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The effect of dietary energy levels on the sexual puberty of ram lambs

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Abstract

Aim of study: To evaluate the effect of different feeding levels on body weight changes, sexual behavior activities, and spermatogenesis characteristics of testis tissue in immature Iranian breed lambs.

Area of study: University of Tabriz, Iran.

Material and methods: A total of 40 (start of experiment, 4; after 6 months, 12; 9 months, 12; sexual behavior, 12) two-month-old immature ram lambs were divided into three equal groups and were fed for eight months with three different concentrate mixtures formulated using conventional ingredients based on barley grain. Lambs received the same amount of crude protein with three levels of energy. The diet of the low-energy (LE) group had an energy of 10% lower than the control group, which received a diet with optimum energy of 100% according to the NRC. Lambs in the high-energy (HE) group were fed diets with an energy of 10% higher than the optimum energy group. In each group, four lambs were castrated in three steps, including the ages of 2 (start of treatment), 6, and 9 months. Body weight and scrotal circumference were recorded monthly. Sexual behaviors toward estrus ewes were evaluated for 30 min, and the testes were analyzed histologically.

Main results: The lambs in the LE group had lower body weight and smaller scrotal circumference than HE group ($p<0.05$). Sexual behaviors in the HE group occurred at an earlier age, such as mount and mounts with ejaculation, as the external presentations of puberty ($p<0.05$).

Research highlights: Underfeeding of pre-pubertal animals delayed puberty, while high-plane feeding advanced puberty.

Additional key words: nutrition; postnatal feeding; testis; spermatogenesis.

Abbreviations used: GEH (germinal epithelium height); GnRH (gonadotropin-releasing hormone); HE (high energy); LE (low energy); RI (repopulation index); SPI (spermatogenesis index); STD (seminiferous tubules diameter); TDI (tubular differentiation index).

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Introduction

Photoperiod, metabolism and nutrition impinge upon the genetic blueprint and affect function of gonadotropin-releasing hormone (GnRH) secretory system in mature Merino rams. They have been reviewed by Hötzel *et al*. (1998); Mattos *et al*. (2000) or Martin *et al*. (2010; 2011). It has been shown that changing the nutrition alters the total mass of testicular tissue, hypothalamo-pituitary axis and gonadotropin levels, semen quantity and quality in mature

rams (Bielli *et al*., 2001; Tufarelli *et al*., 2011; Aitken *et al*., 2012; Moghaddam *et al*., 2012; Guan *et al*., 2014). However, the nature of such changes depends on the type and timing of the nutritional challenge. In most mammals, post-puberty is critical because there is an inevitable focus on the function of Sertoli cells that are thought to lose their ability to divide and become terminally differentiated at puberty (Guan & Martin, 2017).

Postnatal development of the testis embraces a period of time in which both the somatic and germ cells undergo proliferation and differentiation that will result in the first round of spermatogenesis and set a framework for the future constant sperm production (Oatley *et al*., 2005). Sertoli cells are only formed during specific periods of fetal and postnatal development in sheep and sperm production is directly related to their number. Anything that affects the testicular complement of Sertoli cells has important implications for adult sperm production (Sharpe *et al*., 2003; Sweeney *et al*., 2007).

Nazari-Zenouz *et al*. (2016) reported the proliferation of Sertoli cells in Ghezel lambs up to 3 months of age, that were stabilized at 5 months of age, as well as that the puberty in Ghezel ram lambs began at 4 months of age with an appearance of primary spermatocyte and Leydig cells and was completed in 8-9 months of age. However, attainment of reproductive capacity at puberty, and its maintenance during adulthood, is the final consequence of a complex series of maturation events that involve proper functional organization at the early stages of development of the hypothalamic network governing GnRH secretion, the driving signal for the control of pituitary gonadotropins (Tena-Sempere, 2010; Çeribaşı *et al*., 2020). In sheep, many neuroendocrine networks responsible for the homeostatic control of essential body functions become organized during the perinatal and postnatal stages of development (Roa *et al*., 2010; Valasi *et al*., 2012).

