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Supplementation with dietary linseed oil during peri-puberty stimulates steroidogenesis and testis development in rams

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ABSTRACT

Omega-3 polyunsaturated fatty acids (ω-3 PUFAs), such as α-linolenic acid (ALA), eicosapentaenoic acid, and docosahexaenoic acid, are involved in male reproductive function. In this study, we investigated the effects of linseed oil (LO) as a source of ALA on the steroidogenesis and changes of testicular histology in rams. Sixteen 3-month old rams during peri-puberty were randomly assigned into two groups. Eight rams were assigned as the control group, and the other received LO (4% dry matter of total diet) as the LO treatment group. After an 81-day feeding trial, the rams were slaughtered and investigated. Results revealed that compared with control group, diet containing LO did not affect body weight (36.87 ± 0.53 kg vs. 37.65 ± 0.64 kg, respectively; $P = 0.361$), average daily gain (227.47 ± 5.82 g vs. 237.95 ± 9.22 g, respectively; $P = 0.353$) and epididymis weight (40.77 ± 4.41 g vs. 45.53 ± 4.01 g, respectively; $P = 0.398$), however, it up-regulated PUFAs metabolism and steroidogenesis-related genes mRNA expression ($P < 0.05$), and increased plasma estradiol concentration (14.88 ± 0.67 pg/mL vs. 19.50 ± 1.27 pg/mL, respectively; $P < 0.05$). Therefore, LO stimulated seminiferous tubule development and increased the number of Sertoli cells (19.17 ± 2.14 vs. 27.2 ± 2.39, respectively; $P < 0.01$), germ-cell layers, as well as testis weight (148.65 ± 22.66 g vs. 249.96 ± 30.63 g, respectively; $P < 0.05$). All these results suggested that LO can improve testis development during peri-puberty by regulating steroidogenesis in rams' testes.

1. Introduction

Omega-3 polyunsaturated fatty acids (ω-3 PUFAs), such as α-linolenic acid (ALA) from linseed oil (LO), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) from fish oil, play important roles in reproductive physiological processes, including the regulation of prostaglandin synthesis and membrane properties [1]. The fatty acid composition and metabolism of spermatozoa, Sertoli and Leydig cells in adult male were studied, and found that the lipid composition of the sperm plasma membrane is a major determinant of the mobility characteristics and viability. PUFAs account for nearly 60% of the phospholipids that bind in the total fatty acid of cells and in sperm [2–4]. In several mammalian species including rodents, docosapentenoic acid is present in large concentrations, whereas in others including human, ram, and bull, the DHA content is relative [2–6].

Omega-3 PUFAs are semi-essential and should be sufficiently included in the diet. Omega-3 PUFAs greatly affect the fluidity of the plasma membrane and thus contribute to sperm structure formation, acrosome reaction, and sperm-oocyte fusion [2]. Testis development and spermatogenesis depend on many gonadal hormones. The ω-3 PUFAs, such as DHA, EPA, and their metabolites, in testis greatly affect the prostaglandin synthesis and steroidogenesis via the direct influence on steroid acute regulator (*StAR*) and cytochrome *P450*, which play critical roles in regulating steroid synthesis [7,8]. Omega-3 PUFAs also activate and stimulate the expression of peroxisome proliferator-activated receptor (*PPARγ*) [9], which regulates the gene transcription involved in lipid and glucose metabolism via binding to the peroxisome proliferator response element [10]. Regulated by the degree of saturation of the

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lipid, sterol regulatory element binding protein 1 (*SREBP1*) is a strong regulator of the activity of fatty acid desaturases (*FADS1* and *FADS2*), PUFAs biosynthesis, and steroidogenesis [11].

