NON-ALCOHOLIC FATTY LIVER DISEASE (NAFLD) is a chronic liver condition that has a disease spectrum ranging from simple steatosis (SS) to non-alcoholic steatohepatitis (NASH), liver cirrhosis, and hepatocellular carcinoma (HCC) [1]. NAFLD has a 30% prevalence rate in the general population in western countries [2] and a 12–24% prevalence rate in China [3].

The progression of NAFLD is a complex process modulated by various mechanisms, such as oxidative and endoplasmic reticulum stress, lipotoxicity, and inflammation [1, 4]. The progression from SS to NASH is a key step in the development of NAFLD and individuals with NASH account for approximately 30% of NAFLD patients. Oxidative stress is a major determinant of the pathogenesis and progression of NASH [5, 6].

The suppression of oxidative stress is a key therapeutic goal in the prevention of NASH progression [7-9]. Modulating antioxidant capacity can redress the imbalance between oxidative stress and antioxidant protection in NAFLD. Nuclear factor-erythroid-2-related factor 2 (Nrf2) is a transcription factor that activates antioxidant response elements (AREs), thereby playing a central role in stimulating the expression of several antioxidant-associated genes in order to reduce oxidative stress [10, 11]. As expected, several natural compounds that activate Nrf2 are antioxidants [12].

Vitamin D is a well-documented suppressor of oxidative stress [13-15]. Vitamin D-dependent activation
of Nrf2, which in turn activates the Nrf2-Keap1 pathway, can protect against diabetic nephropathy [12, 16]. We previously demonstrated that the active form of vitamin D (1,25(OH)2D3) mitigated diabetes-related liver injury [17]. However, to our knowledge, there have been no studies investigating whether vitamin D activated the Nrf2 pathway in NAFLD. For this reason, we used 1,25(OH)2D3 to determine whether this vital cofactor attenuated oxidative stress and NAFLD in a Nrf2-dependent manner.

Materials and Methods

Animal models and treatment
Eight-week-old male Sprague-Dawley (SD) rats (Grade SPF) were purchased from Beijing Huafukang Biological Science and Technology Stock Co. Ltd (Beijing, China). The rats were kept under a 12:12-hour light/dark cycle in a room with constant temperature (21 ± 3°C) and had free access to water and food. The animals were randomly assigned to three groups: group CON (n = 10), fed standard rat chow containing 48% corn, 20% soybean meal, 10% wheat bran, 10% flour, 8% fish meal, 2% farina, 1% vegetable oil, 0.5% salt, and 0.5% trace elements; group HFD (n = 13), fed high-fat chow from the 0th week, containing 72% standard rat chow, 20% lard, 2% cholesterol, 5% egg yolk powder, and 1% bile salts, and treated with corn oil (1 mL/kg body weight, intraperitoneal injection, twice per week) from the 4th week; and group HFD+VD (n = 13), fed high-fat chow from the 0th week and treated with 1,25(OH)2D3 (5 μg/kg body weight, intraperitoneal injection, twice per week) from the 4th week. Body weight was recorded every 4 weeks. After 20 weeks, blood was collected from rat femoral arteries after a 14-hour fasting period. The animals were euthanized and the livers were excised, weighed, and the length of the tibias was recorded. One portion of the liver was placed in liquid nitrogen for quantitative RT-PCR, and another into 4% paraformaldehyde for histological analysis. The procedures were approved by the Animal Care and Use Committee of Tianjin Medical University (Tianjin, China) and were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Biochemical parameters
Serum alanine transaminase (ALT), aspartate aminotransferase (AST), triglycerol (TG), total cholesterol (TC), low-density lipoprotein (LDL), high-density lipoprotein (HDL), calcium (Ca), and phosphorus (P) levels were measured using an autoanalyzer (No. 7179A, Automatic Biochemistry Analyzer, Hitachi, Japan).

Intrahepatic triglyceride content analysis
Liver samples (100 mg) were homogenized with 9× phosphate-buffered saline (PBS) in ice water and centrifuged to extract the supernatant. Intrahepatic TG was measured using a triglyceride assay kit (Nanjing JianCheng Bioengineering Institute, Nanjing, China) according to the manufacturer’s protocol. Hepatic TG content was expressed as μmol/g liver protein (gprot liver).