Although the impact of metabolic programming on other physiological functions tightly coupled to energy homeostasis is yet to be fully characterized, compelling evidence has mounted recently, suggesting that puberty onset and reproductive capacity are also sensitive to nutritional influences during the early stages of development (Castellano *et al*., 2011; Kenny & Byrne, 2018). Concerning the effects of nutrition on gamete production, the responses in the ewe (ovulation rate) have been studied extensively (Kaminski *et al*., 2015), but less attention has been paid to male fertility especially in the pre-puberty period. The most influential underfeeding of pre-pubertal animals delay puberty, while high-plane feeding may advance puberty (Valasi *et al*., 2012).

Most of the ruminant studies addressing the impact of early nutritional programming of puberty have focused on the consequences of undernutrition during pregnancy or lactation (Bielli *et al*., 2001, 2002; Da Silva *et al*., 2001; Robinson *et al*., 2006; Bollwein *et al*., 2017). However, these investigations remain scarce and have yielded contradictory findings. In most cases, gestational subnutrition or postnatal underfeeding have been reported to induce a variable delay in puberty, as monitored by the age of mating and/or first ejaculate, as well as other indices of testis maturation (Robinson *et al*., 2006; Guan & Martin, 2017). Nutrition studies conducted in the pre-pubertal period were carried out as supplementation compounds in a short period of time; it was tried to investigate the effect of nutrition throughout the period from weaning to full maturity. Nevertheless, there is little studied about the impact of a changed diet, including an increase or decrease in diet energy supplements than maintenance on the timing of puberty, body and testis weight, scrotal circumference, and spermatogenesis activity in the lambs. Therefore, the present study aimed to evaluate the effect of different feeding levels on body weight changes, sexual behavior activities, and spermatogenesis characteristics of testis tissue in immature Iranian breed lambs reared under intensive conditions.

Material and methods

Animals and experimental design

Ghezel male lambs (n=40) were randomly selected from the experimental farm of the Agriculture Faculty at University of Tabriz (38°05′N, 46°28′W), East Azerbaijan, Iran. All male lambs were born in winter (December 2018) and were transferred to outdoor pens at the age of two months (Fig. 1). Lambs were stratified by weight and randomly assigned to one of the three groups consisting of 12 animals each. The diets of lambs in the low-energy (LE) group had 10% lower energy than the control group with the optimum energy of 100% as recommended by the NRC (2007) (Tables S1-S4 [suppl]). The energy of the diets fed to the lambs in the high energy (HE) group was 10% higher than the control group. The experimental energy levels were maintained in animals by feeding variable levels of concentrate, including barley grain, wheat bran, and calcium bicarbonate. All the lambs were housed in a particular pen of a sheep shed with windows allowing natural light in until 10 months of age. Lambs were fed their diets twice daily at 8 am and 3 pm for eight months. Every month lambs were weighed and their scrotal circumference was recorded with a tape measure at the point of maximum diameter when the skin of the scrotum was stretched taut around both testes.

Tissue collection and histological processing

Tissue samples were taken from the testes at three ages: 2 months (4 lambs from all groups), 6 months (4 lambs from each group, 4×3), and 9 months (4 lambs from each group, 4×3). Four lambs from each group were kept until showing sexual behavior. Testes were removed surgically.

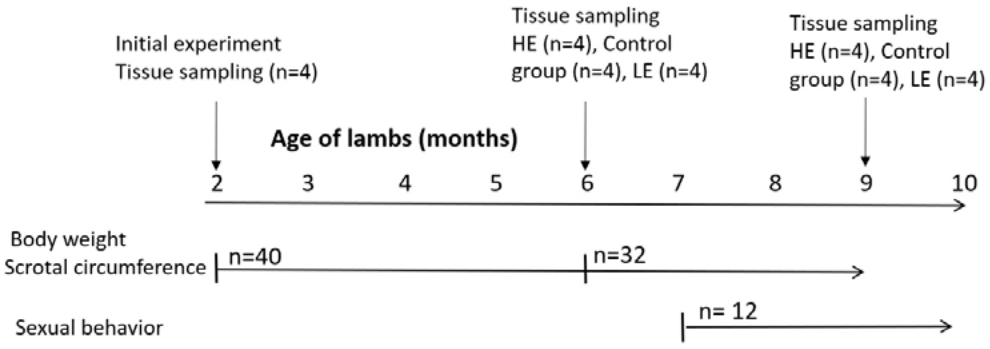

Figure 1. Experimental design. Nutrition groups were initiated 2 months after birth in three groups: control (12 lambs), high-energy diet (HE) (12 lambs), and low-energy diet (LE) (12 lambs) models. Tissue sampling was done in three steps: at 2 (start of experiment; 4 lambs), 6 (12 lambs) and 9 (12 lambs) months of age. In 7 to 10 months of age sexual behavior was recorded. Body and testicular circumference was recorded monthly after treatment to 9 months of age.

