Effects of genistein and genistin on *in vitro* maturation of pig oocytes

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ABSTRACT: The objective of the study was to verify the hypothesis that GEN (genistein – phytoestrogen and an inhibitor of tyrosine protein kinase – TPK) effects on pig oocyte maturation and cumular cell expansion under *in vitro* conditions are connected with its estrogenic activity. Oocytes were cultivated for 24 hours up to the stage of the first meiotic metaphase (MI). Three different doses of GEN (13, 40, 80 μg/ml of medium) and also three doses of GIN, genistin, an analogue of GEN without effects on TPK, (80, 160 and 240 μg/ml of medium) were tested. To verify the reversibility of GEN effects, the oocytes were first cultivated for 24 hours with 80 μg of GEN per 1 ml of medium and then for another 24 hours without any GEN. GEN blocked pig oocyte maturation at the stage of the germinal vesicle (GV), depending on the dose. After rinsing out the GEN the oocyte maturation recovered, but with abnormalities (32%). GIN in a concentration of 80 μg/ml of medium induced a significant blockage at the GV stage (18%). With an increase in the GIN concentration, the number of oocytes blocked at the GV stage significantly decreased, but the abnormal maturation increased (up to 31%). GEN inhibited the cumular cell expansion in proportion to its dose. GIN had a less pronounced effect. As GEN and GIN effects demonstrate similar patterns, it is probable that estrogenic activity is involved.

Keywords: phytoestrogen; isoflavone; oocyte maturation; pig; estrogenic effect; tyrosine protein kinase

Phytoestrogens are natural plant substances similar to endogenous estrogens by their chemical structure. Due to this similarity they can bind to estrogen receptors (Cassidy, 2003). Isoflavones constitute one of the main phytoestrogen groups. They are present mainly in soy beans and clover (Reinli and Block, 1996; Greiner et al., 2001; Moravcová and Kleinová, 2002). In plant tissues phytoestrogens appear especially as sugar derivates – glycosides (Barrett, 1996; Burton and Wells, 2002). Soy genistein (GEN) represents the most frequently investigated isoflavone ever (Knight and Eden, 1996; Scambia et al., 2002). Its glycoside is genistin (GIN) (Steer et al., 2003). Both these substances demonstrate estrogenic activity. However, genistin – unlike genistein – does not exert any inhibitory effects on tyrosine protein kinase (TPK) (Liu et al., 2004).

Phytoestrogens exert many effects on the animal organism – positive or negative ones (Barrett, 1996; Gallo et al., 1999). They act as a weak estrogen, and their antiestrogenic properties have been observed as well – dependent on the hormonal environment of the organism (Wang et al., 1996; Fritsche and

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Steinhart, 1999; Rosselli et al., 2000). In human beings the intake of higher amounts of phytoestrogens in foods is usually connected with positive effects on health concerning some chronic diseases like cancer, osteoporosis or cardiovascular diseases (Boker et al., 2002; Kayisli et al., 2002; Moravcová and Kleinová, 2002). Nevertheless, not all studies have confirmed such a positive concept until now (e.g. Ju et al., 2006a,b). In animals, an increased intake of phytoestrogens induced infertility in ewes (Bennetts et al., 1946), cattle, cheetahs in captivity (Setchell et al., 1987; Benassayag et al., 2002) and birds (Leopold et al., 1976; Viglietti-Panzica et al., 2007).

