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Effects of supplemental flaxseed on the ovarian and uterine functions of adult cycling mice

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Abstract. Dietary fatty acids (FA) have an effect on animal reproduction. The purpose of the study was to find out how supplemental flaxseed can modulate the FA metabolism and how FA can influence the release of ovarian hormones and functions of the uteri through the modulation of their specific receptors. Cycling mice were fed a basal diet (control) and basal diet fortified with 10% flaxseed for 6 weeks to examine its influence on the structure and function of the ovaries and uteri, and serum levels of FA. Unlike controls (30%), 100% of mice fed flaxseed exposed oestrus at the end of the supplemental period. Serum FA were analysed using gas chromatography. The ovaries and uteri underwent histological and immunohistochemical analyses, respectively. The ovarian fragments were cultured with or without follicle-stimulating hormone and culture media were analysed for progesterone (P4) and oestradiol-17β (E2) using immunoassays. Dietary flaxseed increased the serum FA concentration, sizes of the ovaries and primary follicles, the release of P4 and E2, the thickness of endometrium and myometrium, and altered the expression of oestrogen and progesterone receptors in all uterine compartments. Dietary flaxseed can promote ovarian steroidogenesis and uterine activity in the mouse.

Key words: Flaxseed — Hormones — Ovary — Oestrogen and progesterone receptors — Uterus

Introduction

Flaxseed is one of the richest plant sources of both the essential polyunsaturated fatty acids (PUFA) and lignans (Patade et al. 2008; Andrejčáková et al. 2016; Sopková et al. 2017). These components can affect the functions of each part of the reproductive system of animals and humans (Petit et al. 2001; Sirotkin and Harrath 2014) including the release of steroid hormones by the ovaries and inhibit their response to follicle-stimulating hormone (FSH) and insulin-like growth factor (IGF-I) (Sirotkin 2016). Major flaxseed lignans, secoisolariciresinol and matairesinol, when consumed are metabolised by the gut microbiota into enterolignans with phytoestrogenic activity (Singh et al. 2011; Truan et al. 2012), whose structure is very similar to natural oestrogens such as oestradiol-17β (Navarro 2005). Enterolignans can express weak oestrogenic or anti-oestrogenic effects on the ovaries, uterus, and other reproductive and non-reproductive tissues mimicking the effects of natural oestrogens (Gustafsson 2003; Retana-Márquez et al. 2012; Michel et al. 2013).

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The effect of oestrogens is mediated *via* the oestrogen receptors (ER) α and β located on the cell membrane and in the nucleus regulating mitosis and other cell functions, e.g. proliferation and differentiation of cells (Wang et al. 1999; Lecomte et al. 2017). Both ER subtypes (but mainly ERβ) are involved greatly in the action of the ovaries. However, mainly ERα, and only poorly ERβ, is involved in the function of the uterus (Couse and Korach 1999). Progesterone attenuates the sensitivity of cells to oestrogen by decreasing its receptor levels (Clarke and Sutherland 1990) *via* direct interaction of progesterone receptors (PR) and ERα (Martin 1980). ERα is a predominant ER subtype in the rodent uterus and ERβ plays a minor and non-specific role in regulation of the uterine function (Wang et al. 1999). Phytoestrogens can bind to both ER subtypes, but appear higher affinity for ERβ (Benassayang et al. 2002; Rietjens et al. 2013; Paterni et al. 2014).

PUFA are the integral components of cell membranes influencing their physical-chemical properties and fluidity (Yu et al. 2014). Phytoestrogens can stimulate lipid metabolism including PUFA, in the liver by favouring the metabolism of n-3 PUFA over n-6 PUFA (Sopková et al. 2017; Vlčková et al. 2018; Rezaei et al. 2020). Decreased activity of Δ-6-desaturase, the enzyme common for metabolism of both n-6 and n-3 PUFA (Stoffel et al. 2008), results in the incorporation of n-3 PUFA derivatives in membranes of all the body cells (Hulbert et al. 2005) and in modulation of cell cycle and apoptosis (Vlčková et al. 2018). Many studies reported that flaxseeds are applicable in the suppression of menopausal symptoms (Akhbari Torkestani et al. 2011; Colli et al. 2012) and cancers of reproductive organs and breasts in postmenopausal women (Thompson et al. 2005; Lowcock et al. 2013; Cetisli et al. 2015). Most studies assign the effects of flaxseed on reproduction to enterolignans or PUFA but only few referred a link between them.

As dietary FA can modulate the production and the release of ovarian hormones and influence their effects on the uterine functions through their specific receptors, the objective of this study was to examine the influence of long-term dietary flaxseed supplementation on the morphophysiological properties of the ovaries and uteri of healthy adult mice. Particular purposes were to evaluate the course of oestrous cycles using vaginal cytology and analyse morphometric parameters of the ovarian and uterine tissue. The effect of dietary flaxseed on the ovarian endocrine function was evaluated *in vitro* for the release of progesterone, oestradiol-17β, and IGF-I by the ovarian tissues and their responsiveness to FSH. Uterine function was evaluated using immunohistochemistry techniques for the detection and distribution of the ERα and ERβ, as well as PRB. The levels of selected fatty acids in blood serum of mice were analysed in relation to investigate the link between high intake of flaxseed n-3 PUFA and functions of the ovaries and uteri.

Materials and Methods

Animals, housing, and diets

The experiment on mice was performed at the Institute of Microbiology and Gnotobiology (IMG), University of Veterinary Medicine and Pharmacy (UVMP) in Košice, Slovak Republic. The experiment was approved by the State Veterinary and Food Administration of the Slovak Republic (Approval No. 1177/14-221) and the animals were handled in accordance with the guidelines established by the relevant commission. The experiment was carried out on 40 SPF (specific pathogen free) adult female mice at the age of 42 days of BALB/c line (VELAZ s.r.o., Prague, Czech Republic) divided into two groups after being transported to the experimental housing at the IMG UVMP in Košice, where the trial continued, differing in the diet supplementation. The mice were housed in a barrier breeding system (6/7 mice in each cage, receiving 24.5 g of feed mixture *per* day divided into two doses) with the temperatures maintained between 20–24°C, relative humidity of 45–65%, and a 12-h light/dark regimen. Animals of both groups were fed a standard diet for mice (Altromin 1311; VELAZ s.r.o.). The diet composition is the same as was published previously by Andrejčáková et al. (2020). The animals had free access to water kept in glass bottles. The diet of the control group (C; $n = 20$) consisted of the standard diet alone and the diet of the experimental group (F; $n = 20$) consisted of the standard diet supplemented with 10% milled flax seed (cultivar Flanders, AGRITEC s.r.o., Šumperk, Czech Republic). Composition (in %) of FA in flaxseeds was the following: lipids (dry matter basis) 45.78, palmitic FA (C16:0) 5.1, stearic FA (C18:0) 3.7, oleic FA (C18:1) 18.4, linoleic FA (C18:2) 16.1, and α-linolenic FA (C18:3) 56.8. The corresponding diets were fed to mice for 6 weeks.

