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Identification of candidate genes in regulation of spermatogenesis in sheep testis following dietary vitamin E supplementation

Yang-Hua Qu[a,1], Lu-Yang Jian[a,1], Ce Liu[a], Yong Ma[a], Chen-Chen Xu[a], Yue-Feng Gao[a], Zoltan Machaty[b], Hai-Ling Luo[a,*]

[a] State Key Laboratory of Animal Nutrition, College of Animal Science and Technology, China Agricultural University, Beijing 100193, PR China
[b] Purdue University, Department of Animal Sciences, West Lafayette, IN, 47907, USA

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ABSTRACT

Dietary vitamin E supplementation is beneficial to semen quality in different sheep and goat breeds. The aim of this research was to further investigate the effect of vitamin E in sheep on spermatogenesis and its regulatory mechanisms using RNA-seq. Thirty male Hu lambs were randomly divided into three groups. The animals received 0, 200 or 2000 IU/day vitamin E dietary supplementation for 105 days, and its effects were subsequently evaluated. The results indicate vitamin E supplementation increased the number of germ cells in the testes and epididymides. The positive effects were reduced, however, in animals that received 2000 IU/d vitamin E. Using the RNA-seq procedure, there was detection of a number of differentially expressed genes such as *NDRG1*, *FSCN3* and *CYP26B1* with these genes being mainly related to the regulation of spermatogenesis. Supplementation with 2000 IU/d vitamin E supplementation resulted in a lesser abundance of skeleton-related transcripts such as *TUBB*, *VIM* and different subtypes of collagen, and there was also an effect on the ECM-receptor interaction pathway. These changes appear to be responsible for the lesser beneficial effect of the greater vitamin E concentrations. The results provide a novel insight into the regulation of spermatogenesis by vitamin E at the molecular level, however, for a precise understanding of functions of the affected genes there needs to be further study.

1. Introduction

Mammalian spermatogenesis involves a series of complex physiological processes that germ cells undergo at the onset of puberty (Feng et al., 2014; Kotaja, 2014). In male animals, functional gametes are produced only if spermatogenesis proceeds normally. It is believed that the hypothalamic - pituitary - gonadal axis and a number of regulatory factors have central roles in the control of spermatogenesis (Anderson et al., 2008; Matson et al., 2010; Saleela et al., 2010). In addition, nutrition and the environment also have considerable influence on the process.

Vitamin E is a micronutrient essential for mammals. It was first described as a fat-soluble compound that was essential for the reproduction of rats (Evans and Bishop, 1922). Extensive investigations resulted in it being recognized as a potent antioxidant which

* Corresponding author at: Yuanmingyuan West Road No. 2, Haidian District, Beijing, PR China.
E-mail addresses: quyanghua@cau.edu.cn (Y.-H. Qu), jianluyang@cau.edu.cn (L.-Y. Jian), 344196303@qq.com (L. Ce), 376220572@qq.com (Y. Ma), chenxu30510@cau.edu.cn (C.-C. Xu), nexhyf@cau.edu.cn (Y.-F. Gao), zmachaty@purdue.edu (Z. Machaty), luohailing@cau.edu.cn (H.-L. Luo).
[1] These authors contributed equally to this work.

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functioned to scavenge free radicals (Niki, 2014). Naturally occurring vitamin E exists in eight different forms, of which α-tocopherol has the greatest biological activity. In mammals, α-tocopherol is the major form of vitamin E distributed in the cytoplasm in various tissues (Schneider, 2005; Jiang, 2014; Kono and Arai, 2015).

Results of previous studies indicated dietary vitamin E supplementation in animals was beneficial to semen quality, especially when there were conditions associated with environmental stress (Kaur and Bansal, 2015). It is generally accepted that the improved antioxidative status resulting from vitamin E dietary supplementation was responsible for the positive effects, as the sperm plasma membrane is rich in polyunsaturated fatty acids that are susceptible to lipid peroxidation (Ghosh et al., 2002; Zhu et al., 2015). Even in the absence of increased oxidative stress, however, supplementation with vitamin E also has a favorable effect on semen quality. (Bensoussan et al., 1998). There has continued to be a focus on male reproductive performance of different sheep and goat breeds receiving vitamin E treatments. In Jintang black goats and Aohan fine-wool sheep, dietary supplementation of vitamin E intake improved semen quality (Yue et al., 2010). Histological analysis of the testes of Boer goats following vitamin E supplementation indicated there was an increased number of germ cells as well as Sertoli cells, indicating vitamin E was beneficial to the spermatogenic process (Zhu et al., 2009). Because there was less stress as a component of conducting these studies, it was hypothesized that there might be factors other than antioxidation that were involved in the regulation of spermatogenesis by vitamin E. In addition, results of these experiments also indicated vitamin E did not have effects in a dose-dependent manner. The benefits of vitamin E were incrementally less if the dose exceeded a certain threshold (Liu et al., 2005; Zhu et al., 2009; Yue et al., 2010). Further studies have, therefore, been needed to clarify the reason for these previous findings.

Recently, the use of RNA-seq analysis has become increasingly popular in the field of animal science, as the technology offers a transient assessment of the relative abundance of transcripts for all genes. In the present study, there was collection of testis samples from male Hu lambs following the supplementation of diets with different amounts of vitamin E. The RNA-seq technique was conducted to investigate the mechanism through which vitamin E regulates spermatogenesis in sheep, and also, to determine the possible reason for the lesser positive effects of the larger amounts of vitamin E supplementation.

2. Materials and methods

2.1. Animals, experimental design and sample collection

Thirty healthy male Hu lambs (three months of age) with a uniform genetic composition and a similar initial body weight (BW) of 22.63 ± 1.41 kg (mean ± SD) were allocated to three groups using a randomized complete block design. Each group contained ten animals. The animals were housed individually in a pen and water was supplied *ad libitum*. Diets were formulated on the basis of the National Research Council (NRC, 2007) standard (Table 1). Lambs in the different groups were fed the basal diets supplemented with 0 IU (CK), 200 IU (VE200) or 2000 IU (VE2000) vitamin E (in the form of α-tocopherol) per day. The experiment was conducted for 105 days, until the lambs were of a slaughter weight of 40 kg.

All animals were slaughtered using Muslim procedures after fasting for 12 h with free access to water. The testes and epididymides of each lamb were then removed from the scrotum. A small piece of the testis was immediately placed in a centrifuge tube and stored in liquid nitrogen until total RNA was extracted, and another sample of testes tissue was stored at −20 °C and used for the determination of vitamin E concentration. Additional pieces of the testicular and epididymal tissues were fixed in 4% formaldehyde at room temperature to process tissue sections embedded in paraffin to determine the number of germ cells in the tissues.