The negative impact of phytoestrogens on reproductive processes was also shown under *in vitro* conditions. GEN is capable of significantly influencing the *in vitro* maturation of mammalian oocytes. Depending on the dosage, it blocks the germinal vesicle breakdown (GVBD) in mouse (Makarevich et al., 1997) and pig oocytes (Jung et al., 1993). Tatemoto and Terada (1996) did not find any significant inhibition of GVBD in cattle oocytes cultivated with GEN, but depending on the dosage, there was an increase in the number of oocytes that blocked meiotic maturation in the stages of late diakinesis (LD) or premetaphase I (PMI). Van Cauwenberge and Alexandre (2000) reported that in oocytes exposed to GEN only after GVBD completion there was no effect on MAP kinase activation, metaphase spindle formation or on metaphase I (MI) to anaphase I (AI) transition. However, the oocytes did not expel the first polar bodies. GEN was able to induce parthenogenetic activation in up to 50% of mouse oocytes (Sun et al., 1998; Van Cauwenberge and Alexandre, 2000), but this metaphase – interphase transition was not accompanied by exocytosis of cortical granules or by expulsion of the second polar body (Van Cauwenberge and Alexandre, 2000). Another known effect of GEN is the inhibition of the expansion of cumular cells of mouse (Tirone et al., 1997) and pig oocytes (Ježová et al., 2001).

GEN is characterised as a specific inhibitor of tyrosine protein kinase (TPK) with estrogenic activity (Akiyama et al., 1987; Makarevich et al., 1997). The authors of the above-mentioned studies connect the observed effects precisely with TPK inhibition for the most part. Jung et al. (1993) demonstrated the inhibition of protein phosphorylation in the affected oocytes. Moore and Kinsey (1994) observed comparable effects of GEN with those of erbstatin and lavendustin A, other TPK inhibitors, in sea urchin oocytes. The effects on nuclear maturation are explained by the resulting inhibition of MAP kinase (Jung et al., 1993; Sun et al., 1998) and directly of $p34^{cdc2}$ kinase (Tatemoto and Terada, 1996). The suppression of cumular expansion might be connected with the blocking of the function of receptors for the epidermal growth factor – EGF and partially the follicle stimulating hormone – FSH (Tirone et al., 1997; Ježová et al., 2001; Procházka et al., 2003). Nevertheless, not all studies comparing the effects of GEN with TPK inhibitors show completely the same effects of the substances compared. Although Makarevich et al. (1997) found an agreement between GEN and lavendustin A in the majority of parameters observed, they also noticed some differences. Van Cauwenberge and Alexandre (2000) pointed out that GVBD inhibition in mouse oocytes was found in GEN but not after the addition of TPK inhibitors tyrphostin B46 or lavendustin A. Singh et al. (1993) did not confirm the ability of TPK inhibitors to block the activation of EGF receptor.

It is obvious that in addition to TPK inhibition GEN can also act in other ways. The DNA topoisomerase II (Markovits et al., 1989) and S 6 kinase (Linassier et al., 1990) inhibition has also been described. The possibility of direct or indirect inhibition of protein kinase A and C (Makarevich et al., 1997; Van Cauwenberge and Alexandre, 2000) and of some other enzymes, among others aromatase or hydroxysteroid dehydrogenases, has been discussed as well (Bolego et al., 2003). The estrogenic activity of GEN demonstrated in somatic cells is also mentioned because it is not possible to exclude the simultaneous activity of GEN by means of estrogenic receptors in oocytes, either (Makarevich et al., 1997).

Estrogenic receptors are present partly on the cytoplasmic membrane (m-ER) and partly on the nuclear membrane (n-ER) in cells (Rhen and Cidlowski, 2004). Although m-ERs trigger several signal pathways including even influencing TPKs (Rhen and Cidlowski, 2004; Schiff et al., 2005), Beker-van Woudenberg et al. (2004) stated that the effects of estradiol (E_2) on maturation of denuded bovine oocytes were not based on interaction with m-ERs, but instead, probably direct effects on the genome or micro-skeleton were involved. In addition, E_2- m-ERs interaction is connected rather with the activation of TPKs (Rhen and Cidlowski, 2004).