Oestrus induction and cycling

The mice of both groups were induced to oestrus by transferring male urine-contaminated bedding – the "Whitten effect", on the first day of feed supplementation. Oestrus occurred in females by the third night from exposure. Vaginal smears were obtained in 5-day intervals over the experimental period using a saline-dampened plastic swab. The smears were stained by using the Pappenheim stain (May-Grünwald and Giemsa-Romanovski dyes combination; Merck KGaA, Darmstadt, Germany; Hrubiško and Hule 1970) and evaluated under the microscope (Nikon Eclipse E200; Tokyo, Japan) using the classification system described by Byers et al. (2012). Shortly: proestrus – mostly round nucleated and some cornified epithelial cells; oestrus – mostly cornified cells of irregular shape; metestrus – cornified epithelial cells and polymorphonuclear leukocytes;

diestrus – mostly polymorphonuclear leukocytes and a few nuclear epithelial cells.

Necropsy and morphometric analysis

At the end of experiment, only animals showing oestrus (n = 6 for each group) were humanely euthanized using cervical dislocation and their ovaries and uterine horns were collected. The ovaries were taken for the culture processing and histological analysis. The ovaries and uterine horns for routine histology (haematoxylin-eosin staining, H-E; AppliChem GmbH, Darmstadt, Germany; n = 6 for each organ) and excisions of the uterine horns for immunohistochemistry were fixed in 4% paraformaldehyde for 24 h, dehydrated, embedded in paraffin and cut into 5 μm thick sections by use of Leica RM2255 microtome (Leica Microsystems Nussloch GmbH, Nussloch, Germany).

Ovarian fragment culture preparation and processing

The ovaries (n = 6) were transported to the tissue culture laboratory in cooling containers (4°C) in phosphate-buffered saline (PBS; Sigma, St. Louis, MO, USA) within 15 min of slaughter. The ovaries were washed several times in PBS and a sterile physiological solution (0.9% NaCl) and dissected in halves. About 0.5-mm wedge ovarian tissue sections (1 fragment *per* well) were placed into sterile 24-well plates (Beckton Dikinson, Mountain View, CA, USA; 1 ml *per* well) with sterile culture medium (DMEM/F12 1:1, BioWhittakerTM Verviers, Belgium; 1 ml) supplemented with 10% foetal bovine serum (FBS; BioWhittakerTM) and 1% antibiotic-antimycotic solution (Sigma), incubated at 37.5°C in 5% CO_2 humidified air for 24 h. The ovarian fragments of animals fed (experimental group) or not fed (control group) supplemental flaxseed were incubated with or without FSH (follicle stimulating hormone from porcine pituitary; Sigma). Active substances of this preparation represented 50 IU FSH, which was dissolved in culture medium and added to the culture plates immediately before the experiment at 0, 2.48, 28.4, 284 and 2840 mIU/ml. After incubation, media from the 24-well plates were aspirated and frozen at −20°C to await immunoassays.

Immunoassay

Concentrations of progesterone (P4), oestradiol-17β (E2), and insulin-like growth factor-I (IGF-I) were determined in 25–100 μl of culture medium using radioimmunoassay (RIA; P4 and E2 measured in ng/ml and pg/ml, respectively) or immunoradiometric assay (IRMA; IGF-I measured in ng/ml) according to the manufacturer's instructions in duplicate, described in our previous studies (Vlčková et al. 2017, 2018).

Histological analysis

Stained sections of both the ovaries and uterine horns were embedded in Canadian balsam and evaluated using basic software for ProgRes® CapturePro ver. 2.8.0 WIN (Jenoptik, Jena, Germany) motion-picture camera and microscope Eclipse E200 (Nikon). Sizes of the ovaries, average diameters of follicles, and corpora lutea (CL) in μm, as well as the numbers of three-category follicles (primary, secondary, and tertiary follicles including ovulatory follicles) and numbers of CL. The follicle size categories were identified according to Griffin et al. (2006) as follows: primary follicles – oocytes surrounded by a single layer of cuboidal granulosa cells and diameters in the range of 50–100 μm; secondary (preantral) follicles – oocytes surrounded by double or stratified layer of granulosa cells without visible space between the cells (without formation of antral cavity) and of diameter in the range of 100–180 μm; tertiary (antral) follicles – follicles with multi-layered cells containing any antrum with diameter ranged at 180–300 μm; ovulatory (Graafian) follicles – the largest antral follicles (350–450 μm) containing one continuous antral cavity and oocyte with cumulus cells pushed to periphery of the antrum. Diameters of all ovaries, follicles, and CL were calculated from two perpendicular measurements and expressed in μm. Excisions from the basal portion of the uterine horns were evaluated for the thickness of the myometrium and endometrium (perpendicular distance from the luminal epithelium to myometrium) with particular parts: the height of the luminal epithelium, *lamina propria mucosae*, and *stratum subglandulare*. Numbers of tubular glands in the endometrium were evaluated in four quadrants (with calculation of mean value for each analysed tissue section) as well as their lengths. All measurable parameters were expressed in μm.

Immunohistochemical analysis (IHC)

Paraffin sections of ovaries were deparaffinised and rehydrated. For detection, antigen retrieval was performed by boiling the slides in 10-mM citrate buffer (pH 6.0) for 2 min. To block endogenous peroxidase activity, the slides were incubated in TBS (0.05 M Tris-HCl plus 0.15 M NaCl, pH 7.6) with 0.3% H_2O_2 addition for 20 min. To block non-specific binding, the sections were incubated for one hour with 8% bovine serum albumin (BSA). In addition, monoclonal mouse anti-PRB (dilution 1:200; ThermoFisher Scientific, Waltham, MA, USA), monoclonal mouse anti-human ERα (dilution 1:30), and monoclonal mouse anti-human ERβ (dilution 1:20) antibodies (Dako, Glostrup, Denmark) were applied and incubated overnight at 4°C. After rinsing with TBST (TBS containing 0.1% Tween20), the sections were incubated with goat anti-mouse secondary antibodies (Dako REAL™ EnVision™/HRP, Rabbit/Mouse (ENV), ready-to-use, Dako) for 2 h. Following the incubation, the specimens were rinsed

in TBST followed by TBS. A colour reaction was visualised by diaminobenzidine as a chromogen (Dako REAL™ DAB+ Chromogen, Dako). The stained sections were counterstained with haematoxylin for stereological assessment, rinsed in distilled water, dehydrated and immersed in DPX (Distyrene Plasticiser and Xylene; Buchs, Switzerland). In preparing a negative control for each sample, the primary antibody was omitted. Photographic documentation was obtained by using an optical microscope (Olympus BX43, Olympus Corporation, Tokyo, Japan) coupled to a camera (Olympus UC30, Olympus Corporation) and computer. To evaluate the intensity of the IHC reaction quantitatively, approximately six images from sections of each examined animal ($n = 6$ for each group) were analysed by using a public domain ImageJ software (National Institutes of Health, Bethesda, MD, USA). The outlines of all cells, which showed immunopositive signal in the uteri were marked manually and then a grey level (GL) of marked areas was measured. The intensity of the IHC reaction was expressed as the relative optical density (ROD) of the DAB brown reaction products and was calculated using the formula described by Smolen (1990), where GL is the grey level of the stained area (specimen) and unstained area (background) and blank is the GL measured after the slide was removed from the light path.

