Active vitamin D deficiency mediated by extracellular calcium and phosphorus results in male infertility in young mice

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Sun W, Chen L, Zhang W, Wang R, Goltzman D, Miao D. Active vitamin D deficiency mediated by extracellular calcium and phosphorus results in male infertility in young mice. Am J Physiol Endocrinol Metab 308: E51–E62, 2015. First published November 4, 2014; doi:10.1152/ajpendo.00076.2014.—We used mice with targeted deletion of 25-hydroxyvitamin D-1α-hydroxylase [1α(OH)ase−/−] to investigate whether 1,25(OH)2D3 deficiency results in male infertility mediated by 1,25(OH)2D3 or extracellular calcium and phosphorus. Male 1α(Oh)ase−/− and their wild-type littermates fed either a normal diet or a rescue diet from weaning were mated at 6–14 wk of age with female wild-type mice on the same diet. The fertility efficiency of females was analyzed, and the reproductive phenotypes of males were evaluated by histopathological and molecular techniques. Hypocalcemic and hypocalsephatemic male 1α(Oh)ase−/− mice on a normal diet developed infertility characterized by hypergonadotropic hypogonadism, with downregulation of testicular calcium channels, lower intracellular calcium levels, decreased sperm count and motility, and histological abnormalities of the testes. The proliferation of spermatogenic cells was decreased with downregulation of cyclin E and CDK2 and upregulation of p53 and p21 expression, whereas apoptosis of spermatogenic cells was increased with upregulation of Bax and p-caspase 3 expression and downregulation of Bcl-xl expression. When serum calcium and phosphorus were normalized by the rescue diet, the defective reproductive phenotype in the male 1α(Oh)ase−/− mice, including the hypergonadotrophic hypogonadism, decreased sperm count and motility, histological abnormalities of testis, and defective spermatogenesis, was reversed. These results indicate that the infertility seen in male 1,25(OH)2D3−/− deficient mice is not a direct effect of active vitamin D deficiency on the reproductive system but is an indirect effect mediated by extracellular calcium and phosphorus.

vitamin D; male infertility; spermatogenesis

Although vitamin D is most closely associated with regulation of calcium and bone homeostasis, it has been proposed to have many other actions, including effects on the immune system, diabetes, and cancer prevention (29). One of the recently identified target areas of vitamin D is male reproductive function. Expression of the vitamin D receptor (VDR) and vitamin D metabolizing enzymes has been demonstrated in ejaculatory duct, germ cells, and mature spermatozoa, which suggests that vitamin D has an important role in spermatogenesis and sperm function (2, 5). It has been shown that 19 of 2,483 testis-specific genes in mouse testis can be upregulated by treatment with the active form of vitamin D, 1,25-dihydroxyvitamin D3 [1,25(OH)2D3] (17). Vitamin D deficiency results in reduced sperm counts in male rats and lower fertility rates in female rats inseminated with semen from vitamin D-deficient male rats (17). The impaired reproductive performance of male rats that is induced by vitamin D deficiency is reversible and seems to be mediated predominantly through calcium imbalance, because it can be corrected either by supplying vitamin D or by normalizing calcium levels (36). VDR-knockout mice have significant gonadal insufficiency, with decreased sperm counts and motility, and histological abnormalities of the testis (19). In VDR-knockout mice, feeding high-calcium diets partly restores fertility and increases the rate of conception but does not normalize the number or weight of viable fetuses (18, 19). Therefore, results from previous studies suggest that the defect in the reproductive capacity of male VDR-deficient mice results from both hypocalcemia and the lack of a direct effect of 1,25(OH)2D3 on reproductive function.

