TÜBİTAK

Turkish Journal of Veterinary and Animal Sciences

http://journals.tubitak.gov.tr/veterinary/

Research Article

Turk J Vet Anim Sci
(2014) 38: 686-692

doi:10.3906/vet-1404-23

The effect of short-term lupin (Lupinus angustifolius) feed supplementation on serum steroid hormones, insulin-like growth factor I, and ovarian follicular development and atresia in Merino ewes

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Received: 07.04.2014 • **Accepted:** 26.06.2014 • **Published Online:** 24.10.2014 • **Printed:** 21.11.2014

Abstract: The effects of short-term lupin supplementation on ovulation rate in ewes during oestrous season are well known. In this study, we tested the effects of an 8-day lupin supplementation on folliculogenesis during anoestrus applied over the hormonal treatment of ewes. Fourteen anoestrous Merino ewes were assigned into 2 groups (n = 7 for each) in which the control group was fed a basal diet and the experimental group was supplemented with lupin groats (500 g per head per day). Both groups were treated intramuscularly with 12.5 µg/head lecirelinum (luteinising hormone releasing hormone super analogue) on the first day of supplementation and 5 days later with 37.5 µg/head cloprostenolum D (prostaglandin $F_2\alpha$ analogue). Serum sexual steroid and insulin-like growth factor I (IGF-I) concentrations were assessed using radioimmunoassay and enzyme-linked immunosorbent assay methods, respectively. Folliculogenesis was evaluated using a light microscope. Lupin feeding did not affect progesterone or IGF-I, but it significantly decreased the concentration of oestradiol-17β. Lupin significantly increased the number of follicles of 3–5 mm in diameter and the total rate of atresia. The follicles mainly collapsed and underwent late atresia. The size of healthy and collapsed follicles increased in the lupin-fed group. These results indicated that 8-day lupin supplementation affected follicular development and atresia through an IGF-I/oestradiol feedback system.

Key words: Lupin feeding, ovarian follicle, atresia, oestradiol-17β, insulin-like growth factor I

1. Introduction

Lupin grain is a good source of protein (1) used for livestock feeding of ruminants, pigs, and poultry (2). It is low in fat and starch and high in nonstarch polysaccharides (3). Lupin beneficially influences satiety and energy balance in humans (4) and glycaemic control in rats (5), and it improves blood lipids in pigs (6) and rats (7). High protein nutrition has been widely discussed as a major factor affecting reproduction in cyclic sheep (8–10), but only a few studies clarify its effects as fed in the anoestrous period (11). The effects of lupin supplementation can increase ovulation rate in ewes (8,11,12) through the increase of follicle-stimulating hormone (FSH) concentrations and decreased concentrations of ovarian steroids (13). It was determined that feeding with lupin for 4 to 6 days can increase the ovulation rate in cyclic ewes (9,14). When applied during the luteal phase of the oestrous cycle, it suppresses oestradiol secretion, stimulates the growth of follicles, and enhances the number and quality of ovulations (9–11,15).

The main effect of lupin feeding on folliculogenesis is associated with the action of metabolic hormones such as insulin, insulin-like growth factor I (IGF-I) (16), and growth hormone (17), which control the growth of follicles (11) and mediate the action of key reproductive hormones produced and released in the hypothalamic–pituitary–ovarian axis (15). Ovarian steroids can also modulate metabolic hormones in positive and negative feedback loops (18). IGF-I has direct effects at all levels of the hypothalamic–pituitary–ovarian axis (19) and paracrine effects that participate in folliculogenesis, follicular steroidogenesis (18), and control of ovulation (20). Higher concentrations of insulin and IGF-I suppressed the production of follicular oestradiol, thus decreasing negative feedback of the ovary to FSH and limiting reduced responsiveness of the follicles to luteinising hormone (LH),

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resulting in an increase of follicles saved for ovulation (15). Follicles that do not produce enough steroid hormones, IGF-I, and FSH receptors are not able to respond to low amounts of FSH to continue development and they are subjected to atresia (21,22). Follicular atresia is determined by a dynamic balance among cell division, differentiation, and death (21). The most important attribute of atresia is the activation of apoptosis in the oocyte and granulosa cells (17,21). Follicles undergoing atresia are deficient in granulosa cells (17). Several types of ovarian follicular atresia are characterised by specific signs well described in cows (23) but poorly described in ewes (24).