It is interesting that E_2 shows similar effects on oocyte meiotic maturation to those of GEN. Li et al. (2004) found that after the addition of E_2, there was a statistically significant decrease in the number of porcine oocytes which went through GVBD and reached the second meiotic metaphase (MII) and a statistically significant suppression of their cumular cell expansion. Other authors (Smith and Tenney, 1980 – mouse; Singh et al., 1993; Bing et al., 2001 – pig; Beker-van Woudenberg et al., 2004 – cattle) also recorded similar effects of E_2. In addition, Van Cauwenberge and Alexandre (2000) reported the inhibition of GVBD, although of lower intensity, in another soy phytoestrogen, daidzein (DAN), a structural analogue of GEN without the ability to inhibit TPK.

Obviously, in scientific literature there is no agreement on the mechanism by which GEN acts on oocyte meiotic maturation.

The objective of our studies was to verify the hypothesis that the effect of GEN on pig oocyte maturation and cumulus cell expansion under *in vitro* conditions is connected, at least partially, with its estrogenic activity. To this end the effects of GEN and those of its glycoside GIN, which does not demonstrate the characteristics of a TPK inhibitor, are compared in this study.

MATERIAL AND METHODS

Oocytes were obtained and handled according to methods used by Petrová et al. (2005):

Excision of ovaries

Ovaries were excised from gilts slaughtered in an unknown phase of the estral cycle. After excision the ovaries were maintained in saline (0.9% NaCl) at a constant temperature of 39°C and transported without delay to the laboratory.

Oocyte isolation

Fully-grown oocytes were isolated from follicles 2–5 mm in diameter by aspiration with a 20G needle. Only oocytes with a compact layer of cumulus cells and intact cytoplasm were used for the experiment.

Oocyte cultivation

Oocytes were rinsed 3 times in a culture medium before cultivation. The cultivation took place in Petri dishes 3.5 cm in diameter (Nunc, Roskilde, Denmark), in 3 ml of modified M199 medium (composition given below) at 39°C in a mixture of 5% CO_2 with air.

The composition of the modified M199 medium: 3.9 ml 7% sodium carbohydrate; 60 mg calcium lactate; 25 mg sodium pyruvate; 2.5 mg gentamycin; 150 mg HEPES (all Sigma-Aldrich, Germany); 8 ml M199 medium 10× concentrated (Gibco BRL, Life Technologies, Scotland); distilled water ad 100 ml.

Before cultivation the medium was enriched with 13.4 IU eCG + 6.6 IU hCG/ml (P.G. 600, Intervet Boxmeer, the Netherlands) and 10 mg/ml bovine foetal serum growth proteins (Alseva, the Czech Republic).

The oocytes were cultivated for 24 hours up to the first meiotic metaphase (MI). The control group was cultivated in a pure medium, the experimental groups in media with a certain dose of appropriate phytoestrogen (GEN, GIN – Sigma-Aldrich, Germany).

Evaluation of cumulus-oocyte complexes and nuclear maturation

After the established cultivation period, the complexes of oocytes and their cumular cells (COCs) were photographed with a digital video camera. The area of COC was subsequently measured using the program for the analysis and processing of digital images L.U.C.I.A. (Laboratory Universal Computer Image Analysis) version 4.71 (Laboratory Imaging, the Czech Republic).

After image recording the oocytes were removed from the culture medium and cumular cells were removed with a glass micropipette. The oocytes were fixed in a solution of acetic acid and ethanol (1:3) for a period of 24 hours at least and stained with 1% orcein. The oocytes were evaluated under a light microscope using the phase contrast.

The oocytes were submitted to further evaluation only if at least 85% of the control group oocytes reached MI.

Experimental scheme

GEN and GIN effects on nuclear maturation. Three different doses of GEN (13, 40, 80 μg/ml of

medium) and also three doses of GIN (80, 160 and 240 µg/ml of medium) were tested. The oocytes were cultivated for 24 hours.

GEN and GIN effects on cumulus expansion. Three different doses of GEN (13, 40, 80 µg/ml of medium) and two doses of GIN (80 and 240 µg/ml of medium) were tested. The oocytes were cultivated for 24 hours.

Reversibility of GEN effects. The oocytes were first cultivated for 24 hours with 80 µg of GEN per 1 ml of medium, then rinsed, and subsequently cultivated for another 24 hours without any GEN in the medium.