To investigate the effects of 1,25(OH)2D3 and its molecular targets, we (28) and others (9) have previously generated a mouse model deficient in 1,25(OH)2D3 by targeted gene ablation of Cyp27b1, the gene encoding 25-hydroxyvitamin D-1α-hydroxylase [1α(Oh)ase]. The mutant [1α(Oh)ase−/−] mice developed hypocalcemia, hypophosphatemia, hyperparathyroidism, retarded growth, and the skeletal abnormalities characteristic of rickets when fed a diet of regular mouse chow after weaning. Previously, we characterized functions of 1,25(OH)2D3 in the reproductive system of female 1α(Oh)ase−/− mice and demonstrated that 1α(Oh)ase−/− female mice develop infertility accompanied by decreased estrogen and progesterone levels, elevated FSH and LH levels, and defects in follicular development and corpus luteum formation as well as uterine hypoplasia (34). However, the defective reproductive phenotype was normalized when the mice were fed a rescue diet (containing high calcium, phosphate, and lactose). These studies indicated that the infertility seen in 1,25(OH)2D3−/− deficient female mice is not a direct effect of active vitamin D deficiency on the reproductive system but rather an indirect effect mediated by extracellular calcium and phosphorus. However, unlike the female counterpart, our initial examination of gonadal histology in 1α(Oh)ase−/− male mice did not detect gross abnormalities (28), although a detailed evaluation of the reproductive phenotype, including testis histology, was not performed.

To address this issue, in the present study, male 1α(Oh)ase−/− and their wild-type littermates were fed either a normal diet or a rescue diet from weaning and then mated from 6 to 14 wk of age with female wild-type mice on the same diet. The fertility efficiency of females was analyzed, gonadotropins and gonadal hormones in male mice were measured, and spermatogenesis
and sperm motility were examined by histopathological and molecular techniques.

**MATERIALS AND METHODS**

**Animals and treatments.** Generation and characterization of 1α(OH)ase−/− mice were performed as described previously (28). 1α(OH)ase−/− mice were generated through breeding of heterozygous mice. The genotype of the mice was confirmed by PCR using mouse tail samples. Wild-type littersmates 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. Age- and sex-matched 1α(OH)ase−/− and wild-type littersmates were randomly divided into two groups. After weaning they were fed 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.

**Assessment of serum calcium, phosphorus, and reproductive hormones.** Blood was collected intraorbitally from 3-mo-old male mice, and sera were stored at −80°C. Five samples were obtained for each group. Serum calcium and phosphorus levels were determined by an autoanalyzer (Beckman Synchrone 67; Beckman Instruments). Serum testosterone and estrogen levels were measured with a radioimmunoassay (Diagnostic Products, Los Angeles, CA). Serum LH and 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 reproducible.

**Fertility, sperm count, and sperm motility.** To determine fertility, male 1α(OH)ase−/− and their wild-type littersmates were fed either a normal diet or a rescue diet from weaning, and from 6 to 14 wk of age, each was allowed to mate with two wild-type females for 1 mo and then with another two wild-type females for another month on the same diet. The female wild-type mice were monitored during the time between mating and parturition. The number of pups produced was counted the morning after parturition. To examine the effect of the rate of mating success on fertility in 1α(OH)ase−/− male mice, 8-wk-old wild-type and 1α(OH)ase−/− male mice (n ≥ 5 males each group) maintained on the normal diet or the rescue diet were mated continuously with wild-type females at a 1:2 male/female sex ratio for 1 mo. The presence of a vaginal plug was determined every morning as evidence of a successful mating (32). Any plugged female was continuously with wild-type females at a 1:2 male/female sex ratio for 1 mo. Thirty-microgram protein samples were fractionated by SDS-PAGE and transferred to nitrocellulose membranes. Immunoblotting was carried out as described previously (37), with antibodies against VDR, transient receptor potential vanilloid type 5 (TRPV5), p21, caspase-3, p53 (Cell Signaling Technology), and phosphatase inhibitor. Sections were counterstained with methyl green and mounted with Kaiser’s glycerol jelly.