While the effects and mechanisms of ovulation rate increase in ewes momentarily fed on lupin grain during the oestrous season are well discussed, the present study was designed to verify these effects when fed in the anoestrous period over the hormonal treatment of ewes. The aims of this study were: 1) to test whether an 8-day lupin supplementation, applied over the hormonal treatment of ewes, affected follicular development and ovulation rate through decreased oestradiol-17β and increased IGF-I, and 2) to ascertain how follicular atresia was affected by the lupin supplementation.

2. Materials and methods

2.1. Animals

The experiment was conducted under standard conditions at the Experimental Station of the University of Veterinary Medicine and Pharmacy in Košice, Slovakia, during the sheep anoestrous period (May to June) under standard conditions. Fourteen Merino ewes of age 4–6 years were delivered from a nearby sheep farm (about 1 h of transport) and stabled in pens with the possibility of pasture. Water was offered to ewes ad libitum. All procedures were approved by the Ethical Committee of the State Veterinary and Food Administration of the Slovak Republic (Approval No. 2371/08-221).

2.2. Experimental design

Ewes were divided into 2 groups; the diet of the control group (C; n = 7) consisted of trefoil-grass silage and hay, while the diet of the experimental group (L; n = 7) was supplemented with lupin groats (*Lupinus angustifolius*, var. Sonet; 500 g per head per day). Sheep were fed lupin once a day at about 0500 hours. A schematic representation of the experimental design is shown in Figure 1. Ewes of both groups were induced to ovulate with an intramuscular injection of lecirelinum (luteinising hormone releasing hormone [LHRH] super analogue; 12.5 μg/head; Supergestran inj. a.u.v. Nordic Pharma, Jesenice, Czech Republic) on day 0 relative to lupin supplementation to start the release of endogenous LH from adenohypophysis and were also injected on day 5 with prostaglandin analogue (PG analogue; cloprostenolum D 37.5 μg/head; Remophan inj. a.u.v. Bioveta, Ivanovice na Hané, Czech Republic) to cause luteolysis. Ewes of the experimental group were fed lupin for 8 days, from day 0 to day 8.

2.3. Blood collection

Blood was collected routinely from the jugular vein on days –4, 0, 3, 5, 8, and 10 of the experiment for hormonal and IGF-I profiles. Samples were centrifuged for 15 min at 200 × *g* after coagulation at room temperature (18 to 22 °C). Blood serum was stored at –20 °C until assayed.

2.4. Hormone assay

The concentration of progesterone in the blood serum was assessed using a radioimmunoassay (RIA) method (Architect Progesterone, Abbott Ireland Diagnostics Division, Lisnamuck, Longford, Ireland). Samples were assessed in duplicate. The analytical sensitivity (limit of detection) of progesterone was ≤0.1 ng/mL and the intraassay and interassay coefficients of variation were ≤7% and ≤10%, respectively. Results are expressed in ng/mL.