Statistical Analysis. Data from all experiments were subjected to statistical analysis. Each experiment was performed three times at least. The results were pooled for presentation and evaluated by chi-square analysis (Snedecor and Cochran, 1980). The *P*-value less than 0.05 was considered significant.

RESULTS AND DISCUSSION

The stage of nuclear maturation of oocytes exposed to GEN is shown in Tables 1 and 2, whereas Table 3 contains the data on oocytes exposed to GIN. The rate of cumular cell expansion expressed by the area of COC is described in Table 4.

GEN blocked pig oocyte maturation at the stage of GV, depending on the dose used. The concentration of 80 µg/ml of culture medium was 100% effective (Table 1). After rinsing out the GEN and subsequent 24-hour cultivation in the medium without GEN (Table 2) we observed 100% recovery of oocyte maturation. However, a significantly lower percentage of oocytes reached the MI stage (62%) compared to simple 24-hour cultivation without GEN in the medium (95%). 32% of the oocytes showed abnormal

Table 1. Nuclear maturation (in %) of oocytes exposed to genistein for 24 hours

Phase of meiotic maturation	GEN concentration in culture medium (µg/ml)				
	0	1	13	40	80
GV	2^{A}	2^{A}	26^{B}	36^{B}	97^{C}
LD	2^{A}	5^{A}	8^{A}	2^{A}	0^{A}
MI	95^{A}	86^{A}	66^{B}	62^{B}	3^{C}
AI	1^{A}	5^{A}	0^{A}	0^{A}	0^{A}
Ab	0^{A}	2^{A}	0^{A}	0^{A}	0^{A}
Number of oocytes	100	100	92	85	83

^{A,B}figures with the same superscripts in rows do not differ significantly ($P > 0.05$)

Table 2. Recovery (in %) of oocyte nuclear maturation after 24-hour exposure to GEN and subsequent 24-hour cultivation in a free medium

Phase of meiotic maturation	Control (free medium)		GEN	
	24 hours	48 hours	80 µg/ml 24 hours	+ 0 µg/ml 24 hours
GV	2^{A}	0^{A}	97^{C}	0^{A}
LD	2^{A}	0^{A}	0^{A}	0^{A}
MI	95^{A}	2^{C}	3^{C}	62^{B}
AI	1^{A}	0^{A}	0^{A}	0^{A}
MII	0^{A}	98^{B}	0^{A}	6^{A}
Ab	0^{A}	0^{A}	0^{A}	32^{B}
Number of oocytes	100	100	83	71

^{A,B}figures with the same superscripts in rows do not differ significantly ($P > 0.05$)

Table 3. Nuclear maturation (in %) of oocytes exposed to genistin for 24 hours

Phase of meiotic maturation (%)	GIN concentration in culture medium (μg/ml)			
	0	80	160	240
GV	2^{A}	18^{B}	17^{B}	5^{A}
LD	2^{A}	10^{A}	13^{A}	14^{A}
MI	95^{A}	62^{B}	56^{BC}	47^{C}
AI	1^{A}	4^{A}	3^{A}	3^{A}
MII	0^{A}	2^{A}	0^{A}	0^{A}
Ab	0^{A}	4^{A}	11^{A}	31^{B}
Number of oocytes	100	92	94	94

^{A,B}figures with the same superscripts in rows do not differ significantly ($P > 0.05$)

maturation (dissipation of metaphase chromosomes in cytoplasm, formation of two metaphase figures or metaphase chromosomes accompanied by an irregular chromatin aggregation).