LO also contributes to testis development and steroidogenesis. LO contains up to 50% ω-3 PUFAs, especially the ALA that can be converted into EPA and DHA through the alternating steps of elongation and desaturation involving ELOVL and FADS enzymes in mammals [12]. Most published data focused on the dietary fish oil as source of ω-3 PUFAs in improving the fresh or post-thaw semen quality in livestock [3,13–15]. However, the ω-3 PUFAs in the testis have dual effects on spermatogenesis: on the one hand, mammalian spermatozoa are sensitive to lipid peroxidation due to the phospholipid content of sperm membranes with high ω-3 PUFAs sidechains; on the other hand, the ω-3 PUFAs in testis provide some protection against peroxidation via its constituent antioxidants [16,17]. The function of LO on testis development during rams peripuberty is poorly reported. The number of Sertoli cells in testes determines the reproductive capacity of the adult male. Sertoli cells proliferation occurred during the early fetal and neonatal period and particularly the greatest during peri-pubertal stage [18,19]. The early stage is the most important period for testis development in rams. The present study aimed to investigate the effect of dietary LO on steroidogenesis and changes of testis development during peri-pubertal in rams.

2. Materials and methods

2.1. Reagents

Trizol reagent, transScript one-step gDNA removal and cDNA synthesis super-mix kit, and transStart tip green qPCR super-mix were purchased from Transgen Biotech (Beijing, China). Architect 2nd generation testosterone kit, Architect estradiol assay kit, Architect 2nd generation testosterone calibrators and estradiol calibrators were purchased from Abbott (Abbott Park, USA). Hematoxylin and eosin (H&E) staining kit was purchased from Boster Biotech (Wuhan, China). All the other reagents were purchased from Sangon Biotech (Shanghai, China).

2.2. Animals and feeding trial

Chinese Hu sheep is famous for its early sexual maturity and high fecundity with starting estrus and gestation in all seasons of the year. Hu sheep reached puberty at a mean age of 120 days. Yue [14] mentioned that spermatids and spermatozoa are first observed in seminiferous tubules after 80 days, and many mature spermatozoa are found in the epididymis after 120 days. In this study, sixteen Hu rams (average body weight of 22.84 ± 0.26 kg) aged 3 months were housed in individual pens (1 m × 1.5 m) and randomly assigned into two groups. Eight rams were used as the control group, whereas the remaining received LO (4% dry matter of total diet) as the LO treatment group. The diets were prepared to complete diet pellets with 6 mm in diameter. The levels of crude protein and calculated metabolized energy content were similar in the control and LO diet groups (Table 1).

The entire experimental period lasted for 81 days. The parasites were expelled, and disinfection and epidemic prevention were performed during the adaptation period from D0 to D20 to ensure that the rams adapt to the diets and surroundings. The normal commencing trial period was 60 days from D21 to D80, during which all rams were fed thrice a day at 8:00, 14:00, and 19:00 with ad libitum access to fresh water and multi-nutrient blocks. The daily feed offerings and refusals from D21 to D30 and D71 to D80 were recorded to calculate the dry matter intake (DMI). All rams were weighted at the beginning and the end of the 60-day commencing trial prior to feeding.

The trial was performed at the Minqin Zhongtian Sheep Industry Co., Ltd. (Minqin, China) from August to November 2015. This study was conducted in strict accordance with the recommendations from the Guide for the Animal Care and Use Committee of Lanzhou University. No ram was harmed during the feeding trial.

2.3. Sample collection

At the end of the feeding trial, blood samples were collected in heparinized vials through jugular venepuncture. The samples were centrifuged at × 1000 g at 4 °C for 30 min and the blood plasma was stored at −20 °C. Immediately after blood sampling, the rams were humanely slaughtered by a licensed slaughter man in a way of severing the carotid artery, ingular vein, trachea, esophagus, and vagus nerve according to the halal slaughter procedure as outlined in the Chinese Local Standards DB13/T 963–2008 [20] at the Minqin Zhongtian Sheep Industry Co., Ltd. Their body, testes, and epididymis weight without spermatic cord were measured. Testes tissues were rapidly collected from the left testis without tunica albuginea using liquid nitrogen for frozen tissues and 10% formalin for fixed tissues.

2.4. RNA isolation and cDNA synthesis

Total RNA was isolated using Trizol reagent according to the manufacturer's instructions. The RNA quantity and quality was detected using Nano Drop 2000 (Thermo Fisher, Waltham, USA). Total RNA (2.5 μg) was reverse-transcribed to cDNA using TransScript one-step gDNA removal and cDNA synthesis super-mix kit at 42 °C for 15 min following the manufacturer's instructions. The cDNA was diluted 1:10 with nuclease-free water and stored at −80 °C.