The concentration of oestradiol-17β in the blood serum was assessed using a RIA method (E2-RIA-CT Kit KIP0629; DIAsource ImmunoAssays S.A., Nivelles, Bel-

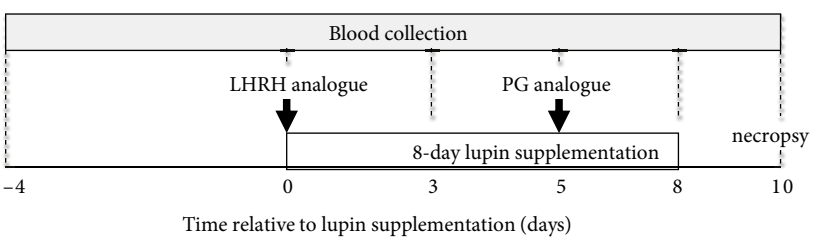

Figure 1. Schematic representation of the experimental design. Day 0 is the beginning of lupin supplementation, which lasted 8 days. Anoestrous ewes fed and not fed on lupin were treated for oestrous induction with 12.5 μg/head of LHRH intramuscular injection on day 0 and then with 37.5 μg/head of prostaglandin analogue on day 5 of the experiment to synchronise them. Blood was collected on days –4, 0, 3, 5, 8, and 10 of the experiment (dotted lines). On day 10, necropsy was carried out.

gium). The analytical sensitivity was 2 pg/mL and the intraassay and interassay coefficients of variation were ≤5.9% and ≤10.1%, respectively. Results are expressed in pg/mL.

The concentration of IGF-I in blood serum was assessed using an enzyme-linked immunosorbent assay (ELISA) method (IGF-I 600 ELISA Kit EIA-4140; DRG Instruments GmbH, Marburg, Germany). Samples were assayed in triplicate. Absorbance was measured in the microplate by the ELISA reader at a wavelength of 450 nm. The analytical sensitivity was 1.292 ng/mL and the intraassay and interassay coefficients of variation were ≤4.72% and ≤7.22%, respectively. Results are expressed in ng/mL.

2.5. Necropsy and macroscopic analysis

On the last day of the experiment (day 10), the ewes were euthanised by intravenous application of T61 inj. a.u.v. (4–6 mL/head; Intervet International B.V., Boxmeer, the Netherlands) for collection of ovaries. The animals were deprived of food for 12 to 18 h before necropsy. Ovaries with oviducts were carefully pulled into an abdominal incision and cut for other processing. Oviducts and ovaries were weighed separately with a sensitivity of 0.01 g. The ovaries were measured (length, width, and thickness for volume calculation) with a manual Empire Vernier calliper (Germany). The ovaries were then cut into halves. One half of each ovary was used for preparing follicular fragments for in vitro experiment (data not included). The other half of each ovary was used for histological analysis.

2.6. Histological analyses and image processing

The ovary halves were processed using standard histological methods as described previously (24). Sections of ovarian tissues were stained with Mayer haematoxylin and eosin (25). The stained sections were fixed in Canadian balsam.

The ovarian sections were observed using PC System for Image Processing LUCIA-G version 4.71 connected to a PAL GKB CCD camera CC-8603 and ZEISS Axiolab light microscope (Carl Zeiss Co., Jena, Germany). Every 20th section was evaluated. All antral follicles were counted and classified into 3 categories according to their diameter: follicles of <1 mm, 1 to <3 mm, and 3–5 mm. There was only 1 follicle larger than 5 mm found in the sections. The follicular diameter was calculated as a mean of 2 perpendicular diameters. Each antral follicle was evaluated as healthy or atretic according to criteria described by Marion et al. (23); atretic follicles were evaluated with respect to the type of atresia (early atresia; definite atresia – collapsing, contracting, or cystic; and late atresia). The thicknesses of the granulosa and thecal layers in healthy, early atretic, contracting, and collapsing atretic follicles were calculated as means from 5 measurements of a particular layer perpendicular to the basal membrane of the follicle. Single layers in the late atretic follicles appeared mixed and were not distinguishable; therefore, these follicles were excluded from such measurements. Follicles with cystic atresia were excluded, too, in instances of a very thin thecal layer (<30 μm) and granulosa reduced to a single row connected into a chain.