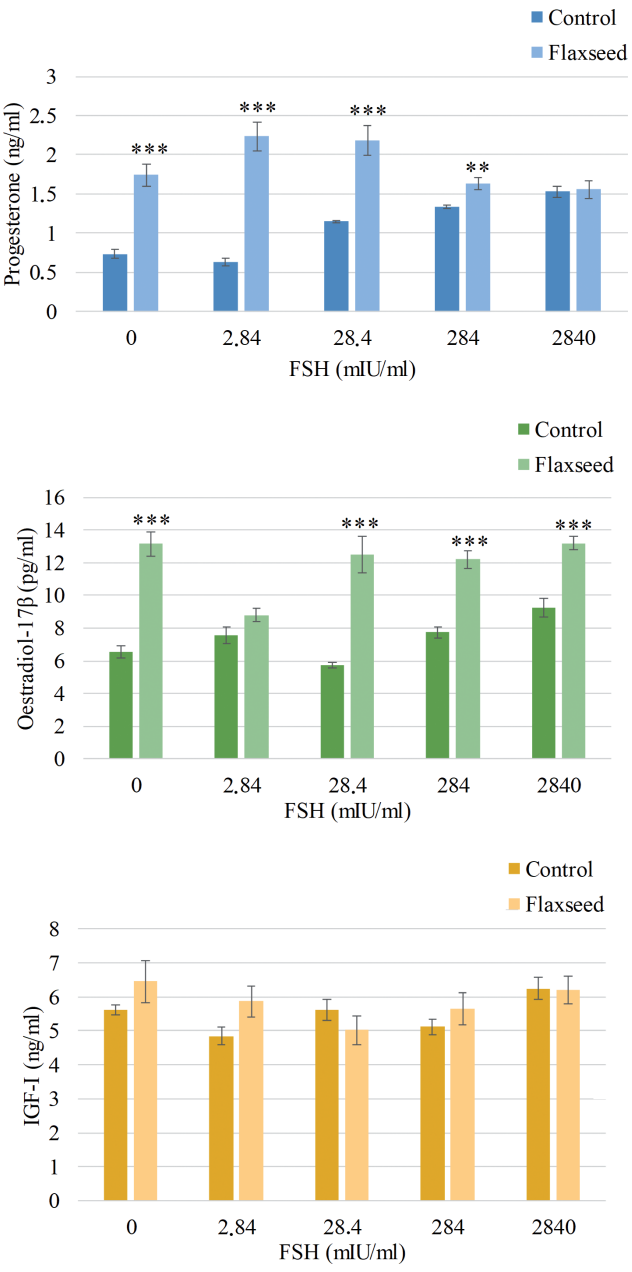

Figure 1. Effect of flaxseed feeding on the release of progesterone (P4), oestradiol-17β (E2), and insulin-like growth factor (IGF-I) by isolated murine ovaries cultured with or without the addition of follicle-stimulating hormone (FSH). Values are means ± SEM. Mean values within columns with the asterisks differ significantly from according controls (**$p < 0.01$; *** $p < 0.001$).

$$ROD = \frac{OD\ specimen}{OD\ background} = \frac{\log\left(\frac{GL_{blank}}{GL_{specimen}}\right)}{\log\left(\frac{GL_{blank}}{GL_{background}}\right)}$$

The positive DAB brown reaction is compared to negative control in all evaluated parameters. The intensity of colour reaction of all receptor markers was concentrated in nuclei of endometrial (including luminal epithelium, glandular epithelium, and stroma) and myometrial cells and evaluated separately in the corresponding compartments of the uterine wall.

Gas chromatography

The serum FA extraction and gas chromatography were performed based on the method of Folch et al. (1957). Extracted lipids were transesterified to FA methyl esters (FAME) with sodium methanolate. The profile of FAME was established by the use of a gas chromatograph (Schimadzu GC 17, Schimadzu, Kyoto, Japan) with a flame ionization detector. The measurements were expressed in mol%.

Statistical analyses

The follicular and uterine parameters, the ROD values as well as the concentrations of hormones released by the cultured ovaries and serum FA are expressed as mean ± SEM. Each measured parameter was calculated *per* one animal ($n = 6$ for each group). Differences between the groups were compared using an unpaired *t*-test (GraphPad Prism 5.0 for Windows, GraphPad Software, San Diego, CA, USA) at all parameters measured. Differences from controls or between the doses at least $p < 0.05$ were considered significant and marked with asterisks.

Results

Oestrous cycling

The course of oestrous cycles in mice during supplemental period was more or less regular. At the end of the monitored period, vaginal cytology revealed that most mice of the control group were in diestrus (60%), 30% mice in oestrus, and remaining 10% in proestrus. In contrast, all mice with the diet supplemented with flaxseed showed oestrus (100%). Based on these data, only mice showing oestrus were selected for analysis of the ovaries and uteri.

Ovarian tissue culture and hormones output

The effect of flaxseed feeding on the release of P4, E2, and IGF-I by the isolated murine ovarian fragments cultured with or without the addition of FSH is shown in Figure 1. Dietary flaxseed significantly increased ($p < 0.0001$) the release of P4 and E2, but not IGF-I release by the ovarian tissue cultured without FSH. The FSH promoted the release of P4 at doses of 2.84 ($p < 0.0001$), 28.4 ($p < 0.001$), and 284 ($p < 0.01$) mIU/ml, but not at the highest dose (2840 mIU/ml) and release of E2 at doses of 28.4 ($p < 0.0001$), 284 ($p < 0.0001$), and 2840 ($p < 0.001$) mIU/ml, but not at the lowest dose (2.84 mIU/ml) by the cultured ovarian tissue of mice fed the diet supplemented with flaxseed, although, it did not markedly affect the release of IGF-I.

Table 1. Measurements of the ovaries and ovarian structures of mice fed or not fed flaxseed

	Parameter	Control	Flaxseed
Ovary	Size (μm)	1076.00 ± 42.41	1408.00 ± 79.01***
Primary follicles	Size (μm)	86.30 ± 2.64	95.84 ± 1.25*
	Mean number^{+}	0.94 ± 0.31	0.60 ± 0.24
Secondary follicles	Size (μm)	131.80 ± 2.99	132.50 ± 4.06
	Mean number	3.89 ± 0.57	3.13 ± 0.57
Antral follicles	Size (μm)	238.70 ± 7.04	225.50 ± 6.01
	Mean number	1.17 ± 0.25	1.93 ± 0.36
Preovulatory follicles	Size (μm)	337.7 ± 9.57	362.50 ± 5.66
	Mean number	0.28 ± 0.11	0.21 ± 0.12
Corpora lutea	Size (μm)	212.20 ± 8.15	236.30 ± 15.06
	Mean number	1.94 ± 0.33	1.73 ± 0.27

Values are means ± SEM. * $p < 0.05$, *** $p < 0.001$ *vs.* Control. $^{+}$ mean number *per* slide.

Histological analysis of the ovaries

The ovaries of mice in oestrus contained degenerating CLs and small newly formed CLs (Fig. 2). The ovaries and primary follicles of mice receiving flaxseed-supplemented diet were larger ($p < 0.001$ and $p < 0.05$, respectively) than the ovaries of control animals (Table 1). Neither sizes nor numbers of secondary and tertiary, including ovulatory, follicles and CLs were affected by the diet.

