The Effect of Flaxseed on the Porcine Uterus

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Abstract
Flaxseed is an important supplement in animal feed. It contains high levels of polyunsaturated fatty acids and lignans acting as phytoestrogens, which can alter cell functions in various organs. The aim of this work was to analyse the effect of supplemental flaxseed in pigs on the morphophysiological properties of the uterus. The experiment included 12 Landras fattening pigs. The control group was fed with a standard feeding mixture for fattening pigs and the experimental group was fed a standard mixture with an addition of 10 % milled flaxseed. After six weeks of fattening, six animals of each group were slaughtered and samples of the uterine horns were taken for the routine histological (height of the endometrium and its compartments, number of endometrial glands, and myometrium height) and for immunohistochemical (expression of oestrogen and progesterone receptor markers) analyses. In the experimental group fed a flaxseed supplement, significant thickening of the superficial epithelium, endometrium, and *lamina propria* layer was found. The number of endometrial glands was higher than in the control uteri by about 1/3. Moreover, a flaxseed-enriched diet significantly increased the expression of oestrogen receptors α and β, as well as progesterone B receptors in all uterine compartments. The addition of flaxseed in the feed for six weeks had a stimulating effect on the monitored parameters of the uterus of fattening pigs.

Keywords: fattening pig, flaxseed, oestrogen receptors, progesterone receptor, uterus

1. Introduction

Flaxseed is widely used in diet supplementation programs in farm animals to improve meat or fat characteristics. The advantage of flaxseed is its composition of nutrients and low amount of anti-nutritive substances [1]. It is a good source of dietary fibre and the best plant source of polyunsaturated fatty acids (PUFAs) [2]. A high content of n-3 PUFAs (α-linolenic acid, ALA) metabolize in the gut into timnodonic (EPA) and cervonic (DHA) acids, which have many positive effects on lipid metabolism [3], digestive, cardiovascular, neural and other systemic functions [4]. Flaxseed lignans are a component of dietary fibre and after their ingestion, they are metabolised in the gut into phytoestrogenic enterolignans with weak oestrogenic or anti-oestrogenic activity [5]. Together with enterolignans, PUFAs and some other FAs influence reproductive functions via modifying the composition, integrity, and fluidity of cell membranes [6] and thus the affinity of phytoestrogens to steroid receptors [7]. In the uterus, the effect of oestrogens is mediated mainly through its receptor α (Erα) [8], while the phytoestrogen affinity is higher for the receptor β (Erβ) [9]. The sensitivity of uterine cells to ERs is attenuated by the decrease in the number of progesterone receptors (PR) [10]. Supplemental flaxseed increased the thickness of the endometrium and myometrium, and altered the expression of ERs and PR in the mouse uterus [7] however, its effect on the porcine uterus is not clear. Therefore, the purpose of this study was to observe the structural changes in the endometrium

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and myometrium as well as to localize and quantify ERs and PR using an immunohistochemical method in the uterus of fattening gilts fed supplemental flaxseed.