**Assessment of apoptosis.** Dewedged paraffin sections were stained with an In Situ Cell Death Detection Kit (Roche Diagnostics), using a previously described protocol (25). Briefly, after treatment with 3 μg/ml proteinase K for 20 min at room temperature, the sections were incubated with a reaction mixture for TUNEL of DNA strand breaks for 60 min at 37°C. Sections were then incubated with Converter-AP (Sigma-Aldrich) for 30 min at 37°C, and alkaline phosphatase was visualized after 10–15 min of treatment with Fast Red TR/Naphthol AS-MX phosphate (Sigma-Aldrich) containing 1 mM levamisole as an endogenous alkaline phosphatase inhibitor. Sections were counterstained with methyl green and mounted with Kaiser’s glycerol jelly.

**Statistical analysis.** Data from image analysis are presented as means ± SE. Statistical comparisons were made using a two-way ANOVA, with P < 0.05 considered significant.

**RESULTS**

**Effects of 1,25(OH)2D3 deficiency and dietary minerals on male fertility.** To determine whether 1,25(OH)2D3 deficiency resulted in male infertility mediated by extracellular calcium and phosphorus, 1α(OH)ase−/− and wild-type mice were fed either a normal diet or a rescue diet after weaning. Each 6-wk-old 1α(OH)ase−/− or wild-type male mouse was mated
Effects of 1,25(OH)₂D₃ deficiency and dietary minerals on extracellular and intracellular calcium concentrations and Ca²⁺ transporters. To determine whether the normalization of male reproductive defects that occurred in 1α(OH)ase⁻/⁻ mice by rescue diet was associated with disorders of calcium transport, we examined serum calcium and phosphorus levels, intracellular calcium concentrations, and the expression levels of the CaSR, CaV3.1 (a voltage-gated Ca²⁺ channel), and TRPV5 (an excitomotor-receptor gated Ca²⁺ channel). Results showed that serum calcium and phosphorus levels (Fig. 1, A–C), intracellular calcium concentrations, and the expression levels of CaSR, CaV3.1, and TRPV5 in testes were downregulated dramatically (Fig. 1, D–G) in 1α(OH)ase⁻/⁻ mice compared with the wild-type mice fed the normal diet. 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; furthermore, the expression levels of CaSR, CaV3.1, and TRPV5 in testes were all upregulated significantly in 1α(OH)ase⁻/⁻ mice fed the rescue diet (Fig. 1, A–G). The expression levels of TRPV5 were upregulated significantly, but other parameters were not altered significantly in wild-type mice fed the rescue diet compared with the mice fed the normal diet (Fig. 1, A–G).

Effects of 1,25(OH)₂D₃ deficiency and dietary minerals on the function of the hypothalamic-pituitary-gonadal axis. To determine whether impaired spermatogenesis caused by 1,25(OH)₂D₃ deficiency was associated with impaired function of the hypothalamic-pituitary-gonadal axis, we examined serum levels of FSH, LH, estradiol, and testosterone and expression levels of hydroxysteroid dehydrogenases and of the LH receptor in mice of both 1α(OH)ase⁻/⁻ and wild-type genotypes fed either the normal diet or the rescue diet. Results showed that serum FSH and LH levels were raised significantly (Fig. 2, A and B), whereas serum estradiol and testosterone levels and the expression levels of 3β-hydroxysteroid dehydrogenase type VI (3β-HSD VI), 17β-hydroxysteroid dehydrogenase type III (17β-HSD III), and the LH receptor in testes were decreased significantly in 1α(OH)ase⁻/⁻ mice compared with the wild-type mice fed the normal diet (Fig. 2, C–H). On the rescue diet, serum FSH, LH, estradiol, and testosterone levels and the expression levels of 3β-HSD VI, 17β-HSD III, and the LH

Table 2. Effects of 1,25(OH)₂D₃ deficiency and dietary minerals on male mating and fertility (n = 5)