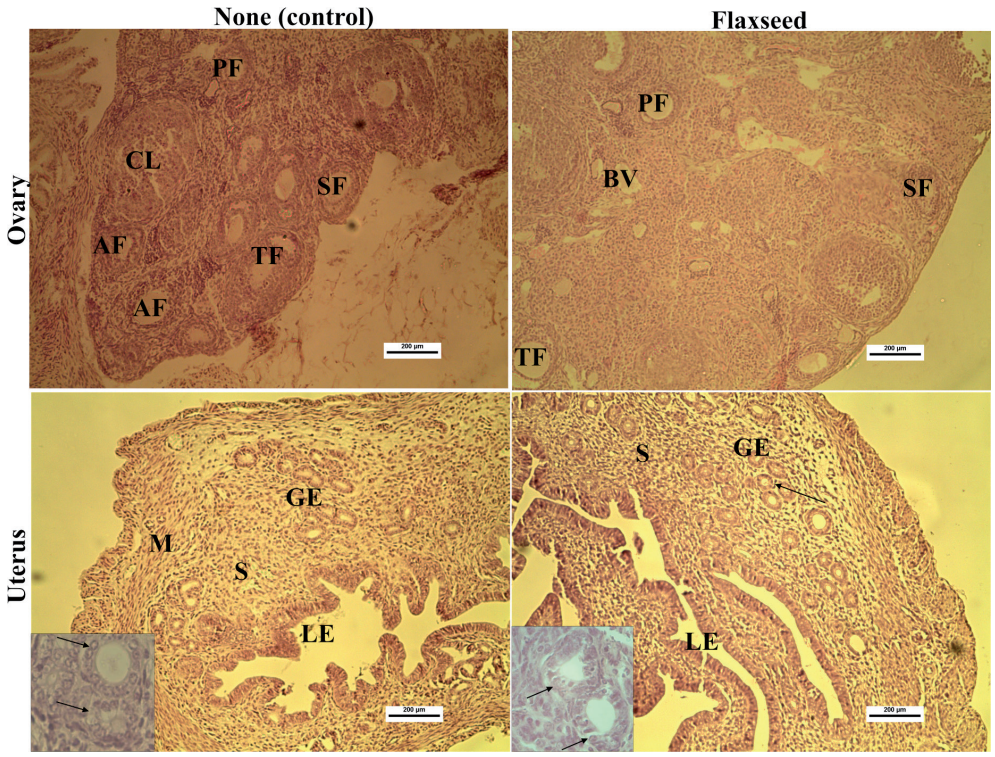

Figure 2. Histological structure of the ovary and uterine wall of oestrous mice fed or not fed flaxseed. Ovary: PF, primary follicle; SF, secondary follicle; TF, tertiary follicle; AF, atretic follicle; CL, small corpus luteum with basophilic cells; BV, blood vessel; Uterus: LE, luminal epithelium; S, stroma; GE, glandular epithelium; M, myometrium. Scale bar = 200 μm; magnification 100×. Incorporated pictures at magnification 400× show glandular epithelium with the appearance of cellular degeneration (arrows).

Table 2. Histological structure of the uterine wall of mice fed or not fed flaxseed

Part of the uterine wall	Control	Flaxseed
Luminal epithelium (μm)	18.35 ± 0.23	19.38 ± 0.57
Endometrium (μm)	215.00 ± 5.38	411.10 ± 14.08***
Myometrium (μm)	56.84 ± 0.80	69.70 ± 3.40**
Glandular epithelium (μm)	254.50 ± 9.68	371.00 ± 16.33***
Length of glands (μm)	18.66 ± 0.36	25.94 ± 0.72***
Number of glands	22.38 ± 0.67	44.23 ± 2.10***

Values are means ± SEM. ** $p < 0.01$, *** $p < 0.001$ *vs.* Control.

Histological analysis of the uterine horns

The uterine horns of mice in oestrus were more voluminous with luminal dilatation and larger number of mucosal notches in the flaxseed-fed group compared to controls. In the luminal and glandular epithelium appeared cellular degeneration and stroma was infiltrated by leukocytes (Fig. 2). Moreover, the overall thickness of the endometrium ($p < 0.0001$), height of the glandular epithelium ($p < 0.001$), diameter and number of endometrial glands ($p < 0.001$), and thickness of the myometrium ($p < 0.01$) were affected by flax-seed supplementation (Table 2). On the other hand, the height of the luminal epithelium was not affected by the diet.

Immunohistochemistry of the uterine horns

Immunohistochemical analysis revealed that markers of ERα, ERβ, and PRB were expressed in nuclei of cells throughout all the uterine compartments, including luminal and glandular epithelium, stroma, and myometrium (Fig. 3). The diet supplementation of flaxseed modulated the receptors for