2.7. Statistical analyses

The 2 groups of ewes fed and not fed lupin consisted of 7 animals each (n = 7). Variances in hormonal concentrations between the groups were assessed by repeated measures ANOVA with Tukey's post hoc analysis (GraphPad Prism 3.0 for Windows, GraphPad Software, San Diego, CA, USA). The numbers of follicles, the weights and volumes of the ovaries, and the weights of the oviducts were calculated for each group of animals (n = 7). The lupin-supplemented group was compared to the control group using a paired t-test. The mean sizes of ovarian follicles and the mean thicknesses of granulosa and theca interna of healthy and atretic follicles in lupin-fed ewes were compared to those of control ones using an unpaired t-test. The distribution (in percentage) of healthy and atretic follicles was examined using a chi-square test. All data are means with standard error of the mean (SEM). Differences from controls were considered to be significant at levels of $P < 0.05$ and $P < 0.01$.

3. Results

3.1. Progesterone, oestradiol, and IGF-I concentrations

The serum concentrations of progesterone, oestradiol-17β, and IGF-I in Merino ewes fed and not fed on lupin groats for 8 days are shown in Figure 2. In the control ewes, the concentration of oestradiol-17β was decreased ($P < 0.05$) on day 3 relative to day –4. There was no effect of lupin feeding on progesterone, oestradiol-17β, and IGF-I concentrations, although oestradiol-17β remained relatively greater during lupin feeding when it was depressed in the control ewes.

3.2. Macroscopic analysis of ovaries and oviducts

The volumes of ovaries and weights of ovaries and oviducts in the control and lupin-fed ewes are shown in Table 1. The volume of ovaries was greater ($P < 0.05$) in the lupin-fed ewes than in the controls, while the lupin supplementation did not affect the weight of ovaries and oviducts.

3.3. Histological analysis of follicles

The numbers and diameters of antral follicles of the 3 diameter classes in Merino ewes fed and not fed on lupin groats for 8 days are shown in Table 2. Lupin supplementation significantly increased the number of follicles of 3–5 mm in diameter ($P = 0.0167$), the total mean size of follicles ($P = 0.0015$), the size of follicles <1 mm in diameter ($P = 0.0255$), and the size of follicles of 1 to <3 mm in diameter ($P = 0.0134$). There was only 1 antral follicle of 8 mm in diameter, and it was observed in the ovary of 1 lupin-fed ewe.

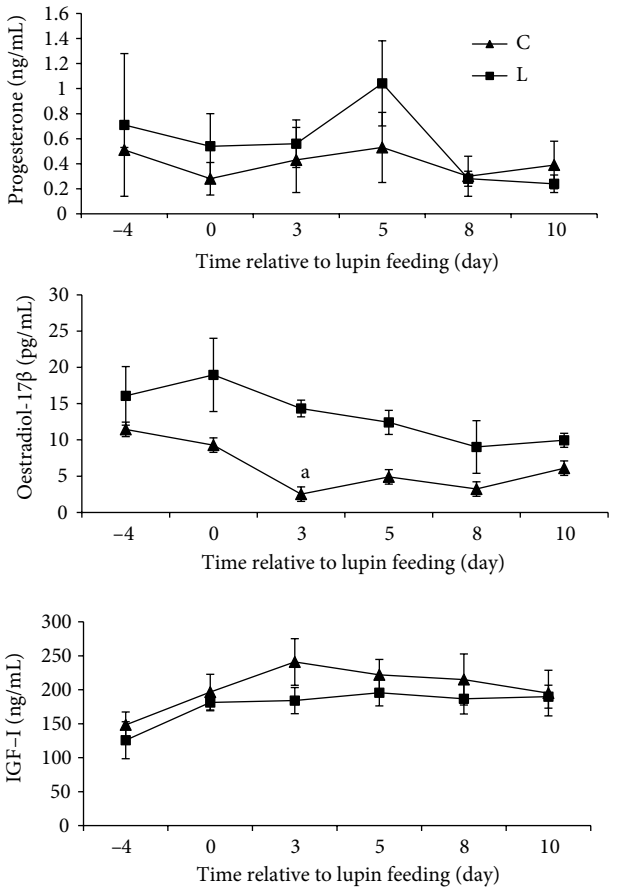

Figure 2. Serum concentrations of progesterone, oestradiol-17β, and IGF-I in Merino ewes supplemented (L) or not (C) with lupin groats for 8 days. Different letters indicate significantly different mean values at $^{a}P < 0.05$ for control group on day 3 compared to control group on day –4.

