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Vitamin D deficiency impairs testicular development and spermatogenesis in mice

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ABSTRACT

Vitamin D deficiency is prevalent especially in men. Nevertheless, whether vitamin D deficiency impairs male reproduction remains under debate. The aim of this study is to investigate whether vitamin D deficiency has an impact on testicular development and spermatogenesis in mice. In the control group and vitamin D deficient (VDD) diet group, dams and their pups were fed with standard-chow diet and VDD diet, respectively. Interestingly, testicular weight and sperm quality are reduced, testicular germ cell proliferation is suppressed, and the percentage of mature seminiferous tubules is decreased in VDD diet-fed mice. Moreover, testicular testosterone (T) synthesis enzymes are down-regulated in VDD diet-fed mice. Correspondingly, serum and testicular T levels are reduced in VDD diet-fed mice. Importantly, fertility index is reduced and live fetuses are decreased when both males and females are fed with VDD diet. These results provide evidence that vitamin D deficiency impairs testicular development and spermatogenesis.

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Vitamin D is a secosteroid hormone and essential for the maintenance of calcium uptake and bone metabolism, with severe vitamin D deficiency in children resulting in the occurrence of rickets [1–3]. Vitamin D deficiency (25-(OH)D lower than 20 ng/ml) is increasingly recognized as a public health problem [4,5]. Several reports showed that vitamin D deficiency was associated with adverse pregnant outcomes [6–8]. Moreover, maternal vitamin D deficiency programs demonstrate reproductive dysfunction in adult female offspring through adverse effects on hypothalamic function [9]. On the other hand, numerous studies demonstrated that vitamin D deficiency was associated with increased risk of metabolic disorders, such as insulin resistance and non-alcoholic fatty liver disease among overweight children [10–13].

It is increasingly recognized that vitamin D itself is no activity. Vitamin D is converted to 25-(OH)D by cytochrome P450 (CYP)2R1 and is then converted into 1,25(OH)2D3, the active form of vitamin D, by CYP27B1 [14,15]. The actions of vitamin D are mediated by vitamin D receptor (VDR) that binds 1,25(OH)2D3 [16]. Several studies demonstrate that all components that mediate vitamin D activity, such as VDR, CYP2R1 and CYP27B1, are highly expressed in testis, epididymis, seminal vesicle, prostate and spermatozoa [17,18], suggesting that vitamin D is important for spermatogenesis and sperm maturation. Nevertheless, whether vitamin D deficiency impairs spermatogenesis and steroidogenesis remains under debate. According to a small cross-sectional study, low vitamin D status was not a risk factor for poor semen quality in a population of young healthy men [19]. By contrast, a recent study showed that vitamin D deficiency was linked with semen quality and sex steroid levels in infertile men [20].

The aim of this study was to investigate the effects of vitamin D deficiency on testicular development and spermatogenesis in mice. Our results showed that testicular weight, mature seminiferous tubules and sperm quality were reduced in vitamin D deficient (VDD) diet-fed mice. Moreover, testicular testosterone (T) synthesis enzymes were downregulated. In addition, serum and testicular T levels were reduced. Importantly, fertility index and live fetuses were decreased when both males and females were fed with VDD

Abbreviations: VitD3, Vitamin D3; VDD, Vitamin D deficiency; FSH, follicle-stimulating hormone; LH, luteinizing hormone; T, testosterone; StAR, steroidogenic acute regulatory protein; CYP11A1, P450scc; CYP17A1, Cytochrome P450 17A1; 17βHSD, 17β-Hydroxysteroid dehydrogenase; 3β-HSD, 3β-Hydroxysteroid dehydrogenase.

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diet. The present study provides evidence that vitamin D deficiency impairs testicular development and spermatogenesis.

1. Materials and methods

1.1. Chemicals and reagents

StAR, CYP11A1, CYP17A1, 17 β -HSD, 3 β -HSD and β -actin antibodies were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA, USA). Chemiluminescence (ECL) detection kit was from Pierce Biotechnology (Rockford, IL, USA). Mouse luteinizing hormone (LH) and follicle-stimulating hormone (FSH) enzyme-linked immunosorbent assay (ELISA) kits were purchased from USCN Life Science & Technology Co. (Wuhan, China). 125I-based T radioimmunoassay (RIA) kits were purchased from Beijing North Institute of Biological Technology (Beijing, China). All the other reagents were from Sigma or as indicated in the specified methods.