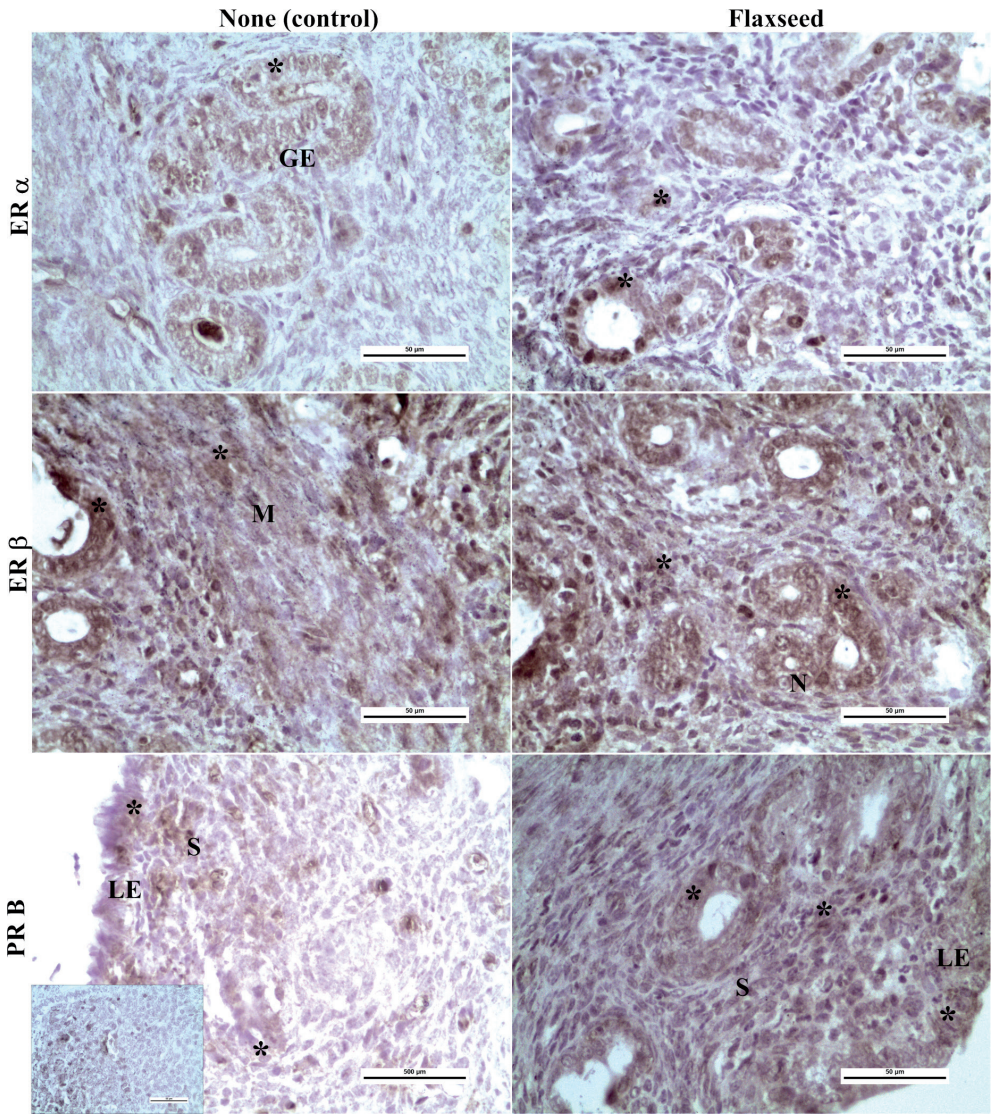

Figure 3. Immunohistochemical reaction of ERα, ERß, and PRB in the uterus of oestrous mice fed or not fed flaxseed. LE, luminal epithelium; S, stroma; GE, glandular epithelium; M, myometrium; N, necrosis; *, positive reaction. Scale bar = 50 μm; magnification 400×. Incorporated picture at magnification 400× is an example of negative control where primary antibodies were omitted.

oestrogen (ERα, ERβ) and progesterone (PRB) in the uterus of adult mice compared to control animals (Table 3). The colour intensity (ROD) of the ERα was significantly higher ($p < 0.001$) in the endometrial glands and myometrium, but lower in the cells of the luminal ($p < 0.05$) and glandular epithelium ($p < 0.01$). Flaxseed feeding also increased the ROD of ERβ in both the cells of glandular epithelium and endometrial glands ($p < 0.001$), but lowered in myometrium ($p < 0.001$), and it did not affect the colour intensity in the luminal epithelial cells. In contrast to oestrogen receptors, flaxseed feeding lowered the ROD of PRB in the cells of glandular epithelium ($p < 0.001$), endometrial glands ($p < 0.01$), and myometrium ($p < 0.001$), but did not affect markedly the ROD in the cells of the luminal epithelium. The flaxseed feeding resulted in higher colour intensity of ERα to ERβ ratio in the myometrium ($p < 0.01$) and lower colour intensity in the stroma ($p < 0.05$), however it did not alter the ratio of these receptors in other parts of the endometrium.

Fatty acids

The effect of flaxseed feeding on the levels of selected FAs in the blood serum of mice is shown in Table 4. Flaxseed consumption increased the level of saturated FAs (SFA; $p < 0.001$), mostly palmitic acid (C16:0; $p < 0.001$), although myristic acid decreased (C14:0; $p < 0.01$). Flaxseed decreased the levels of unsaturated FAs (USFA; $p < 0.001$), from which the levels of monounsaturated FAs (MUFA; $p < 0.001$) were lower and levels of PUFAs was higher ($p < 0.001$) than in control animals. The MUFAs levels were specifically altered as follows: palmitoleic acid (C16:1 n-7), vaccenic acid (C18:1 n-7) and oleic acid (C18:1 n-9) decreased ($p < 0.001$), cis-7-hexadecenoic acid (C16:1 n-9; $p < 0.001$) increased, and myristoleic acid did not alter markedly. From the PUFAs, both n-3 and n-6 groups increased ($p < 0.001$) their levels by flaxseed feeding. From n-3 PUFAs measured, only alphalinolenic (ALA; $p < 0.001$) and timnodonic (EPA; $p < 0.05$) acids increased in levels, although clupanodonic (DPA n-3) and cervonic (DHA) acids levels did not alter significantly by the special diet. On the other hand, all n-6 PUFAs decreased ($p < 0.001$), including γ-linolenic (GLA), cis,cis-11,14-eikosadienoic (EDA), dihomo-γ-linolenic (DGLA), arachidonic (AA), adrenic (DTA) as well as osbond (DPA n-6) acid, although linoleic acid (LA) increased ($p < 0.01$) after dietary flaxseed. Moreover, this diet decreased the ratio of n-6 to n-3 FAs ($p < 0.001$) by about two fold.

Discussion

In the recent study, adult cycling mice fed or not fed flaxseeds for 6 weeks were observed in oestrus reflecting the follicular phase on the ovaries and proliferative phase in the uterus. As to why flaxseeds have an effect on reproduction in animals, several mechanisms have been mentioned arising from the composition of its active substances, lignans and oil rich in n-3 PUFA.

This study revealed that all mice (100%) were synchronized to oestrus after a 42-day diet supplemented with 10% milled flaxseed. A similar effect was referred to in the study of Tou et al. (1998) in mice fed the same concentration of flaxseed where about 20% females showed persistent oestrus and prolonged oestrous cycle. Prolonged oestrous cycles were also found by Orcheson et al. (1998) after application of supplemental flaxseed or its component secoisolariciresinol diglucoside (SDG) to cycling rat dams in a dose-dependent manner. Generally, the effect of SDG is explained as the effect modulating oestrogen receptors and so can influence the course of oestrous cycles *via* affecting the plasma concentration of oestrogens (Delman et al. 2015). Daily intake of flaxseeds prolonged luteal phase in normally cycling women but with no change in the length of follicular phase (Phipps et al. 1993).

We found significantly larger ovaries in the flaxseed fed mice, correlated to our previous findings in weanlings after 24-day supplemental period (Vlčková et al. 2018). Several other studies have found that the weight of the ovaries have been increased after feeding flaxseed supplemented diets

Table 3. Intensity of immunohistochemical reaction of ERα, ERß, and PRß in the uterus of mice fed or not fed flaxseed

Receptor	Part of the uterine wall	ROD	
		Control	Flaxseed
ERα	LE	2.94 ± 0.06	2.72 ± 0.06*
	S	3.65 ± 0.14	2.89 ± 0.15**
	GE	2.40 ± 0.09	3.41 ± 0.18***
	M	1.59 ± 0.02	1.91 ± 0.03***
ERß	LE	2.72 ± 0.10	2.80 ± 0.11
	S	2.66 ± 0.07	3.17 ± 0.06***
	GE	1.91 ± 0.04	2.84 ± 0.18***
	M	1.59 ± 0.02	1.26 ± 0.01***
PRß	LE	6.37 ± 0.07	6.67 ± 0.21
	S	7.15 ± 0.11	6.50 ± 0.04***
	GE	7.34 ± 0.09	7.02 ± 0.06**
	M	2.50 ± 0.02	2.14 ± 0.01***
ERα/ERß	LE	1.1 : 1	1 : 1
	S	1.4 : 1	1 : 1.1*
	GE	1.3 : 1	1.2 : 1
	M	1 : 1	1.5 : 1**

ROD, relative optical density expressing intensity of immunohistochemical reaction; ER, estrogen receptor; PR, progesterone receptor; LE, luminal epithelium; S, stroma; GE, endometrial glands; M, myometrium. Values are means ± SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ *vs.* Control.