Histology and immunohistochemistry
Formalin-fixed liver tissue sections were stained with hematoxylin-eosin (H&E) and Masson’s trichrome. Histological scoring analysis was performed by two blinded hepatopathologists using the NASH Clinical Research Network scoring system [18]. The NAFLD activity score (NAS) was calculated by assigning scores for steatosis (0 to 3), lobular inflammation (0 to 3), and hepatocellular ballooning (0 to 2).

Immunohistochemical staining with anti-NRF2 antibodies (No. sc-722, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was performed to observe changes in NRF2. Hematoxylin counter staining was used for NRF2 immunostaining. NRF2 nuclear translocation was quantified by both positive and negative staining counts of hepatocyte nuclei in ten fields of view at scale bar of 20 μm. The positive and negative nuclei counts determined the total number of hepatocytes and the percentage of positive nuclei was calculated [19].

Quantitative RT-PCR
Quantitative RT-PCR was performed for the quantification of gene expression. In brief, total RNA was extracted from the liver using Trizol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer’s instructions. The first strand cDNA was synthesized using a TIANScript RT Kit (Tiangen Biotech Co. Ltd, Beijing, China) and then PCR-amplified using a SYBR Green fluorescent PCR kit (Sangon Biotech, Shanghai, China) on a real time fluorescent PCR cycler using gene specific primers. The primer sequences are shown in Table 1. Relative mRNA levels were normalized to the internal reference gene Actb. Fold expression was calculated by the 2−ΔΔCt method.
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and 20th week, but the difference was not significant. In addition, this trend was not observed at the 8th week (Fig. 1A). The liver weight and liver weight:tibia length ratio was increased in group HFD compared with group CON and was significantly lower in group HFD+VD compared with group HFD (Fig. 1B and C).

Biochemical parameters

The results of the biochemical parameter analysis are shown in Table 2. Serum TGs, TC, and LDL were considerably increased in group HFD rats compared to control and considerably reduced in group HFD+VD compared to group HFD. However, serum HDL was considerably reduced in group HFD rats compared with group CON and increased in group HFD+VD. Both serum ALT and AST in group HFD+VD rats were considerably reduced compared to those in group HFD rats. There were no changes in serum calcium and phosphate levels.

Hepatic histology and intrahepatic lipid

As shown in Fig. 2A, hepatocytes of control rats were in a cord-like arrangement and the lobular structure was intact. No obvious infiltration of inflammatory cells or necrosis in the portal area was observed, with the exception of minor hepatic steatosis. However, in group HFD rats, hepatocytes were arranged in a disordered manner and obvious hepatic steatosis and hepatocellular ballooning were observed. Additionally, marked inflammatory cell infiltration around hepatocytes and diffuse infiltration around the portal vein were observed. Compared to group HFD rats, lesions were less frequent in group HFD+VD animals; the latter group had incidences of pathology similar to group CON rats (Fig. 2A).

Table 1  Sequences of primers for Quantitative RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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<tr>
<td>Actb</td>
<td>5′-CTCCTATGCCATCCTGCTGCTG-3′</td>
<td>5′-TTGCTCGAAGTCTAGGGCAACATAG-3′</td>
</tr>
<tr>
<td>Nrf2</td>
<td>5′-CAGCACATCCAGACAGACAC-3′</td>
<td>5′-CCAGAGACCTAGCTAGGCAACAC-3′</td>
</tr>
<tr>
<td>Gclc</td>
<td>5′-TTGAGAATCTCTGCTATGTC-3′</td>
<td>5′-CACTTTGATGTTTCTGTCGCAAC-3′</td>
</tr>
<tr>
<td>Nqo1</td>
<td>5′-CAATTCAGATGGCAATTCTCGG-3′</td>
<td>5′-GTTCGAACCTGGAAGCCAC-3′</td>
</tr>
<tr>
<td>Hmox-1</td>
<td>5′-AGTTCAACAGCTCTCATCGTG-3′</td>
<td>5′-TTTGTGTCTCTGTCGCAAC-3′</td>
</tr>
<tr>
<td>Sod2</td>
<td>5′-GGAGAACCAAGGAGATGTTG-3′</td>
<td>5′-AATCTGTAAGCGACCTTGCTC-3′</td>
</tr>
<tr>
<td>Cat</td>
<td>5′-TGTTGCTCCCAACTACTAC-3′</td>
<td>5′-ATAGAATGTCCGCACCTGAG-3′</td>
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</tbody>
</table>
**Table 2** Biochemical parameters of rats