2. Materials and methods

The procedure used 12 fattening pigs of the Landras breed with an average weight of 76±5.48 kg. The females were housed at the Pig Fattening and Slaughter Station, s.r.o. in Spišské Vlachy, Slovak Republic. In the 6-week fattening period, control pigs (n=6) were fed standard feed for fattening pigs OŠ-06 (Dom krmív, Spišské Vlachy) at a dose of 3 kg/head/day. Experimental pigs (n = 6) were fed the same diet enriched with flaxseed (variety Libra, 57% α-linolenic acid). The animals had free access to water. After 6 weeks of fattening, the pigs of both groups were slaughtered. Excisions from the right uterine horn (basal part; n=6 for each group) were fixed in 4% paraformaldehyde. After 24 hours, they were dehydrated, embedded in paraffin and cut into 5 μm sections (Leica RM2255 microtome, Leica Germany). For routine histological examination, paraffin sections were stained with hematoxylin and eosin (H-E) and evaluated using a light microscope (Nikon Eclipse E200; Japan) with a digital camera (ProgRes Capture Pro 2.7.7; Jena Germany) and NIS Elements Br software (Imaging Software, Nikon Laboratory Imaging, Japan). At ×100 magnification, endometrial thickness was determined by measuring the distance from the superficial epithelium perpendicular to the myometrium and myometrial thickness by measuring the distance from the endometrium perpendicular to the perimetrium as the average of 3 measurements in different areas of the uterine horn. We proceeded the same in determining the thicknesses of individual parts of the endometrium, i.e. superficial cylindrical epithelium, *lamina propria mucosae* and *stratum subglandulare*. All parameters were measured in μm. The tubular glands of the uterus were counted in four quadrants expressing their average number. Immunohistochemical analysis was performed according to our previous study [7]. Shortly, uterine paraffin sections were deparaffinised and rehydrated followed by the antigen retrieval (slides boiling in 10 mM citrate buffer; pH 6.0 for 2 min), blocking of endogenous peroxidase activity (incubation in TBS [0.05M Tris–HCl plus 0.15M NaCl, pH 7.6] + 0.3% H_2O_2 for 20 min) and non-specific binding (1-h incubation with 8% bovine serum albumin). Subsequently, polyclonal rabbit anti-ER α and β (both 1:100 dilution; ThermoFisher Scientific, Waltham, MA, USA) monoclonal mouse anti-PRB (1:100; Invitrogen ThermoFisher) were added and preparations were incubated overnight at 4 °C. The sections were incubated with goat anti-mouse secondary antibodies (Dako REAL™ EnVision™/HRP, Rabbit/Mouse (ENV), ready-to-use, Dako, Denmark) for 2 h after rinsing in TBST (TBS + 0.1% Tween20). Following incubation, specimens were rinsed in TBST followed by TBS. The colour reaction was induced with diaminobenzidine (Dako REAL-DAB + Chromogen, Dako), sections were counterstained with hematoxylin and embedded in DPX (Distyrene Plasticizer and Xylene; Buchs, Switzerland). There was a negative control (primary antibody omitted) on each slide. Photomicrographs were obtained using a light microscope connected to a computer and a camera (Olympus UC30, Olympus Corporation, Japan) to evaluate the intensity of the immunoreaction. Quantification of the IHC reaction was performed as a relative optical density (ROD) of immunopositive (DAB brown reaction products) signal in uterine cells at grey level by using ImageJ software (National Institutes of Health, Bethesda, MD, USA). Six images from sections of each examined animal (n=6 for both groups) were analysed and ROD was calculated using the formula described by Smolen [11].

3. Results and discussion

Morphometric properties of the fattening gilts' uterine horn tissue in control animals and animals after six weeks of 10% flaxseed supplementation are shown in Figure 1. Flaxseed-fed animals had higher endometrium ($P<0.01$), including lamina propria mucosae ($P<0.05$) and luminal epithelium ($P<0.05$) as well as a higher number of tubular glands when compared to the controls. The stratum subglandulare and myometrial cells were not affected significantly by the diet. Tou et al. [12] reported similar results in pregnant mice fed a diet enriched with flaxseed and phytoestrogen cumestrolate. There was an increase in uterine

relative weight, severe estrogenization, and increased embryonic mortality. Polyunsaturated fatty acids are one of the most important components of flaxseed [6, 3], which can have a significant effect on oestrogen metabolism and function [13].