1.2. Animals and treatments

Adult male ICR mice (10 week-old, 34–36 g) and female ICR mice (10 week-old, 32–34 g) were purchased from Beijing Vital River whose foundation colonies were all introduced from Charles River Laboratories, Inc (Wilmington, MA, USA). The animals were allowed free access to fodder and water at all times and were maintained on a 12 h light/dark cycle in a controlled temperature (20–25 °C) and humidity (50 \pm 5%) environment. After acclimatization and quarantine, four female mice were mated with two males in a cage. All pregnant mice were naturally delivered their pups. Within 24 h after birth, excess pups were removed, so that only eight pups (four males and four females) per dam were kept. Dams and their pups were then randomly assigned into two groups (each group is comprised of 6 dams, 48 male pups and 48 female pups) as follows. In the control group, dams and their pups were fed with standard-chow diets (Standard AIN93G Rodent diet with 1000 IU vitamin D₃/kg). In the vitamin D deficient (VDD) diet group, dams and their pups were fed with VDD diet (lower than 25 IU vitamin D₃/kg). Food composition is shown in Supplemental Table 1. Total sugars, total fat, total protein, total calories, calorie from standard-chow and vitamin D3 content are shown in Supplemental Table 2. Mice were inspected daily for food intake and weighted weekly. Seven weeks after diet intervention (10 weeks old), 12 males each group were anesthetized using a mixture of isoflurane and oxygen and then sacrificed with dislocation of cervical vertebrae, followed by cervical dislocation upon confirming lack of toe pinch reflex. Blood samples from eye socket were collected for 25(OH)D and hormones. The cauda epididymides were collected for sperm analysis. Testes were excised, dissected, weighed, and then divided into two parts: left one was frozen immediately in liquid nitrogen for immunoblot and testicular T measurement; the other part of the testes was preserved in mDF for 6 h and then fixed using 4% PFA for 18 h. To investigate the effects of vitamin D deficiency on mating behavior, fertility index and pregnant outcomes, 24 female pups each group were mated with 12 male pups (two females to one male) either from the VDD diet group or the control group for five consecutive days. Females were checked daily in the morning and the presence of a vaginal plug was designated as successful mating. Mating index was calculated as follow: mating index (%) = number of vaginal plug-positive females/number of mating females \times 100. Fertility index were calculated as follow: fertility index (%) = number of pregnant females/vaginal plug-positive females \times 100. All pregnant mice were anesthetized with a mixture of isoflurane and oxygen and sacrificed on GD13. The uterine horns were exposed. Live, dead and resorbed fetuses were counted. All procedures on animals followed the guidelines for humane treatment set by the Association

of Laboratory Animal Sciences and the Center for Laboratory Animal Sciences at Anhui Medical University (Permit Number: 15-0013). In the study, all mice were monitored at least twice per day. In addition, the rules of humane endpoints were strictly performed to determine when mice should be euthanized. All efforts were taken to minimize suffering when mice met our euthanasia criteria.

1.3. Serum 25-(OH)D

Radioimmunoassay (RIA) kits were used to measure the concentration of serum 25-(OH)D [8]. The repeatability was determined through measuring the concentration of quality controls. The controls fell within the acceptable range given by the manufacturer. The 25-(OH)D RIA kits' detection limits is from 1.5 to 100 ng/ml. In the present study, all values fell within the detection limits.

1.4. Sperm analysis

Male offspring were sacrificed at 10 weeks old. Whole cauda epididymis was collected for sperm analysis and the sperm unit was 10⁶ per gram of tissue weight. Sperm were counted according to a previous protocol [21]. Sperm viability was evaluated by staining with eosin-nigrosin according to a method described in a previous study [22]. Unstained (intact membrane) and red-stained (disrupted membrane) spermatozoa were counted under oil-immersion light microscopy at \times 100 magnification. Sperm viability is expressed as the percentage of cells with an intact membrane.

1.5. Testicular histology

Male offspring were sacrificed at 10 weeks old. Testes were excised and were fixed using mDF for 6 h and then 4% PFA for 18 h. Paraffin-embedded testes were cut 5 μ m thick and were mounted upon glass slides. Sections were stained by hematoxylin and eosin (H&E) for morphological analysis. The seminiferous tubules were classified according to a previous study [23]. Each experiment was repeated three times.

1.6. Immunohistochemistry

Testicular germ cell proliferation was identified by staining for PCNA. Sections of 5 μ m thick were dewaxed and rehydrated. Antigen retrieval was performed by pressure cooking slides. Non-specific binding sites were blocked with 5% (w/v) BSA before the addition of goat polyclonal antibody against PCNA (1: 200, Santa Cruz, USA). Immunostaining was developed by application of diaminobenzidine, and slides were counterstained with hematoxylin, dehydrated. The number of PCNA-positive cells was counted in twelve randomly selected fields from each slide at a magnification of 400 \times .

1.7. Terminal dUTP nick-end labeling (TUNEL) staining

Testicular germ cell apoptosis was detected with the TUNEL technique using an in situ apoptosis detection kit (Cat# G7130; Promega, Madison, WI, USA) according to protocols as described by previous study with minor modification [24]. The number of TUNEL-positive cells was counted in twelve randomly selected fields from each slide at a magnification of 400 \times .

1.8. Immunoblot

Testicular samples were homogenized in lysis buffer and then were centrifuged at 15,000g for 15 min. Supernatants from each

Table 1
Primers for Real-Time PCR.