<table>
<thead>
<tr>
<th>Females</th>
<th>Plugs</th>
<th>Pregnancy</th>
<th>FCP (%)*</th>
<th>FC (%)#</th>
</tr>
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<tr>
<td>1α(OH)ase⁺/⁺</td>
<td>72</td>
<td>22</td>
<td>18</td>
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<tr>
<td>1α(OH)ase⁻/⁻</td>
<td>59</td>
<td>9</td>
<td>0</td>
<td>15.2</td>
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<td>Rescue diet</td>
<td>1α(OH)ase⁺/⁺</td>
<td>81</td>
<td>31</td>
<td>27</td>
</tr>
<tr>
<td>1α(OH)ase⁻/⁻</td>
<td>78</td>
<td>28</td>
<td>24</td>
<td>35.9</td>
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</tbody>
</table>

FCP, frequency of copulatory plug; FC, frequency of conception. *FCP was calculated as the ratio of the no. of plugged females to the total no. of females mating with different genotypes of mice fed a normal diet or a rescue diet; #FC was calculated as the ratio of the no. of pregnant females to plugged females.

with two wild-type females for 1 mo and then with another two wild-type females for another month. When maintained on the normal diet, 1α(OH)ase⁻/⁻ males sired no pups (n = 5 males, n = 20 females), whereas their wild-type littermates sired 149 pups; the average number of pups sired by every wild-type male mouse was 29.8 ± 1.158 (Table 1). When fed a rescue diet, 1α(OH)ase⁻/⁻ and wild-type males displayed equal fertility, with the total number of offspring varying between 147 and 153 and the average number of pups obtained varying between 29.4 ± 0.87 and 30.6 ± 1.077 for 1α(OH)ase⁻/⁻ and wild-type males, respectively (Table 1).

To determine whether the infertility that was observed in 1α(OH)ase⁻/⁻ male mice on the normal diet was associated with a reduction in mating, 8-wk-old wild-type and 1α(OH)ase⁻/⁻ male mice fed a normal diet or a rescue diet were mated continuously with wild-type females at a 1:2 male/female sex ratio for 1 mo, and the presence of a vaginal plug was assessed every morning as evidence of a successful mating. The number of total females, plugged females, litters, and offspring was calculated as the ratio of the no. of pregnant females to plugged females.

Table 1. Effects of 1,25(OH)₂D₃ deficiency and dietary minerals on male fertility

<table>
<thead>
<tr>
<th>Male Mice</th>
<th>WT</th>
<th>No. of pups</th>
<th>Total no. of pups</th>
<th>Average no. of pups</th>
<th>No. of pups</th>
<th>Total no. of pups</th>
<th>Average no. of pups</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal diet (n = 5)</td>
<td>Mouse no. 1</td>
<td>29</td>
<td>149</td>
<td>29.8 ± 1.16</td>
<td>0</td>
<td>0</td>
<td>0*</td>
</tr>
<tr>
<td>Mouse no. 2</td>
<td>33</td>
<td></td>
<td></td>
<td></td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse no. 3</td>
<td>27</td>
<td></td>
<td></td>
<td></td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse no. 4</td>
<td>28</td>
<td></td>
<td></td>
<td></td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse no. 5</td>
<td>32</td>
<td></td>
<td></td>
<td></td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rescue diet (n = 5)</td>
<td>Mouse no. 1</td>
<td>30</td>
<td>153</td>
<td>30.6 ± 1.08</td>
<td>28</td>
<td>147</td>
<td>29.4 ± 0.87†</td>
</tr>
<tr>
<td>Mouse no. 2</td>
<td>34</td>
<td></td>
<td></td>
<td></td>
<td>32</td>
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<tr>
<td>Mouse no. 5</td>
<td>32</td>
<td></td>
<td></td>
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<td>32</td>
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</tbody>
</table>

1.25(OH)₂D₃, 1,25-dihydroxyvitamin D₃; 1α(OH)ase, 25-hydroxyvitamin D-1 α-hydroxylase; WT, wild type. Average no. of pups is presented as means ± SE. *P < 0.001, different from WT littermates; †P < 0.001, different from genotype-matched mice on the normal diet.
receptor in testes were all normal in 1α(OH)ase−/− mice compared with wild-type controls (Fig. 2, A–H).