(Tou et al. 1998) or diets supplemented with aqueous extract from flaxseeds (Nazir et al. 2011; Dilshad et al. 2012). All mentioned studies reported that this increase in weight might be due to the weak oestrogenic effect of flaxseeds (Nazir et al. 2011; Dilshad et al. 2012; Kiyama 2016), the effect of polyunsaturated fatty acids (Vlčková et al. 2018) or a combination of several mechanisms.

We found that the numbers of follicles and CLs of mice fed flaxseed did not differ markedly from the controls. Similar results were found after flaxseed feeding in the studies performed on rats (Ward et al. 2001; Collins et al. 2003), but not on cows (Petit et al. 2004) in which the number of medium sized follicles was even lower. However, other studies contradicted that (Robinson et al. 2002; Moallem et al. 2013). In weanling gilts, lower number of primary follicles was found, but there was no effect on the number of other follicle types (Vlčková et al. 2018).

In relation to the effect of flaxseeds on the size of follicles and CLs, we found that flaxseed feeding in mice resulted in larger primary follicles, but sizes of other follicle types including ovulatory follicles, and CLs were not affected markedly. This finding correlates with our previous results obtained from weaning pigs (Vlčková et al. 2018) and with the studies performed on rats (Nazir et al. 2011; Dilshad et al. 2012) and dairy cows (Moallem et al. 2013) fed flaxseed-enriched diet. On contrary, some studies in cows (Petit et al. 2004) and ewes (Wonnacott et al. 2010) showed no significant effect on the mean follicle size of the flaxseed or PUFA supplemented diet, respectively, even though Ambrose et al. (2006) found even larger preovulatory follicles in cows fed flaxseed. The knowledge about the effects of flaxseed or its components is quite controversial and may depend on the animal species and age, dose and length of flaxseed feeding or some other influences.

Table 4. Effect of flaxseed on the levels of selected fatty acids in the blood serum of mice

Fatty acids (mol%)	Trivial name (Abbreviation)	Control	Flaxseed
14:0n	Myristic	0.40 ± 0.08	0.05 ± 0.01**
14:1n-5	Myristoleic	0.15 ± 0.09	0.05 ± 0.01
16:0n	Palmitic	19.41 ± 0.46	27.25 ± 0.99***
16:1n-7	Palmitoleic	6.53 ± 0.11	1.47 ± 0.11***
16:1n-9	7-hexadecenoic	0.52 ± 0.01	0.77 ± 0.05***
18:1n-7	Vaccenic	1.81 ± 0.07	1.38 ± 0.04***
18:1n-9	Oleic	40.50 ± 0.87	21.78 ± 0.48***
18:2n-6	Linoleic (LA)	16.22 ± 1.82	23.49 ± 0.97**
18:3n-6	γ-linoleic (GLA)	0.265 ± 0.03	0.05 ± 0.01***
18:3n-3	α-linolenic (ALA)	0.525 ± 0.15	4.15 ± 0.54***
20:1n-9	Gondoic	0.48 ± 0.06	0.99 ± 0.05***
20:2n-6	Eicosadienoic (EDA)	0.76 ± 0.06	0.18 ± 0.01***
20:3n-6	Dihomo-γ-linoleic (DGLA)	0.72 ± 0.07	0.31 ± 0.03***
20:4n-6	Arachidonic (AA)	3.01 ± 0.08	1.46 ± 0.26***
20:5n-3	Timnodonic (EPA)	0.16 ± 0.08	0.41 ± 0.07*
22:4n-6	Adrenic (DTA-6)	2.88 ± 0.02	0.02 ± 0.00***
22:5n-6	Osbond (DPA-6)	0.69 ± 0.13	0.02 ± 0.01***
22:5n-3	Clupanodonic (DPA-3)	0.29 ± 0.04	0.19 ± 0.05
22:6n-3	Cervonic (DHA)	0.88 ± 0.12	0.60 ± 0.21
SFA		25.20 ± 1.15	43.16 ± 1.27***
USFA		74.67 ± 1.18	59.01 ± 1.15***
MUFA		49.53 ± 0.98	26.47 ± 0.49***
PUFA		21.19 ± 0.70	31.87 ± 1.15***
n-3		2.18 ± 0.19	5.68 ± 0.43***
n-6		23.98 ± 2.30	25.52 ± 0.16
n-6/n-3		11 : 1	4.5 : 1***
EPA/AA		1 : 6	1 : 3.5**

SFA, saturated fatty acids; USFA, unsaturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids. Values are means ± SEM. *$p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ *vs.* Control.

This study is first to report about the secretion of steroid and protein hormones and their response to FSH addition by the cultured ovarian fragments of mice fed flaxseed. The 6-week diet supplementation with flaxseed caused the ovaries to increase the release of both the P4 and E2 as was found in plasma of ewes offered n-3 diet (Wonnacott et al. 2010). Even though IGF-I can promote androgen and oestrogen secretion by granulosa cells (Ulloa-Aguire et al. 2007; Sirotkin 2014), its release by the murine ovaries did not differ markedly among the groups. The dietary increase in steroid hormones, but not IGF-I, was even supported by the addition of FSH to the culture medium. Follicle-stimulating hormone is a key gonadotropin, which stimulates the aromatisation of androgens to oestrogens in granulosa cells promoting steroid hormones and IGF-I release (Sirotkin et al. 2003) to support development and maturation of antral follicles in follicular phase (Ulloa-Aguire et al. 2007; Sirotkin et al. 2008). Both high E2 and later slight increase in P4 concentrations are essential for the induction of preovulatory luteinizing hormone (LH) surge and triggering of ovulation (Drummond 2006). We may thus assume that dietary flaxseed can promote ovulation process by promoting luteinisation of follicles. Studies performed on cows (Robinson et al. 2002) and ewes (Wonnacott et al. 2010) fed a diet fortified with ALA (n-3 PUFA) can support our results. We can postulate that ovarian steroidogenesis in mice was influenced by the interaction between phytoestrogenic mechanism and a change in FA metabolism. One reason is that PUFA may change cell membrane composition and fluidity, and thus the transfer of nutrients in and out of the cell as well as the enzymatic and receptor activities (Moallem et al. 2013; Yu et al. 2014). PUFA alone or together with enterolignans bound to oestrogen receptors (ER) was found to decrease the activity of key enzyme, 17-β-OH dehydrogenase in oestrogen metabolism (Kurzer and Xu 1997). High intake of ALA increases serum concentrations of EPA and DHA, which, together with lower concentrations of several saturated fatty acids, induce apoptosis and suppress proliferation of ovarian cells (Vlčková et al. 2018), the opposite effect of E2 action in the ovaries (Wang et al. 1999; Lecomte et al. 2017).

Long-term feeding of flaxseed to mice almost doubled the thickness of endometrium and increased the thickness of myometrium, height of the glandular epithelium, tubular gland diameter and number as well. The height of the luminal tall columnar epithelium was not affected by the diet. We believe that these changes in the uterus, especially its thickening, could be mediated through the effect of phytoestrogens contained in flaxseed via modulation of ER and progesterone receptors and that such receptor modulation is linked with changes in FA metabolism.