Genes	Sequences	Sizes(bp)
18S	Forward: 5'-GTAACCGTTGAACCCATT-3' Reverse: 5'-CCATCCAATCGGTAGTAGCG-3'	151
17 β -hsd	Forward: 5'-TCACGATCGGAGCTGAATCC-3' Reverse: 5'-CAGGAGGAATCGTTGAGCGG-3'	113
3 β -hsd	Forward: 5'-TTTTCAGCCACCACCATCTCA-3' Reverse: 5'-GGTCTGTCTTCCAGTGATT-3'	137
Cyp11a1	Forward: 5'-AAGACCTGGAAGGACCATGC-3' Reverse: 5'-CACCAGGTAAGTGGCTGAAG-3'	118
Cyp17a1	Forward: 5'-GCTTCTGGTGACCAATCC-3' Reverse: 5'-AGGAGTGAGTCCGGTCATT-3'	107
Star	Forward: 5'-ATGGCCACACATTTGGGGA-3' Reverse: 5'-ACTGAGCAGCCAAGTGAGTT-3'	129

sample were added to a gel loading buffer and boiled for 5 min. Proteins in loading buffer were subjected to electrophoresis in 10% SDS polyacrylamide gel for 3 h. The gel was transferred electrophoretically onto a polyvinylidene fluoride membrane (Immobilon-P; Millipore, Bedford, MA) and blocked in 5% nonfat powdered milk in Dulbecco's PBS (DPBS) overnight at 4 °C. The membranes were then incubated for 2 h with rabbit polyclonal antibodies against mouse StAR, CYP11A1, CYP17A1, 17 β -HSD, 3 β -HSD or α -tubulin at room temperature. After incubate with goat anti-rabbit IgG antibody, the membranes were colored using an enhanced ECL detection kit.

1.9. Isolation of total RNA and real-time RT-PCR

Total RNA in mouse testes was extracted using TRI reagent. RNase-free DNase treated total RNA (1.0 mg) was reverse-transcribed with AMV (Pregmaga). Real-time RT-PCR was performed with a LightCycler 480 SYBR Green I kit (Roche Diagnostics GmbH) using gene-specific primers as listed in Table 1. The amplification reactions were carried out on a LightCycler 480 Instrument (Roche Diagnostics GmbH) with an initial hold step (95C for 5 min) and 45 cycles of a three step PCR (95C for 15 s, 60C for 15 s, 72C for 30 s).

1.10. Radioimmunoassay (RIA)

Serum was separated by centrifugation and stored at –80 °C. Steroids were solubilized in PBS. Serum and testicular T concentrations were measured using ¹²⁵I-based T RIA kits according to the manufacturer's protocol. The T RIA kits' detection limits is from 10 to 800 pg/ml. In the present study, all values fell within the detection limits.

1.11. Enzyme-linked immunosorbent assay (ELISA)

A competitive inhibition enzyme immunoassay was used to determine levels of serum LH and FSH according to the manufacturer's protocol. The LH ELISA kits' detection limits is from 0.1 to 8 mIU/ml. The FSH ELISA kits' detection limits is from 1.0 to 80 mIU/ml. In the present study, all values fell within the detection limits.

1.12. Statistical analysis

Shapiro-Wilk test was used to determine normality of the data. In the present study, all data were normally distributed and were expressed as means \pm SEM. ANOVA and Student *t* test were used to determine differences between two groups. *P* < 0.05 was considered statistically significant.

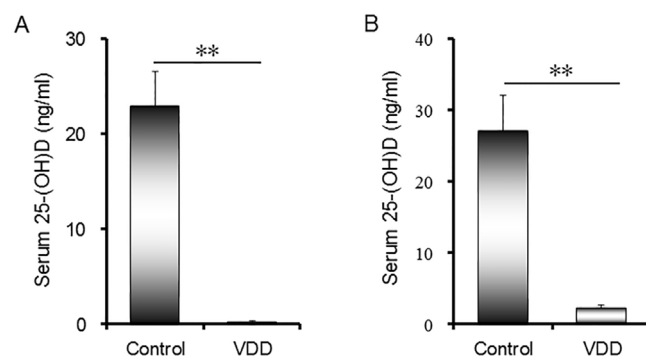


Fig. 1. The effects of vitamin D deficiency diet on serum 25-(OH)D concentration. In the control group, dams and their pups were fed with standard-chow diets. In the VDD diet group, dams and their pups were fed with vitamin D deficient diets, in which vitamin D was depleted. (A) Serum 25-(OH)D of male offspring was measured at seven weeks after diet intervention. (N = 12). (B) Female mice were mated and all pregnant mice were sacrificed on gestational day 13. Serum 25-(OH)D of female mice was measured (N = 24). Data were expressed as means \pm SEM. **P* < 0.05, ***P* < 0.01.

2. Results

2.1. Serum 25-(OH)D concentration

Serum 25-(OH)D concentration of male offspring was measured at seven weeks after diet intervention. As shown in Fig. 1A, serum 25-(OH)D concentration of male offspring was significantly reduced in VDD diet-fed mice. Serum 25-(OH)D concentration of female mice was then measured on gestational day 13. As expected, serum 25-(OH)D concentration of female mice was significantly reduced in VDD diet-fed mice (Fig. 1B).

2.2. Gonadal weight and sperm quality in adult male offspring

The effects of vitamin D deficiency on body weight, liver weight and kidney weight were analyzed. As shown in Table 2, the absolute and relative of kidney weight were significantly reduced in VDD-fed mice as compared with controls. No significant difference on body weight and liver weight was observed between two groups. The effects of vitamin D deficiency on the weights of testes and epididymides in adulthood were then analyzed. As shown in Table 2, the absolute and relative weights of testes were significantly reduced in VDD-fed mice. No significant difference on the absolute and relative weights of epididymides was observed between two groups (Table 2). The effects of vitamin D deficiency on sperm quality were also analyzed. As shown in Table 2, the number of spermatozoa in the cauda epididymidis was significantly

Table 2

Body weight, liver weight, reproductive organs weights and sperm count in adult male offspring.