Effects of 1,25(OH)2D3 deficiency and dietary minerals on spermatogenesis. We next examined alterations of the morphology of the testis and epididymis and the number of sperm in mice of both genotypes fed either the normal diet (ND) or a rescue diet (RD). In 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 calcium-sensing receptor (CaSR), CaV3.1, and transient receptor potential vanilloid type 5 (TRPV5). β-Actin was used as loading control for Western blots. E–G: densitometric analysis of CaSR (E), CaV3.1 (F), and TRPV5 (G) protein levels relative to β-actin protein level expressed relative to the levels of WT mice on ND. Each value is the mean ± SE of determinations in 5 mice of the same genotype. *P < 0.05, **P < 0.01, and ***P < 0.001, difference from WT mice on the same diet; #P < 0.05, ##P < 0.01, and ###P < 0.001, difference from genotype-matched mice on ND. KO, knockout.

Fig. 1. Effects of 1,25-dihydroxyvitamin D3 [1,25(OH)2D3] deficiency and dietary minerals on extracellular and intracellular calcium concentrations and protein levels of Ca2+ channels. A and B: serum calcium (A) and phosphorus (B) were determined in 3-mo-old wild-type (WT) and 25-hydroxyvitamin D-1α-hydroxylase [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 calcium-sensing receptor (CaSR), CaV3.1, and transient receptor potential vanilloid type 5 (TRPV5). β-Actin was used as loading control for Western blots. E–G: densitometric analysis of CaSR (E), CaV3.1 (F), and TRPV5 (G) protein levels relative to β-actin protein level expressed relative to the levels of WT mice on ND. Each value is the mean ± SE of determinations in 5 mice of the same genotype. *P < 0.05, **P < 0.01, and ***P < 0.001, difference from WT mice on the same diet; #P < 0.05, ##P < 0.01, and ###P < 0.001, difference from genotype-matched mice on ND. KO, knockout.
Effects of 1,25(OH)₂D₃ deficiency and dietary minerals on sperm morphology and motility. We next examined alterations of sperm morphology and motility in both mouse genotypes fed either the normal diet or the rescue diet using transmission electron microscopy and CASA assay. By transmission electron microscopy we detected distinct ultrastructural abnormalities in the sperm heads of 1α(OH)ase⁻/⁻ mice fed the normal diet, including misshapen apical parts of sperm heads and swollen cell membranes (Fig. 4A). However, no distinct ultrastructural abnormalities were detected on either sagittal sections or on cross-sections of the fibrous sheath structures of the sperm principal piece (Fig. 4, B and C). CASA assay showed that the percentage of motile sperm and the average path velocity (VAP) and mean straight-line velocities (VSL) of sperm were reduced dramatically in 1α(OH)ase⁻/⁻ mice compared with their wild-type littermates maintained on the normal diet (Fig. 4, D–F). On

**Fig. 2.** Effects of 1,25(OH)₂D₃ deficiency and dietary minerals on the function of hypothalamic-pituitary-gonadal axis. A–D: concentrations of FSH (A), LH (B), estradiol (C), and testosterone (D) levels were determined in 3-mo-old WT and 1α(OH)ase⁻/⁻ male mice fed ND or RD. E: representative Western blots of testis extracts for expression of 3β-hydroxysteroid dehydrogenase type VI (3β-HSD VI), 17β-hydroxysteroid dehydrogenase type III (17β-HSD III), and LH receptor (LHR). β-Actin was used as loading control for Western blots. F–H: densitometric analysis of 3β-HSD VI (F), 17β-HSD III (G), and LHR (H) protein levels relative to β-actin protein level expressed relative to the levels of WT mice on ND. Each value is the mean ± SE of determinations in 5 mice of the same genotype. #P < 0.05, **P < 0.01, and ***P < 0.001, difference from WT mice on the same diet; ##P < 0.01 and ###P < 0.001, difference from genotype-matched mice on ND.
the rescue diet, the sperm morphology and motility were all normal in 1α(OH)ase⁻/⁻ mice (Fig. 4, A–F).