2.2. RNA extraction, library construction and sequencing

Three testis samples in each group were randomly selected and total RNA was isolated using the Trizol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. The concentration and purity of the extracted RNA was determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The integrity of the RNA was evaluated using a Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, CA, USA). To meet the library construction requirements, the

Table 1
Ingredients and chemical composition of the formulation.

Ingredients (DM basis)	Ratio (%)	Chemical composition (DM basis)	Ratio (%)
Corn silage	22.00	Metabolizable energy (MJ/kg)[2]	10.14
Peanut vine	28.00	Crude protein	15.05
Corn	29.00	Ether extract	2.38
Soybean meal	14.50	Neutral detergent fiber	27.22
Wheat bran	3.00	Acid detergent fiber	18.00
Sodium chloride	0.50	Calcium	0.75
Sodium bicarbonate	0.50	Phosphorus	0.36
Minerals and vitamin premix[1]	2.50		

[1] Per kilogram of premix: 220000 IU vitamin A; 70000 IU vitamin D; 1.6 g Fe; 0.3 g Cu; 1.2 g Mn; 1.8 g Zn; 26.0 mg I; 5.6 mg Se; 10.0 mg Co; Premix contained no vitamin E.

[2] All values were measured except metabolizable energy.

concentration of the extracted RNA was no less than 100 ng/μL, and the A260/A280 ratio ranged from 1.8 to 2.1. The RIN values of all samples were more than 8.0. A total of 5 μg high-quality RNA was delivered to Gene Denovo Biotechnology Co. (Guangzhou, China) for library construction and sequencing.

Following total RNA extraction, oligo (dT) beads were used for mRNA enrichment. The mRNA was then fragmented into pieces using fragmentation buffer and reverse transcribed into first-strand cDNA using random primers, followed by second-strand cDNA synthesis by adding buffer, dNTP, DNA polymerase I and RNase H. The cDNA was then ligated to Illumina sequencing adapters after purification with the QiaQuick PCR extraction kit, followed by end repair and Poly(A) tail addition. The products were separated by size using agarose gel electrophoresis, amplified using PCR, and finally sequenced using Illumina HiSeq™ 2500 by Gene Denovo Biotechnology Co. (Guangzhou, China).

2.3. Quality control and alignment with a reference genome

First, raw reads were acquired from the sequencing platforms. This was followed by filtering, to remove adapters and low-quality bases that negatively affect the process of assembly and analysis. Ribosomal RNA (rRNA) was also removed using mapping reads to an rRNA database using Bowtie2 (Langmead and Salzberg, 2012) to ensure high quality for further analysis. Clean reads of each sample were then aligned to the sheep reference genome (Oar_v3.1) using Tophat2 (version 2.0.3.12) (Kim et al., 2013). The data were mapped according to the following principles: a) maximum read mismatch is 2; b) the distance between mate-pair reads is 50 bp; c) the error of distance between mate-pair reads is ± 80 bp.

2.4. Transcript assembly and gene abundance quantification

The alignments were used for the reconstruction of transcripts by means of the Cufflinks software package (Trapnell et al., 2012). The program constructed faux reads based on the reference to remedy the influence of low-coverage sequencing. The reassembled fragments were mapped with reference genes and the similar ones were removed. After this, the Cuffmerge software was used to merge transcripts from each replica of one group into an integrated set of transcripts, the transcripts from the various treatment groups were then merged again into an integrated set of transcripts for further analysis.

Gene abundances were quantified by RSEM (Li and Dewey, 2011). The relative abundance of genes was normalized using the FPKM (fragments per kilobase of transcript per million mapped reads) value. This calculation method can be used to eliminate the effects of different gene lengths and amounts of sequencing data, which assures that the gene expression obtained can be immediately used for the analysis of differentially expressed genes (DEGs) among the samples.

2.5. Identification of DEGs with gene ontology (GO) and pathway analysis

The edgeR package (http://www.rproject.org/) was used to analyze DEGs among the treatment groups. GO and pathway enrichment analyses were then applied to the DEGs. First, all DEGs were aligned to GO terms in the Gene Ontology database (http://www.geneontology.org/). Gene numbers were then calculated for each term, and the significantly enriched terms were defined using the hypergeometric test. A *P*-value of 0.05 was considered as a threshold. Pathway enrichment analysis was also performed based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (Kanehisa et al., 2008). The calculation method was the same as that in the GO analysis. Those meeting the $P < 0.05$ requirement were considered as significantly enriched pathways.

2.6. Validation of RNA-seq data

Eight DEGs were selected for quantitative real-time polymerase chain reaction (qRT-PCR) to validate the RNA-seq data. Six samples in each group, including three samples from the RNA-seq analysis, were chosen for qRT-PCR. Total RNA was extracted from all samples using the Trizol reagent. The cDNA was then synthesized using a FastQuant RT Kit (with gDNase) according to the

Table 2
Information about primers used for qRT-PCR.

Gene	Forward primer	Reverse primer	Product size (bp)	R^2	E%
GAPDH	5’ GGCGTGAACCACGAGAAGTA	5’ GGCGTGGACAGTGGTCATAA	141	0.988	95.03
NDRG1	5’ TGCCTACATCCTCACTCGC 3’	5’ GACTACCTCCACGTTATTCTGC 3’	199	0.997	102.2
FSCN3	5’ ATGCGATCCGACCGAAGT 3’	5’ GGGCTCCTGTTTGGTTTAGTGT 3’	147	0.999	96.7
CYP26B1	5’ CCCCAAAGGCTGGAGTGT 3’	5’ CGCCGAAAGGGAGGTAAT 3’	149	0.996	106.1
STRA8	5’ GGCTGTTTACTCCCACTCT 3’	5’ ATGCTGGCATATTCTTTCTT 3’	179	0.996	91.2
COL1 A1	5’ CCCAGTTGTCTTACGGCTATG 3’	5’ GACCACGAGGACCAGAAGG 3’	81	0.998	106.4
COL4A1	5’ TGCGGAAGTTCAGCACCAT 3’	5’ CGAGTAGTCGTTGCGGGAG 3’	80	0.996	110.9
TUBB	5’ CCCTCGTGCTATCTTGGT 3’	5’ GCTCGGCTCCCTCTGTAT 3’	155	0.992	106.0
VIM	5’ CGCTTCGCCAACTACATC 3’	5’ ACTTGCCCTGTCCCTTGA 3’	94	0.993	92.2

GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; NDRG1, N-myc downstream regulated 1; FSCN3, Fascin actin-bundling protein 3; CYP26B1, Cytochrome P450, family 26, subfamily B, polypeptide 1; STRA8, Stimulated by retinoic acid gene 8; COL1 A1, Collagen, type I, alpha 1; COL4A1, Collagen, type IV, alpha 1; TUBB, Tubulin, beta class I; VIM, Vimentin.