<table>
<thead>
<tr>
<th></th>
<th>CON (n = 10)</th>
<th>HFD (n = 13)</th>
<th>HFD+VD (n = 13)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triglycerol (mmol/L)</td>
<td>0.65 ± 0.07</td>
<td>0.81 ± 0.04 **</td>
<td>0.68 ± 0.03 ***</td>
</tr>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>1.63 ± 0.14</td>
<td>1.91 ± 0.18 **</td>
<td>1.67 ± 0.14 **</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/L)</td>
<td>1.13 ± 0.08</td>
<td>0.66 ± 0.06 **</td>
<td>0.96 ± 0.08 **</td>
</tr>
<tr>
<td>LDL cholesterol (mmol/L)</td>
<td>0.20 ± 0.03</td>
<td>0.38 ± 0.04 **</td>
<td>0.21 ± 0.04 **</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>44.00 ± 3.37</td>
<td>60.00 ± 5.67 **</td>
<td>47.83 ± 3.97 **</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>134.75 ± 13.05</td>
<td>189.01 ± 15.96 **</td>
<td>167.12 ± 10.38 ***</td>
</tr>
<tr>
<td>Serum calcium (mmol/L)</td>
<td>2.25 ± 0.15</td>
<td>2.24 ± 0.16</td>
<td>2.37 ± 0.17</td>
</tr>
<tr>
<td>Serum phosphate (mmol/L)</td>
<td>2.14 ± 0.21</td>
<td>1.71 ± 0.24</td>
<td>2.00 ± 0.28</td>
</tr>
</tbody>
</table>

* = P < 0.05, ** = P < 0.01 vs group CON; * = P < 0.05, *** = P < 0.01 vs group HFD. Serum alanine transaminase (ALT), aspartate aminotransferase (AST), low-density lipoprotein (LDL), high-density lipoprotein (HDL), and high-fat diet (HFD) and control (CON) groups.

**Fig. 1** Effects of 1,25(OH)₂D₃ therapy on body weight and liver weight.

(A) The body weight of rats in group HFD was significantly increased at the 4th week over that of group CON. At the 8th, 12th, 16th, and 20th week, the body weight of rats in group HFD appeared to be increased compared with that in group CON, but the increase was not significant. Additionally, the body weight of rats in group HFD+VD did not significantly decrease compared to that in group HFD at the 12th, 16th, and 20th week. (B, C) Liver weight and liver weight:tibia length ratio of group HFD+VD rats decreased compared to those of group HFD. There were 10 to 13 rats in each group. * = P < 0.05, ** = P < 0.01 vs group CON, * = P < 0.05, ## = P < 0.01 vs group HFD.
As shown in Fig. 2B (Masson’s trichrome staining), no obvious fibrosis (indicated by green coloration) was found in the hepatic sinus and around hepatic cells of control animals. However, obvious coloration around sinusoidal spaces and in the portal area was observed in group HFD. Liver lobules in rats of this group were disordered and contained clumped fibrous tissue deposits. In group HFD+VD, the level of fibrosis was lower than in group HFD; liver structure in the former group was almost the same as in group CON. No pseudolobule formation was evident in any of the three groups.

Compared with those of group CON, liver fat, lobular inflammation, ballooning, and NAS scores were considerably elevated in group HFD animals. These scores were markedly reduced in group HFD+VD animals (Fig. 2C and 2D).

Oil Red O staining revealed small lipid droplets scattered in a small number of liver cells (< 5%) in group CON. In contrast, liver cells in group HFD animals were of different sizes and full of lipid droplets.
A diffuse distribution of hepatocytes with fewer lipid droplets per cell was observed in group HFD+VD (Fig. 3A). In addition, the intrahepatic TG level was considerably reduced in group HFD+VD compared to group HFD (Fig. 3B). No significant differences in serum calcium and phosphate were observed among the three groups (Table 2).

**Hepatic MDA and F2α-isoprostane content**

Hepatic MDA and F2α-isoprostane concentrations were increased in group HFD, confirming that the high-fat diet had indeed induced hepatic oxidative stress. Group HFD+VD rats had reduced hepatic MDA and F2α-isoprostane concentrations, compared to group HFD rats (Fig. 4).

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Fig. 3 (A) Liver sections stained with Oil Red O revealed hepatic lipid accumulation in all three groups. Scale bars: 50 μm. (B) Intrahepatic TG levels of group HFD+VD rats decreased compared to those of group HFD. There were 9 rats in each group. * = P < 0.05, ** = P < 0.01 vs group CON; † = P < 0.05, ‡ = P < 0.01 vs group HFD.

