Absence of 1,25(OH)2D3 results in male mice infertility mediated by extracellular calcium and phosphonium

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Abstract: To identify the effects of 1,25(OH)2D3 deficient and calcium and phosphorus supplement on male reproductive system, mice with targeted deletion of 25-hydroxyvitamin D 1α-hydroxylase [1α(OH)ase-/-] were used in this paper. The 1α(OH)ase-/- mice and their wild-type littermates were fed either a normal diet or a rescue diet (high calcium, phosphate, and lactose) starting from weaning until 3 mo of age, then sperm spermatogenesis and motility were explored. Results showed that 1α(OH)ase-/- male mice displayed hypocalcemia and hypophosphatemia, lower concentration of intracellular calcium accompanied with reduced protein levels of calcium transport channels in testis, less sperm count, weaken sperm motility and abnormal sperm morphology at ultrastructure. Furthermore, the deficient spermatogenesis in 1α(OH)ase-/- male mice were deeply found to be concerned with decreased spermatogenic cells proliferation and increased spermatogenic cells apoptosis which might be caused by unusual serum gonadal hormone (estrogen and testosterone) and gonadotropic hormone (follicle-stimulating hormone and luteotrophic hormone) , unbalanced expression of apoptotic and pro-apoptotic proteins. When serum calcium and phosphorus were normalized by the rescue diet, the concentration of intracellular calcium in spermatogenic cells returned to normal level and the defective reproductive phenotype including impaired spermatogenesis and sperm motility in the 1α(OH)ase-/- male mice were reversed. These results indicate that the infertility seen in 1,25(OH)2D-deficient male mice is not a direct effect of active vitamin D deficiency on the reproductive system but is an indirect effect mediated by decreased extracellular /and or intracellular calcium concentration.

Key words: 1,25(OH)2D3; spermatogenesis; sperm motility

0 Introduction

Although Vitamin D is most closely associated with the control of calcium and bone metabolism, it is proposed many other effects on immune system, diabetes, cancer prevention and etc[1]. The importance of VD for male reproduction has been demonstrated in several studies[2, 3].The active form of vitamin D is $1,25(OH)_2D_3$, which is produced by 1 alpha hydroxylase (CYP27b1) from its precursor 25(OH)D. The production of the hormonally active form of vitamin D was traditionally thought to be accomplished exclusively within the kidney. Once produced in the kidney, $1,25(OH)_2D_3$ is released into the serum and acts as an endocrine hormone on the intestine, bone, and kidney to control calcium metabolism.

$1,25(OH)_2D_3$ is traditionally thought to exert its function by binding to the vitamin D receptor (VD-VDR). This complex forms a heterodimer with the retinoid receptor (VD-VDR-RXR) and binds to a vitamin D responsive element(VDRE) to regulate gene transcription[4]. However, there is growing evidence that $1,25(OH)_2D_3$ also has rapid actions by bindind to plasma membrane such as vitamin D binding protein or a membrane associated pool of the traditional VDR[5, 6].

In human, VDR and vitamin D metabolizing enzymes have been demonstrated to express in

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ejaculatory duct, germ cells and mature spermatozoa, which suggested vitamin D as an important role in spermatogenesis and sperm function[7, 8].VD deficiency in male rats results in reduced sperm counts, and female rats inseminated with semen from VD deficient male rats have lower fertility rates[3]. The impaired reproductive performance induced by VD deficiency is reversible and seems to be mediated predominantly through calcium imbalance, because it can be corrected either by supplying VD or by normalizing calcium levels[9]. As well as VD deficient male rats, VDR knockout mice models is characterized by an infertile phenotype including lower fertility, reduced sperm counts and a low number of motile spermatozoa. However, the infertile phenotype only could be partly restored by calcium supplement [10].

To identify the actions of vitamin D on male reproductive function and explore the mechanisms, 1α(OH)ase$^{-/-}$ male mice were used first time in the present study. 1α(OH)ase$^{-/-}$ male mice and their wild-type male littermates were fed either a normal diet or a rescue diet from weaning and then mated with wild-type female mice on the same diet until 3-mon old. The fertility efficiency of females was analyzed, spermatogenesis and sperm motility were examined by histopathological and molecular techniques.

1 Materials and methods

1.1 Animals and treatment

Generation and characterization of 1α(OH)ase$^{-/-}$mice were performed as previously described [11].1α(OH)ase$^{-/-}$mice were generated through breeding of heterozygousmice. The genotype of the mice was confirmed by PCR using mouse tail samples. Wild-type littermates were used as control animals in all experiments. The use of animals in this study was approved by the Institutional Animal Care and Use Committee of Nanjing Medical University. Five pairs of age- and sex-matched1α(OH)ase$^{-/-}$ and wildtype littermates were randomly divided into two groups. After weaning they were fed with a normal diet containing 1% calcium and 0.67% phosphorus or a rescue diet (TD96348 Teklad, Madison, WI) containing 2% calcium, 1.25% phosphorus, and 20% lactose for 3 mo.

1.2 Assessment of serum calcium 、phosphorus and reproductive hormones

Blood was collected intraorbitally 3-mon aged male mice.and sera were stored at -80℃. Five samples for each group. Serum calcium and phosphorus levels were analyzed by autoanalyzer (Beckman Synchron 67; Beckman Instruments). Serum testosterone and estrogen levels were measured with radioimmunoassay (Diagnostic Products, Los Angeles, CA). Serum luteinizing hormone (LH) and follicle-stimulating hormone (FSH) levels were measured by enzyme-linked immunosorbent assays (Amersham Pharmacia Biotech, Aylesbury, UK). The overall experimental design was performed twice to ensure that the data were repeatable.

1.3 Fertility ,sperm count and sperm motility

To determine fertility, individual WT or 1α(OH)ase$^{-/-}$ males were mated continuously with two WT females for 1 month, followed by a second month with two different females. All females were maintained for 1 month after the mating period.

Cauda epididymal sperm were collected from 3-mon aged mice, washed and uspended in PBS (140mMNaCl, 10mMphosphate buffer, pH 7.2), and then counted in a hemocytometer.

Quantitative parameters of sperm motility were determined by computer-assisted sperm analysis (CASA). Cauda epididymides were removed and transported in M16 medium on ice, and sperm were collected within 60 min. Sperm were collected in M16 (37 ℃, 5% CO2 and air), and CASA parameters were determined immediately after collection and after incubation for 1 h. Statistical analyses comparing WT and 1α(OH)ase$^{-/-}$. Differences were considered significant when $P < 0.05$.