2.5. Quantitative real-time PCR analysis

Relative mRNA abundance of luteinizing hormone receptor (*LHR*), follicle stimulating hormone receptor (*FSHR*), androgen-binding protein (*ABP*), *StAR*, aromatase (*P450scc* and *P450arom*),

Table 1
Chemical characteristics of the experimental diets.

Item (% of DM)	Control	LO
Ingredient, % of DM		
Barley straw	26.00	26.00
Malt root	8.00	8.00
Corn	45.25	39.55
Concentrate feed[a]	20.75	22.45
LO	–	4.00
Chemical composition, % of DM		
Metabolizable energy (MJ/kg) (calculated)	12.60	12.60
Crude protein	16.30	16.30
Neutral detergent fibre	35.10	35.10
Calcium	0.65	0.65
Phosphorus	0.34	0.34
Fatty acid composition (% of FAs)		
Total saturates	39.02	25.31
Total MUFAs	25.85	40.77
Total PUFAs	35.12	33.91
ω-3 PUFAs	3.24	6.19
ω-6 PUFAs	31.88	27.02

LO = linseed oil, DM = dry matter, FAs = Fatty acids, MUFAs = Monounsaturated fatty acids, PUFAs = Polyunsaturated fatty acids.

[a] Concentrate feed composed with flax meal, soybean meal, cotton seed meal, salt, powder, vitamin and mineral premix. Concentration of vitamin and mineral per kilogram of DM: 2500 IU vitamin A; 23 IU vitamin E; 0.3 mg selenium; 70 mg ferrum; 41 mg zinc; 8 mg copper.

Table 2
Primers sequences and accession number for all genes transcripts analyzed by quantitative RT-PCR experiments.

Target gene	Sense primer, 5'→3'	Antisense primer, 5'→3'	Accession No.
FSHR	ccacaccaaaagccagtacc	gctcaccttcatgtagctgc	XM_015093783.1
LHR	aatggcggtcctcatcttca	atacagaaacggattggcgc	NM_001278566.1
ABP	aggccttagtgctataatccttg	gtgcaatcatcactgaagacg	XM_012190154.1
P450scc	cagggctccggaaagtttgt	acggtacagttctggaggga	NM_001093789.1
StAR	cccagctgcgtggatttatc	ctctccttcttccagccctc	NM_001009243.1
3β-HSD	gaatcggcatggttctgtcc	ccgtagatgtacatgggcct	XM_012183658.1
P450arom	gcatggcaagctctccttct	caccacgtttctcggcaaaa	NM_001123000.1
FADS2	acccattgagtacggcaaga	gtacaaagggatgagcagcg	XM_015103138.1
ELOVL2	acagacctgctctttccctc	tgtagcctccttcccaactg	XM_015093202.1
SREBP1	atggctttgattctcgtggc	ttttcagtgtccgcaactgg	XM_012151742.2
PPARγ	acggcggcttctacaccgact	tgccgcttgacccactcgt	XM_015093026.1
GAPDH	atggtgaaggtcggagtgaa	acttgccatgggtggaatcat	XM_012166462.1

ABP = Androgen-binding protein; *3β-HSD* = 3β-hydroxysteroid dehydrogenase; *ELOVL2* = Fatty acid chain elongase; *FADS2* = Fatty acid desaturase 2; *FSHR* = Follicle stimulating hormone receptor; *GAPDH* = Glyceraldehyde-3-phosphate dehydrogenase; *LHR* = Luteinizing hormone receptor; *PPARγ* = Peroxisome proliferator-activated receptor; *SREBP1* = Sterol regulatory element binding protein 1; *StAR* = Steroid acute regulator.

3β-hydroxysteroid dehydrogenase (*3β-HSD*), *FADS2*, fatty acid chain elongase (*ELOVL2*), *SREBP1* and *PPARγ* genes were detected using Bio-Rad CFX96 real-time system (Bio-Rad, CA, USA) according to the manufacturer's instructions as follows: preheating at 94 °C for 3 min, followed by 40 cycles of denaturation at 94 °C for 5 s and extension at 60 °C for 30 s. Fluorescence was measured at the end of extension step. At the end of each RT-PCR, dissociation-curves analyses were performed to avoid false-positive signals. The reaction system was performed in a 20-μL final volume containing 10 ng cDNA, 10 μL green qPCR super-mix, 0.4 μL of primers (10 μM), and distilled water up to 20 μL. The gene-specific primers used are shown in Table 2. The relative expression value of the target genes were normalized to the expression levels of glyceraldehyde-3-phosphate dehydrogenase. The mRNA levels were expressed as the relative fold change ($2^{-\Delta\Delta CT}$).

