzein

| Meiotic maturation stage (%) | Concentration of daidzein | | | | |
|---|---|---|---|---|---|
| | 0µg/ml | 10µg/ml | 20µg/ml | 40µg/ml | 50µg/ml |
| GV | $3^{A}$ | $4^{A}$ | $14^{B}$ | $12^{B}$ | $14^{B}$ |
| LD+PM | $1^{A}$ | $10^{B}$ | $4^{AB}$ | $8^{B}$ | $11^{B}$ |
| MI | $88^{A}$ | $85^{A}$ | $72^{B}$ | $74^{B}$ | $71^{B}$ |
| MII | $5^{A}$ | $0^{B}$ | $5^{A}$ | $2^{A}$ | $2^{A}$ |
| Ab+deg | $1^{A}$ | $1^{A}$ | $5^{A}$ | $4^{A}$ | $2^{A}$ |
| Number of oocytes | 159 | 97 | 125 | 100 | 104 |

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

2. Influence of daidzein on expansion of cumular cells

The degree of cumular cell expansion is expressed as the mean area of cumulo-oocyte complexes (COC) in percentage compared to the area of COC cultivated in a non-supplemented medium (Table 2).

In oocytes cultivated during meiotic maturation together with daidzein, the COC was smaller in all concentrations tested ($P < 0.05$). The most effective concentrations were the lowest and highest (10 and 50µg/ml), as the oocytes reached 52% and 37% of the area of COC when compared to the control group.

Table 2 – Area of cumulus oocyte complexes (COC) after 24hours cultivation with daidzein

| **Concentration of daidzein (µg/ml)** | **Average of areas COC (%)** |
|---|---|
| 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 row differ on the level (P Y 0,05)*

In this study we demonstrated a negative influence of daidzein on meiotic maturation of pig oocytes under *in vitro* conditions. Nuclear maturation of oocytes was inhibited from the lowest concentration (10µg/ml) used. This concentration inhibited nuclear maturation partially, because only 10% of the oocytes achieved late diakinese, not the MI stage. A higher concentration of daidzein (20µg/ml, if you like 80µM) already prevented the germinal vesicle breakdown in the course of meiotic maturation.

Van Cauwenberge and Alexander (2000) used concentrations similar to our experiment (Dai 50, 100, 200μM) and also noted inhibitory effects of daidzein on nuclear maturation of mouse oocytes, as well as Yoshida and Mizuno (2012) did at a dose of 100μM of daidzein. However, in the experiments mentioned the authors found a markedly higher percent of inhibited oocytes. This can indicate that porcine oocytes might be less sensitive to daidzein's effects than mouse oocytes are. Contemporarily it seems, the lack of daidzein effect on pig oocyte nuclear maturation in the paper by Galeati et al. (2010) was really related to low concentration used not to the properties of daidzein itself.

We also demonstrated the effect of daidzein on cumular cell expansion from the lowest concentrations used. The inhibitory effect was not dose dependent and maximum intensity was reached when using the lowest and highest concentration of the supplement.

The results of studies concerned with the influence of daidzein on cumular cell expansion are lacking. Nevertheless, the inhibition of this phenomenon observed in our study agrees with the decrease of progesterone production during pig oocyte *in vitro* maturation published by Galeati et al. (2010). Also, the results can be compared with another flavonoid with an affinity to estrogen receptors, which is genistein. This substance also significantly suppresses expansion of the cumulus (Vodková et al., 2008). In contrast to daidzein, the inhibitory activity of genistein was observed in a dose dependent manner. The effect of genistin, a glycoside form of genistein, on cumulus expansion was markedly weaker (Vodková et al., 2008) and similarly to daidzein did not suppress cumular cell expansion in a dose dependent manner (Vodková et al., 2008). Neither daidzein nor genistin in contrast to genistein is considered a tyrosine protein kinase inhibitor (Jung et al., 1993). It is possible that this very fact can play a role in the general trend of incidence of these substances. The absence of dose-dependence of daidzein and absence of an effect on cumular expansion can be due to the phenomenon referred to as low-dose effect, which is typical for many endocrine disruptors (Vandenberg et al., 2012).

## Conclusion

Daidzein acted in an inhibitory manner on the indicators we monitored in our study – nuclear maturation and expansion of cumular cells. The inhibitory effects of daidzein were not dose dependent, and the effects of a series of increasing concentrations were manifested more intensively on the expansion of cumular cells than on the nuclear maturation of oocytes.

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