1.4 Fluo-3 loading

The testes from WT or 1α(OH)ase$^{-/-}$ males were decapsulated and finely grinded in spermatogenic cell culture medium . The cells were subjected to serial filtration through Nitex mesh (1000 and 20μm), Miracloth, and glass wool. The resultant suspension was centrifuged and resuspended in Ham's F-12 Dulbecco's modified Eagle's medium, the final concentration is 10^6 cells ml^{-1}.

Changes in intracellular $Ca2^+$ concentration were detected with the fluorescent probe fluo-3AM. Briefly, the cells were loaded for 30 min at 25 ℃ with 5μ M fluo-3AM containing 1μ M pluronic acid F-127 for proper dispersal and 0.25 mM sulfinpyrazone, an organic anion transport inhibitor to reduce leakage of the fluo-3 dye. Just before use, the cells were washed with medium to remove non hydrolyzed fluo-3AM. Fluorescence measurements were performed at an excitation of 488 nm and the obtained mean fluorescence intensity is on behalf of intracellular calcium concentration.

1.5 Histology and Electron Microscopy

The testes and epididymides of WT or 1α(OH)ase$^{-/-}$ males were collected on 3-mon aged mice and were fixed in Bouin's solution. The fixed tissues were then paraffin-embedded, sectioned, and stained with hematoxylin and eosin for histological examination.

For transmission electron microscopy, cauda epididymides were fixed in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.15 M sodium phosphate buffer overnight at 4 ℃., postfixed in 2% osmium tetroxide in cacodylate buffer, and embedded in Lowicryl resin. Sections were stained with uranyl acetate and lead citrate and examined in a JEM-1010 transmission electron microscope at 80 kV. The transmission electron microscopes used were in the Nanjinj Medical University.

1.6 Immunochemistry staining

Immunohistochemical staining was carried out for Ki67 using the avidin-biotin-peroxidase

complex technique with affinity-purified mouse anti-Ki67 monoclonal antibody (Medicorp, Montreal, Canada) as described previously[12] . Briefly, dewaxed and rehydrated paraffin-embedded sections were incubated with methanol-hydrogen peroxide (1:10) to block endogenous peroxidase activity and then washed in Tris-buffered saline (pH 7.6). The slides were then incubated with the primary antibodies overnight at room temperature. After being rinsed with Tris-buffered saline for 15 min, tissues were incubated with secondary antibody for 45 min. Sections were then washed and incubated with the Vectastain Elite ABC reagent (Vector Laboratories, Burlington, ON, Canada) for 45 min. Staining was developed with 3,3=-diaminobenzidine (2.5mg/ml), followed by counterstaining with Mayer's hematoxylin.

1.7 Western blot analysis

Proteins were extracted from both testis and quantified with a protein assay kit (Bio-Rad, Mississauga, ON, Canada). Thirty-microgram protein samples were fractionated by SDS-PAGE and transferred to nitrocellulose membranes. Immunoblotting was carried out as described elsewhere(Xue Y 2005) with antibodies against TRPV5, cyclinE, 3βHSD VI, 17βHSD III, LHR, Bax, Bcl-xL (Santa Cruz) , CaSR (Abcam), CaV3.1(Chemicon), p-caspase3 and β-Actin(Bioworld Technology). Bands were visualized with ECL chemiluminescence (Amersham) and analyzed by Scion Image Beta 4.02 (Scion, National Institutes of Health).

1.8 Assessment of apoptosis

The in situ detection of cells with DNA strand breaks was carried out by the TUNEL method. Firstly, the serial sections were deparaffinized three times in xylene. The sections were then rehydrated through a series of decreasing concentrations of ethanol before the slides were washed in phosphate-buffered saline (PBS). After that, the sections were subjected to partial digestion with proteinase K (20 ḡ/ml) (Merck) at room temperature for 15 min and were washed two times in PBS. Thereafter, the sections were incubated at room temperature in an equilibration buffer. The tissue sections were incubated at 37 ℃ for 60 min with the TUNEL reaction mixture (70% reaction buffer, 30% TdT enzyme) in a humidified chamber in the dark. The slides were agitated and incubated in the stop/wash buffer for 10 min at room temperature. After the incubation, the slides were washed three times with PBS (phosphate-buffered saline). Then, anti-alkaline phosphatase conjugate was applied to the slides and incubated in a humidified chamber for 30 min at room temperature. The slides were washed four times with PBS. 100 mM Tris-maleate buffer containing naphthol AS-MX phosphate (0.2 mg/ml, dissolved in ethylene glycol monomethyl ether, both Sigma) as substrate and Fast Red TR (0.4 mg/ml, Sigma) as a stain for the reaction product. After washing with distilled water, the sections were counterstained with Vector Methyl Green nuclear counterstain (Vector Laboratories, Burlington, Ontario, Canada) and mounted with Kaiser's glycerol jelly.

1.9 Statistical analysis

Data from image analysis are presented as mean ± s.e.m. Statistical comparisons were made using a two-way ANOVA, with $P<0.05$ considered significant.

2 Results

2.1 Effects of $1,25(OH)_2D_3$ deficiency and dietary minerals on male fertility

To determine whether $1,25(OH)_2D_3$ deficiency resulted in male infertility mediated by extracellular calcium and phosphorus, $1\alpha(OH)ase^{-/-}$ or wild-type mice were fed on either the normal diet or the rescue diet after weaning. Each 6-week-old $1\alpha(OH)ase^{-/-}$ or wild-type male mice were mated with 2 wild-type females for 1 month, then with other 2 wild-type females for another month. When maintained on the normal diet, $1\alpha(OH)ase^{-/-}$ males sired no pups (n=5 males, n=20 females), whereas their wild-type littermates sired 149 pups and the average number of pups obtained by every wild-type male mice was 29.8±1.158. When fed with the rescue diet, $1\alpha(OH)ase^{-/-}$ and wild-type males displayed equally fertility, the number of offspring was 153 and 147, and the average number of pups obtained by every wild-type male mice was 30.6±1.077 and 29.4±0.87, respectively (Table 1).

Table 1. Effects of $1,25(OH)_2D_3$ deficiency and dietary minerals on male fertility

		$1\alpha(OH)ase^{+/+}$			$1\alpha(OH)ase^{-/-}$		
		Number of pups	Total number of pups	Average number of pups	Number of pups	Total number of pups	Average number o pups
Normal diet (n=5)	1	29	149	29.8±1.16	0	0	0^{***}
	2	33			0		
	3	27			0		
	4	28			0		
	5	32			0		
Rescue diet (n=5)	1	30	153	30.6±1.08	28	147	$29.4±0.87^{†††}$
	2	34			32		
	3	29			27		
	4	28			28		
	5	32			32		

Average number of pups is presented as means±SEM. ***: $P<0.001$, Different from wild-type littermates; †††: $P<0.001$, different from genotype-matched mice on ND.