All left testes were weighed and the length (longitudinal axis), width (transverse axis), and height (depth axis) of testes were recorded. The testicular volume was calculated by the following formula: $V = (\pi / 6) \times \text{length} \times \text{width} \times \text{height}$, which is the constant generally used in the assumption of ellipsoid testicular shape (Daniel *et al*., 1982; Taskinen *et al*., 1996).

Histological samples were taken from three different regions of the testis, including dorsal (attached to the caput epididymis), middle, and ventral (attached to the caudal epididymis) regions. The microscopic structure of the sex cord or seminiferous tubules was analyzed histologically. These testicular tissue samples were fixed immediately in 10% buffered formalin solution for at least 72 h and were then dehydrated by a graded series of ethanol, cleared by xylene, and impregnated in paraffin wax. The paraffin blocks were cut into serial sections of 5 µm, stained with hematoxylin and eosin, and evaluated by histomorphometric methods.

For the histomorphometric evaluation of testicular tissue, seminiferous tubules diameter (STD), germinal epithelium height (GEH), as well as spermatogenesis indices, including tubular differentiation index (TDI), spermatogenesis index (SPI), and repopulation index (RI) were analyzed in 200 cross-sections of seminiferous tubules (Keyhanmanesh *et al*., 2018; Oghbaei *et al*., 2018). To assess the TDI, seminiferous tubules with more than three layers of differentiated germ cells were obtained. To determine the SPI and RI, the percentage of the seminiferous tubules with normal spermiation and the ratio of active to inactive spermatogonia were calculated, respectively.

Sexual behavior

The first signs of sexual behavior were presented at the age of 7 months. Consequently, the sexual behavior of 12 lambs (four lambs of each group) was evaluated during the age of 7-10 months. The sexual behavior of the lambs was assessed in a 5 m × 5 m pen with an estrus ewe. An ovariectomized ewe was induced to estrus with a hormonal treatment (*i.e*., 5-6 days of intravaginal sponges impregnated with medroxyprogesterone acetate, and 12 h injections of estradiol benzoate) bi-monthly. The incidence of foot-stamping, anogenital sniffing, noising, flehmen response, mount attempts, mounts, and mounts with ejaculations were recorded for a 30-min period (Ramukhithi *et al*., 2017).

Statistical analysis

The data were analyzed using a mixed model by SAS (see Appendix [suppl]). The effects of treatment duration and treatment × time interaction were assessed. Biometrical parameters for three experimental groups were analyzed by the analysis of variance, followed by Duncan's post hoc test to evaluate differences between the groups. For the variables that were not normally distributed, logarithm and square-root transformation were used where appropriate. The probability $p<0.05$ was considered significant.

Results

Body weight, scrotal circumference

Figure 2 shows that the mean age of the three groups, as well as the bodyweight of the lambs at the beginning of the trial did not differ significantly between the three groups. The mean weights of lambs were 20.19±1.2 kg, 20.08±0.8 kg, and 20.8±1.6 kg in the HE, control, and LE groups, respectively. At 9 months of age, the grouping variable showed a clear influence on body weight. There were differences