2.6. Testosterone and estradiol levels

Both testosterone and estradiol (E2) in the blood plasma were detected in the First Hospital of Lanzhou University by using an automated chemiluminescent microparticle immunoassay with Architect 2nd generation testosterone kit and Architect estradiol assay kit on Architect i4000SR system (Abbott Park, USA), according to the manufacturer's instructions [21–23]. The limit of quantification for the Architect 2nd generation testosterone assay was 2.30 ng/dL. The intra-assay and inter-assay coefficient of variations (CVs) were both 10% for the samples with testosterone concentration ranging from 1.44 ng/dL to 1000 ng/dL. The analytical sensitivity of the Architect estradiol assay was ≤10 pg/mL. The intra-assay and inter-assay CVs were both ≤7% at low controls (target 45 pg/mL), ≤3% at medium controls (target 190 pg/mL) and ≤2% at high controls (target 600 pg/mL), respectively. The Architect 2nd generation testosterone calibrators and estradiol calibrators were used to generate the calibration curve. The testosterone and estradiol assay used a four-parameter logistic curve fit data reduction method to generate a calibration curve.

2.7. Testicular histology

Testes tissues were immersed in 10% formalin and dehydrated by increasing the concentration of alcohol. The tissues were immersed in xylene, embedded in paraffin, sectioned at 4 μm, stained with H&E, and evaluated by light microscopy. Image representatives of the typical histological profile in control and LO groups were captured at 100 and 400 times magnification using the Scopeimage 9.0 software (Ningbo yongxin, Ningbo, China). Images with a 100 times magnification were used to measure the diameter of the seminiferous tubules, and with a 400 times magnification were used to count the number of Sertoli cells per seminiferous tubule cross-section based on their shape and location. For each testis, 6 to 10 seminiferous tubules were randomly selected, Sertoli cells number and two diameters (long and short) of seminiferous tubules were measured as Kazemi S et al. described [24].

2.8. Statistical analyses

All experimental data were presented as means ± SEM. Data were evaluated using SPSS program, version 13.0 (SPSS, Chicago, USA). Normality of distribution were analyzed by one-sample Kolmogorov-Smirnov test. The homogeneity of variances were analyzed by Leven's test. Significant differences were analyzed by Student's *t*-test (equal variances) and Welch's *t*-test (unequal variances), P values < 0.05 were considered statistically significant.

3. Results

All the parameters detected in this study were normally distributed. The differences between two groups were analyzed by Student's *t*-test, except for DMI from D21 to D30 (unequal variances, P < 0.05) using Welch's *t*-test. Results indicated that compared with control group, LO did not affect DMI from D21 to D30, D71 to D80, body weight, average daily gain and epididymis weight, after the 60-day commencing trial. However, the testes weights were significantly higher in the LO group than in the control group (P < 0.05) (Table 3).

The differences of PUFAs metabolism-related and steroidogenesis-related genes expression between two groups were analyzed using Student's *t*-test (*StAR*, *ELOVL2*, *FADS2* and *LHR* with equal variances, P > 0.05) and Welch's *t*-test (*SREBP1*, *PPARγ*, *FSHR*, *ABP*, *P450scc* and *3β-HSD* with unequal variances, P < 0.05). The LO treatment up-regulated the *SREBP1*, *PPARγ*, *FSHR*, *ABP*, *StAR*,

Table 3
Comparison of growth performance for finishing rams with control and linseed oil (LO) diet.

Items	Control(n = 8)	LO (n = 8)	P-value
DMI $_{(D21\ to\ D30)}$, kg/day	1.27 ± 0.08	1.32 ± 0.06	0.577
DMI $_{(D71\ to\ D80)}$, kg/day	1.73 ± 0.06	1.63 ± 0.05	0.231
Body weight, kg	36.87 ± 0.53	37.65 ± 0.64	0.361
ADG, g/day	227.47 ± 5.82	237.95 ± 9.22	0.353
Epididymis weight, g	40.77 ± 4.41	45.53 ± 4.01	0.398
Testes weight, g	148.65 ± 22.66	249.96 ± 30.63	<0.05

DMI = Dry matter intake; ADG = average daily gain.