2.2 Effects of $1,25(OH)_2D_3$ deficiency and dietary minerals on spermatogenesis

The process of spermatogenesis which makes stem spermatogonia matured into spermatozoa takes place in the seminiferous tubules of the testis. Persistant function of this process is a requirement for maintaining male fertility. To identify whether male infertility caused by $1,25(OH)_2D_3$ deficiency was associated with impaired spermatogenesis mediated by extracellular calcium and

phosphorus, we examined alterations of the morphology of testis and epididymis and the number of sperm in both genotype mice fed on either the normal diet or the rescue diet. Results showed that the sizes of testis and epididymis, the testis weight, the testis weight/body weight ratios and the number of sperms in the epididymis tails were reduced significantly in 1α(OH)ase$^{-/-}$ mice compared with their wild-type littermates when maintained on the normal diet (Figs. 1A-D). Histological analysis demonstrated that there were abnormalities in mutant seminiferous epithelium in 3-month-old 1α(OH)ase$^{-/-}$ mice. In stage Ⅶ–Ⅷ seminiferous tubules, the elongating spermatids were visible in both genotype males, but the spermatogenic cells were arranged disorder and a number of round spermids were observed to be shed from the seminiferous epithelium in 1α(OH)ase$^{-/-}$ mice (Fig. 1E). Consistent with sperm count results from the cauda epididymis, histological analysis of epididymis revealed that very few mature sperms were observed on the sections of the cauda epididymis from 1α(OH)ase$^{-/-}$ mice fed the normal diet (Fig. 1F). On the rescue diet, compared with genotype-matched male mice on the normal diet, the testis weight, the testis weight/body weight ratios, the number of sperms in the epididymis tails and histology of testis and epididymis were all normal in 3-month-old 1α(OH)ase$^{-/-}$ mice fed the rescue diet (Figs. 1A-F).

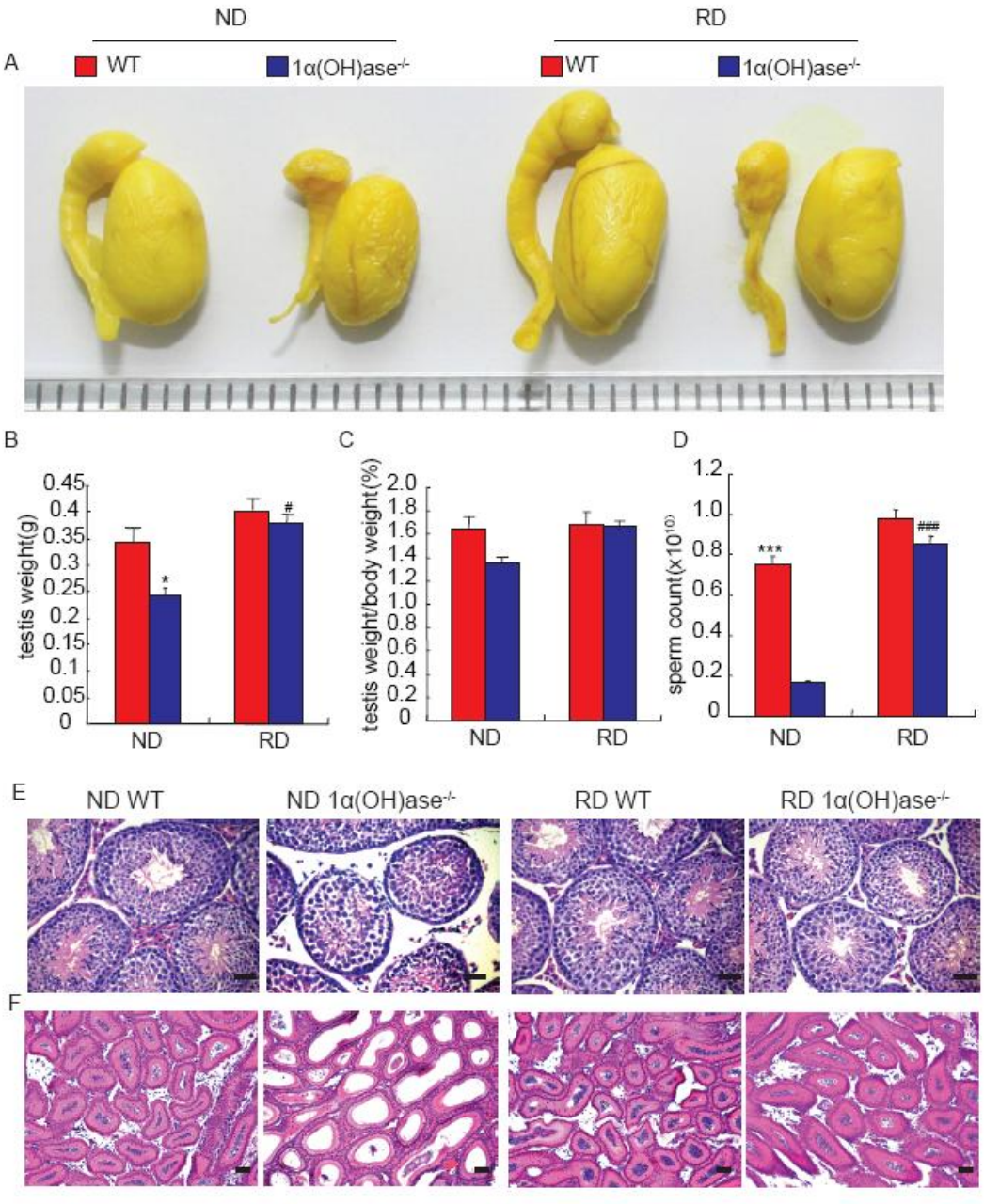

Fig. 1 Effects of $1,25(OH)_2D_3$ deficiency and dietary minerals on spermatogenesis (A) Representative images of testis from 3-month-old wild-type (WT) and 1α(OH)ase$^{-/-}$ male mice fed a normal diet (ND) or a rescue diet (RD). (B) The testes weight. (C) The testes weight/body weight ratios. (D) The sperms were collected from the cauda epididymides and counted. Representative hematoxylin and eosin-stained micrographs of paraffin-embedded sections of (E) testes and (F) the cauda epididymides. Scale bar= 50μm. Different from wild-type mice on the same diet: **P* < 0.05, ****P*<0.001; different from genotype-matched mice on ND: #*P*<0.05, ### *P* <0.001.