Immunohistochemical analysis showed that markers of ERα, ERβ, and PRB were expressed in nuclei of cells throughout all the uterine compartments, including luminal and glandular epithelium, stroma, and myometrium with different immunostaining intensity among the groups, receptors, and uterine compartments. These data support the observations of Wang et al. (1999, 2010) in mice. Flaxseed feeding resulted in a decreased expression of ERα, a dominant receptor subtype in the rat uterus (Wang et al. 1999) in the luminal epithelium and stroma, but increased its expression in the glandular epithelium and myometrium. On the other hand, the expression of ERβ was increased in the stroma and glandular epithelium, but decreased in the myometrium and was not affected markedly in the luminal epithelium. High oestrogen concentrations in the proliferative phase of the cycle are responsible for the protection against uterine epithelial apoptosis and for promoting its proliferation resulting in complete uterine growth response (Wood et al. 2007; Winuthayanon et al. 2010) and increase in myometrial contractions (Kunz et al. 1996). In the proliferative phase of uterine cycle, E2 induces ERα and PR isoforms to increase expression of genes necessary for cell division and tissue growth (Talbi et al. 2006; Patel et al. 2015). On the other hand, ERβ can suppress transcription of ERα and expression of PR at lower plasma E2 concentration inducing apoptosis in epithelial cells (Winuthayanon et al. 2010; Böttner et al. 2014; Hapangama et al. 2015). According to Hapangama et al. (2015), ERβ is the main safety mechanism for restriction of the potent mitogenic action of E2 in the healthy endometrium. Progesterone is a key hormonal regulator of the female reproduction playing a major role in preparing the uterus for embryo implantation and pregnancy. Progesterone receptors, isoforms A and B, mediate P4 actions in the uterine tissue, principally stromal and epithelial cells of endometrium and smooth muscle cells of myometrium (Patel et al. 2015). Flaxseed fed to mice decreased the expression of PRB almost in all uterine compartments. These results indicate that flaxseed lignans must compete with endogenous E2 and increase expression of ERβ in the endometrium, thus repressing epithelial PR expression (Wada-Hiraike et al. 2006). In addition, these data indicate that flaxseed can suppress epithelial proliferation and stromal decidualization in preparation for embryo implantation (Critchley and Saunders 2009). Progesterone and oestradiol promote myometrial growth by stimulation of hyperplasia and hypertrophy of myometrial cells. During proliferative phase, myometrial contractions increase in intensity and frequency and decrease after ovulation with P4 dominance (Kunz and al. 1996). In our study, flaxseed increased expression of ERα, but decreased ERβ and PRB. As activation of ERα results in the contracting activity of uterine smooth muscle cells, we postulate that flaxseed diet can support the contraction of myometrium.

The data in the present study also revealed lower ratio of ERα/ERβ in the uterine stroma, but increased such ratio in the myometrium after 6-week diet fortification with flaxseed.

The ERα/ERβ ratio has been used to evaluate the imbalance in the relative ER isoforms expression in endometrial malignancies (Šmuc and Rižner 2009), to predict disease outcome (Sastre-Serra et al. 2013), and to determine phytoestrogenic effects of enterolignans in the target tissues (Jordan 2007). High ratio is associated with a bad effect of phytoestrogens, while low ratio is associated with good effect of phytoestrogens. Our results therefore show positive effect of flaxseed ingestion on the endometrial health preventing tumour formation or other uterine abnormalities (Böttner et al. 2014).

The effects of phytoestrogens on reproductive organs were studied elsewhere (Wang et al. 1999; Jordan 2007; Šmuc and Rižner 2009; Wang et al. 2010; Nazir et al. 2011; Dilshad et al. 2012). Phytoestrogens can stimulate lipid metabolism in the liver (Böttner et al. 2014; Sopková et al. 2017; Rezaei et al. 2020). However, whether the changes in ovarian functions or in uterine receptor expression of mouse may result from changes in FA metabolism is limited.

Although flaxseed oil is low in SFA (Cunnane et al. 1993), the serum concentrations of palmitic but not myristic acid were found increased in mice fed flaxseed for 6 weeks. In addition, the fatty acid analysis revealed that dietary flaxseed lowered the concentrations of USFA reflecting a decrease in MUFA, although PUFA concentration was increased. High content of ALA in the diet of mice elevated the serum concentrations of n-3 PUFA (ALA and EPA but not DHA) but did not markedly affect total n-6 PUFA, although LA was higher. The activity of Δ-6-desaturase, the enzyme responsible for the metabolism of both the n-6 and n-3 PUFA (Stoffel et al. 2008), was decreased resulting in the lower conversion of n-6 PUFA and in favour of n-3 PUFA conversion. Therefore, all the derivatives down the metabolic pathway of LA were decreased (EDA, GLA, DGLA, AA, DTA n-6, and DPA n-6) and these findings are in line with several studies (Perini et al. 2011; Gustone 2012). By inhibiting the elongation and desaturation of LA, its con-centration maintains high in the blood. Unlike our previous results obtained from piglets (Vlčková et al. 2018) and data of several other studies (Stoffel et al. 2008; Perini et al. 2011), DPA n-3 and DHA serum concentrations in mice were not markedly affected by flaxseed feeding. However, our results agree with that of Petit et al. (2004) who found that ALA supplementation in cows enhanced the concentra-tion of EPA but not of other longer n-3 PUFA in plasma. Generally, USFA may influence cell growth and proliferation by modifying membrane fluidity and receptor activities (Moallem et al. 2013; Yu et al. 2014). The secretion of E2 and P4 increased by the ovaries of oestrous mice fed flaxseed in this study indicates that such a diet can affect the process of ovulation. Ovulation has many attributes in common with inflammation including increased production of reac-tive oxygen species (ROS) and prostaglandins (PGs) in ovulatory follicles (Shkolnik et al. 2011; Agarwal et al. 2012). Fatty acids (mainly n-6 AA) work as precursors of pro-inflammatory PGs of series 2 necessary for successful ovulation process and for maintaining normal reproductive functions (Dikshit et al. 2015). Prostaglandin E2 is involved in cumulus expansion of oocyte at ovulation (Edson et al. 2009). It means that enough substrate (AA n-6) ensures maintaining of normal ovulation process. Omega-3 PUFA inhibit the synthesis of pro-inflammatory AA-derived PGE2, which can be elevated in many cancers, including ovarian (Eilati et al. 2013) and thus inhibit neutrophilic inflammatory responses (Sourinejad et al. 2019) via increased production of anti-inflammatory PGs of series 3 (Tvrzická et al. 2009). Moreover, flaxseed's lignan SDG shows antioxidant properties that inhibit production of ROS (Adolphe et al. 2010; Kasote 2013; Mbemya et al. 2017) and therefore we suggest that the diet enriched with flaxseed fed for 6 weeks may suppress ovulation.

Whether the changes in uterine receptors expression could be modulated by the changes in FA metabolism caused by high intake of dietary ALA in flaxseed was observed in this study. Flaxseed increased serum concentrations of palmitic (C16:0), 7-hexadecenoic (C16:1 n-9) and gondoic (C20:1 n-9) acids, LA, ALA, and EPA. It has been reported that USFA (mainly PUFA AA n-6 and DHA n-3) dramatically potentiated the binding of E2 to human uterine tissue via irreversible binding to oestrogen receptors (Benassayag et al. 1986). However, USFA inhibited or potentiated the binding between E2 and ER, or inhibited the binding between P4 and PR in rat uterus depending on the amount of hormone or FA (Kato 1989). This inhibitory mechanism of PUFA is not clear, although inhibitory action strengthened with elongation of PUFA. Moreover, binding of P4 to PR was also inhibited by palmitic (C16:0) and palmitoleic acids (C16:1 n-7) (Mitsuhashi et al. 1986). In this study, we have not been analysed which FA particularly affected the binding of hormone to its receptor. As DHA was not affected by flax-seed and AA and palmitoleic acid were even decreased, we can postulate that flaxseed could alter the affinity of E2 to ER or P4 to PR *via i*nhibition by LA, ALA, EPA, palmitic acid or selected MUFA (C16:1 n-9 and C20:1 n-9).