	Control	VDD
No of male offspring (n)	12	12
Body weight (g)	43.2 \pm 0.92	44.0 \pm 0.92
Absolute liver weight (g)	1.955 \pm 0.038	1.927 \pm 0.086
Relative liver weight (%)	45.17 \pm 0.36	43.80 \pm 0.24
Absolute kidney weight (mg)	745.5 \pm 33.6	642.9 \pm 34.4*
Relative kidney weight (%)	16.95 \pm 0.73	14.08 \pm 0.32*
Absolute testis weight (mg)	274.8 \pm 12.72	248.7 \pm 5.30*
Relative testis weight (%)	0.64 \pm 0.089	0.57 \pm 0.010*
Absolute epididymides weight (mg)	135.1 \pm 8.49	142.2 \pm 7.14
Relative epididymides weight (%)	0.32 \pm 0.025	0.32 \pm 0.017
Sperm number ($\times 10^6$)	24.75 \pm 0.68	19.21 \pm 1.79**
Sperm viability (%)	56.38 \pm 6.38	38.89 \pm 6.32*

All data were expressed as means \pm SEM. **P* < 0.05, ***P* < 0.01 as compared with Control M \times Control F.

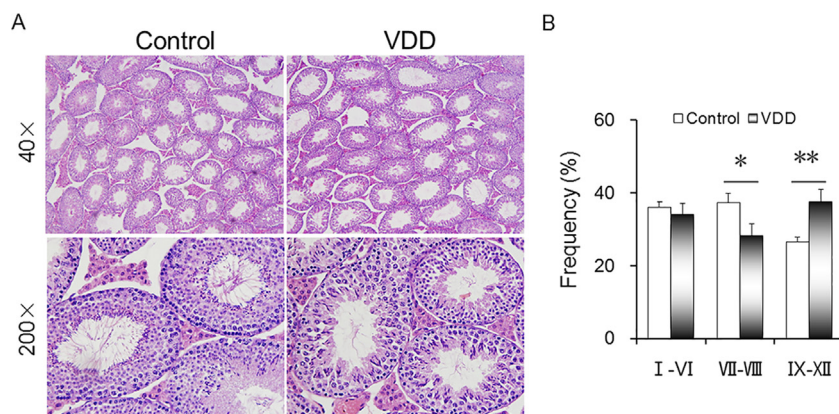


Fig. 2. The effects of vitamin D deficiency on testicular histology in adult male offspring. In the control group, dams and their pups were fed with standard-chow diets. In the VDD diet group, dams and their pups were fed with vitamin D deficient diets, in which vitamin D was depleted. Half of male offspring ($N = 12$) were sacrificed at seven weeks after diet intervention. Testicular cross-sections were stained by hematoxylin/eosin staining. (A) Representative photomicrographs of testicular histology are shown. (B) The stages of the seminiferous tubules were classified into three stage groups: stages I–VI, stages VII–VIII and stages IX–XII. The percent of the cycle of seminiferous tubules in different stages are compared between control group and VDD group. Data were expressed as mean \pm SEM ($N = 12$). More than 100 tubules per mice were examined. * $P < 0.05$, ** $P < 0.01$.

Table 3
Effects of vitamin D deficiency in early life on mating behavior and fertility index.

Groups	Females	Males	Plugs	Pregnancy	Mating index (%)	Fertility index (%)
Control M \times Control F	24	12	23	20	95.8	83.3
Control M \times VDD F	24	12	19	17	79.2	70.8
VDD M \times Control F	24	12	22	18	91.2	75.0
VDD M \times VDD F	24	12	19	15	79.1	62.5

Mating index was calculated as follow: mating index (%) = number of vaginal plug-positive females/number of mated females \times 100.

Fertility index were calculated as follow: fertility index (%) = number of pregnant females/number of mated females \times 100.

All data were expressed as means \pm SEM. * $P < 0.05$, ** $P < 0.01$ as compared with Control M \times Control F.

cantly reduced in VDD-fed mice. In addition, sperm viability was significantly reduced in VDD-fed mice (Table 2).

2.3. Testicular histology in adult male offspring

Testicular histology was assessed by H&E staining. As shown in Fig. 2A, vitamin D deficiency disturbed the array of spermatogenic cells in testicular sections of male offspring at adulthood. The percent of the cycle of seminiferous tubules in different stages are presented in Fig. 2B. In control males, 37.9%, 38.6% and 23.5% of the seminiferous tubules per section were in stages I–VI, VII–VIII and IX–XII, respectively. In VDD-fed males, 35.6%, 24.9% and 39.5% of the seminiferous tubules were in stages I–VI, VII–VIII and IX–XII, respectively (Fig. 2B). Further analysis showed that vitamin D deficiency significantly reduced the percent of seminiferous tubules in stages VII–VIII. By contrast, the percent of seminiferous tubules in stages IX–XII were markedly elevated in VDD-fed males (Fig. 2B).