2.3 Effects of $1,25(OH)_2D_3$ deficiency and dietary minerals on sperm morphology and motility

To identify whether male infertility caused by $1,25(OH)_2D_3$ deficiency was associated with sperm morphological abnormalities and asthenospermia mediated by extracellular calcium and

phosphorus, we examined alterations of sperm morphology and motility in both genotype mice fed on either the normal diet or the rescue diet using transmission electron microscopy and CASA assay. The observations of transmission electron microscopy detected distinctly ultrastructural abnormalities in sperm head including misshaped apical parts of sperm head and cell membrane swelled in 1α(OH)ase$^{-/-}$ mice fed the normal diet (Fig. 2A). However, no distinctly ultrastructural abnormalities were detected in fibrous sheath structures of sperm principal piece on either in sagittal sections or in cross sections in 1α(OH)ase$^{-/-}$ mice fed the normal diet (Figs. 2B and C). Results from CASA assay showed that the percentage of motile sperms, Average path velocity (VAP) and mean straight-line velocities (VSL) of sperms were reduced dramatically in 1α(OH)ase$^{-/-}$ mice compared with their wild-type littermates when maintained on the normal diet (Figs. 2D-F). On the rescue diet, compared with genotype-matched female mice on the normal diet, the sperm morphology and motility were all normal in 1α(OH)ase$^{-/-}$ mice fed the rescue diet (Figs. 2A-F).

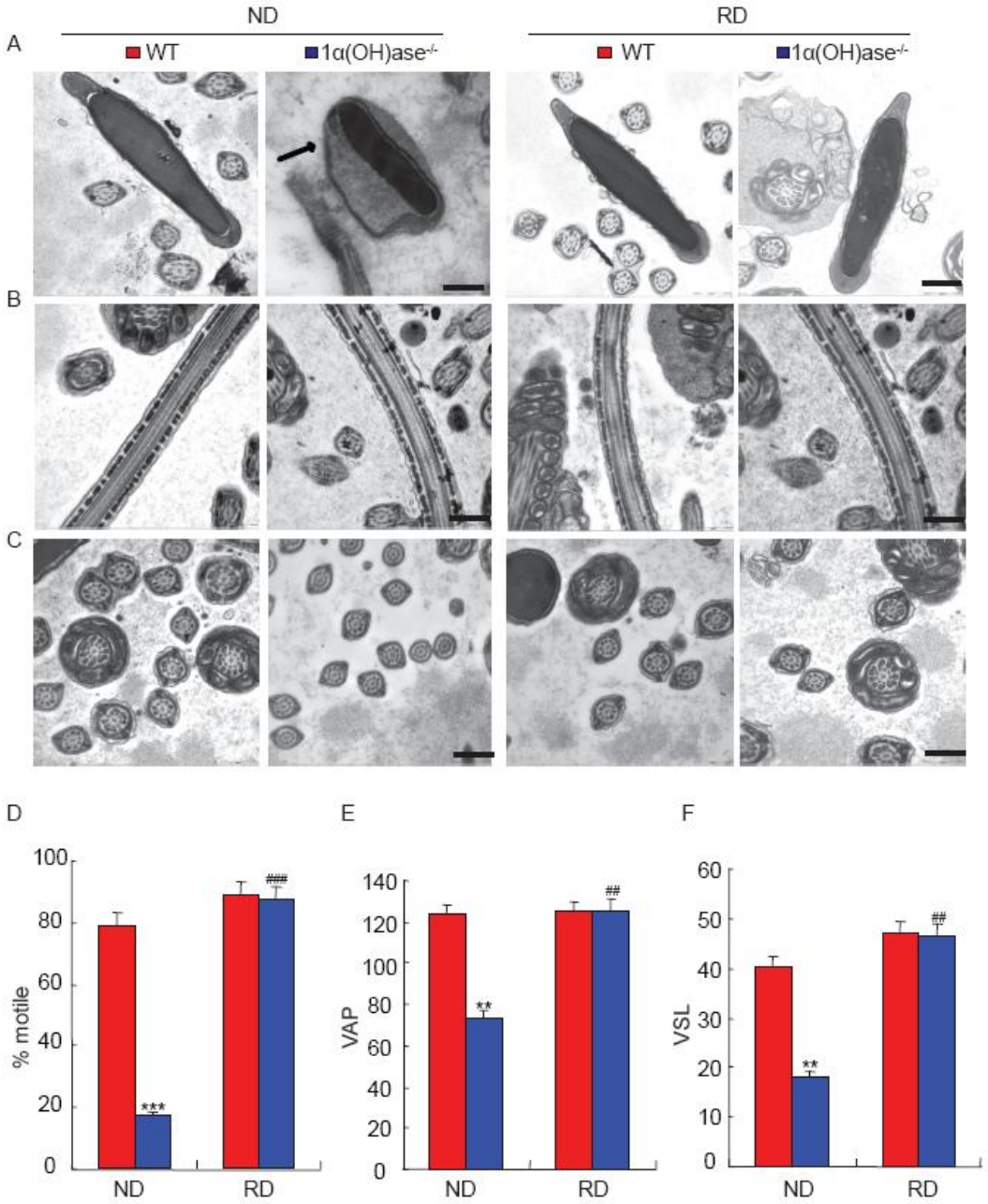

Fig. 2 Effects of 1,25(OH)$_2$D$_3$ deficiency and dietary minerals on sperm morphology and motility (A-C) Representative transmission electron micrographs of (A) sperm heads and sperm principal piece on (B) sagittal sections and (C) cross-sections from 3-month-old wild-type (WT) and 1α(OH)ase$^{-/-}$ male mice fed a normal diet (ND) or a rescue diet (RD). Scale bar=1μm. (D-F) Sperms were collected from 3-month-old WT and 1α(OH)ase$^{-/-}$ mice on either ND or RD and were analyzed using CASA assay and presented sperm motility parameters as (D) % motile, (E) VAP and (F) VSL. Different from wild-type mice on the same diet: ** $P < 0.01$, *** $P<0.001$; different from genotype-matched mice on ND: ## $P<0.01$, ### $P <0.001$.

2.4 Effects of 1,25(OH)$_2$D$_3$ deficiency and dietary minerals on the proliferation of spermatogenic cells

Maintenance of normal spermatogenesis is determined by a dynamic balance between spermatogenic cells proliferation and apoptosis[13] . To determine whether impaired spermatogenesis caused by 1,25(OH)$_2$D$_3$ deficiency was associated with defects in the proliferation of spermatogenic cells mediated by extracellular calcium and phosphorus, we examined alterations of the proliferation of spermatogenic cells and expression levels of cell proliferation regulating molecules in both genotype mice fed on either the normal diet or the rescue diet by immunohistochemistry and Western blots. Results showed that the number of Ki67 positive spermatogenic cells (Fig. 3A-B). On the rescue diet, compared with genotype-matched female mice on the normal diet, the proliferation of spermatogenic cells was all normal in 1α(OH)ase$^{-/-}$ mice fed the rescue diet (Fig. 3).