P450scc, and *P450arom* mRNA expression levels ($P < 0.05$) and stimulated the *ELOVL2* ($P = 0.092$) except for *FADS2* ($P = 0.439$), *LHR* ($P = 0.327$) and *3β-HSD* ($P = 0.667$) genes expression (Fig. 1), respectively.

The results of Student's *t*-test suggested that the average plasma testosterone concentration in LO group (0.67 ± 0.09 ng/mL) was not different ($P = 0.382$) from that in control group (0.56 ± 0.08 ng/mL) (Fig. 2A), however, the plasma E2 concentration in LO group (19.50 ± 1.27 pg/mL) was significant higher ($P < 0.05$) than that in the control group (14.88 ± 0.67 pg/mL) (Fig. 2B).

The testicular histology was examined in this study. The LO induced gross morphological changes in the testes, compact and regular arrangement of germ cells was observed in their seminiferous epithelium with five or more layers of different stages of germ cells. Numerous elongated spermatozoids were also observed in the luminal of seminiferous tubules (Fig. 3A and C). In the control group (Fig. 3B and D), most seminiferous tubules showed vacuolation, and the number of germ cells were significantly less. The number of Sertoli cell per seminiferous tubule cross-section was also increased after LO treated compared with control group (27.2 ± 2.39 *vs.* 19.17 ± 2.14, respectively; $P < 0.01$). The diameter of the seminiferous tubule ranged from 123.79 μm to 181.65 μm in the LO treatment group and from 95.05 μm to 145.53 μm in the control group. The mean diameter of the seminiferous tubules in the LO and control groups were 149.49 ± 4.92 μm and 121.53 ± 3.35 μm, respectively, and the difference was significant ($P < 0.01$).

4. Discussion

In this study, LO in diet did not significantly affect average daily gain after trail due to there is no difference of the levels of crude protein, calculated metabolized energy, digestibility of DM or organic matter (*data not shown*) between the controls and LO groups. The lipid content strongly affects the testicular histology and physiology. Essential fatty acid alterations in fatty acid metabolism are associated with testicular spermatogenesis in animals. In bulls, the reduced sperm quality with age is correlated with the reduced activity of FADS activity, which are necessary for PUFAs synthesis [8]. In a previous report, a DHA-rich diet stimulated the *FADS1* and *FADS2* mRNA expressions in the liver of rams [25]. In the current study, LO up-regulated the *SREBP1*, *PPARγ*, and *ELOVL2* expressions.

Spermatogenesis and steroidogenesis in the testes are regulated by complex endocrine mechanisms. PUFAs also modulate steroidogenesis as indicated in previous reports, which stated that supplemental fat increases the LH, progesterone, and estradiol

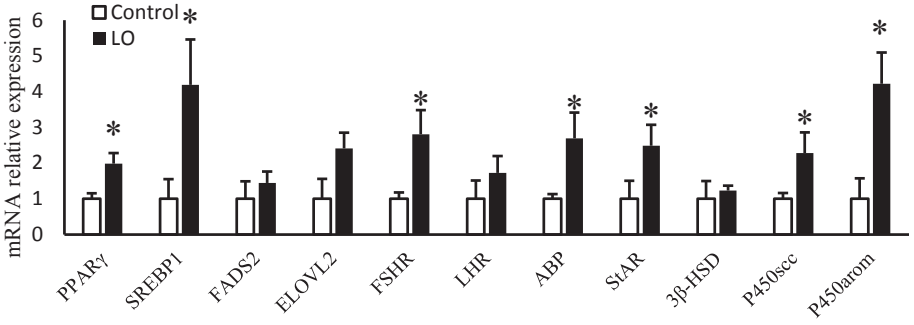

Fig. 1. Effect of dietary linseed oil (LO) on the mRNA levels of enzymes involved in PUFAs metabolism and steroidogenesis in rams. The expression levels of peroxisome proliferator-activated receptor (*PPARγ*), sterol regulatory element binding protein 1 (*SREBP1*), follicle stimulating hormone receptor (*FSHR*), androgen-binding protein (*ABP*), aromatase (*P450scc* and *P450arom*), and steroidogenic acute regulatory protein (*StAR*) in LO ($n = 8$) group were significantly higher than those in the controls ($n = 8$). Values were shown as means ± SME. *$P < 0.05$.