2.4. Testicular germ cell proliferation and apoptosis in adult male offspring

Testicular germ cell proliferation was determined by immunohistochemistry. As shown in Fig. 3A, numerous PCNA-positive cells were observed in seminiferous tubules. Interestingly, the percentage of PCNA-positive cells in seminiferous tubules was significantly reduced in VDD-fed mice as compared with controls (Fig. 3C). Testicular germ cell apoptosis was determined using TUNEL assay (Fig. 3B). No significant difference on the number of seminiferous tubules with TUNEL-positive cells was observed between VDD-fed mice and controls (Fig. 3D).

2.5. Serum and testicular hormones in adult male offspring

The effects of vitamin D deficiency on serum FSH and LH in adult male offspring were analyzed. As shown in Fig. 4A and B, no significant difference on serum FSH and LH levels was observed between VDD-fed mice and controls. The effects of vitamin D deficiency on serum and testicular T in adult male offspring were then analyzed. As shown in Fig. 4C, serum T concentration was significantly reduced in VDD-fed mice. Correspondingly, testicular T concentration was significantly decreased in VDD-fed mice as compared with controls (Fig. 4D).

2.6. Testicular StAR and T biosynthetic enzymes in adult male offspring

The effects of vitamin D deficiency on the mRNA levels of StAR and genes of testicular T biosynthetic enzymes are presented in Fig. 5. As shown Fig. 5A and D, no significant difference on the testicular *17 β -hsd* and *Cyp17a1* mRNA level was observed between VDD-fed mice and controls (Fig. 5A and D). Interestingly, mRNA levels of testicular *3 β -hsd*, *Cyp11a1* and *Star*, were obviously decreased in VDD group (Fig. 5B, C and E). The effects of vitamin D deficiency on the levels of StAR and testicular T biosynthetic enzymes protein are presented in Fig. 6. As shown in Fig. 6A and B, no significant difference on testicular 17 β -HSD was observed between VDD-fed mice and controls. Interestingly, the levels of testicular 3 β -HSD, CYP11A1, CYP17A1 and StAR protein were significantly reduced in adult male pups that were fed with VDD (Fig. 6A, C–F).

2.7. Mating behavior, fertility index and pregnant outcomes

The effects of vitamin D deficiency on mating behavior and fertility index were analyzed. As shown in Table 3, mating index was