The mean numbers of healthy and atretic follicles in the ovaries of Merino ewes fed and not fed on lupin groats are shown in Table 3. Lupin feeding nearly doubled (P = 0.0203) the total number of atretic follicles and the number of atretic follicles of 1 to <3 mm in diameter (P = 0.0401).

The mean numbers and diameters of several types of healthy and atretic follicles are shown in Table 4. Lupin supplementation affected the size of healthy follicles, which were larger (P = 0.0185) than those in the control ewes. Collapse and late atresia occurred in the lupin-fed group more frequently (P = 0.0208 and P = 0.0334, respectively) than in the control group, and the measured diameters of the collapsing atretic follicles were larger (P = 0.0078) in the lupin-fed group than the control ewes. Collapsing atresia appeared with the typical folding of both granulosa and theca interna; however, these layers were not seriously degenerated and the basal membrane was not broken.

Lupin supplementation did not affect the thickness of the granulosa and theca interna of follicles; however, the stratum granulosum of contracting atretic follicles was reduced (P = 0.0094; Table 5).

4. Discussion

In the present study, about double the number of follicles of 1 to <3 mm in diameter underwent atresia, triple the number of follicles underwent a collapsing type of atresia, and about double the number of late atresia incidences were found in ewes fed lupin. Most follicles in the terminal stages of atresia had small diameters (<1 mm); therefore, these follicles could increase the number of follicles of <1 mm in diameter. In this study, most of the collapsing follicles with typical folding were not seriously degenerated and the basal membrane, granulosa cells, and oocytes seemed to be healthy, which is partially in agreement with observations of the study of Marion et al. (23), except for the basal membrane, which should be broken and missing in some areas. We suggest that such follicles could collapse in association with the growth of a new cohort of follicles.

The volume of ovaries was increased by lupin supplementation and may result from a higher number of large follicles and increased diameters of smaller follicles. The main influence of lupin feeding was on follicles of 3–5 mm in diameter, the numbers of which were approximately doubled, and on the sizes of follicles of <1 mm and 1 to <3 mm

Table 1. Mean ± SE volumes and weights of the ovaries and weights of the oviducts in ewes fed (n = 7)$^{+}$ and not fed (n = 7)$^{+}$ on lupin groats for 8 days*.

Treatment	None (control)	Lupin supplement
Ovary volume (cm^3)	1.36 ± 0.10	1.84 ± 0.15^{a}
Ovary weight (g)	1.32 ± 0.11	1.28 ± 0.08
Oviduct weight (g)	1.10 ± 0.03	1.13 ± 0.04

*Different superscript letters in a row indicate significantly different mean values at $^{a}P = 0.0343$ for lupin-supplemented group compared to control group.
$^{+}$Total number of ewes.

Table 2. Numbers and diameters (mean ± SE) of surface ovarian follicles in the 3 diameter classes in Merino ewes fed (n = 7) and not fed (n = 7) on lupin groats for 8 days*.

Treatment	None (control)		Lupin supplement	
Follicles	Number+	Size (mm)	Number+	Size (mm)
Total	20.71 ± 4.39	1.06 ± 0.06 (n = 146)#	32.71 ± 4.87	1.35 ± 0.06[b] (n = 206)#
<1 mm	11.14 ± 2.36	0.59 ± 0.03 (n = 76)	13.71 ± 2.28	0.68 ± 0.02[c] (n = 86)
1 to <3 mm	8.43 ± 2.30	1.35 ± 0.04 (n = 62)	16.14 ± 2.21	1.50 ± 0.05[d] (n = 102)
3–5 mm	1.14 ± 0.46	3.41 ± 0.15 (n = 8)	2.86 ± 0.63[a]	3.65 ± 0.10 (n = 18)

*Different superscript letters in a row indicate significantly different mean values at [a]P = 0.0167, [b]P = 0.0015, [c]P = 0.0255, and [d]P = 0.0134 for lupin-supplemented group compared to control group. +Total number of follicles per ewe.
#Total number of values of follicle diameters per follicle category.