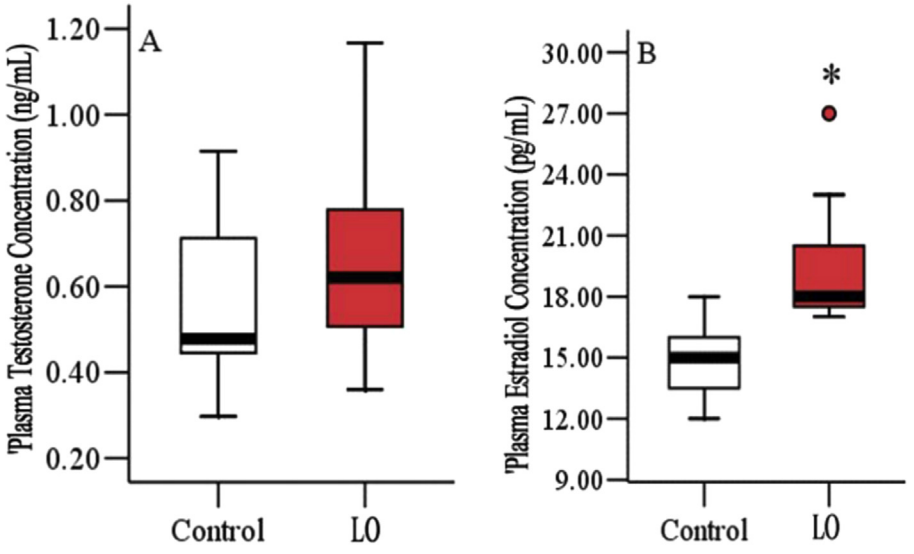

Fig. 2. Effect of the dietary linseed oil (LO) on the testosterone and E2 concentration in peripheral blood. Box plots showing minimum, maximum, and quartiles of concentration of testosterone and E2 in the control ($n = 8$) and LO groups ($n = 8$). Supplementation with 4% LO did not increase the testosterone secretion in rams (A), however, the plasma E2 concentration in LO group was significantly higher than that in the controls (B). *$P < 0.05$.