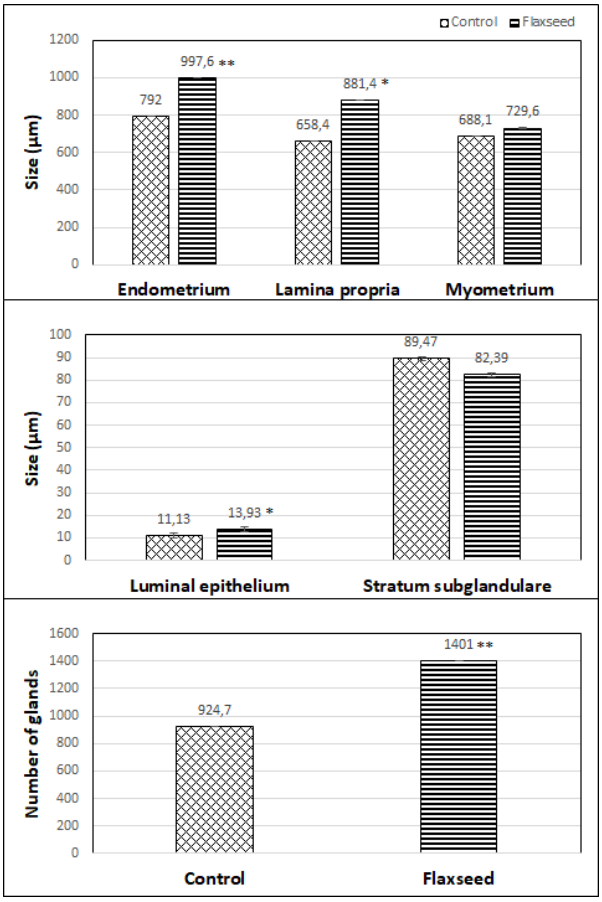

Figure 1. Morphometric properties of the fattening pigs' uterine horn tissue after flaxseed supplementation

In general, oestradiol-17β and progesterone bind to cytosolic receptors and exert their effects by binding to nuclear receptors [14, 15]. Immunohistochemical analysis showed that the ERα and PRB markers were localized in the cell nuclei of all uterine compartments of both groups (Figure 2). Several studies [7, 16] have reported similar results in mice. In contrast, we found cytosolic immunolocalization of the ERβ marker in endometrial and myometrial cells of both groups, while in uterine smooth muscle cells of flaxseed-fed gilts, it was also observed in the nuclei. These results show that flaxseed exerted its effects only in the myometrium and not in the endometrium, in which seems to have opposing action to ERα in response to oestrogens [17].

The effect of supplemental flaxseed on the expression of those receptors based on the quantitative analysis of relative optical density (ROD) is summarized in Table 1. We found more intensive immunoreaction of all mentioned steroid receptors in the flaxseed group compared to the control uteri, particularly ROD of ERα, ERβ, and PRB in the luminal epithelium ($P<0.05$, $P<0.01$, $P<0.05$, respectively), stroma ($P<0.001$, $P<0.01$, $P<0.001$, respectively), endometrial glands ($P<0.01$, $P<0.001$, $P<0.001$, respectively) as well as in myometrium ($P<0.001$ for all). These results are not consistent with our previous observations in mice [7] in which 10% flaxseed feeding for 6 weeks suppressed the expression of ERα in the luminal epithelium and stroma, while increased in glands and myometrium. In the same study, on the other hand, ERβ immunolocalization was increased in the stroma and glands, while decreased in the myometrium and luminal epithelium. Of the porcine uterine compartments, the stroma showed the weakest colour intensity at all receptor markers. Moreover, the ROD of ERβ was lower than that of ERα suggesting the flaxseed applied only weak oestrogenic activity [9] in porcine uteri. The proliferative oestrogen-dominant phase of the uterine cycle is characterised by oestrogenic induction of higher gene expression of ERα and PR isoforms necessary for cell division and tissue growth [15], which relates to our study. However, ERβ may suppress both at lower plasma oestrogen levels restricting potent mitogenic action of oestrogens in the healthy endometrium [14]. Phytoestrogenic effects of enterolignans contained in the flaxseed on the target tissues can be determined by the ERα/ERβ ratio [18]. A high ratio relates to a bad effect, while a low ratio to a good effect of phytoestrogens. We found higher ERα/ERβ ratio in the stroma ($P<0.05$), whilst it was not markedly affected in other parts of the uterus in the flaxseed group compared to the controls. These results are not in line with the results of our previous study on mice, in which the ratio was lower in the stroma and higher in the myometrium after 6-week diet supplementation with 10% flaxseed [7]. Progesterone has a major role in preparing the uterus for blastocyst implantation and proceeding pregnancy. Progesterone receptors (i.e. isoforms A and B) mediate hormone effects mainly in stromal and epithelial cells of endometrium and smooth muscle cells of myometrium [15]. In the current

study, the flaxseed diet resulted in higher ROD of PRB in all uterine compartments compared to the control uteri with the most intensive immune signal in myometrial and stromal cells.

