**DIRECT INHIBITORY EFFECT OF FLAXSEED ON PORCINE
OVARIAN GRANULOSA CELL FUNCTIONS**

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DIRECT INHIBITORY EFFECT OF FLAXSEED ON PORCINE OVARIAN GRANULOSA CELL FUNCTIONS

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Abstract

Flaxseed is useful as a functional food and alternative medicine owing to its beneficial health effects. Its action on ovarian cell functions and interrelationships with the upstream hormonal regulators remain unknown. Our aim was to examine the direct influence of flaxseed extract on basal porcine ovarian functions (proliferation, apoptosis), leptin release, and response to insulin-like growth factor I (IGF-I). First, we examined the effect of flaxseed extract on the accumulation of proliferation (PCNA) and apoptosis (bax) marker and on leptin release in cultured porcine ovarian granulosa cells. Next, granulosa cells were cultured with IGF-I with and without flaxseed extract and analyzed for PCNA and bax accumulation by quantitative immunocytochemistry, whereas leptin was analyzed by RIA. Flaxseeds decreased the accumulation of proliferation marker and increased that of the apoptosis marker at all doses and reduced leptin output at 100 $\mu\text{g/ml}$. On the contrary, IGF-I promoted PCNA and suppressed bax. Flaxseed did not modify IGF-I action on these parameters.

Thus, we showed flaxseed action on porcine reproductive processes, with a direct effect on the ovary and flaxseed ability to affect ovarian cell proliferation, apoptosis, and leptin release. Furthermore, we confirmed the pro-proliferating and anti-apoptosis action of IGF-I, but indicated that flaxseed action on ovarian cell proliferation and apoptosis are not due to changes in their response to IGF-I. The potential direct anti-reproductive action of flaxseed needs to be considered during its application in nutrition, medicine, and animal production.

Keywords: flaxseed, IGF-I, ovary, proliferation, apoptosis, leptin.

INTRODUCTION

Flaxseed is cultivated in many parts of the world for fiber, oil, as a nutritional product, as well as for medicinal purposes. It is a rich source of biologically active health-promoting components such as the ω -3 fatty acid, α -linolenic acid, the phytoestrogen and antioxidant lignan secoisolariciresinol diglucoside, fiber, amino acids, proteins, and microelements (Goyal et al., 2014; Kajla et al., 2015; Parikh et al., 2018). These components are beneficial against cardiovascular diseases (they have anti-hypertensive, anti-atherogenic, anti-arrhythmic, cholesterol, and atherosclerosis lowering effects), diabetes, cancer, arthritis, osteoporosis, and autoimmune and neurological disorders. Flaxseed proteins help in the prevention and treatment of heart diseases and support the immune system (Goyal et al., 2014; Parikh et al., 2018). Flaxseed, owing to its phytoestrogenic effect, can prevent or weaken some symptoms of menopause (hot flashes, vaginal atrophy, lowering bone mineral density, and bone turnover) without promotion of estrogen-associated cancers (Dew and Williamson, 2013; Ghazanfarpour et al., 2016). In addition, flaxseed intervention increased the urinary 2α -hydroxyestrone/ 16α -hydroxyestrone ratio, which has been associated with a lower risk of breast cancer (Dew and Williamson, 2013).

Some *in-vivo* studies were performed to examine flaxseed effect on reproductive processes. Female rats, fed with low doses of flaxseed exhibited delayed onset of puberty, and induced anestrus, but exposure to flaxseed at high doses caused early onset of puberty, higher relative ovarian weight, higher serum estradiol levels, and lengthened estrous cycles up to persistent estrus in rats and their