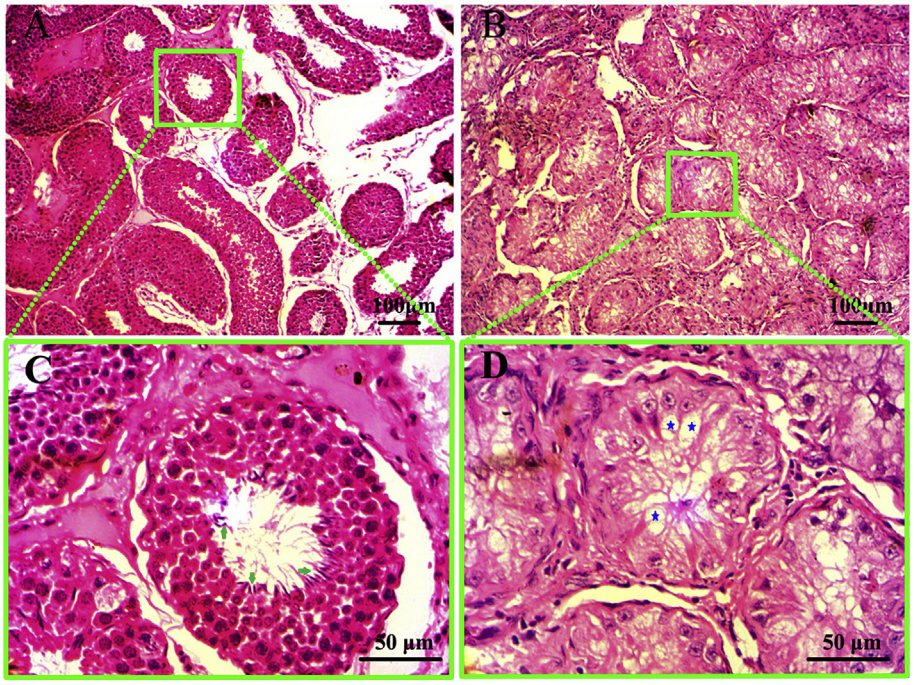

Fig. 3. Histological images of testes from control and linseed oil (LO) groups. Hematoxylin and eosin (H&E) staining, 100 × (Green row: multiple changes were presented in a further magnification (400 ×) of the green bordered box.). Testes from the LO group showed a compact and regular arrangement of germ cells and elongated spermatozoids (green arrow) in their seminiferous epithelium (A and C). Control group showed vacuolation (★) of seminiferous epithelium (B and D). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

concentrations in the plasma [8,26–29]. LH stimulated the testosterone secretion by Leydig cells via binding to *LHR*. LH also stimulated the StAR protein synthesis and phosphorylation in Leydig cells and induced the histone acetylation in *StAR* and *P450scc* gene promoter region via a LHR-stimulated cAMP pathway [30,31]. StAR activation is the first step in testosterone synthesis, which modulates the translocation of cholesterol from outer to inner mitochondrial membrane. Cholesterol was metabolized to the pregnenolone by *P450scc* [32]. In this study, *StAR* and *P450scc* mRNA levels were up-regulated in the LO group. Testosterone concentration was also detected and verified that the LO can stimulate steroidogenesis. Nevertheless, the two groups had the same plasma testosterone level. The mechanism on how dietary ω-3 PUFAs modulate testicular steroidogenesis is poorly understood. One possible explanation is that the finding of some authors studying fish and pig [4,33], in which the ALA (ω-3 PUFA) in LO is converted into EPA and inhibits the arachidonic acid (ω-6 PUFA) metabolism into prostaglandin-2, which stimulates the testosterone production. Another explanation is that the testosterone synthesized is metabolized to E2 via the P450arom enzyme. ALA can enhance progesterone synthesis by theca cells and COCs *in vitro* [34], and the diet supplementation with linseed and Menhaden oil also stimulate E2 synthesis [4,35]. To test this hypothesis, we detected the *FSHR*, *ABP*, and *P450arom* expression levels in testes tissues and E2 concentration in plasma. The results confirmed our hypothesis that LO significantly up-regulates *FSHR*, *ABP*, and *P450arom* expression levels and also the E2 concentration. The sensitivity of Sertoli cells to FSH was increased by the LO treatment, which further stimulated *ABP* expression and E2 synthesis.

The steroids secreted by Sertoli and Leydig cells contribute to the development of sexual organs [36]. The number of Sertoli cells in testis determine the testicular size, and both FSH and E2 have proliferation effect on Sertoli cells [37,38]. In our study, LO treatment also increased the number of Sertoli cells. In male, FSH stimulates *ABP* expression and contributes to spermatogenesis and the growth of testes. The accumulation of testosterone via binding to ABP causes high local testosterone concentration in the seminiferous tubules, which is a fundamental factor in normal spermatogenesis [36]. E2 is notably involved in the initiation and maintenance of spermatogenesis and it promotes the survival of germ cells [37]. Previous studies showed that PUFAs can contribute to spermatogenesis in bulls, boars, and roosters [8,14,15]. Our study produced similar result for testes weight and spermatogenesis. The testes weight in the LO group were greater than that in the controls. The morphology of the testes in the present study showed that LO also stimulated spermatogenesis with five or more layers of different stages of germ cells in their seminiferous epithelium, which resulted in the seminiferous tubules with bigger diameter than that in the controls.

5. Conclusion

Dietary treatment of rams with LO increased the expression levels of genes involved in PUFAs metabolism and steroidogenesis, stimulated the E2 secretion, and thus improved the testis development. These findings provide a sound basis for the beneficial effect of LO to male sheep reproduction. However, PUFAs are also associated with increased oxidative stress, which might have a detrimental effect on testes and reduce the semen quality. Hence, further studies are needed to verify whether the difference in testis development will be permanent or improve the sperm production.

Conflict of interest statements

The authors declare there is no conflict of interest.

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