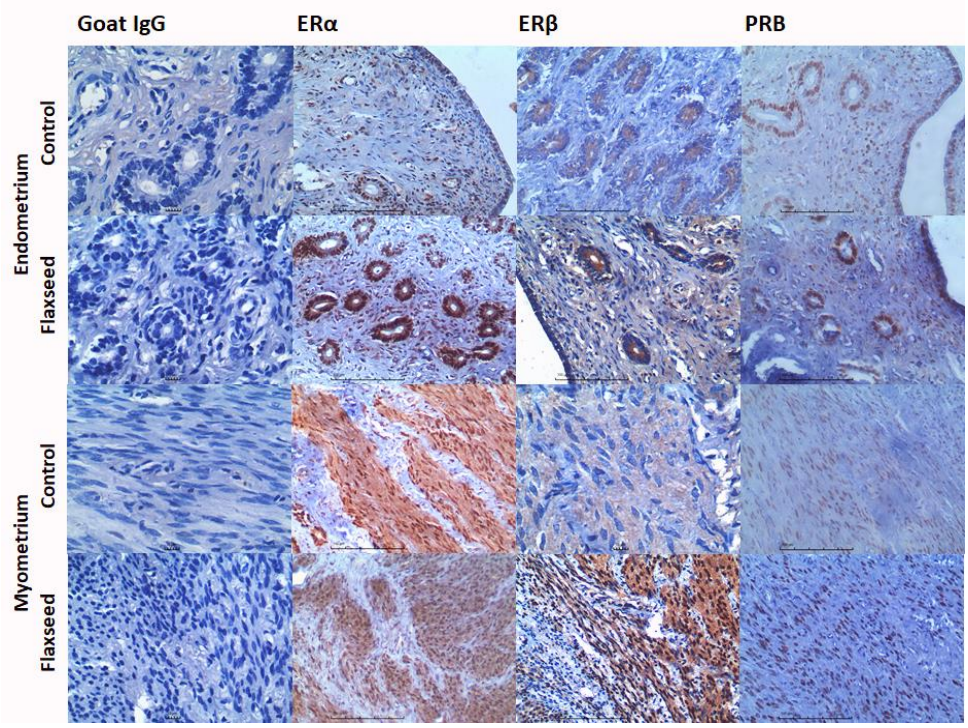

Figure 2. Representative samples showing expression of ERα, ERβ, and PRB in the uterine tissue of fattening pigs after 10% flaxseed diet supplementation

Table 1. Relative optical density of immunoreaction in the uterine tissue of the fattening pigs after flaxseed supplementation

Supplement/Receptor	ROD			
	Luminal epithelium	Stroma	Endometrial glands	Myometrium
ERα				
None (Control)	1.13±0.04	0.82±0.04	1.14±0.04	0.95±0.04
Flaxseed	1.36±0.06*	1.07±0.01***	1.29±0.03**	1.18±0.01***
ERβ				
None (Control)	1.12±0.02	0.75±0.02	0.97±0.01	0.84±0.03
Flaxseed	1.37±0.06**	0.84±0.02**	1.27±0.02***	1.27±0.04***
ERα/ERβ				
None (Control)	1.00±0.02	1.09±0.02	1.17±0.03	1.13±0.01
Flaxseed	0.99±0.01	1.27±0.02*	1.01±0.02	0.93±0.03
PRB				
None (Control)	1.10±0.07	0.82±0.04	1.06±0.02	0.88±0.01
Flaxseed	1.27±0.02*	1.17±0.01***	1.20±0.02***	1.38±0.03***

*P<0.05; **P<0.01; ***P<0.001, [1]ROD = relative optical density, [2]ER = oestrogen receptor, [3]PR = progesterone receptor.

These data are not in line with the previous study on mice with the same diet [7], where the expression of PRB was found the opposite in all uterine compartments except for the luminal epithelium.

4. Conclusions

The observed results indicate that flaxseed as a 10% diet supplement fed for six weeks to fattening gilts applied its supporting effects on the endometrium thickness (including luminal epithelium, *lamina propria* and number of endometrial glands) as the effect on the steroid hormone receptors expression. Moreover, its effect on the myometrium was supported by binding phytoestrogens to nuclear ERβ receptors.