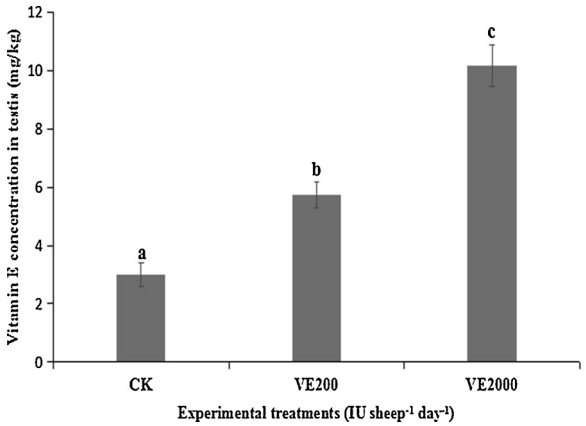

Fig. 1. Vitamin E concentration in sheep testis fed basal diets supplemented with 0 IU (CK), 200 IU (VE200) or 2000 IU (VE2000) vitamin E per day; One-way ANOVA with Tukey's test was applied; Data are expressed as mean ± SD; Different letters indicate differences ($P < 0.05$).

manufacturer's instructions (TIANGEN, Beijing, China). The house-keeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as a reference. Information of primer sequences for the selected genes, including the R^2 and E%, is reported in Table 2. The qRT-PCR was performed at 95 °C for 15 min, followed by 40 cycles at 95 °C for 10 s and 60 °C for 30 s in a FQD-96 A real-time PCR system (BIOER, Hongzhou, China) using SYBR Green SuperReal PreMix Plus (TIANGEN, Beijing, China), and finished with the melting curve analysis.

2.7. Statistical analyses

The vitamin E concentration and germ cell number were analyzed using an one-way ANOVA using IBM SPSS statistics 20.0 (SPSS Inc., Chicago, IL, USA). A Tukey's test was used to compare the means among groups when there were significant differences. A difference was considered to have occurred with $P < 0.05$ and a tendency at $0.05 \leq P < 0.10$.

The significant DEGs in the RNA-seq were identified following the rules of a false discovery rate (FDR) of < 0.05 using the Benjamini and Hochberg approach. For the qRT-PCR data, relative mRNA transcript abundances were calculated using the $2^{-\triangle\triangle Ct}$ method (Livak and Schmittgen, 2001).

3. Results

3.1. Vitamin E concentration in the sheep testis

Testicular vitamin E concentrations in the various groups are depicted in Fig. 1, and the data for results of the RNA-seq analysis within each group are included in Supplemental File 1. Dietary vitamin E supplementation resulted in an increase in vitamin E concentrations in the sheep testis compared with the control group ($P < 0.05$). A greater incremental increase in the vitamin E supplementation resulted in a more substantial effect, as the average testicular vitamin E concentration in the VE2000 group was greater than that in the VE200 group ($P < 0.05$).

3.2. Germ cell number in the sheep testis and epididymis

Fig. 2a and b includes sections of sheep testes and epididymides, while the average germ cell numbers are listed in Table 3, and the data for results of the RNA-seq analysis are included in Supplemental File 1. The total number of germ cells and number of spermatids in the vitamin E-supplemented groups tended to be greater ($P = 0.063$ and $P = 0.062$, respectively), whereas the number of spermatocytes was greater ($P = 0.001$) than those in the control group. There was no difference in the number of spermatogonia in the testes in response to vitamin E supplementation. In addition, supplementation with vitamin E resulted in a marked increase in number of spermatozoa in the epididymis compared with the control group ($P < 0.001$).

3.3. Summary of the RNA-seq data

There was a total of 21.48, 43.07, 36.98 million raw reads for the CK group (CK-1, CK-2, CK-3), 34.65 41.61, 31.35 million raw reads for the VE200 group (VE200-1, VE200-2, VE200-3), and 34.91, 31.57, 34.37 million raw reads for the VE2000 group (VE2000-1, VE2000-2, VE2000-3). The information about total RNA quality and clean reads after filtering and mapping to the sheep reference genome is included in Supplemental File 2.

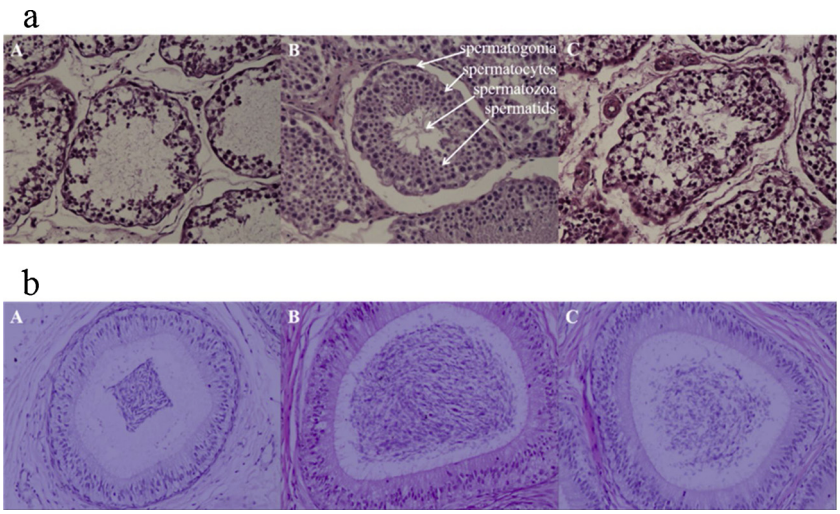

Fig. 2. Paraffin sections of sheep testis (2a) and epididymis (2b) tissues containing spermatogonia, spermatocytes, spermatids and spermatozoa fed basal diets supplemented with 0 IU (A), 200 IU (B) or 2000 IU (C) vitamin E, respectively.

Table 3
Effect of vitamin E on the germ cell number in sheep testis and epididymis (average numbers are shown).