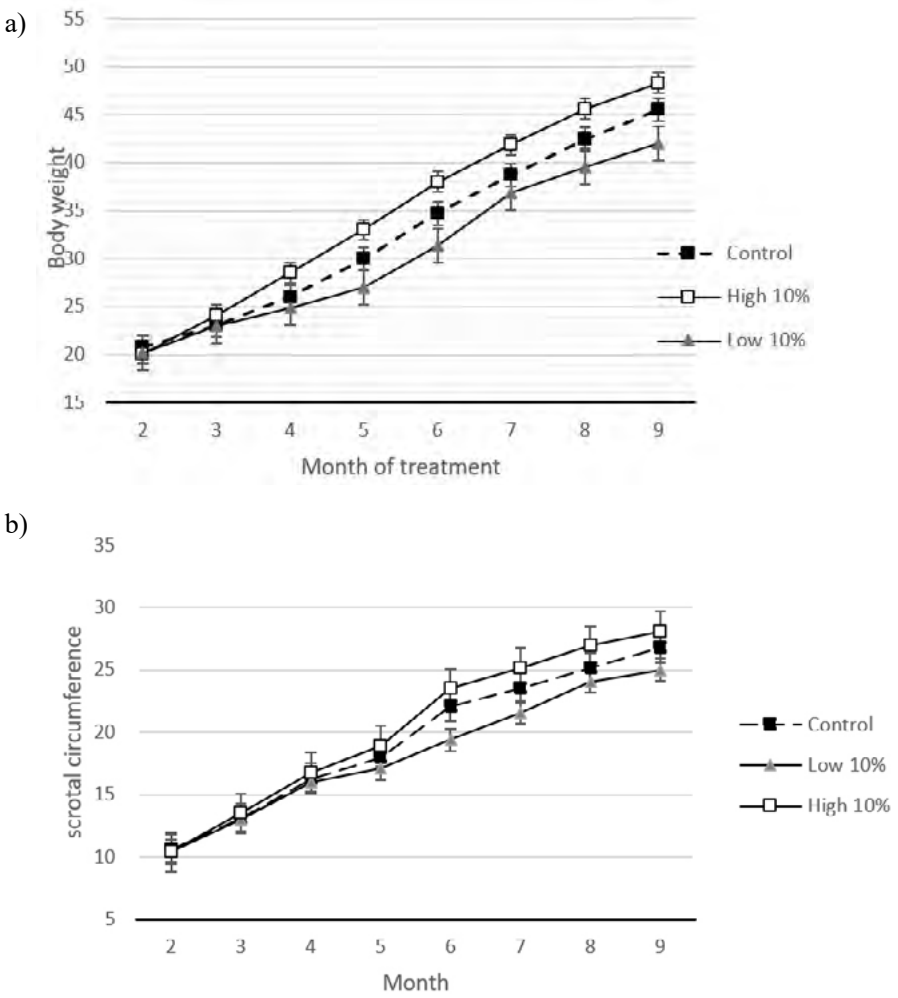

Figure 2. Body weight (a) and scrotal circumference (b) affected by feeding treatment in Ghezel ram lambs. * (p<0.05, Tukey's test) for high and control *vs* low diet.

(p<0.05) of 6.27 kg between the HE and LE dietary treatments, 2.77 kg between the HE and control groups, and 3.5 kg between the control and LE dietary treatments. In terms of scrotal circumference, the dietary treatments had similar effects after an initial lag of 6 months. Scrotal circumference was larger (p<0.05) in lambs fed HE and control diets than in the lambs fed LE diet at all ages of 6-9 months. Therefore, they differed 3.1 cm at the end of the treatment (9 months of age), but differences between the HE and control groups were not significant (Fig. 2).

Testis weight, length, width, height and volume

Our results indicated a significant effect of treatment on left testis weight at the age of 6 and 9 months as the HE and control groups were significantly different from the LE group (p<0.05). As shown in Fig. 3, the left testis in the HE group was heavier than in the control and LE diet groups at 6 months and at 9 months of age but no differences were observed between HE and control group. Furthermore, the length at 6 and 9 months was lower in the LE group than in the HE and control groups (p<0.05). Width at 6 months was lower in the LE group than in the HE and control groups; however, at 9 months no differences between control and LE groups were observed but were lower than in the HE group. The height of left testis at 6 months was lower in the LE group than in the HE and control groups, but there were no differences at 9 months between groups. Specifically, lambs in the LE group had a lower testis volume (p<0.05) than in the HE group. However, the difference between the control and LE groups was not statistically significant at 9 months of age (Table 1).