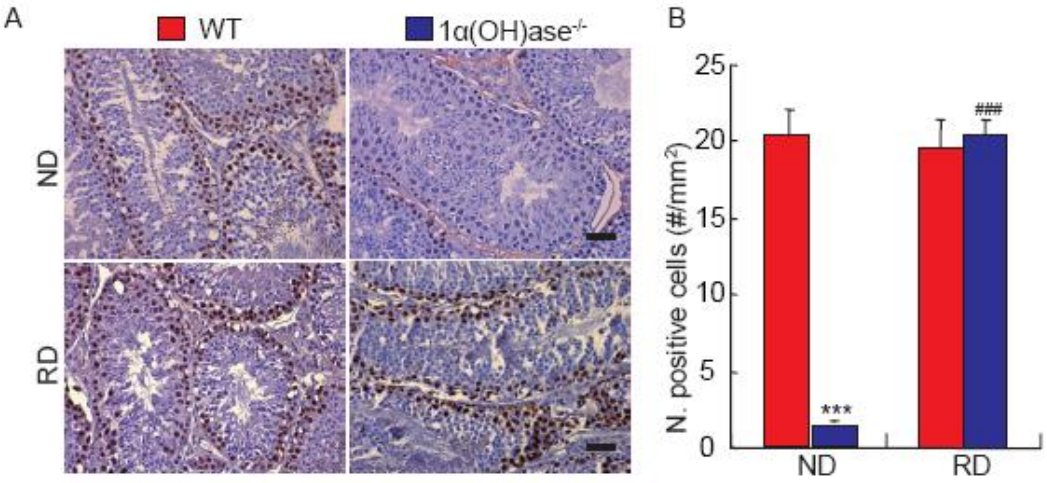

Fig. 3. Effects of 1,25(OH)$_2$D$_3$ deficiency and dietary minerals on the proliferation of spermatogenic cells
(A) Representative micrographs of testis paraffin sections stained immunohistochemically for Ki67 from 3-month-old wild-type (WT) and 1α(OH)ase$^{-/-}$ male mice fed a normal diet (ND) or a rescue diet (RD). Scale bar=50μm (B) The number of Ki67 positive spermatogenic cells in unit area (#/mm^2). Different from wild-type mice on the same diet: ***P*< 0.01, ****P*<0.001; different from genotype-matched mice on ND: ##*P* < 0.01, ### *P* <0.001.

2.5 Effects of 1,25(OH)$_2$D$_3$ deficiency and dietary minerals on the apoptosis of spermatogenic cells

To determine whether impaired spermatogenesis caused by 1,25(OH)$_2$D$_3$ deficiency was associated with increased spermatogenic cell apoptosis mediated by extracellular calcium and phosphorus, we examined alterations of the apoptosis of spermatogenic cells and expression levels of cell apoptosis regulating molecules in both genotype mice fed on either the normal diet or the rescue diet by TUNEL and Western blots. Results showed that the number of apoptotic spermatogenic cells (Fig. 4A-B) and the pro-apoptotic protein expression levels of Bax and p-caspase 3 were increased significantly, whereas anti-apoptotic protein expression levels of

Bcl-xl were down-regulated dramatically in testes from 1α(OH)ase$^{-/-}$ mice compared with those from wild-type mice fed the normal diet (Figs. 4C-F). On the rescue diet, compared with genotype-matched female mice on the normal diet, the apoptosis of spermatogenic cells and expression levels of Bax, p-caspase 3 and Bcl-xl were all normal in 1α(OH)ase$^{-/-}$ mice fed the rescue diet (Fig. 4).