Items	Treatments[1]			SEM	*P*-value
	CK	VE200	VE2000		
Number of total germ cells (per convoluted seminiferous tubule)	118.0	147.5	133.0	12.2	0.063
Number of spermatogonia (per convoluted seminiferous tubule)	15.4	16.7	16.9	1.1	0.340
Number of spermatocytes (per convoluted seminiferous tubule)	10.7^{a}	15.7^{b}	12.7^{a}	1.2	0.001
Number of spermatids (per convoluted seminiferous tubule)	91.9	115.1	103.4	9.5	0.062
Number of spermatozoa (per ductus epididymis)	264.1^{a}	647.5^{b}	562.4^{c}	33.7	< 0.001

a, b, c Values associated with different superscript letters in the same row are different ($P < 0.05$).
[1] Supplementation of 0 IU (CK), 200 IU (VE200) and 2000 IU (VE2000) vitamin E per day.

3.4. Number of genes and differentially expressed genes

The total number of genes detected in the experiments is reported in Supplemental File 3. There were 22076, 21595 and 21880 genes for the CK, VE200 and VE2000 group, respectively. The number of genes recognized were 17555, 17443 and 17367, respectively; while the remaining genes are thought to be genes that were first detected in the present study.

The FPKM was used to determine the extent of expression of each gene. The information of DEGs between the two groups is presented in Supplemental Files 4 to 6. The DEGs were considered significant if the FDR was less than 0.05. The number of significant DEGs among the three groups is depicted in Fig. 3. There was identification of 36 significant DEGs between the VE200 and CK groups, of which there was upregulation of 13 and downregulation of 23 (Supplemental File 7). A total of 145 DEGs were identified between the VE2000 and CK groups, of which there was upregulation of 39 and downregulation of 106 (Supplemental File 8). There were also 33 DEGs between the VE200 and VE2000 groups, including 16 in which there was upregulation and 17 in which there was downregulation (Supplemental File 9).

3.5. GO analysis of DEGs

All DEGs were annotated using three GO terms: biological process (BP), cellular component (CC) and molecular function (MF). The DEGs between the CK and VE200 groups were enriched into 73 BP, 21 CC and 25 MF, for which there was a total of 12 enrichments associated with a *P*-value of < 0.05 (Supplemental File 10). The 20 GO terms for DEGs with the greatest ranking between the CK and VE200 groups are included in Fig. 4. The data from the GO enrichment analysis indicated that the DEGs were mainly involved in developmental processes, including embryo development, single-organism development, multicellular organism development and anatomical structure development. The *N-myc downstream regulated 1* (*NDRG1*) gene was downregulated 2.38-fold in the VE200 group being one of the DEG affecting developmental processes.

The DEGs between the CK and VE2000 groups were enriched into 95 BP, 41 CC and 44 MF, of which a total of 18 were significantly enriched (Supplemental File 11). The 20 GO terms for DEGs with the greatest ranking are included in Fig. 5. The GO analysis revealed that the cell differentiation and developmental processes were among those that were enriched. Genes such as *NDRG1* (downregulated 2.48-fold in the VE2000 group) and *fascin actin-bundling protein 3* (*FSCN3*, upregulated 1.88-fold) are some of

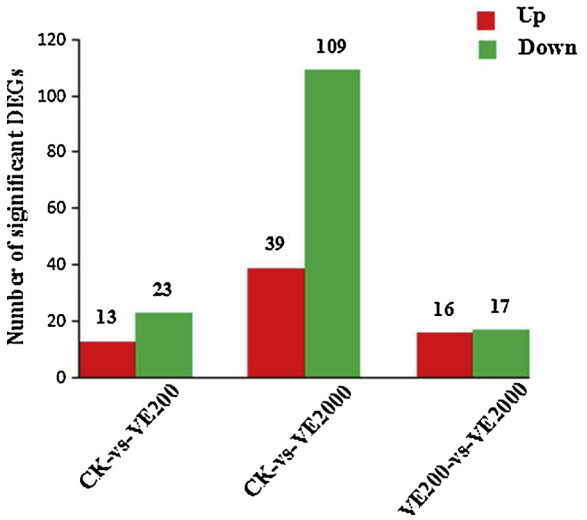

Fig. 3. Number of DEGs in the three groups; DEGs were identified using RNA-seq following the rules of a false discovery rate (FDR) of < 0.05.

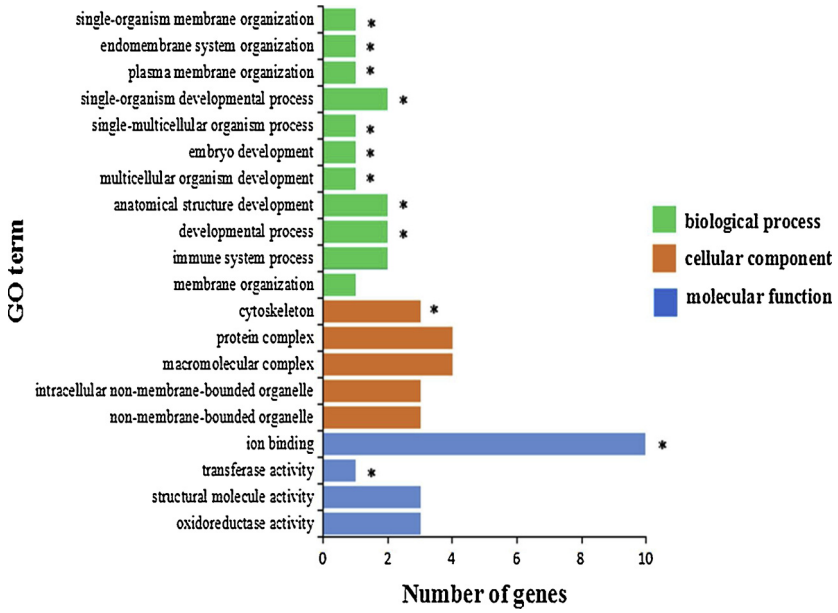

Fig. 4. Twenty GO terms for DEGs with the greatest rankings for the CK and VE200 groups.

the more relevant in this regard. In addition, genes involved in cellular component organization, cytoskeleton organization and structural molecular functions were identified as well, with relevant genes being *tubulin, beta class I* (*TUBB*, downregulated 1.80-fold), *vimentin* (*VIM*, downregulated 2.06-fold), *microtubule-associated protein 1 A* (*MAP1 A*, downregulated 1.86-fold) and several different subtypes of collagen. Interestingly, the relative extent of gene expression of these cytoskeleton-related genes in the VE2000 group were all less compared to those in the CK group.