Possible effects of flaxseed on the ovarian and uterine functions resulting from the observed results are shown in Figure 4. The possible mechanisms of action of selected fatty acids on steroidogenesis, ovulation, and uterine performance as well as functional interrelationships between fatty acids and phytoestrogens from flaxseed require further investigation.

Conclusion

The obtained results show that the 6-week intake of dietary flaxseed can alter ovulation via promoted steroidogenesis (E2 and P4) by the cultured mouse ovarian fragments as well as

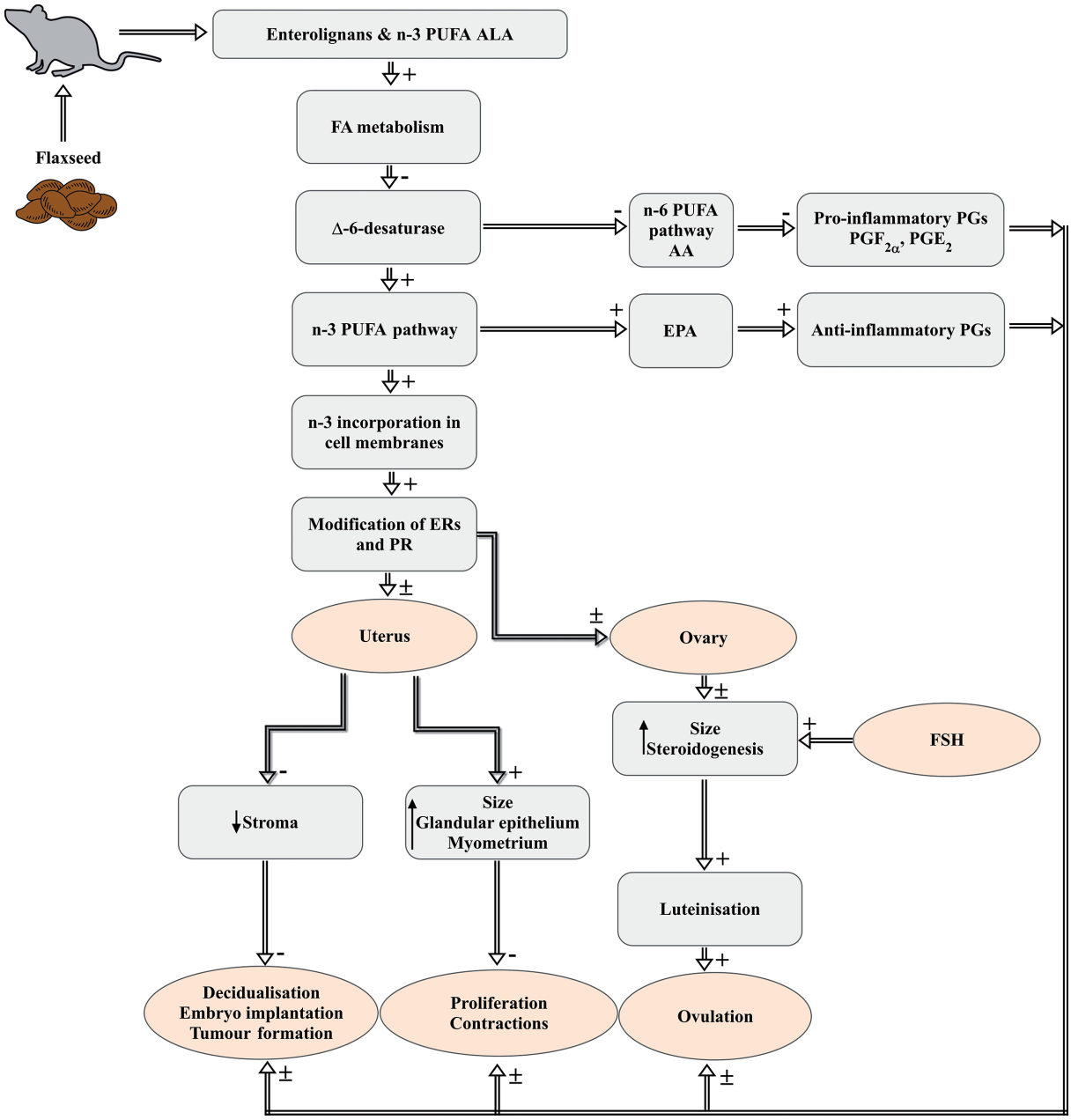

Figure 4. Possible effects and mechanism actions of dietary flaxseed on the ovarian and uterine functions of oestrous mice. Flaxseed can exert its effects through the high content of n-3 PUFA ALA and lignans. High levels of ALA favour the n-3 PUFA metabolic pathway over the n-6 PUFA pathway *via* the Δ-6-desaturase, a common enzyme for both metabolic pathways resulting in higher production of EPA and EPA-derived anti-inflammatory PGs. High n-3 PUFA are incorporated in cell membranes altering their properties, integrity, fluidity and modifying steroid receptors (ERs, PR). Both the ovary and uterus are under the action of FSH during oestrus and it seems the gonadotropin exerts its effect despite the intervention of phytoestrogens and PGs. The FSH supports steroidogenesis before ovulation resulting in proliferation, massive luteinisation of granulosa cells and enlargement of ovaries altering the process of ovulation. The own process of ovulation can be altered by the anti-inflammatory effects of enterolignans and PGs. Increased steroidogenesis in the ovaries supports the proliferation of glandular epithelium of the endometrium and in myometrium (enlargement of the uterus) *via* expression of ERα supporting the action of E2 and contractility of smooth muscle cells. Flaxseed components increase the expression of ERβ in the glandular epithelium and endometrial stroma. Lower expression of ERα in the stroma can result in lower stromal decidualisation in preparation for embryo implantation, but also can prevent tumour formation. Flaxseed can block P4 binding to its receptors in the proliferative phase by lowering their expression. AA, arachidonic acid; ALA, α-linolenic acid; EPA, eicosapentaenoic acid; ER, oestrogen receptors; FA, fatty acids; PG, prostaglandins; PR, progesterone receptor; PUFA, polyunsaturated fatty acids.

their response to FSH. In addition, binding of steroids on the uterine ER and PR can be potentiated or inhibited by the modification of PUFA metabolism caused by intake of high portion of ALA from flaxseed and changes in several SFA and MUFA. Those FA can be incorporated in cell membranes of ovaries and uteri modulating their fluidity and affecting the production of pro-inflammatory and anti-inflammatory prostaglandins.

Animal welfare statement. The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to and the appropriate ethical review committee approval has been received. The authors confirm that they have followed EU standards for the protection of animals used for scientific purposes.

Conflict of interest. The authors declare no conflict of interest.

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