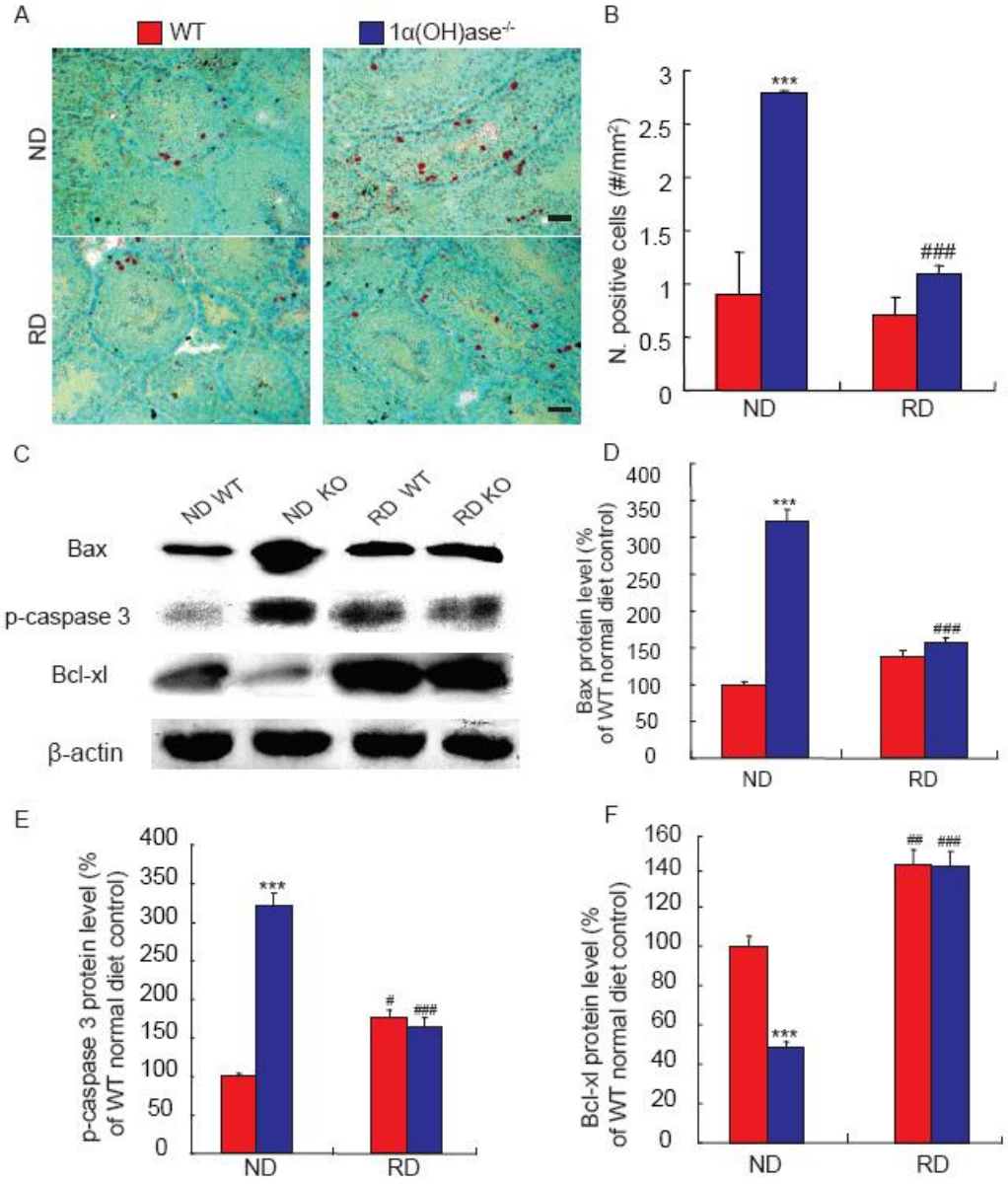

Fig. 4. Effects of 1,25(OH)$_2$D$_3$ deficiency and dietary minerals on the apoptosis of spermatogenic cells (A) Representative micrographs of testis paraffin sections stained with TUNEL from 3-month-old wild-type (WT) and 1α(OH)ase$^{-/-}$ male mice fed a normal diet (ND) or a rescue diet (RD). Scale bar=50μm (B) The number of TUNEL positive spermatogenic cells in unit area (#/mm^2). (C) Representative Western blots of testis extracts for expression of Bax, p-caspase 3 and Bcl-xl. Actin was used as loading control for Western blots. (D) Bax, (E) p-caspase 3, (F) Bcl-xl protein levels relative to actin protein level were assessed by densitometric analysis and expressed relative to the levels of WT mice on ND. Different from wild-type mice on the same diet: **P< 0.01, ***P<0.001; different from genotype-matched mice on ND: # P < 0.05, ##P < 0.01, ### P <0.001.

2.6 Effects of $1,25(OH)_2D_3$ deficiency and dietary minerals on the function of hypothalamic-pituitary-gonadal axis

To determine whether impaired spermatogenesis caused by $1,25(OH)_2D_3$ deficiency was associated with impaired function of hypothalamic-pituitary-gonadal axis mediated by extracellular calcium and phosphorus, we examined alterations of serum follicle stimulating hormone (FSH), luteinizing hormone (LH), oestradiol and testosterone and expression levels of hydroxysteroid dehydrogenases and LH receptor in both genotype mice fed on either the normal diet or the rescue diet. Results showed that serum FSH and LH levels were raised significantly (Figs. 5A-B), whereas serum oestradiol and testosterone levels and the expression levels of 3β-hydroxysteroid dehydrogenase type VI (3βHSD VI), 17β-hydroxysteroid dehydrogenase type type III (17βHSD III) and LH receptor in testes were decreased significantly in $1\alpha(OH)ase^{-/-}$ mice compared with the wild-type mice fed the normal diet (Figs. 5C-H). On the rescue diet, compared with genotype-matched female mice on the normal diet, serum FSH, LH, oestradiol and testosterone levels and the expression levels of 3β-hydroxysteroid dehydrogenase type VI (3βHSD VI), 17β-hydroxysteroid dehydrogenase type type III (17βHSD III) and LH receptor in testes were all normal in $1\alpha(OH)ase^{-/-}$ mice fed the rescue diet (Fig. 5).

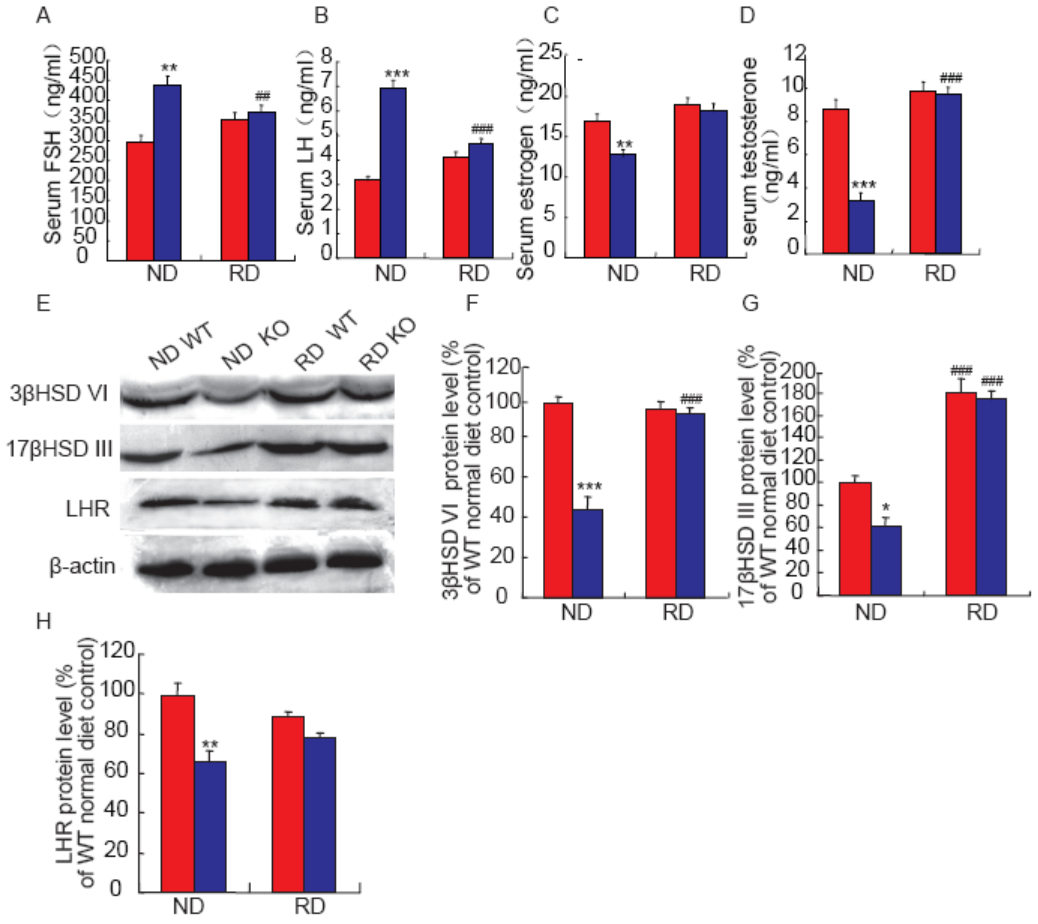

Fig. 5. Effects of $1,25(OH)_2D_3$ deficiency and dietary minerals on the function of hypothalamic-pituitary-gonadal axis (A) follicle stimulating hormone (FSH), (B) luteinizing hormone (LH), (C)

oestradiol, (D) testosterone levels were determined in 3-month-old wild-type (WT) and 1α(OH)ase$^{-/-}$ male mice fed a normal diet (ND) or a rescue diet (RD). (E) Representative Western blots of testis extracts for expression of 3βHSD VI, 17βHSD III and LHR. Actin was used as loading control for Western blots. (F) 3βHSD VI, (G) 17βHSD III and (H) LHR protein levels relative to actin protein level were assessed by densitometric analysis and expressed relative to the levels of WT mice on ND. Different from wild-type mice on the same diet: *P < 0.05, **P< 0.01, ***P<0.001; different from genotype-matched mice on ND: ##P < 0.01, ### P <0.001.