3.6. KEGG pathway analysis of DEGs

Pathway annotation of DEGs was performed using the KEGG database. Data for all enriched pathways of DEGs in the CK and VE200 groups are included in Supplemental File 12. The results indicate that there were 14 enriched pathways with a *P*-value of < 0.05. The listing of the five pathways (those related to 2-oxocarboxylic acid metabolism, prostate cancer, valine, leucine and isoleucine degradation, retinol metabolism and drug metabolism - cytochrome P450) with the greatest ranking is included in Table 4. The DEGs in the five pathways included *glutamic pyruvate transaminase* (*GPT2*, downregulated 3.23-fold in the VE200 group), *platelet-derived growth factor receptor, alpha polypeptide* (*PDGFRA*, downregulated 2.17-fold), *branched chain amino-acid transaminase 1* (*BCAT1*, upregulated 4.00-fold) and *cytochrome P450, family 26, subfamily B, polypeptide 1* (*CYP26B1*, downregulated 1.79-fold).

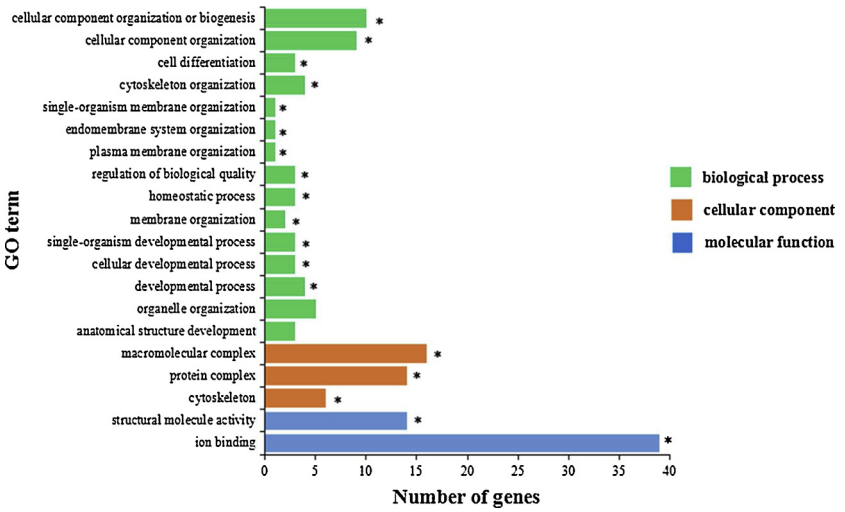

Fig. 5. Twenty GO terms for DEGs with the greatest rankings for the CK and VE2000 groups.

Table 4
KEGG pathway analysis of DEGs between the CK and VE200 groups (Five greatest values).

Pathway name	Pathway ID	Number of DEGs with pathway annotation	Percentage of DEGs with pathway annotation (%)	*P*-value
2-Oxocarboxylic acid metabolism	ko01210	2	13.33	< 0.001
Prostate cancer	ko05215	3	20	0.002
Valine, leucine and isoleucine degradation	ko00280	2	13.33	0.005
Retinol metabolism	ko00830	2	13.33	0.006
Drug metabolism - cytochrome P450	ko00982	2	13.33	0.006

All data for enriched pathways among the DEGs in the CK and VE2000 groups are included in Supplemental File 13. There were 13 enriched pathways, with the five with the greatest ranking being extracellular matrix (ECM) receptor interaction, protein digestion and absorption, focal adhesion, PI3K-Akt signaling pathway and the AGE-RAGE signaling pathway (Table 5). The DEGs enriched in these pathways were similar, including different subtypes of collagen, such as *collagen, type I, alpha 1* (*COL1 A1*, downregulated 2.66-fold in the VE2000 group), *collagen, type I, alpha 2* (*COL1 A2*, downregulated 2.07-fold), *collagen, type IV, alpha 1* (*COL4A1*, downregulated 1.68-fold), *collagen, type IV, alpha 3* (*COL4A3*, downregulated 1.75-fold), *collagen, type IV, alpha 4* (*COL4A4*, downregulated 2.03-fold), *collagen, type VI, alpha 1* (*COL6A1*, downregulated 2.03-fold), *collagen, type VI, alpha 2* (*COL6A2*, downregulated 2.76-fold) and *collagen, type VI, alpha 3* (*COL6A3*, downregulated 2.32-fold), which indicates the ECM function was affected by the 2000 IU/d dietary vitamin E supplementation.

3.7. Validation of genes by qRT-PCR

Eight DEGs were selected for further qRT-PCR analysis, including *NDRG1*, *FSCN3*, *CYP26B1*, *TUBB*, *VIM*, *COL1 A1*, *COL4A1* and *STRA8* (Fig. 6). The results indicated there was a trend similar to that for the RNA-seq data, even though there were some inconsistencies when *P*-values were assessed. These findings indicated that the accuracy of RNA-seq was acceptable.

Table 5
KEGG pathway analysis of DEGs between the CK and VE2000 groups (Five greatest values).

Pathway name	Pathway ID	Number of DEGs with pathway annotation	Percentage of DEGs with pathway annotation (%)	*P*-value
ECM-receptor interaction	ko04512	11	18.97	< 0.00001
Protein digestion and absorption	ko04974	10	17.24	< 0.00001
Focal adhesion	ko04510	13	22.41	< 0.00001
PI3K-Akt signaling pathway	ko04151	12	20.69	0.00002
AGE-RAGE signaling pathway in diabetic complications	ko04933	7	12.07	0.00004