Effects of 1,25(OH)₂D₃ deficiency and dietary minerals on the proliferation of spermatogenic cells. We next examined, by immunohistochemistry and Western blots, the proliferation of spermatogenic cells and expression levels of cell proliferation-regulating molecules in both mouse genotypes fed either the normal diet or the rescue diet. In testes from 1α(OH)ase⁻/⁻ mice compared with those from wild-type mice fed the normal diet, we found that the number of Ki-67-positive spermatogenic cells (Fig. 5, A and B) and the protein expression levels of cyclin E and cyclin-dependent kinase 2 (CDK2) were decreased significantly, whereas cell cycle-dependent kinase inhibitors, including p53 and p21 expression levels, were upregulated dramatically (Fig. 5,
In 1α(OH)ase−/− mice fed the rescue diet, the proliferation of spermatogenic cells and expression levels of cyclin E, CDK2, p53, and p21 were all normal (Fig. 5).

**Effects of 1,25(OH)2D3 deficiency and dietary minerals on the apoptosis of spermatogenic cells.** To determine whether impaired spermatogenesis caused by 1,25(OH)2D3 deficiency was associated with increased spermatogenic cell apoptosis mediated by extracellular calcium and phosphorus, in mice of both genotypes fed either the normal diet or the rescue diet, we compared alterations of the apoptosis of spermatogenic cells and expression levels of cell apoptosis-regulating molecules by TUNEL and Western blots.
those from wild-type mice fed the normal diet, we found that the number of apoptotic spermatogenic cells (Fig. 6, A and B) and the proapoptotic protein expression levels of Bax and p-caspase 3 were increased significantly, whereas antiapoptotic protein expression levels of Bcl-xl were downregulated dramatically (Fig. 6, C–F). On the rescue 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. 6).
DISCUSSION

In the present study, we employed a combination of genetic and dietary manipulations to assess the effects of 1,25(OH)2D3 deficiency vs. calcium and phosphorus deficiency on murine male reproductive capacity. We found that the absence of 1,25(OH)2D3 resulted in male infertility that could be corrected by normalizing serum calcium and phosphorus alone. These results employing a 1α(OH)ase−/− mouse model are consistent with previous studies that reported that calcium and phosphorus normalization in vitamin D-deficient rats (20) and in VDR deficient mice (18) corrects infertility, implying that mineral ion deficiency and likely hypocalcemia per se are the major driving factors for reduced fertility.

A previous study demonstrated that the impaired male fertility caused by vitamin D deficiency also resulted in reducing successful matings (20). To determine whether the infertility that occurred in 1α(OH)ase−/− male mice on the normal diet
was associated with a reduction in matings, vaginal plugging was assessed every morning as evidence of a successful mating. Our results demonstrated that the FCP of 1α(OH)ase−/− male mice was ~50% of wild-type littersmates when maintained on the normal diet. When maintained on the rescue diet, the reduced numbers of successful matings in 1α(OH)ase−/− male mice were normalized. These results suggest that active vitamin D, mediated by extracellular calcium and phosphate, is necessary not only for fertility, but also for successful matings.