Fig. 4 Effects of 1,25(OH)2D3 therapy on hepatic oxidative stress in NAFLD rats. (A) Hepatic MDA content was significantly increased in group HFD and reduced in group HFD+VD. (B) Hepatic F2α-isoprostane content was reduced in group HFD+VD compared with group HFD. There were 9 rats in each group. * = P < 0.05, ** = P < 0.01 vs group CON; † = P < 0.05, ‡ = P < 0.01 vs group HFD.
Quantitative RT-PCR
We investigated Actb mRNA levels in liver tissues and used this as an internal control. The relative expression of Nrf2 mRNA and its target genes in liver tissues were determined. Nrf2 mRNA expression was not increased in either group HFD or group HFD+VD (Fig. 5A). However, the expression of Gclc, Nqo1, and Cat mRNA in group HFD was considerably increased compared to that in group CON. In addition, transcription levels of these genes were considerably increased in group HFD+VD rats compared to those in group HFD (Fig. 5B, 5C, 5F). Hmox1 expression levels appeared to increase in group HFD+VD animals; however, the change was not statistically significant (Fig. 5D). Finally, expression levels of Sod2 mRNA were considerably increased in group HFD+VD compared to those in groups CON and HFD (Fig. 5E).

![Graphs showing mRNA expression levels for Nrf2, Gclc, Nqo1, Hmox1, Sod2, and Cat for different groups.](image-url)
**NRF2 nuclear translocation**

Rat liver samples were immunohistochemically stained for NRF2. In group CON, no obvious positive staining was observed, whereas positive staining of NRF2 in the cytosol and negative staining of NRF2 in the nucleus (indicated by arrows) were observed in cells from HFD rats. Positive nuclear NRF2 staining (indicated by arrows) was observed in cells of HFD+VD rats (Fig. 6). Whereas 2.35% of group CON hepatocyte nuclei were NRF2-positive, this figure rose to 17.6% and 52.3% in group HFD and HFD+VD rats, respectively (Table 3).

**Discussion**

In NAFLD progression, the “two-hit” theory is widely accepted [21]. This theory posits that the first hit is lipid accumulation in the hepatocytes and the second hit is oxidative stress-induced lipotoxicity, which promotes cellular injury leading to NASH [22, 23]. In the present study, body weights, liver enzymes, serum TGs, and intrahepatic TGs were significantly elevated in group HFD rats. Liver pathology results demonstrated that our model faithfully recapitulated the tissue changes associated with NAFLD. Furthermore, significant elevation of MDA and F2α-isoprostane indicated the presence of hepatic oxidative stress, which is closely associated with NAFLD progression.

The relationship between vitamin D deficiency (VDD) and NAFLD has been investigated. VDD is prevalent among NAFLD patients and is independently associated with NASH [24-26]. Nakano et al. demonstrated that sunlight therapy increased serum 1,25(OH)_{2}D_{3} levels and ameliorated NASH progression in a choline-deficient and iron-supplemented l-amino acid-defined (CDAA) diet-induced NASH rat model [27]. Yin et al. suggested that 1,25(OH)_{2}D_{3} protected against HFD-induced hepatic steatosis by...
preventing FA oxidation and restoring lipogenesis [28]. A randomized clinical trial showed that vitamin D reduced serum MDA and liver enzymes, decreased oxidative stress, and reduced the expression of inflammatory biomarkers in adults with NAFLD [29]. In our previous study, the active form of vitamin D down-regulated the NF-κB pathway via Toll-like receptor 4; this was associated with a strong anti-inflammatory effect in rats subjected to liver injury [17]. Consistent with this, our current results showed that 1,25(OH)2D3 treatment reduced serum TG, TC, LDL, and intrahepatic TG and attenuated NAFLD-associated pathological changes in rats on a high-fat diet. In addition, the changes in serum TG, TC, LDL, and intrahepatic TG paralleled the changes to liver weight and the liver weight:tibia length ratio. The effects of vitamin D on liver weight may be related to the regulation of lipid metabolism-related gene expression in the liver [28]. The body weight of rats in group HFD+VD displayed the same trend as liver weight and the biochemical markers on 12th, 16th and 20th week, but no significant difference was found between groups HFD and HFD+VD. One reason may be that the different organs (i.e., adipose tissue) had different responses to vitamin D. Further studies are required to investigate the effects of vitamin D on different tissues. In addition, body weight can be affected by many factors (i.e., food intake). Therefore, the liver weight and the liver weight:tibia ratio were used as primary markers for evaluating the effects of vitamin D.