Histomorphometrical and spermatogenesis indices

In the two-month-old animals (start of the experiment), sex cords were observed as solid cords without any lumen. These solid sex cords contained peripheral primitive Sertoli cells and gonocytes, which were limited with basal lamina and peritubular myoid cells (Fig. 4). The mean STD at this age was 49.33±2.96. The histomorphometric data of semi-

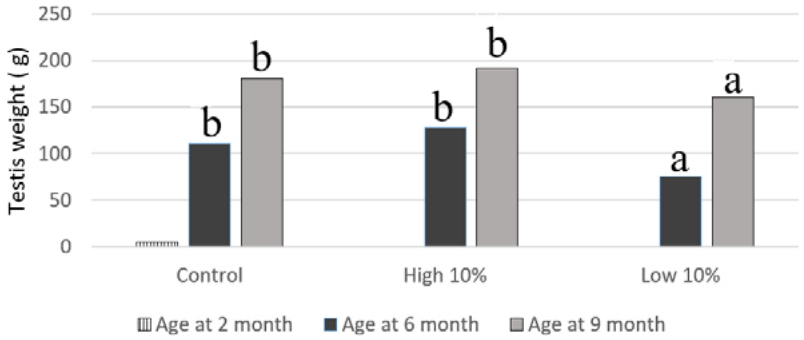

Figure 3. Weight of left testis in lambs fed high diet, control diet or low diet in 2, 6 and 9 months of age. Different letters above the histogram bars indicate significant differences between groups (p<0.05, F test).

niferous tubules at the ages of 6 and 9 months are presented in Table 2. At 6 months of age, the treatments significantly affected the mean of STD and GEH. There was a significant difference between the control and LE groups (p<0.01) and also between the HE and LE diet groups (p<0.01). The mean of STD and GEH in the HE group had no significant difference from the control group. There was a similar status in the lambs of 9 months of age, specifically, lambs in the LE group had a narrower STD (p<0.01) and lower GEH (p<0.01) than the HE or control groups.

Treatment imposed a significant impact on spermatogenesis indices, as indicated by the SPI (p<0.01), RI (p<0.01), and TDI (p<0.01) in the lambs of 6 and 9 months of age (Table 2). The 6-month lambs in the LE group had a lower values (34.33%, 38.66%, and 30.66%) for SPI, RI and TDI, respectively than those of control (71%, 73.33%, and 67%; p<0.01) and HE groups (52.16%, 56.33%, and 52.66%; p<0.01). At the age of 9 months SPI, RI and TDI, respectively, were lower in the LE group 49%, 44%, and 36% (p<0.01), compared to the control (81.66%, 85.33%, and 75.33%; p<0.01) and HE groups (63.69%, 66%, and 59.5%; p<0.01). Late spermatids were absent in the seminiferous tubules of testis in the LE group with only a few early spermatids in some tubules of the LE diet lambs. The SPI, RI, and TDI indices in the lambs fed HE diet were lower than the control group (p<0.01). The spermatogenesis process in the lambs fed HE diet was better than in the lambs fed LE diet, whereas this procedure in the HE diet had less quality, compared to the control group (Fig. 4).

Sexual behavior activities

Sexual behavior activities of unimproved indigenous lambs are illustrated in Table S5 [suppl]. No sexual activities were observed before at the age of 28 weeks. At the age

Table 1. Biometric size of left testis in treated groups.

	Age	High diet		Control diet		Low diet		p-value[3]
		Means[1]	SEM[2]	Means	SEM	Means	SEM	
Length (cm)	2			2.13	0.04			
	6	7.64 ^{b}	0.93	6.94 ^{b}	0.23	6.53 ^{a}	0.63	<0.05
	9	8.14 ^{b}	0.18	8.05 ^{b}	0.24	7.88 ^{a}	0.63	<0.05
Width (cm)	2			1.11	0.03			
	6	4.24 ^{b}	0.40	4.11 ^{b}	0.14	3.86 ^{a}	0.42	<0.05
	9	5.68 ^{b}	0.45	5.23 ^{a}	0.43	5.13 ^{a}	0.26	<0.05
Height (cm)	2			1.26	0.05			
	6	5.21 ^{b}	0.39	5.32 ^{b}	0.28	4.58 ^{a}	0.44	<0.05
	9	5.87	0.20	5.84	0.22	5.81	0.08	
Volume (cm^3)	2			1.59	0.13			
	6	87.82 ^{b}	7.07	80.20 ^{b}	9.09	64.30 ^{a}	9.22	<0.05
	9	141.13 ^{b}	9.50	128.06 ^{a}	10.02	125.56 ^{a}	8.66	<0.05

[1] Means (n=4). [2] SEM: standard error of the mean. [3] $^{a-c:}$ p<0.05 for different letters (within row).