offsprings (Tou et al., 1998, 1999). Flaxseed intake decreased androgen levels with a concomitant reduction in hirsutism in women with polycystic ovarian syndrome (Nowak et al., 2007). Flaxseed and its components could down-regulate estrogen receptors, alter estrogen metabolism and prostaglandin E2 production in both, healthy and tumor ovarian cells of chicken (Eilati et al., 2013; Dikshit et al., 2015, 2017). Feeding flaxseed containing phytoestrogens was found to promote bovine ovarian cycle, blood progesterone level, ovarian follicle and *corpus luteum* growth, and developmental competence of oocytes, but did not affect the pregnancy rate (Petit and Twagiramungu, 2006; Colazo et al., 2009; Moallem et al., 2013; Jahani-Moghadam et al., 2015; Ulfina et al., 2015). Other authors however, did not observe these effects (Ambrose et al., 2006) or even reported the inhibitory action of flaxseed intake or linolenic acid on bovine folliculogenesis, luteogenesis, plasma progesterone level (Hutchinson et al., 2012) and onset of estrus (Zachut et al., 2010, 2011). The oocytes from flaxseed-fed cows showed increased developmental capacity (Petit and Twagiramungu, 2006; Zachut et al., 2010). Taken together, these *in-vivo* studies performed on chicken, humans, rats, and cows demonstrated that flaxseed molecules could affect the production of gonadal steroid hormones and prostaglandins, which in turn could influence ovarian folliculogenesis and oogenesis. The effect of flaxseed intake has not been tested on other species and on other hormones. Previous *in-vivo* studies failed to detect the site of action of flaxseed in organisms; it remains unclear whether flaxseeds affect the ovaries directly or via upstream hypothalamo-hypophysial ovarian regulators. Finally, the mechanism of flaxseed effect on ovarian folliculogenesis remains to be established. Follicle development, growth, selection, and atresia depend on the equilibration between follicular cell proliferation and apoptosis (Sirotkin, 2014). Influence of flaxseed on these two processes has not yet been studied.

The effect of flaxseed on ovarian response to upstream regulators has also not yet been examined. One such key ovarian stimulator could be insulin-like growth factor I (IGF-I), which is a known promoter of ovarian cell proliferation and follicle survival and development, inhibitor of ovarian cell apoptosis, stimulator of ovarian hormone release, and a local mediator of gonadotropin and other hormonal regulators of the ovary (Guidice, 2001; Sirotkin, 2014).

The objective of the present study was to examine the direct effect of flaxseed on proliferation, apoptosis, release of peptide hormone leptin, and response to IGF-I by cultured ovarian granulosa cells of pig. These processes and species have not been studied in this respect.

MATERIAL AND METHODS

Isolation and culture of granulosa cells

Ovaries of non-cycling pubertal Slovakian White gilts, about 180 days of age, were obtained, processed, and their granulosa cells were isolated and cultured as described previously (Stochmalova et al., 2017). Briefly, the granulosa cells were aspirated with a syringe and sterile needles from follicles having diameters of 3-5 mm. The granulosa cells were then isolated by centrifugation for 10 min at $200 \times g$, and subsequently rinsed with sterile DMEM/F12 1:1 medium (BioWhittaker™, Verviers, Belgium), followed by resuspension in the same medium, supplemented with 10 % fetal calf serum (BioWhittaker™) and 1 % antibiotic-antimycotic solution (Sigma, St. Louis, MO, USA), at a final density of 10^6 cells/ml. Aliquots of the cell suspension were then dispensed to 16-well chamber slides (Nunc Inc., International, Naperville, USA; 200 μ l/well; for immunocytochemistry) and to 24-well culture plates (Nunc™, Roskilde, Denmark; 1ml suspension/well; for RIA) and incubated at 37.5 °C in 5 % CO₂ in humidified air

until a monolayer having a confluence of 60–75 % was formed after 3–5 d, at which point the medium was renewed. After replacing the medium, in the 1st series of experiments, cells were cultured for 2 days with flaxseed extract (MEDU s.r.o., Čenkovce, Slovakia) at concentrations of 1, 10, and 100 µg/ml. To obtain this extract, flaxseed was ground in a coffee mill, and portions of the flaxseed powder were added to 50 µl of dimethyl sulphoxide (DMSO) (AppliChem GmbH, Darmstadt, Germany) to achieve a stock solution of 1 mg/ml of plant extract. After 2 days extraction, the appropriate concentrations i.e 1, 10, and 100 µg/ml of the flaxseed solutions were dissolved in the culture medium immediately before its addition to the cells, such that the final concentration of DMSO did not exceed 0.001%. Previous studies did not reveal substantial effects of 0.001% DMSO on ovarian cell function and viability (data not shown). Controls included ovarian cells cultured in the incubation medium (with 0.001% DMSO) without flaxseed treatment and the medium incubated without cells (blank control). In the 2nd series of experiments, granulosa cells were cultured with IGF-I (Sigma, St. Louis, USA) at concentrations of 1, 10, and 100 ng/ml alone or in combination with flaxseed extract mentioned above at a dose of 10 µg/ml. IGF-I, like flaxseed extract, was dissolved in culture medium immediately before its addition to the cells. Control was represented by cells or medium cultured without flaxseed extract or IGF-I.