Vitamin D inhibits oxidative stress in animal models [13, 30, 31]. The synthetic variant, 1,25(OH)2D3, reduces lipid peroxidation and enhances superoxide dismutase, catalase, and glutathione peroxidase activity in diabetic rats [13]. The inhibition of cardiac NADPH oxidase activity in uremic rats may be associated with the effects of vitamin D [30]. However, prior to the current report, the relationship between vitamin D and oxidative stress in NAFLD had not been studied. We showed that hepatic oxidative stress markers were significantly reduced by vitamin D treatment, implying that this cofactor prevented HFD-induced oxidative stress in NAFLD.

Antioxidant enzymes served to protect against oxidative stress and the expression of antioxidant genes was induced by the Nrf2/ARE pathway [10]. We hypothesized that 1,25(OH)2D3 would enhance antioxidant capacity by modulating Nrf2 to combat oxidative stress. Therefore, we determined the expression of antioxidant genes, including Gclc, Nqo1, Homx1, Sod2, and Cat. As expected, the mRNAs encoding antioxidant enzymes, except Homx1, were up-regulated in group HFD+VD, indicating that 1,25(OH)2D3 therapy directly inhibited oxidative stress and enhanced the antioxidant capacity of the liver in NAFLD rats. Hmox1 gene expression was different from the others, possibly because Heme oxygenase-1 (HO-1) plays a role primarily in the early stages of NAFLD, such as hepatic steatosis [32]. However, hepatic Masson’s trichrome staining for rats in group HFD indicated liver fibrosis, which is the late stage of NAFLD. Karaman’s study suggested that the intensity of HO-1 expression was reduced during the development of liver fibrosis [33]. In addition, insufficient numbers of rats in our experiment may have been another reason for the statistical insignificance of Hmox1 expression between groups HFD and HFD+VD. Therefore, further studies are required to investigate the function of HO-1 in different stages of NAFLD.

The transcription factor Nrf2 plays an important role in protecting against NAFLD [10, 11, 34]. Nrf2 binds to Keap1 in the cytoplasm and enters the nucleus to regulate the expression of downstream antioxidant enzymes through binding to AREs. We hypothesized that 1,25(OH)2D3 would affect Nrf2 expression, thereby enhancing antioxidant capacity. Nrf2 expression is often up-regulated by antioxidants [7, 16, 35]. In this study, we found no changes in Nrf2 mRNA expression across all three groups; this was inconsistent with the results reported by Nakai et al. [16]. However, we did observe increased nuclear NRF2 immunohistochemical staining in samples from NAFLD rats treated with 1,25(OH)2D3, which implied that this therapy promoted NRF2 nuclear translocation in order to enhance antioxidant gene expression. Therefore, vitamin D-dependent modulation of Nrf2 translocation, rather than transcription, is more likely to play a role in protecting the liver from oxidative stress.

In our study, rats were treated with a high dose of 1,25(OH)2D3. Because high doses of 1,25(OH)2D3 may lead to disorders such as hypercalcemia, we tested serum calcium and phosphate levels. Serum calcium and phosphate levels in rats treated with 1,25(OH)2D3 were not significantly different from those of normal rats. One limitation of our study was that we were technically unable to determine the serum levels of 25(OH)D3 and vitamin D binding protein (DBP) in
the different experimental groups. Further studies are therefore required to clarify the relationship between 1,25(OH)2D3, VDR, and Nrf2, and to define the mechanisms by which vitamin D regulates Nrf2 translocation.

In conclusion, we found that 1,25(OH)2D3 therapy partially ameliorated hepatic histological damage in NAFLD rats. The protective effects of this molecule may be related to a reduction in oxidative stress and an increase in antioxidant capacity. Nrf2 nuclear translocation regulated by 1,25(OH)2D3 may be involved in the observed protective effects.

Acknowledgments

This work was supported by Grant No.30973040 to M.Z. from the Natural Science Foundation of China, and Grant No.ZYYFY2014040 to C.Z. from TMUGH funding.

Disclosures

None of the authors have any potential conflicts of interest associated with this research.

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