Table 3. Numbers of healthy and atretic follicles of the 3 follicular size classes in ovaries in Merino ewes fed (n = 7) and not fed on lupin (n = 7)*.

Treatment		None (control)		Lupin supplement	
Follicles		Mean ± SE+	%	Mean ± SE+	%
Total	Healthy	6.00 ± 2.10	29	6.29 ±1.25	19
	Atretic	14.43 ± 2.55	71	27.43 ± 4.14[a]	81
<1 mm	Healthy	3.86 ± 1.30	36	2.86 ± 0.77	21
	Atretic	6.86 ± 1.50	64	11.00 ± 2.06	79
1 to <3 mm	Healthy	1.57 ± 0.81	19	2.43 ± 0.61	16
	Atretic	6.71 ± 1.72	81	13.00 ± 1.85[b]	84
3–5 mm	Healthy	0.29 ± 0.18	25	0.71 ± 0.42	27
	Atretic	0.86 ± 0.40	75	1.86 ±0.60	73

*Different superscript letters in a row indicate significantly different mean values at [a]P = 0.0203 and [b]P = 0.0401 for lupin-supplemented group compared to control group. +Total number of follicles per ewe.

Table 4. Numbers and diameters of healthy follicles and follicles in various stages of atresia in anoestrous Merino ewes fed on lupin (n = 7) compared with controls (n = 7)*.

Treatment	None (control)		Lupin supplement	
Follicles	Number+	Size (mm)	Number+	Size (mm)
Healthy	6.00 ± 2.10	0.89 ± 0.09 (n = 42)#	6.29 ± 1.25	1.31 ± 0.16[a] (n = 36)#
Early atresia	5.14 ± 1.18	1.35 ± 0.16 (n = 42)	5.14 ± 0.55	1.52 ± 0.18 (n = 37)
Collapsing atresia	2.29 ± 0.75	1.07 ± 0.07 (n = 18)	9.00 ± 2.08[b]	1.66 ± 0.12[d] (n = 51)
Contracting atresia	2.43 ± 0.37	1.31 ± 0.16 (n = 18)	2.00 ± 0.72	1.64 ± 0.23 (n = 14)
Cystic atresia	1.00 ± 0.00	1.57 ± 0.00 (n = 1)	1.00 ± 0.00	1.53 ± 0.00 (n = 1)
Late atresia	4.43 ± 1.11	0.98 ± 0.14 (n = 31)	9.71 ± 2.53[c]	0.93 ± 0.08 (n = 54)

*Different superscript letters in a row indicate significantly different mean values at [a]P = 0.0185, [b]P = 0.0208, [c]P = 0.0334, and [d]P = 0.0078 for lupin-supplemented group compared to control group. +Total number of follicles per ewe.
#Total number of values of follicle diameters per follicle category.

Table 5. Thicknesses of the stratum granulosum (SG) and theca interna (TI) of healthy and atretic follicles on ovaries of ewes fed on lupin, compared with controls.