Immunocytochemical analysis

The cells after being cultured were processed and subjected to immunocytochemical analysis by using mouse monoclonal antibodies against either PCNA (marker of proliferation, Shiomi and Nishitani, 2017) and bax (marker of apoptosis, Peña-Blanco and García-Sáez, 2018) (all from Santa Cruz Biotechnology, Inc., Santa Cruz, USA; dilution 1:500 in PBS) and secondary swine antibody against mouse IgG labeled with horse-radish peroxidase (Servac, Prague, Czech Republic, dilution

1:1000) and DAB-substrate (Roche Diagnostics GmbH, Mannheim, Germany) as was previously reported (Stochmalova et al., 2017). A ratio of DAB-HRP-stained cells to the total cell number was calculated.

Immunoassay

Concentrations of leptin were determined in incubation medium by using a multi-species leptin RIA kit (LINCO Research, St. Charles, USA) according to the instructions of the manufacturer. RIA was validated for use in samples of culture medium. The cross-reactivity of leptin antiserum to human leptin was 100%, while to porcine, rat, mouse, and canine leptins was 67%, 61%, 73%, and 3%, respectively. Its cross reactivity to human insulin, human proinsulin, human C-peptide, rat insulin, glucagon, and IGF-I was less than 0.001%. Leptin assay sensitivity was 0.801 ng/ml. Intra and interassay coefficients of variation did not exceed 3.6% and 8.7 %, respectively. RIA was validated for use in samples of culture medium by dilution tests.

Statistical analysis

Each experimental group was represented by three chamber-slide wells and by 4 cell culture wells. The proportions of cells containing specific immunoactivity were calculated from at least 1000 cells per chamber. The percentage of cells containing antigen in different groups of cells was calculated. Each series of experiments was performed 3 times. The data shown are the means of values obtained in these 3 separate experiments performed on separate days with separate groups of granulosa cells, each obtained from 9-15 animals.

Significant differences between the experiments were evaluated using the one or two-way ANOVA followed by paired Wilcoxon-Mann Whitney test, using the Sigma Plot 11.0 software (Systat Software, GmbH, Erkhart, Germany). Differences from control at $P < 0.05$ were considered significant.

RESULTS

Ovarian granulosa cells after culture contained markers of proliferation (PCNA) and apoptosis (bax), and they secreted the substantial amounts of leptin. PCNA was localized mainly in the cell nuclei, whilst bax was observed both in cytoplasm and nuclei. These parameters were influenced by administration of flaxseed extract and IGF-I.

In the 1st series of experiments, addition of flaxseed extract to culture medium significantly decreased the percentage of cells containing PCNA at all doses (Fig. 1A) and increased the proportion of cells containing bax (Fig. 1 B) in a dose-dependent manner. In addition, flaxseed at a dose of 100 $\mu\text{g/ml}$ decreased leptin release (Fig. 1C).

Results of the 2nd series of experiments confirmed the inhibitory action of flaxseed at 10 $\mu\text{g/ml}$ on the PCNA-positive cells (Fig. 2A) and its stimulatory action on the accumulation of bax (Fig. 2B) (see point IGF-I at dose 0 ng/ml). IGF-I increased the percentage of cells containing PCNA (Fig. 2A) and bax (Fig. 2B) at all doses in a dose-dependent manner. Although flaxseed addition affected basal PCNA and bax accumulation, it did not substantially modify IGF-I effect on these processes (Fig. 1A, B).

DISCUSSION

Our experiments represent the first *in-vitro* studies of flaxseed action on ovarian functions. These studies are the first demonstration that flaxseeds can directly affect the ovary, thereby affecting reproductive processes. This direct effect can explain the previous reports on flaxseed influence on female reproduction *in vivo* (see Introduction). In addition, these studies are the first to demonstrate effect of flaxseeds on ovarian functions in pig.

This is the first observation of flaxseed effect on ovarian cell proliferation and apoptosis. Flaxseed inhibited accumulation of PCNA (marker and promoter of DNA polymerization during S-phase of the cell cycle, Shiomi and Nishitani, 2017) and increased accumulation of bax (marker and promoter of cytoplasmic/mitochondrial apoptosis, Peña-Blanco and García-Sáez, 2018). This downregulation of proliferation and upregulation of apoptosis suggests that flaxseed can dramatically reduce ovarian cell proliferation/apoptosis rate. Furthermore, our observations are the first evidence that flaxseed can affect not only ovarian steroid hormones and prostaglandin, but also the peptide hormone leptin. Leptin is considered an indirect as well as direct stimulator of ovarian functions such as ovarian cell proliferation and folliculogenesis, and inhibitor of ovarian cell apoptosis (Sirotkin, 2011, 2014). Taken together, our observations suggest that flaxseed can directly inhibit ovarian cell functions – suppress their proliferation, promote their apoptosis, and inhibit the release of the peptide hormone leptin. It cannot be excluded, that just reduction in leptin output could induce the changes in proliferation and apoptosis. However, the fact that flaxseeds in low and moderate doses affected cell proliferation and apoptosis, but not leptin concentration, does not support this hypothesis.