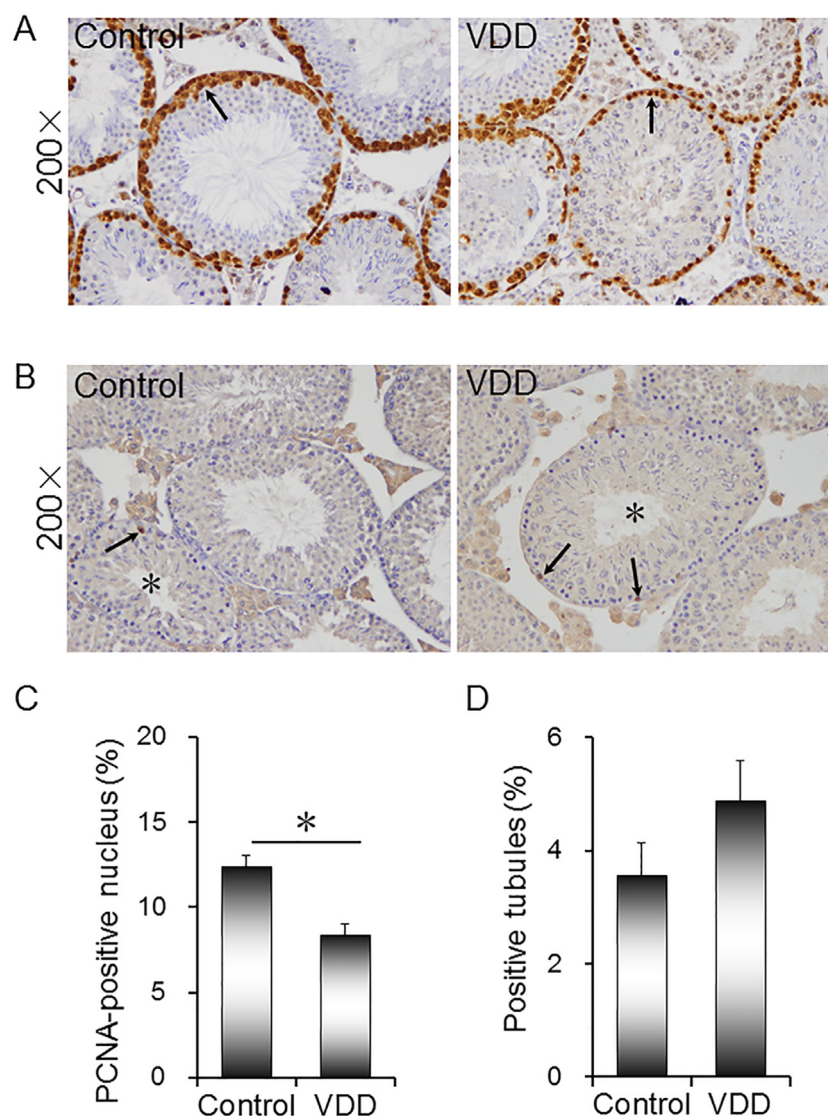


Fig. 3. The effects of vitamin D deficiency on testicular germ cell proliferation and apoptosis in adult male offspring. In the control group, dams and their pups were fed with standard-chow diets. In the VDD diet group, dams and their pups were fed with vitamin D deficient diets, in which vitamin D was depleted. Half of male offspring (N = 12) were sacrificed at seven weeks after diet intervention. (A and C) Testicular germ cell proliferation was determined by staining for PCNA. (A) Arrows indicate PCNA-positive cells. (C) The rate of PCNA-positive cells. (B and D) Testicular germ cell apoptosis was determined using TUNEL. (B) Arrows indicate TUNEL-positive cells. (D) The rate of tubules with TUNEL-positive cells. All data were expressed as mean \pm SEM (N = 12). * $P < 0.05$.

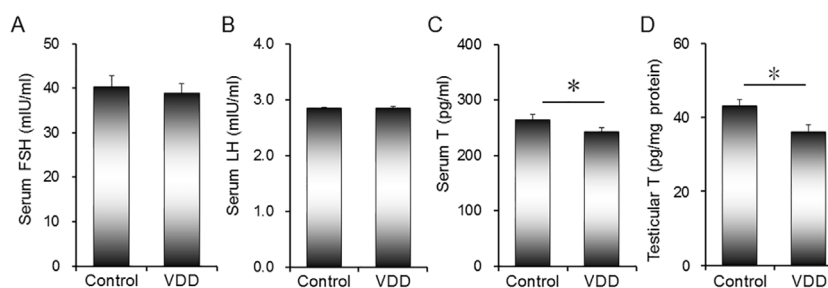


Fig. 4. The effects of vitamin D deficiency on serum and testicular hormones in adult male offspring. In the control group, dams and their pups were fed with standard-chow diets. In the VDD diet group, dams and their pups were fed with vitamin D deficient diets, in which vitamin D was depleted. Half of male offspring (N = 12) were sacrificed at seven weeks after diet intervention. Serum (A) FSH and (B) LH were measured using ELISA. (C) Serum and (D) testicular T were measured by RIA. Data were expressed as means \pm SEM (N = 12). * $P < 0.05$.

decreased in VDD-fed female mice, whereas vitamin D deficiency had little effect on fertility index in female mice. By contrast, vitamin D deficiency had little effect on mating index in male offspring, whereas fertility index was reduced in VDD-fed male offspring

(Table 3). The effects of vitamin D deficiency on pregnant outcomes were analyzed. As shown in Table 4, there was no significant difference on the numbers of resorptions per litter and dead fetuses. Interestingly, there was a downstream trend on the numbers of

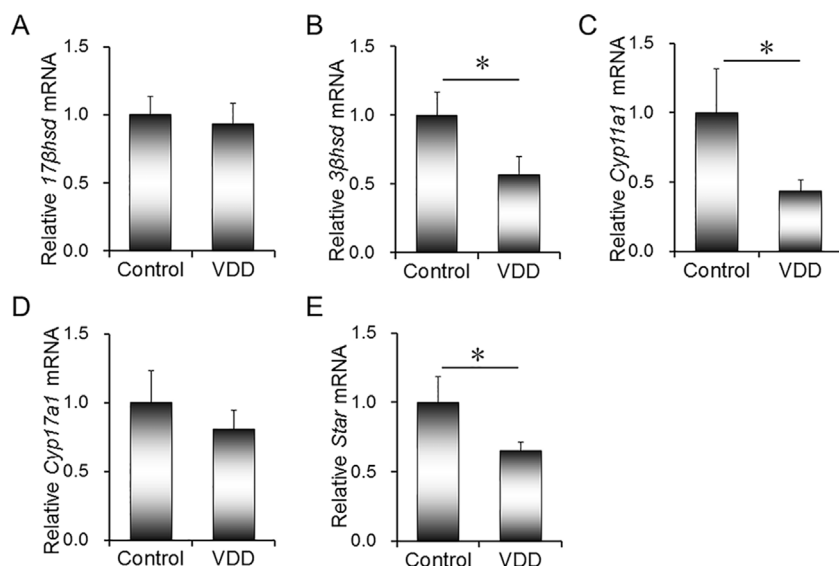


Fig. 5. The effects of vitamin D deficiency on the mRNA levels of StAR and genes of testicular T biosynthetic enzymes in male offspring. In the control group, dams and their pups were fed with standard-chow diets. In the VDD diet group, dams and their pups were fed with vitamin D deficient diets, in which vitamin D was depleted. Half of male offspring (N = 12) were sacrificed at seven weeks after diet intervention. Testicular *17β-hsd*, *3β-hsd*, *Cyp11a1*, *Cyp17a1* and *Star* mRNAs were measured using real-time RT-PCR. (A) *17β-hsd*. (B) *3β-hsd*. (C) *Cyp11a1*. (D) *Cyp17a1*. (E) *Star*. Data were expressed as means ± SEM (N = 12). **P* < 0.05, ***P* < 0.01.