2.7 Effects of 1,25(OH)$_2$D$_3$ deficiency and dietary minerals on extracellular and intracellular calcium concentrations and Ca^{2+} transpoters

To determine whether the normalization of male reproductive defects occurred in 1α(OH)ase$^{-/-}$ mice by rescue diet was associated with disorders of calcium transport, we examined alterations of serum calcium and phosphorus levels and intracellular calcium concentrations and the expression levels of calcium sensing receptor (CaSR), CaV3.1, a voltage gated Ca^{2+} channel, and TRPV5, an excitomotor- receptor gated Ca^{2+} channel in testis tissue. Results showed that serum calcium and phosphorus levels (Figs. 6A-C) and the expression levels of CaSR, CaV3.1 and TRPV5 in testes were down-regulated dramatically (Figs. 6D-G) in 1α(OH)ase$^{-/-}$ mice compared with the wild-type mice fed the normal diet (Figs. 5C-H). On the rescue diet, compared with genotype-matched female mice on the normal diet, serum calcium and phosphorus levels and intracellular calcium concentrations were all normal, whereas the expression levels of CaSR, CaV3.1 and TRPV5 in testes were all up-regulated significantly in 1α(OH)ase$^{-/-}$ mice fed the rescue diet (Fig. 6).

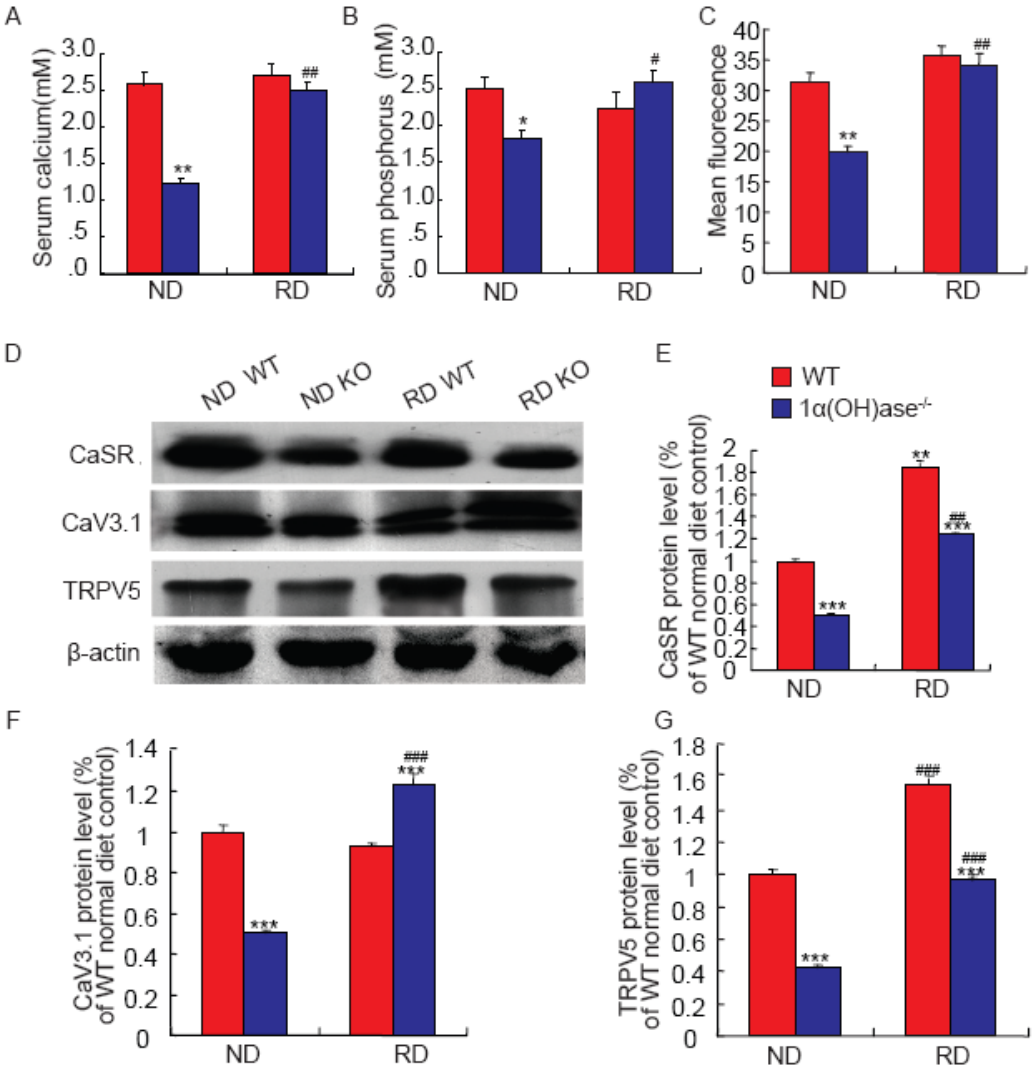

Fig. 6. Effects of 1,25(OH)$_2$D$_3$ deficiency and dietary minerals on extracellular and intracellular calcium concentrations and protein levels of Ca^{2+} channels (A) Serum calcium, (B) phosphorus were determined in 3-month-old wild-type (WT) and 1α(OH)ase$^{-/-}$ male mice fed a normal diet (ND) or a rescue diet (RD). (C) Intracellular calcium concentrations were measured in cells isolated from testes by fluo-3 fluorescence staining and flow cytometric analysis and presented as mean fluorescence intensity. (D) Western blots of testis extracts for the expression of CaSR, CaV3.1 and TRPV5. β-tubulin was used as loading control for Western blots. (E) CaSR, (F) CaV3.1 and (G) TRPV5 protein levels relative to β-tubulin protein level were assessed by densitometric analysis and expressed relative to the levels of WT mice on ND. Different from wild-type mice on the same diet: *P < 0.05, **P< 0.01, ***P<0.001; different from genotype-matched mice on ND: #P<0.05, ##P < 0.01, ### P <0.001.