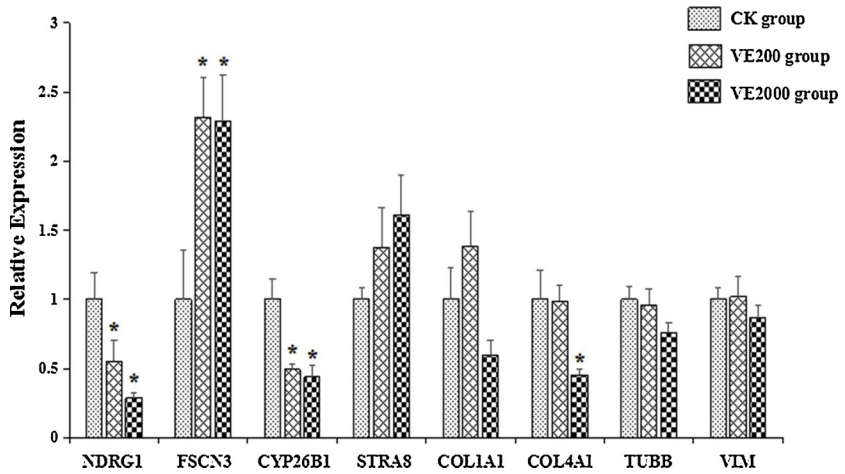

Fig. 6. Relative expression of eight DEGs in the three groups; One-way ANOVA with Tukey's test was applied; Data are expressed as mean ± SD; Difference was considered to exist $P < 0.05$; *NDRG1*, *N-myc downstream regulated 1*; *FSCN3*, *fascin actin-bundling protein 3*; *CYP26B1*, *cytochrome P450, family 26, subfamily B, polypeptide 1*; *STRA8*, *stimulated by retinoic acid gene 8*; *COL1 A1*, *collagen, type I, alpha 1*; *COL4A1*, *collagen, type IV, alpha 1*; *TUBB*, *tubulin, beta class I*; *VIM*, *vimentin* genes.

4. Discussion

In the current study, vitamin E intake increased germ cell numbers in the sheep testis, and this may indicate that vitamin E functions in the progression of mitosis as well as meiosis. There were overlapping DEGs between the CK when there was comparison of VE200 (36 DEGs) and CK as compared with the VE2000 (145 DEGs) group. Eight genes were identified with three of them being *NDRG1*, *CYP26B1* and *FSCN3* having a role in the spermatogenesis.

The NDRG1 has important functions in cell growth and differentiation (Fang et al., 2014). In some studies where there were evaluations of cancer cell lines, there was over-expression of the *NDRG1* gene that resulted in a decreased proliferation rate (Kurdistani et al., 1998; Melotte et al., 2010). Suppression of expression of *NDRG1* gene, however, led to the proliferation of endometrial cancer cells (Lv et al., 2012). It has been suggested that the expression of the *NDRG1* gene might be associated with the development of the reproductive organs in the African frog, because silencing as well as over-expression of the gene disrupted morphogenesis of the pronephric ducts (Kyuno et al., 2003). According to our RNA-seq data, the expression of the *NDRG1* gene in the VE200 and VE2000 groups was downregulated 2.38- and 2.48-fold, respectively, compared with the CK group. This is one possible way in which vitamin E promotes the proliferation of germ cells in the sheep testis. Results of a previous report indicated that for NDRG1 orthologues there was a lesser gene expression in response to testosterone treatment in cell culture (Lin and Chang, 1997), while Leydig cells were sensitive to vitamin E supplementations with an increased testosterone synthesis (Glade et al., 2015). There needs to be further studies of the precise regulatory mechanisms of vitamin E on testicular functions if there is to be precise elucidation of actions on testicular tissues.

In the present study, the relative abundance of *FSCN3* transcript was 1.75-fold and 1.88-fold greater in the VE200 and VE2000 groups, respectively. There is a highly specific pattern of gene expression in the testis, primarily in spermatids and mature spermatozoa (Tubb et al., 2002; Kollers et al., 2006). Results of immunofluorescence studies further indicate that the FSCN3 protein is distributed in the anterior region of the head of elongated spermatids, indicating the gene has functions in the morphological changes the spermatids undergo as these cells develop into mature spermatozoa (Tubb et al., 2002). Mice with a knocked-out *fascin* gene had a relatively lesser sperm production being only 10% of that of wild-type animals (Robaire, 2003). In the present study, the relatively greater numbers of spermatids in the sheep testis and mature spermatozoa in the epididymis could be attributed to the greater expression of the *FSCN3* gene in the vitamin E-supplemented groups. It was speculated that some cytokines, testosterone or certain receptors might induce changes in its spatiotemporal gene expression (Gungor-Ordueri and Cheng, 2015), and several factors were also associated with vitamin E supplementation.

In the present study, the function of the retinol metabolism pathway was also enriched in the VE200 group. An important metabolite in this pathway is retinoic acid (RA), which is considered an important regulatory compound of meiosis during spermatogenesis (Raverdeau et al., 2012). The protein encoded by the *CYP26B1* gene is believed to be important for inhibition of the germ cell entry into meiosis as a result of enhancing the oxidized catalysis of RA to its inactive forms (Kumar et al., 2011). Treatment with the CYP26B1 enzyme inhibitor resulted in the neonatal testis having spermatogonial differentiation and there was an induced expression of spermatogenesis-promoting gene such as *STRA8* (Snyder et al., 2010; Griswold, 2016). This indicates that the decrease in *CYP26B1* gene expression can suppress the inhibitory actions that prevent germ cells from entering into meiosis during spermatogenesis. The current results indicate that the *CYP26B1* gene was downregulated 1.79- and 1.69-fold, respectively, in the VE200 and VE2000 groups compared to the control. From these findings, it is hypothesized that the increased vitamin E concentration in sheep testicular tissues inhibited the degradation of RA by downregulating the expression of the *CYP26B1* gene. This action of

vitamin E was also further substantiated because there was a slight enhancement (1.37- and 1.61-fold in both vitamin E supplemented groups, respectively) of the expression of the *STRA8* gene, a downstream target of RA, implying that vitamin E supplementation may have positive functions in the modulation of the spermatogenic process.

There were specific differences in the transcription profiles between the VE200 and VE2000 groups in the current study. There was a decrease in the expression of the *TUBB*, *MAP1 A*, *VIM* and multiple types of collagen genes, such as *COL1 A1*, *COL4A1* and *COL6A1* in the VE2000 group. The expression of these genes are primarily related to cytoskeleton organization and modulation of the ECM structures. The disruption of the microtubule-based cytoskeleton lead to the sloughing of germ cells and retention of spermatids in the seminiferous tubule (Markelewicz et al., 2004; Johnson, 2014; Tang et al., 2016). Results of the studies of the ECM also indicate there is a stimulatory effect on testicular cell proliferation (Akbarinejad et al., 2015). As the major component of the ECM, the type IV collagen is the most abundant collagen form in ruminant testis that is integral in basement membrane structure, normal function of Sertoli cells, and spermatogonia development (Kahsai et al., 1997; Siu and Cheng, 2004). The ECM-receptor interaction pathway was enriched in the present study, indicating that the incremental decrease of effects on spermatogenesis in sheep when there are the largest doses of vitamin E supplementation might be associated with the decreased expression of skeleton- and ECM-related genes. Results of a study in mice indicated the inhibitory effect of vitamin E on the TGF-β signaling pathway, which regulates ECM metabolism (Tasanarong et al., 2011; Wang et al., 2014). This might be a possible mechanism responsible for the incremental decrease of effects of the largest doses of vitamin E supplementation on sheep spermatogenesis.