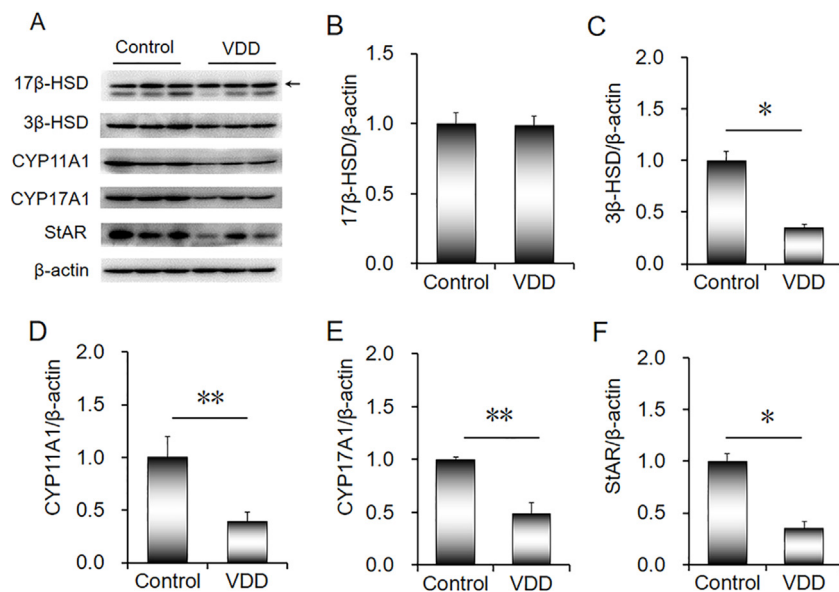


Fig. 6. The effects of vitamin D deficiency on testicular StAR and T biosynthetic enzymes protein in male offspring. In the control group, dams and their pups were fed with standard-chow diets. In the VDD diet group, dams and their pups were fed with vitamin D deficient diets, in which vitamin D was depleted. Half of male offspring (N = 12) were sacrificed at seven weeks after diet intervention. (A) Testicular StAR and T biosynthetic enzymes were measured using immunoblots. (B–F) Quantitative analysis of scanning densitometry at each time point was performed. Testicular *17β-HSD*, *3β-HSD*, *CYP11A1*, *CYP17A1* and *StAR* were normalized to *β-actin* level in the same sample. (B) *17β-HSD*; (C) *3β-HSD*; (D) *CYP11A1*; (E) *CYP17A1*; (F) *StAR*. All experiments were replicated for four times. Data were expressed as means ± SEM (N = 12). **P* < 0.05, ***P* < 0.01.

Table 4
Pregnant outcomes among different groups.

Parameters	Control M × Control F	Control M × VDD F	VDD M × Control F	VDD M × VDD F
No of pregnant mice (n)	19	17	18	15
Resorptions per litter (n)	0.4 ± 0.1	0.8 ± 0.2	0.6 ± 0.2	0.5 ± 0.2
Dead fetuses per litter (n)	0.1 ± 0.1	0.2 ± 0.2	0.1 ± 0.1	0.2 ± 0.1
Live fetuses per litter (n)	15.3 ± 0.3	14.6 ± 0.8	14.7 ± 0.9	13.4 ± 0.8*
Implantation sites of per litter	15.8 ± 0.3	15.6 ± 0.8	15.4 ± 0.9	14.1 ± 0.9

All data were expressed as means ± SEM. **P* < 0.05, ***P* < 0.01 as compared with Control M × Control F.

implantation sites per litter when both males and females were fed with VDD diet in early life. Moreover, the number of live fetuses was significantly reduced when both males and females were fed with VDD diet in early life (Table 4).

3. Discussion

Several cross-sectional clinical investigations showed a positive association between vitamin D deficiency and a poor semen

quality in both fertile and infertile men [25,26,17]. A clinic observation found a lower gene and protein expression of CYP2R1, highly expressed in the testis and encoding vitamin D 25-hydroxylase, in samples with hypospermatogenesis and Sertoli-cell-only syndrome [27]. However, whether these associations reflect a causal effect needs to be further determined [28,29]. In the present study, we investigated the effects of vitamin D deficiency on testicular development and spermatogenesis. Our results showed that the absolute and relative weights of testes were reduced in VDD-fed mice as compared with controls. Further analysis found that vitamin D deficiency significantly reduced the percent of seminiferous tubules in stages VII–VIII. Correspondingly, the percent of seminiferous tubules in stages IX–XII were markedly elevated in VDD-fed males, indicating that the maturation of seminiferous tubules is delayed. Importantly, the number of spermatozoa in cauda epididymidis was reduced in VDD-fed males. These results suggest that vitamin D deficiency impairs testicular development and spermatogenesis.