The results obtained during the 2nd series of experiments confirmed not only the anti-proliferative and pro-apoptotic effects of flaxseed, but also the previous data (Giudice, 2001; Sirotkin, 2014) concerning pro-proliferative and anti-apoptotic action of IGF-I on ovarian cells. On the other hand,

flaxseed addition did not affect IGF-I action on cell proliferation and apoptosis. This suggests that IGF-I and flaxseed have opposing actions on ovarian cell proliferation and apoptosis, but they regulate these processes independently, and flaxseeds do not suppress ovarian functions promoted by IGF-I.

The mechanisms of flaxseed action on ovarian cells remain to be elucidated. The documented antioxidant and phytoestrogenic properties of flaxseed molecules (Dew and Williamson, 2013; Goyal et al., 2014; Kajla et al., 2015; Ghazanfarpour et al., 2016; Parikh et al., 2018) should presumably promote cell proliferation and prevent DNA fragmentation resulting in apoptosis, but in our experiments, flaxseeds exerted the opposite effects. However, its effects might be mediated by steroid hormones or prostaglandins. However, flaxseeds inhibited steroidogenesis (Nowak et al., 2007), estrogen reception, and prostaglandin E2 production (Eilati et al., 2013; Dikshit et al., 2015, 2017), these molecules being promoters of ovarian cell proliferation and suppressors of their apoptosis (Sirotkin, 2014).

Our *in-vitro* studies demonstrated direct suppressive action of flaxseed on ovarian cell functions. They are in line with some previous *in-vivo* data concerning its ability to suppress ovarian functions (Nowak et al., 2007; Hutchinson et al., 2011; Zachut et al., 2010, 2011; Eilati et al., 2013; Dikshit et al., 2015, 2017), and not their promotion (Tou et al., 1998, 1999; Petit and Twagiramungu, 2006; Colazo et al., 2009; Dew and Williamson, 2013; Moallem et al., 2013; Jahani-Moghadam et al., 2015; Ulfina et al., 2015; Ghazanfarpour et al., 2016). The present and previous observations of anti-reproductive flaxseed action should be taken into account when consuming flaxseed as animal and human food or during its medical application. However, if this action could be confirmed *in vivo*, flaxseed could potentially be useful for inhibition of not only pathologic (Nowak et al., 200;

Eilati et al., 2013; Dikshit et al., 2015, 2017) but also of healthy ovarian cells in synchronization of farm animal estrous cycles.

Understanding the mechanisms of action and areas of flaxseed application require further studies. Nevertheless, our observations are the first demonstration of flaxseed action on porcine reproductive processes, of direct effect of flaxseed on the ovary, and of ability of flaxseed to affect ovarian cell proliferation, apoptosis, and leptin release. Furthermore, they confirmed the pro-proliferating and anti-apoptosis action of IGF-I, but indicated that flaxseed actions on ovarian cell proliferation and apoptosis are not due to changes in their response to IGF-I. This potential direct anti-reproductive action of flaxseed needs to be taken into account during its application in nutrition, medicine, and animal production.

CONFLICT OF INTEREST

The authors indicated no potential conflicts of interest.

ETHICAL CONSIDERATION FOR EXPERIMENTAL ANIMALS OR HUMAN

SUBJECTS

Not applicable

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Figure legends

Figure 1. The effect of flaxseed addition at concentrations of 0, 1, 10, and 100 $\mu\text{g/ml}$ on the accumulation of proliferation marker PCNA (A), apoptosis marker bax (B), and the release of leptin (C) by cultured porcine ovarian granulosa cells. Data derived from immunocytochemical (A, B) and radioimmunoassay (C) analyses. a - effect of flaxseed: significant ($P < 0.05$) differences between cells cultured with and without (0 $\mu\text{g/ml}$) flaxseed.

Figure 2. The effect of addition of IGF-I alone at concentrations of 0, 1, 10, and 100 ng/ml and in combination with flaxseed (10 $\mu\text{g/ml}$) on the accumulation of proliferation marker PCNA (A) and apoptosis marker bax (B) in cultured porcine ovarian granulosa cells. Data derived from immunocytochemical analyses. a - effect of flaxseed: significant ($P < 0.05$) differences between corresponding groups of cells cultured with and without flaxseed. b - effect of IGF-I: significant ($P < 0.05$) differences between cells cultured with and without (0 ng/ml) IGF-I.

A.

B.

C.

A.

B.