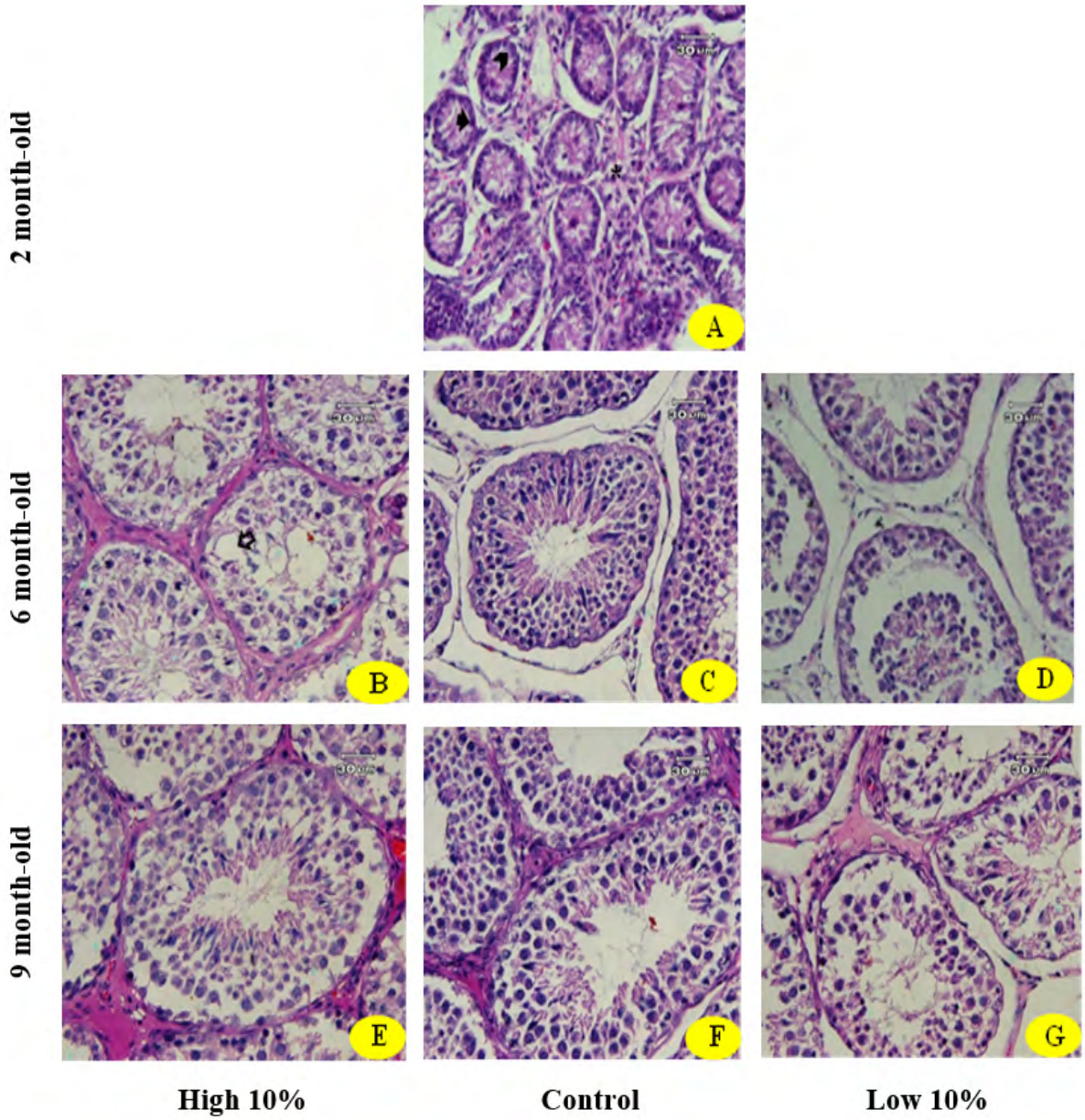

Figure 4. Micrograph of seminiferous tubules in left testis of lambs fed high diet, control diet or low diet in 2 (A), 6 (B, C, D) and 9 (E, F, G) months of age (hematoxylin and eosin, ×400). A) Sex cords are observed without any central lumen. Intertubular spaces are containing mesenchymal cells (*). Each sex cord consists of gonocytes (black arrow) and Sertoli cells (head arrow). B) Seminiferous tubules in lambs fed high diet showing many vacuoles are observed in the germinal epithelium (hollow arrow). C) Seminiferous tubules in control lambs with completed and normal germinal epithelium. D) Seminiferous tubules in lambs fed low diet showing early spermatids shed into the lumen with severe atrophy of germinal epithelium. E) Seminiferous tubules in lambs fed high diet showing complete germinal epithelium with all spermatogenic cells but not well integrated architecture. F) Seminiferous tubules in control lambs showing complete germinal epithelium with full spermatogenesis and spermatozoa in the lumen. G) Seminiferous tubules in lambs fed low diet showing a disrupted and incomplete germinal epithelium.

of 28 weeks, lambs fed the HE and control diets started to show sexual behavior activities with noising behavior. The first mount and mount with ejaculation occurred in lambs of the HE and control groups at the age of 32 weeks. The first mount with real ejaculation in lambs fed LE diet started at the age of 36 weeks with 4 weeks delay, compared to the other two groups. At the age of 38 weeks, the highest proportion of sexual behavior activity was observed in all groups, but it was highest in the control group lambs. There were no significant differences between lambs fed control and HE diets. However, the control and LE groups were significantly different in this regard. Although in the

Table 2. Histomorphometrical and spermatogenesis indices of the left testis in treated groups