5. Conclusions

Dietary vitamin E supplementation appears to be a modulator of the spermatogenic process of Hu lambs. The micronutrient did not, however, exert its effect in a dose-dependent manner. Assessment of the underlying molecular mechanism using RNA-seq procedures indicated vitamin E supplementation affected spermatogenesis in sheep through altering the expression of several related genes, including *NDRG1*, *FSCN3* and *CYP26B1*. In addition, the supplementation with the largest dose of vitamin E resulted in a decreased expression of skeleton- and ECM-related genes, which was probably responsible for the reduced effects of vitamin E when there was the larger supplementations. These results provide a novel insight into the molecular mechanisms of vitamin E in regulation of spermatogenesis in sheep.

Availability of data and materials

The datasets used in the current study are available in NCBI's Sequence Read Archive (SRA) database (https://www.ncbi.nlm.nih.gov/sra/?term=SRP119816) under the accession number SRP119816. The other data related to the study are included in the supplemental files.

Authors' contributions

HLL was the leader who conceived and designed the experiments. YHQ participated in the experimental design, performed the experiments, analyzed the data and wrote the manuscript. LYJ participated in the experimental design, analyzed the data and revised the manuscript. CL participated in the experimental design, helped to perform the experiments and collect the samples. YM participated in the experimental design and helped to analyze the data. CCX participated in the experimental design and provided help for RNA extraction and quality analysis. YFG participated in the experimental design and helped to collect the samples. ZM helped with critical reading and editing the manuscript. All authors read and approved the final manuscript.

Conflict of interests

The authors declare that they have no conflict of interests.

Ethics approval and consent to participate

Each lamb in this study was selected at the Runlin Animal Husbandry Co., Ltd., Linqing city, Shandong province, China. The experiment was conducted in line with the guidelines for experimental animals set by the Ministry of Science and Technology (Beijing, China). All protocols were approved by the China Agricultural University Animal Care and Use Committee.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.anireprosci.

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References

Akbarinejad, V., Tajik, P., Movahedin, M., Youssefi, R., Shafiei, S., Mazaheri, Z., 2015. Effect of extracellular matrix on bovine spermatogonial stem cells and gene expression of niche factors regulating their development in vitro. Anim. Reprod. Sci. 157, 95–102.

Anderson, E.L., Baltus, A.E., Roepers-Gajadien, H.L., Hassold, T.J., de Rooij, D.G., van Pelt, A.M.M., Page, D.C., 2008. Stra8 and its inducer, retinoic acid, regulate meiotic initiation in both spermatogenesis and oogenesis in mice. Proc. Natl. Acad. Sci. U. S. A. 105, 14976–14980.

Bensoussan, K., Morales, C.R., Hermo, L., 1998. Vitamin E deficiency causes incomplete spermatogenesis and affects the structural differentiation of epithelial cells of the epididymis in the rat. J. Androl. 19, 266–288.

Evans, H.M., Bishop, K.S., 1922. On the existence of a hitherto unrecognized dietary factor essential for reproduction. Science 56, 650–651.

Fang, B.A., Kovacevic, Z., Park, K.C., Kalinowski, D.S., Jansson, P.J., Lane, D.J., Sahni, S., Richardson, D.R., 2014. Molecular functions of the iron-regulated metastasis suppressor, NDRG1, and its potential as a molecular target for cancer therapy. Biochim. Biophys. Acta 1845, 1–19.

Feng, C.-W., Bowles, J., Koopman, P., 2014. Control of mammalian germ cell entry into meiosis. Mol. Cell. Endocrinol. 382, 488–497.

Ghosh, D., Das, U.B., Misro, M., 2002. Protective role of alpha-tocopherol-succinate (Provitamin-E) in cyclophosphamide induced testicular gametogenic and steroidogenic disorders: a correlative approach to oxidative stress. Free Radic. Res. 36, 1209–1218.

Glade, M.J., Smith, K., Meguid, M.M., 2015. A glance at nutritional antioxidants and testosterone secretion. Nutrition 31, 1295–1298.

Griswold, M.D., 2016. Spermatogenesis: the commitment to meiosis. Physiol. Rev. 96, 1–17.

Gungor-Ordueri, N.E., Cheng, C.Y., 2015. Fascin - an actin binding and bundling protein in the testis and its role in ectoplasmic specialization dynamics. Spermatogenesis 5, e1002733.

Jiang, Q., 2014. Natural forms of vitamin E: metabolism, antioxidant, and anti-inflammatory activities and their role in disease prevention and therapy. Free Radic. Biol. Med. 72, 76–90.

Johnson, K.J., 2014. Testicular histopathology associated with disruption of the Sertoli cell cytoskeleton. Spermatogenesis 4, e979106.

Kahsai, T.Z., Enders, G.C., Gunwar, S., Brunmark, C., Wieslander, J., Kalluri, R., Zhou, J., Noelken, M.E., Hudson, B.G., 1997. Seminiferous tubule basement membrane. Composition and organization of type IV collagen chains, and the linkage of alpha3(IV) and alpha5(IV) chains. J. Biol. Chem. 272, 17023–17032.

Kanehisa, M., Araki, M., Goto, S., Hattori, M., Hirakawa, M., Itoh, M., Katayama, T., Kawashima, S., Okuda, S., Tokimatsu, T., Yamanishi, Y., 2008. KEGG for linking genomes to life and the environment. Nucleic Acids Res. 36, D480–484.

Kaur, S., Bansal, M.P., 2015. Protective role of dietary-supplemented selenium and vitamin E in heat-induced apoptosis and oxidative stress in mice testes. Andrologia 47, 1109–1119.

Kim, D., Pertea, G., Trapnell, C., Pimentel, H., Kelley, R., Salzberg, S.L., 2013. TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. Genome Biol. 14, R36.

Kollers, S., Day, A., Rocha, D., 2006. Characterization of the porcine FSCN3 gene: cDNA cloning, genomic structure, mapping and polymorphisms. Cytogenet. Genome Res. 115, 189–192.

Kono, N., Arai, H., 2015. Intracellular transport of fat-soluble vitamins a and e. Traffic 16, 19–34.