Unlike our initial examination of gonadal histology in 1α(OH)ase−/− male mice, in which we did not detect gross abnormalities (28), the present study demonstrates a complete (100%) loss of male fertility in 1α(OH)ase-deficient male mice. This apparent inconsistency may have resulted from our initial examination of gonadal histology in 1α(OH)ase−/− male mice at 4 wk of age, which is a sexually immature stage (28). In the present study, a more detailed evaluation of testis histology and reproductive phenotype were performed in 14-wk-old 1α(OH)ase−/− male mice on the normal diet, revealing that 1α(OH)ase−/− mice developed infertility with decreased sperm count and motility and histological abnormalities of the testes. A previous study reported only a partial suppression of male fertility in vitamin D-deficient rats (20); however, vitamin D deficiency was induced by a vitamin D-deficient diet, and although serum 25(OH)D3 was undetectable, serum 1,25(OH)2D3 was still measurable at 10 ± 4 ng/ml (20). In contrast, in our 1α(OH)ase-deficient model, serum 1,25(OH)2D3 was completely absent. This difference may account for the complete loss of male fertility in our study but only partial suppression of male fertility in the previous study. In addition, previous studies using VDR-knockout mice reported that impaired fertility and reduced litter size are dependent on the strain of mice (19, 22, 40). It is possible that the different reproductive phenotypes of different strains of mice may result from different protocols for targeted deletion and the procedures used for phenotyping. To avoid these variations, standard knockout protocols and standard operating procedures for genotyping should be established and employed when assessing differences in strain responses.

We also explored possible mechanisms of 1,25(OH)2D3 and mineral action on the male reproductive system that might underlie the infertility. We found that hypocalcemic and hypophosphatemic 1,25(OH)2D3-deficient male mice developed multiple disorders, including hypergonadotropic hypogonadism, decreased sperm count and motility, histological abnormalities of the testes, decreased proliferation of spermatogenic cells, and increased apoptosis of spermatogenic cells. Because of the strong association between vitamin D deficiency and hypocalcemia (6, 27, 33), we also examined the effect of mineral repletion through dietary sources on defective male reproductive function occurring in 1,25(OH)2D3-deficient male mice. It has been well documented that Ca2+ influx via calcium channels is required for acrosome reaction and sperm motility (3, 11, 23), and it has been reported that calcium channel blockers, by inhibiting calcium currents, may be responsible for the infertility side effects of these drugs (14). In this study, we found that the low ambient extracellular calcium in 1,25(OH)2D3 deficiency was also associated with low intracellular calcium due to downregulation of CaSR and Ca2+ channels, including CaV3.1 and TRPV5, and could be corrected by normalizing serum mineral concentrations.

In primary hypogonadism, testicular function is compromised, and in response the anterior pituitary releases above normal levels of LH/FSH in an attempt to increase output from Sertoli and Leydig cells (35). Gonadal steroid hormone production is an enzymatically mediated process catalyzed by several enzymes from two main categories: the cytochrome P450 enzymes (CYP11A and CYP17A) and HSD enzymes (3β-HSD and 17β-HSD) (26). 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 coexpression of VDR and the metabolizing enzymes in Leydig cells (5) suggest that 1,25(OH)2D3 might affect male reproductive hormone production. In the present study, we found that estrogen and testosterone levels were reduced significantly, and although FSH and LH levels were elevated in infertile 1α(OH)ase−/− male mice, the testes were not responsive to the elevated gonadotropins. Consistent with the reduced serum testosterone levels, the expression of 3β-HSD VI and 17β-HSD III was downregulated significantly in mutant testes, which was associated with reduced expression of the LH receptor. Inasmuch as intracellular calcium may also be required to mediate LH actions (21), reduced intracellular calcium may also have contributed to LH resistance. These alterations that occurred in 1α(OH)ase−/− mice could be completely corrected by normalizing serum calcium and phosphorus. In contrast, in the VDR−/− model, hypergonadotrophic hypogonadism was only partly corrected by increasing calcium and phosphorus levels to nearly normal with a rescue diet (19). Whether this implies that there are some VDR-dependent but mineral ion-independent effects on the hypothalamic-pituitary-testicular axis, as may occur in skin (24), remains to be determined.