Treatment	None (control)		Lupin supplement	
Follicles	Layer of follicular wall			
	SG (μm)	TI (μm)	SG (μm)	TI (μm)
Healthy	53.26 ± 5.36 (n = 41)[#]	62.67 ± 5.79 (n = 41)[#]	60.59 ± 8.81 (n = 19)[#]	62.53 ± 5.68 (n = 19)[#]
Early atresia	53.01 ± 7.99 (n = 34)	62.72 ± 7.70 (n = 33)	46.26 ± 6.71 (n = 33)	52.70 ± 5.80 (n = 35)
Contracting atresia	50.57 ± 5.54 (n = 9)	101.9 ± 13.52 (n = 16)	29.60 ± 4.72[a] (n = 12)	68.12 ± 9.66 (n = 13)
Collapsing atresia	99.86 ± 22.84 (n = 14)	95.91 ± 15.88 (n = 16)	73.27 ± 6.13 (n = 39)	82.41 ± 5.25 (n = 41)

Different superscript letters in a row indicate significantly different mean values at [a]P = 0.0094 for lupin-supplemented group compared to control group. [#]Total number of follicles measured for layer depth.

in diameter, which were significantly increased; however, the percentage of healthy follicles did not change. Thus, this change results from greater recruitment beyond the 2-mm stage independent of gonadotropin requirements (26). A single antral follicle of 8 mm in diameter was observed only in the ovary of 1 lupin-fed ewe, which is similar to the observation of Muñoz-Gutiérrez et al. (11), who showed that follicles of larger than 6 mm were seen only in lupin-supplemented ewes.

Lupin supplementation in hormonally treated ewes during anoestrus increased IGF-I concentrations at the beginning of feeding and these stayed nearly at the same level over the whole period of supplementation; however, they were similar to the control ewes, as shown in another study (8). The concentration of oestradiol-17β was suppressed in the control ewes on day 3 relative to day –4, but lupin did not affect this hormone concentration in this study, which is consistent with the observations of Somchit et al. (14) after an intravaginal progestagen sponge treatment, but contrary to the studies of Viñoles et al. (27), who observed increased concentrations of oestradiol using a different experimental model. It can be suggested that oestradiol is affected by IGF-I and that these hormones are reciprocally modulated, as observed in ewes in oestrous season (15,18,20). The actions of ovarian steroids and metabolic hormones can modulate the effects of each other in positive and negative feedback loops, resulting in a higher number of follicles saved for ovulation.

These mechanisms could be based on the regulation of IGF-I by binding proteins produced at the follicle level (16) and IGF-I receptors in a membrane (28). Lower numbers of these receptors reduced IGF-I-stimulated steroidogenesis in the follicle, leading to reduced secretion of oestradiol (16) and transient increases in FSH secretion (29) and resulting in a selection of additional dominant follicles (16). Lupin supplementation increased the concentration of insulin-like growth factor-binding protein 2 (IGFBP-2) in follicles of 1.5–2.5 mm in diameter and resulted in increased atresia in such follicles (16), which can explain the significantly greater number of atretic medium follicles in this study. IGFBP-2 locally sequesters IGF and reduces its ability to stimulate steroidogenesis in the follicle (20). Oestradiol secretion could also be decreased by a low concentration of a substrate for oestradiol production, cholesterol, which was attenuated by lupin (30).

In conclusion, the effect of 8-day lupin supplementation on follicular development during the anoestrous period of ewes over the hormonal treatment was mediated by decreased concentrations of oestradiol-17β, which could be suppressed by an increase in peripheral IGF-I concentrations, resulting in a higher number of large follicles. Lupin supplementation increased the size of small-sized, medium-sized, and healthy follicles as well as the number of medium-sized follicles, which underwent mostly collapsing and late atresia. It is suggested that short-term lupin supplementation may affect follicular development and atresia through the IGF system.

Acknowledgements

The authors would like to express their special thanks to Assoc Prof A Sirotkin for comments on the manuscript, Dr J Kaľatová for technical assistance and IGF-I assessment, and RIA Laboratory Košice for steroid hormone analyses. This study was supported by the Research Agency of the Ministry of Education, Science, Research, and Sport of the Slovak Republic (VEGA, Grant No. 1/0860/09 and 1/0613/13) and the Agency for the Promotion of Research and Development of the Slovak Republic (APVV, Grant No. 0854-11).

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