3 Discussion

In the present study, we showed here for the first time that 1α(OH)ase mutant male mice accompanied with hypocalcemia and hypophosphatemia displayed injured fertile rates, histological abnormalities of the testis and abnormal sperm parameters including decreased sperm counts, reduced sperm motility and increased aberrant sperm morphology. Unlike the VDR knockout male mice, impaired reproductive performance in 1α(OH)ase$^{-/-}$ male mice could be

restored by calcium and phosphate supplements completly. Although it is a primary descriptive study, our findings expand on earlier analyses by measuring spermatogenesis and motility, further demonstrate that the sperm population decreased in 1α(OH)ase$^{-/-}$ male mice is resulted from inhibition of proliferation and elevated apoptosis occuring in the seminiferous tubules and the reduced sperm motility is due largely to lower concentration of calcium.

As we know, the quality and quantity of produced semen depend on normal function of the testicular structures and reproductive hormones. The steroid hormone production is an enzymatic-mediated process catalyzed by several enzymes from two main categories: the cytochrome P450 enzymes (CYP11A and CYP17A), and hydroxysteroid dehydrogenase (HSD) enzymes (3β-HSD and 17β-HSD)[14]. The altered serum levels of these steroid hormones may cause subsequent reproductive dysfunction by interfering with the feedback regulatory mechanisms of the hypothalamic-pituitary-gonadal axis. In addition, the cytoplasmic co-expression of VDR and the metabolizing enzymes in Leydig cells suggest that VD might affect male reproductive hormone production. In the present study, plasma concentrations of oestradiol and testosterone were reduced in 1a(OH)ase mutant male mice, while FSH and LH levels were increased in large part related to hypothalamic- pituitary-testicular axis function. As an indicator of Leydig cell steroidogenic activity, we measured the the protein levels of 3βHSD VI and 17βHSD III expressed in testes. In accordance with serum testosterone levels, the expressions of 3βHSD VI and 17βHSD III were significantly down-regulated in mutant testes.

The Bcl-2 family of proteins, which contains both pro-apoptotic (Bax, Bak, Bcl-xs, Bad) and anti-apoptotic (Bcl-2, Bcl-xL, Mcl, A1) proteins, constitutes a critical, intracellular checkpoint within a common cell-death pathway that determines the susceptibility. It is generally believed that the ratio of pro-apoptotic to anti-apoptotic Bcl-2 family proteins is the critical determinant of cell fate, with an excess of Bcl-2 resulting in cell survival but an excess of Bax resulting in cell death. We show here that the anti-apoptotic protein such as Bcl-xL is down-regulated and the pro-apoptotic protein Bax is up-regulated in 1a(OH)ase mutant male mice. These results imply that the reduced sperm count in 1α(OH)ase$^{-/-}$ male mice might be resulted from the abnormal genic expressions which induced imbalance of proliferation and apoptosis among spermatogenic cells.

The motility of mammalian sperm is known to be mediated by the motor protein dynein[15]. The action of dynein causes shear displacement between the microtubule outer doublets of the sperm axoneme, which in turn causes the flagellum to bend. The bending of the flagellum in this manner is the basis of the flagellar beat. Beyond that, the basic underlying motor process is subject to regulatory control from Ca^{2+} . Ca^{2+} has the effect of converting sperm from a relatively linear, symmetrical swimming pattern to an asymmetrical form of motility called hyperactivated motility, which is essential for spermatogenesis, sperm motility, hyperactivation and acrosome reaction [16].

$1,25(OH)_2D_3$ plays not only a pivotal role in systemic Ca^{2+} homeostasis but also in the intracellular Ca^{2+} homeostasis. Calcium can be mobilized into sperm from the external milieu by plasma membrane channels, and it is also released internally from intracellular stores, such as the redundant nuclear envelope located at the base of sperm flagellum or the acrosome[17, 18]. A number of calcium channel proteins have been identified in sperm by immunohistochemisty or by measuring specific channel activities, and are regulated by voltage or other mechanisms[19]. In this article, we didn't detect the calcium in seminal fluid for technic limitation. However, the lower concentration of calcium in seminal fluid from 1a(OH)ase mutant male mice could be infered from significantly reduced serum calcium concentration and decreased intracellular calcium concentration was confirmed by the results of the fluo-3 loading experiment and reduced expressions of calcium transports such as TRPV5、CaV3.1 and CaSR. Combined with the normal morphology of sperm flagellum, we concluded that the impaired movement patterns observed in t1α(OH)ase$^{-/-}$ male mice might be majorly resulted from the lower calcium concertration.

In summary, the data presented here suggests that the absence of $1,25(OH)_2D_3$ results in male infertility with impaired spermatogenesis and sperm motility, due to an increased spermatogenic cells apoptosis and reduced calcium concertration. These abnormalities in the mouse male reproductive system were normalized by dietary supplementation of calcium and phosphate. These results support the concept that the regulation of $1,25(OH)_2D_3$ in the male reproductive systen is mediated through extracellular calcium and phosphate.

4 Conclusion

In this paper, we confirmed that absence of $1,25(OH)_2D_3$ resulted in deficient male reproduction through reduced concentrations of extracellular calcium and phosphate.

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活性维生素 D 缺乏通过细胞外钙磷介导导致雄性小鼠不育

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摘要：探讨活性维生素 D 缺乏以及钙磷补充对青年雄性小鼠生殖系统功能的影响。方法 将同窝野生型和 1α-羟化酶基因敲除纯合子（1α(OH)ase-/-）小鼠分笼后分别给予正常饮食和纠正饮食至 3 月龄后观察其生殖能力，并从精子发生和精子运动能力两方面对生育能力变化的原因和机制进行研究。结果 同正常饮食的野生型小鼠相比，1α(OH)ase-/-小鼠表现为低钙、低磷血症，睾丸组织细胞内游离钙离子浓度减少，钙通道蛋白表达减少；睾丸、附睾形态异常；精子数量减少，运动能力降低，电镜结果显示精子的超微结构并未有明显变化。通过进一步研究发现 1α(OH)ase-/-小鼠血清性腺激素（雌激素、睾酮）以及促性腺激素（卵泡刺激素、黄体生成素）的水平异常，睾丸组织内促凋亡蛋白表达增加，凋亡抑制蛋白表达减少，最终导致生精细胞增殖减少，凋亡增加，精子发生异常。给予纠正饮食后，1α(OH)ase-/-雄性小鼠生殖功能的异常得到纠正。结论 活性维生素 D 通过细胞外钙磷介导导致青年雄鼠不育。

关键词：活性维生素 D；精子发生；精子运动
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