The results from this study demonstrated that mineral ion-deficient and 1,25(OH)2D3-deficient mice displayed smaller testes, characterized by histological abnormalities, and significantly lower sperm counts; consequently, we examined whether there was an impairment of spermatogenesis. The process of spermatogenesis, in which stem spermatagonia mature into spermatozoa occurs in the seminiferous tubules of the testis, is a requirement for maintaining male fertility and is determined by a dynamic balance between spermatogenic cell proliferation and apoptosis (30). We found that the proliferation of spermatogenic cells was decreased with downregulation of cyclin E and CDK2 expression and upregulation of p53 and p21 expression, whereas apoptosis of spermatogenic cells was increased with upregulation of Bax and p-caspase 3 expression and downregulation of Bcl-xL expression. When serum calcium and phosphorus were normalized by the rescue diet, the defective spermatogenic parameters were reversed. The testicular expression of p53 is localized mainly to tetraploid spermatocytes undergoing meiotic division and is involved in the meiotic process during spermatogenesis in mice (1, 31). p53 overexpression in the testis has been reported to impair spermatogenesis (12). P21/waf1 is a p53-inducible protein and inhibits the formation of cyclin E-CDK2 complexes (4, 7), blocking cell cycle progression in the G1/S transition (10, 15). Additionally, p53 mediates spontaneous testicular germ cell apoptosis, and in p53-knockout mice, spontaneous apoptosis was less frequent than in wild-type mice (38). Failure to remove defective germ cells by apoptosis results in reduced
fertility with increased percentages of abnormal sperm. The ratio of proapoptotic to antiapoptotic 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. Previous reports have confirmed that overexpression of Bcl-2 and Bcl-xL resulted in reduced spermatogenic apoptosis and accumulation of spermatogonia and spermatocytes (13). However, overexpression of Bax arrested the spermatogenesis by inhibiting first wave of spermatogenesis (30). In mineral deficient 1,25(OH)2D3 deficiency, defective spermatogenesis occurred by activation of p53/p21 signaling to inhibit the proliferation of spermatogenic cells and to stimulate the apoptosis of spermatogenic cells with upregulation of the proapoptotic protein Bax and downregulation of the antiapoptotic protein Bcl-xL. This appeared to be mediated by low extracellular calcium and phosphorus per se.

Reduced sperm motility is a primary cause of infertility in male populations. We detected ultrastructural abnormalities in the sperm head but not in fibrous sheath structures of the sperm principal piece in 1α(OH)2D3−/− mice fed the normal diet, which could contribute to decreased sperm motility. When we analyzed sperm motility directly using the CASA assay, we found that the percentage of motile sperm, VAP, and mean VSL of sperm were reduced dramatically. However, the impaired motility could be corrected by normalizing serum calcium and phosphorus. Calcium can be mobilized into sperm from the external milieu by plasma membrane channels and is also released internally from intracellular stores such as the redundant nuclear envelope located at the base of sperm flagellum or the acrosome (8, 16). A number of calcium channel proteins have been identified in sperm by immunohistochemistry or by measuring specific channel activities and are regulated by voltage or other mechanisms (29). Increased intracellular calcium may convert sperm from a relatively linear, symmetrical swimming pattern to an asymmetrical form of motility called hyperactivated motility, which is essential for sperm fertility (39). In this study, we found that 1,25(OH)2D3 deficiency impaired sperm motility, which was mediated mainly by its effect on modulating extracellular calcium.

In summary, the data presented here suggest that the absence of 1,25(OH)2D3 results in abnormalities in the mouse male reproductive system that were normalized by dietary supplementation of calcium and phosphate. These results support the concept that the regulation of 1,25(OH)2D3 in the male reproductive system is mediated through extracellular calcium and phosphate.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

W.S., L.C., W.Z., and R.W. performed experiments; W.S., L.C., W.Z., and R.W. analyzed data; W.S., L.C., W.Z., and R.W. prepared figures; W.S. drafted manuscript; W.S., L.C., W.Z., R.W., D.G., and D.M. approved final version of manuscript; D.G. and D.M. edited and revised manuscript; D.M. conception and design of research; D.M. interpreted results of experiments.

REFERENCES


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