The mechanism through which vitamin D deficiency impairs testicular development and spermatogenesis remains obscure. Accumulating evidence demonstrates that germ cell proliferation plays a vital role in testicular development and spermatogenesis [30,31]. Numerous studies indicate that vitamin D3 regulates cell proliferation and differentiation through activating vitamin D receptor (VDR) signaling [32,33]. Indeed, several studies found that VDR was highly expressed in human and rodent mature spermatozoa and male reproductive tract [34,35]. An early study showed that a significant decrease on sperm count and motility and histological abnormalities of the testis were observed in *Vdr*-null mutant mice [36]. We showed that the percentage of PCNA-positive cells in the testis was significantly reduced in VDD diet-fed mice, indicating that vitamin D deficiency inhibits testicular germ cell proliferation. Therefore, it is reasonable to assume that vitamin D deficiency impairs testicular development and spermatogenesis through down-regulating VDR signaling and subsequent germ cell proliferation. Several reports indicate that vitamin D has pro-apoptotic and anti-apoptotic effects [37,38]. The present study also investigated the effect of vitamin D deficiency on testicular germ cell apoptosis using TUNEL assay. We found that there was no significant difference in the number of TUNEL-positive cells between vitamin D deficiency group and control group. To get more insights into the apoptosis by vitamin D deficiency, more tests for apoptosis are required to be performed in further studies.

Numerous studies indicate that androgens, primarily T, which act through the somatic cells to regulate germ cell differentiation, are a prerequisite for normal testicular development and spermatogenesis [39–41]. Indeed, several epidemiological reports suggest a positive correlation between serum 25-(OH)D and T levels in middle-aged and older men [42,43]. Recently, an *in vitro* study showed that active vitamin D3 increased T synthesis in human testicular cell cultures [44]. The present study investigated the effects of vitamin D deficiency on serum and testicular T levels in adult mice. Although vitamin D deficiency had no effect on serum LH and FSH, serum and testicular T levels were significantly decreased in VDD diet-fed male offspring. These results suggest for the first time that vitamin D deficiency disturbs testicular T synthesis.

Testicular steroidogenic acute regulatory protein (StAR) and T synthetic enzymes are essential and limiting factors in testicular T synthesis. Testicular StAR is responsible for the transport of cholesterol into mitochondria [45]. On the other hand, T synthetic enzymes, primarily 17 β -HSD, 3 β -HSD, CYP11A1 and CYP17A1, play a critical role on T synthesis in Leydig cells [46]. An *in vitro* study showed that active vitamin D3 up-regulated CYP11A1 and CYP17A1 in human adrenocortical NCI-H295R cells [47]. Another *in vitro* report found that active vitamin D3 elevated 3 β -HSD mRNA human granulosa cells [48]. The present study analyzed the effects of vita-

min D deficiency on testicular StAR and T synthetic enzymes. We showed that the level of testicular StAR protein was decreased in VDD diet-fed mice. In addition, testicular 3 β -HSD, CYP11A1 and CYP17A1 were down-regulated in the VDD diet group. These results suggest that the decreased testicular T synthesis in male pups that were fed with VDD diet is, at least partially, attributed to down-regulation of testicular StAR and T synthetic enzymes.

Increasing evidence indicates that vitamin D deficiency is associated with female infertility [49–51]. Relatively few studies have investigated potentially adverse effects of vitamin D deficiency on male fertility. In the present study, we investigated the effects of vitamin D deficiency on male fertility and pregnant outcomes. Although vitamin D deficiency had little effect on fertility index in male offspring, mating index was significantly reduced in VDD-fed male offspring. Further observation found that there was a downstream trend on the numbers of implantation sites per litter when both males and females were fed with VDD diet. In addition, the number of live fetuses was significantly reduced when both males and females were fed with VDD diet. The present study suggests that vitamin D deficiency impairs male fertility in adulthood. However, there are still a few flaws in the present study. Firstly, these results need to be demonstrated in an epidemiological investigation. Secondly, further researches are necessary to determine the mechanism through which vitamin D deficiency impairs male reproduction. In addition, the present study had not explored the effects of vitamin D supplementation on vitamin D deficiency impairs testicular development and spermatogenesis. Indeed, VDR was highly expressed in human and rodent germ cell and male reproductive tract [34,35]. Several epidemiological studies found that serum vitamin D level was positively associated with sperm motility [52,53]. Thus, additional research is required to analyze whether vitamin D supplementation prevent impairment of testicular development and spermatogenesis.

In summary, the present study investigated the effects of vitamin D deficiency on male reproduction. Our results showed that vitamin D deficiency reduced testicular weight, put off maturation of seminiferous tubules, and impaired sperm quality in adult mice. In addition, vitamin D deficiency disturbed testicular steroidogenesis in adulthood period. Importantly, vitamin D deficiency impaired male fertility. These results provide the first evidence that vitamin D deficiency impairs testicular development and spermatogenesis. Indeed, vitamin D deficiency is prevalent especially in men and is increasingly recognized as a global public health problem [54,55]. Thus, supplementation with vitamin D may be a potential strategy for preventing the impairment of testicular development and spermatogenesis especially in high-risk situations of having vitamin D deficiency.

Author contributions

D.X.X. designed research; L.F. performed experiments; L.F., Y.H.C., S.X. and H.W. analyzed data; D.X.Y., Y.H.C., C.Z. and Y.L.J. contributed reagents and materials; D.X.X. and Y.H.C. wrote the paper.

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Conflict of interest

All authors declare that they have no conflicts of interest.

Ethical approval

All procedures on animals followed the guidelines for humane treatment set by the Association of Laboratory Animal Sciences and the Center for Laboratory Animal Sciences at Anhui Medical University.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.reprotox.2017.06.047>.

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