Acknowledgements

We would like to thank Dr. Jana Kaľatová for technical collaboration. This work was financially supported by the Research Grant Agency of the Ministry of Education, Science, Research and Sport of the Slovak Republic; grant number VEGA 1/0204/20.

References

1. Morris, D. 2007. Flax - A Health and NutritionPrimer. Fourthedition. Winnipeg : Flaxcouncil of Canada. 140 s. ISBN: 978-096-9607-35-9.
2. Patade A, Devareddy L, Lucas EA, Korlagunta K, Daggy BP, Arjamandi BH (2008): Flaxseed reduces total and LDL cholesterol concentrations in Native American postmenopausal women. J Womens Health (Larchmt) **17,** 355-366
3. Sopková D, Hertelyová Z, Andrejčáková Z, Vlčková R, Gancarčíková S, Petrilla V, Ondrašovičová S, Krešáková L (2017): The application of probiotics and flaxseed promotes metabolism of n-3 polyunsaturated fatty acids in pigs. J Appl Anim Res **45,** 93-98
4. Adkins, Y., Darhan, S. K. Mechanisms underlying the cardioprotective effects of omega-3 polyunsaturated fatty acids. Journal of Nutritional Biochemistry. 2010, 21(9), 781-792
5. Michel T, Halabalaki M, Skatsounis AL (2013): New concepts, experimental approaches, and dereplication strategies for the discovery of novel phytoestrogens from natural sources. Planta Med **79,** 514-532
6. Andrejčáková Z, Sopková D, Vlčková R, Kulichová L, Gancarčíková S, Almášiová V, Holovská K, Petrilla V, Krešáková L (2016): Synbiotics suppress the release of lactate dehydrogenase, promote non-specific immunity and integrity of jejunum mucosa in piglets. Anim Sci J **87,** 1157-1166
7. Vlčková, R., Andrejčáková, Z., Sopková, D., Kozioł. K., Hertelyová, Z., Koziorowska, A., Gancarčíková, S. 2022. Effects of supplemental flaxseed on the ovarian and uterine functions of adult cycling mice. General Physiology and Biophysics (Accepted) Doi: 10.4149/gpb_2022003
8. Couse JF, Korach KS (1999): Estrogen receptor null mice: what have we learned and where will they lead us? Endocr Rev **20,** 358-417
9. Paterni I, Granchi C, Katzenellenbogen JA, Minutolo F (2014): Estrogen receptors alpha (ERα) and beta (ERβ): Subtype selective ligands and clinical potential. Steroids **90,** 13-29
10. Clarke CL, Sutherland RL (1990): Progestin regulation of cellular proliferation. Endocr Rev **11,** 266-302
11. Smolen AJ (1990): Image analytic techniques for quantification of immunocytochemical staining in the nervous system. In Methods in Neurosciences; Conn, P.M. Ed.; p. 208–229, Academic Press, New York, USA
12. Tou JCL, Chen J, Thompson LU (1998): Flaxseed and its lignan precursor, secoisolariciresinol diglycoside, affect pregnancy outcome and reproductive development in rats. J Nutr **128,** 1861-1868
13. Prasad, K. Secoisolariciresinol diglucoside from flaxseed delays the development of type 2 diabetes in Zucker rat. In J LabClin Med 2001, 138(1), 32-39
14. Hapangama DK, Kamal AM, Bulmer JN (2015): Estrogen receptor β: the guardian of the endometrium. Hum Reprod Update **21,** 174-193
15. Patel B, Elguero S, Thakore S, Dahoud W, Bedaiwy M, Mesiano S (2015): Role of nuclear progesterone receptor isoforms in uterine pathophysiology. Hum Reprod Update **21,** 155-173
16. Wang DF, Zhang NY, Peng YZ, Qi DS (2010): Interaction of zearalenone and soybean isoflavone on the development of reproductive organs, reproductive hormones and estrogen receptor expression in prepubertal gilts. Anim Reprod Sci **122,** 317-323
17. Thomas, C., Gustafsson,J.-A. The different roles of ER subtypes in cancer biology and therapy. Nat Rev Cancer 2011, 11(8):597-608
18. Jordan V (2007): Chemoprevention of breast cancer with selective oestrogen-receptor modulators. Nat Rev Cancer **7,** 46-53