Indices [1]	Age (month)	High diet		Control diet		Low diet		*p*-value[4]
		Means[2]	SEM[3]	Means	SEM	Means	SEM	
Histomorphometrical indices								
STD (μm)	2			49.33	2.96			
	6	182.00 ^{b}	10.06	176.00 ^{b}	5.56	145.60 ^{a}	2.96	0.01
	9	233.30 ^{b}	5.78	232.00 ^{b}	7.51	172.30 ^{a}	4.80	0.01
GEH (μm)	6	52.00 ^{b}	3.05	57.00 ^{b}	2.08	38.00 ^{a}	1.73	0.001
	9	62.60 ^{b}	1.45	72.00 ^{c}	2.30	47.30 ^{a}	0.66	0.01
Spermatogenesis indices								
TDI (%)								
	6	52.66 ^{b}	2.02	67.00 ^{c}	2.64	30.66 ^{a}	1.45	0.01
	9	59.50 ^{b}	1.73	75.33 ^{c}	1.45	36.00 ^{a}	1.00	0.01
RI (%)								
	6	56.33 ^{b}	0.88	73.33 ^{c}	2.40	38.66 ^{a}	1.85	0.01
	9	66.00 ^{b}	1.66	85.33 ^{c}	0.66	44.00 ^{a}	1.00	0.001
SPI (%)								
	6	52.16 ^{b}	1.45	71.00 ^{c}	1.52	34.33 ^{a}	2.33	0.01
	9	63.69 ^{b}	2.02	81.66 ^{c}	0.88	49.00 ^{a}	2.08	0.01

[1] STD: Seminiferous tubule diameter. GEH: Germinal epithelium height. TDI: Tubular differentiation index. RI: Repopulation index. SPI: Spermiation index. [2] Means (n=4). [3] SEM: standard error of the mean. [4] $^{a\text{-}c}$: $p<0.05$ for different letters (within row).

early onset of puberty, foot-stamping, anogenital sniffing, noising, flehmen response, mount attempts, mounts, and mounts with ejaculations were more frequent in lambs fed the HE diet, compared to other groups ($p<0.05$), in 40 weeks of age, sexual behavior rate in control lambs was higher than in the other two groups ($p<0.05$).

Discussion

Puberty is the stage of development during which an individual becomes capable of sexual reproduction. This is characterized by the maturation of the genital organs, the development of sexual characteristics, and changes in behavior (Nieto *et al*., 2018; Moghaddam *et al*., 2019). The macroscopical growth of the testis along with the microscopical development of seminiferous tubules and their germinal epithelium is the first typical anatomical signs of the beginning of puberty (Rawlings *et al*., 2008; Kastelic, 2014).