Kotaja, N., 2014. MicroRNAs and spermatogenesis. Fertil. Steril. 101, 1552–1562.

Kumar, S., Chatzi, C., Brade, T., Cunningham, T.J., Zhao, X., Duester, G., 2011. Sex-specific timing of meiotic initiation is regulated by Cyp26b1 independent of retinoic acid signalling. Nat. Commun. 2, 151.

Kurdistani, S.K., Arizti, P., Reimer, C.L., Sugrue, M.M., Aaronson, S.A., Lee, S.W., 1998. Inhibition of tumor cell growth by RTP/rit42 and its responsiveness to p53 and DNA damage. Cancer Res. 58, 4439–4444.

Kyuno, J., Fukui, A., Michiue, T., Asashima, M., 2003. Identification and characterization of Xenopus ndrg1. Biochem. Biophys. Res. Commun. 309, 52–57.

Langmead, B., Salzberg, S.L., 2012. Fast gapped-read alignment with Bowtie 2. Nat. Methods 9, 357–359.

Li, B., Dewey, C.N., 2011. RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. BMC Bioinformatics 12, 323.

Lin, T.M., Chang, C., 1997. Cloning and characterization of TDD5, an androgen target gene that is differentially repressed by testosterone and dihydrotestosterone. Proc. Natl. Acad. Sci. U. S. A. 94, 4988–4993.

Liu, H.B., Luo, H.L., Liu, Z.H., Shi, Z.H., Zhao, H.B., 2005. Effect of dietary vitamin E concentration on semen quality of goat. Chin. J. Anim. Sci. 41, 12–14.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25, 402–408.

Lv, X.H., Chen, J.W., Zhao, G., Feng, Z.Z., Yang, D.H., Sun, W.W., Fan, J.S., Zhu, G.H., 2012. N-myc downstream-regulated gene 1/Cap43 may function as tumor suppressor in endometrial cancer. J. Cancer Res. Clin. Oncol. 138, 1703–1715.

Markelewicz, R.J., Hall, S.J., Boekelheide, K., 2004. 2,5-hexanedione and carbendazim coexposure synergistically disrupts rat spermatogenesis despite opposing molecular effects on microtubules. Toxicol. Sci. 80, 92–100.

Matson, C.K., Murphy, M.W., Griswold, M.D., Yoshida, S., Bardwell, V.J., Zarkower, D., 2010. The mammalian doublesex homolog DMRT1 is a transcriptional gatekeeper that controls the mitosis versus meiosis decision in male germ cells. Dev. Cell 19, 612–624.

Melotte, V., Qu, X., Ongenaert, M., van Criekinge, W., de Bruine, A.P., Baldwin, H.S., van Engeland, M., 2010. The N-myc downstream regulated gene (NDRG) family: diverse functions, multiple applications. FASEB J. 24, 4153–4166.

Niki, E., 2014. Role of vitamin E as a lipid-soluble peroxyl radical scavenger: in vitro and in vivo evidence. Free Radic. Biol. Med. 66, 3–12.

Raverdeau, M., Gely-Pernot, A., Feret, B., Dennefeld, C., Benoit, G., Davidson, I., Chambon, P., Mark, M., Ghyselinck, N.B., 2012. Retinoic acid induces Sertoli cell paracrine signals for spermatogonia differentiation but cell autonomously drives spermatocyte meiosis. Proc. Natl. Acad. Sci. U. S. A. 109, 16582–16587.

Robaire, B., 2003. Advancing towards a male contraceptive: a novel approach from an unexpected direction. Trends Pharmacol. Sci. 24, 326–328.

Saleela, M.R., Robert, I.M., Sarah, J.M., 2010. Hormonal regulation of male germ cell development. J. Endocrinol. 205, 117–131.

Schneider, C., 2005. Chemistry and biology of vitamin E. Mol. Nutr. Food Res. 49, 7–30.

Siu, M.K., Cheng, C.Y., 2004. Extracellular matrix: recent advances on its role in junction dynamics in the seminiferous epithelium during spermatogenesis. Biol. Reprod. 71, 375–391.

Snyder, E.M., Small, C., Griswold, M.D., 2010. Retinoic acid availability drives the asynchronous initiation of spermatogonial differentiation in the mouse. Biol. Reprod. 83, 783–790.

Tang, E.I., Mruk, D.D., Cheng, C.Y., 2016. Regulation of microtubule (MT)-based cytoskeleton in the seminiferous epithelium during spermatogenesis. Semin. Cell Dev. Biol. 59, 35–45.

Tasanarong, A., Kongkham, S., Duangchana, S., Thitiarchakul, S., Eiam-Ong, S., 2011. Vitamin E ameliorates renal fibrosis by inhibition of TGF-beta/Smad2/3 signaling pathway in UUO mice. J. Med. Assoc. Thai. 94 (Suppl 7), S1–9.

Trapnell, C., Roberts, A., Goff, L., Pertea, G., Kim, D., Kelley, D.R., Pimentel, H., Salzberg, S.L., Rinn, J.L., Pachter, L., 2012. Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. Nat. Protoc. 7, 562–578.

Tubb, B., Mulholland, D.J., Vogl, W., Lan, Z.J., Niederberger, C., Cooney, A., Bryan, J., 2002. Testis fascin (FSCN3): a novel paralog of the actin-bundling protein fascin expressed specifically in the elongate spermatid head. Exp. Cell Res. 275, 92–109.

Wang, H.Y., Chuang, H., Xiao, J.B., Gao, J., 2014. Regulatory effect of TGF- beta 1 antibodies on extracellular matrix reconstruction in rats with lung injury fibrosis. J. Med. Postgrad. 4, 368–372.

Yue, D., Yan, L., Luo, H., Xu, X., Jin, X., 2010. Effect of Vitamin E supplementation on semen quality and the testicular cell membranal and mitochondrial antioxidant abilities in Aohan fine-wool sheep. Anim. Reprod. Sci. 118, 217–222.

Zhu, H., Luo, H.L., Meng, H., Zhang, G.J., 2009. Effect of vitamin E supplementation on development of reproductive organs in Boer goat. Anim. Reprod. Sci. 113, 93–101.

Zhu, Z., Fan, X., Lv, Y., Zhang, N., Fan, C., Zhang, P., Zeng, W., 2015. Vitamin e analogue improves rabbit sperm quality during the process of cryopreservation through its antioxidative action. PLoS One 10, e0145383.