The results of the experiment are in accordance with data reported by Jung et al. (1993) as regards the effect observed – the inhibition of the germinal vesicle breakdown (GVBD) and also the assessment of the highest effective concentration of GEN in the medium. Makarevich et al. (1997), however, reported a stimulating effect of low dosage of GEN (5 μg/ml) on pig oocyte maturation, but this was probably due to reserves of the culture system used – after 40 hours of cultivation only 58% of the control oocytes reached any corresponding stage of nuclear maturation (AI–MII) and 20% of oocytes did not enter the maturation process at all. Although Jung et al. (1993), after reversing 24-hour cultivation, did not determine any effects similar to ours, they also noticed deviations from standards after previous GEN exposure. They reported significantly impaired maturation from MI to MII stages. Abnormalities of the nuclear maturation observed after rinsing out GEN indicate that although the GEN effect is reversible in principle, the function of microtubules in a portion of oocytes remains disturbed, namely in the meiotic spindle. These changes may relate to an anomaly in the constellation of the relevant proteins phosphorylation. Van Cauwenberge and Alexandre (2000), who studied GEN activity regarding the cytoskeleton of mouse oocytes, explain its effects by the inhibitory action on the MAP kinase activating pathway. However, the negative influence of the estrogenic activity of GEN cannot be excluded, either. Beker-van Woudenberg et al. (2004) demonstrated that the exposure of bovine oocytes to estradiol just after the GVBD gave rise to distinct disturbance in the meiotic spindle organization. Recently, Eichenlaub-Ritter et

Table 4. The area of cumulus-oocyte complexes (COC) after 24-hour cultivation in media with different doses of GEN or GIN. Control without any additive

Tested substance	Control groups				GEN or GIN groups		
	dose (μg/ml)	No. of COC	mean area (μm^2)	SD	No. of COC	mean area (μm^2)	SD
GEN	13	11	$131\,565^{A,1}$	29 223	26	$69\,169^{B,3}$	28 000
	40	15	$128\,756^{A,1}$	52 631	40	$48\,874^{B,4}$	13 892
	80	19	$110\,695^{A,1}$	35 637	33	$42\,184^{B,5}$	8 179
GIN	80	19	$116\,537^{A,1}$	37 014	29	$91\,438^{B,2}$	37 283
	240	10	$157\,143^{A,1}$	37 550	28	$108\,209^{B,2}$	36 742

^{A,B}figures with the same superscripts in rows do not differ significantly ($P > 0.05$)

^{1,2}figures with the same superscripts in columns do not differ significantly ($P > 0.05$)

al. (2007) have published similar results in mouse oocytes as well.

GIN (Table 3) in the concentration of 80 μg/ml of medium (e.g. the concentration when GEN completely inhibits oocyte maturation) induced a significant blockage at the GV stage (18% of oocytes at the GV stage). Nevertheless, this effect was substantially lower than that of GEN. With an increase in the GIN concentration, the number of oocytes blocked at the GV stage significantly declined, but concurrently the percentage of MI stage oocytes significantly decreased, and in addition the number of oocytes with abnormal maturation rose (up to 31%). The types of abnormalities are similar to those observed in the experimental reversal of GEN effects – dissipation of metaphase chromosomes in cytoplasm, formation of two metaphase figures or metaphase chromosomes accompanied by an irregular chromatin aggregation.

We do not know of any literature concerning such a study of GIN effects on mammalian oocytes. However, we can still find a certain parallel in the action of daidzein on mouse oocytes: daidzein, depending on the dosage, increased the proportion of oocytes at the GV and GVBD, but its inhibitory effect compared to the same molar concentrations of GEN was less pronounced, and cell death occurred in a small percentage even at the concentration where GEN only inhibited cellular maturation (Van Cauwenberge and Alexandre, 2000).

In proportion to its dose GEN drastically inhibited the cumular cell expansion. GIN had a similar, although less pronounced effect.

The impairment of nuclear maturation of pig oocytes as well as of the expansion of their cumulus cells demonstrates similar patterns in both GEN and GIN although the GIN effects are less pronounced. With regard to the fact that GIN does not have any more significant properties of a kinase inhibitor (Liu et al., 2004) and that similar effects were determined after estradiol exposure (e.g., Li et al., 2004), it is probable that the estrogenic activity of the isoflavones tested is involved in these effects.

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