Our results indicated that feeding immature Ghezel lambs with a diet below the requirements for maintenance clearly affects body weight, sexual behavior, and the testicular parameters of lambs leading to reduced scrotal circumference, testis mass, spermatogenesis quality, and delayed puberty.

The results of the present study in the lambs fed by the LE diet are in line with the literature about the unambiguous delay of the puberty age together with decreased spermatogenesis rate and testis weight at puberty in males subjected to postnatal sub-nutrition. The impact of nutritional manipulations on the development of puberty might depend not only on the magnitude and timing of energy restriction but also on the type/features of such challenge. It could be due to the association of food deprivation with distinct covariables (Roa *et al*., 2010; Nieto *et al*., 2018) that have different influences on the onset of puberty. Spermatogenesis in the testicular tissue of underfed lambs was generally disrupted by lower SPI, RI, and TDI indices, narrower seminiferous tubule diameter, and a smaller proportion of germinal epithelium. These observations confirm previous studies and demonstrate the major effects of nutrition on spermatogenic efficiency in sexually mature Merino rams (Hötzel *et al*., 1998; Guan *et al*., 2014; 2016). Furthermore, the atrophy of the seminiferous tubules, the increased interstitial tissue, and tissue edema are visible in underfed lamb rams. Previous short-term studies in Merino rams showed positive relationships between nutrition, indicators of metabolic homeostasis, and puberty in sheep (Martin *et al*., 2010; Swelum *et al*., 2017; Nieto *et al*., 2018). It was concluded that the decline in the diet energy for lambs during the postnatal period had

a negative influence on sexual behaviors and reproductive traits.

Our study revealed that in the control lambs (6 and 9 months of age) the testicular tissue, structure of the seminiferous tubules, all spermatogenetic cell markers, spermatogenesis process, and sexual behaviors were normal. In the control group, lambs started to show puberty at the age of 7.5 months with mounting being the most frequent behavior, followed by licking at the age of 8 months. This is in agreement with previous studies as it has been shown that mounting of does by lambs is a common activity, which can be used to evaluate the puberty of lambs when introduced to does (Nazari-Zenouz *et al*., 2016; Ramukhithi *et al*., 2017). Our microscopical examinations revealed that the spermatogenesis process in the testis of HE lambs did not have a normal quality. Although it was higher than in the LE group, different degrees of vacuolation was observed in the germinal epithelium of seminiferous tubules. This vacuolation may be attributed to degenerative changes in spermatogenic cells, disturbances in Sertoli cells (*e.g.,* fat accumulation), and also the physical loss of embedded germ cells in the germinal epithelium of seminiferous tubules (Creasy *et al*., 2012). However, a definitive diagnosis on whether or not fat vacuoles were replaced in germinal epithelium needs further evaluation by specific histochemical staining. Moreover, some studies mentioned that offering a long-term HE diet during the postnatal period can have appreciable negative effects on the development of testis (Bollwein *et al*., 2017; Kenny & Byrne, 2018). Nevertheless, the sexual behavior in HE lambs occurred earlier than in the other two groups.

In summary, according to our results, a diet with low energy of under 10% of the optimum in the postnatal period of Ghezel ram lambs elevates the age of puberty and reduces spermatogenesis efficiency. These processes are associated with some alterations in the body and testis mass that delay sexual behavior and puberty. On the other hand, our findings revealed that a diet with a high energy level of 10% more than the optimum energy may improve the age of puberty and sexual behavior. However, it may alter the germinal epithelium of seminiferous tubules in the testis and diminish spermatogenesis.

Authors' contributions

Conceptualization: G. Moghaddam, G. Hamidian, F. Nazari-Zonouz.
Data curation: G. Hamidian, F. Nazari-Zonouz.
Formal analysis: G. Hamidian, F. Nazari-Zonouz.
Funding acquisition: G. Moghaddam.
Investigation: G. Moghaddam, Gh. Hamidian, F. Nazari-Zonouz.
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Software: G. Hamidian, F. Nazari-Zonouz.
Supervision: G. Moghaddam, G. Hamidian.
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Visualization: G. Moghaddam, G. Hamidian, F. Nazari-Zonouz.
Writing – original draft: F. Nazari-Zonouz.
Writing – review & editing: G. Moghaddam, G. Hamidian, F. Nazari-Zonouz.

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