Clinics and Research in Hepatology and Gastroenterology (2019) xxx, xxx–xxx

Available online at ScienceDirect www.sciencedirect.com

Elsevier Masson France EM|consulte www.em-consulte.com/en

ORIGINAL ARTICLE

Active vitamin D impedes the progression of non-alcoholic fatty liver disease by inhibiting cell senescence in a rat model

Ming Ma*, Qi Long, Fei Chen, Ting Zhang, Wenqiao Wang

Department of Clinical Nutrition, the Children's Hospital, Zhejiang University School of Medicine, National Clinical Research Center for Child Health, 310052 Hangzhou, P.R, China

KEYWORDS
Non-alcoholic fatty liver disease;
Active vitamin D;
Oxidative stress;
Cell senescence;
P53-p21 signaling pathway;
Vitamin D receptor

Summary
Objective: Non-alcoholic fatty liver disease (NAFLD) refers to an accumulation of excess fat in liver due to causes other than alcohol use. The relationship between vitamin D (VD) and NAFLD has been previously studied. Therefore, we aimed to explore the mechanism involved active VD regulating the progression of NAFLD by inhibiting cell senescence and to provide a potential approach for further nutritional treatment of NAFLD.
Methods: Following the induction with high-fat diet and intraperitoneal injection of corn oil, the successfully established NAFLD rat models were treated with $1,25(OH)_2D_3$ at 1 μg/kg, 5 μg/kg or 10 μg/kg. Meanwhile, the levels of factors related to oxidative stress, cell senescence, the p53-p21 signaling pathway and inflammation in liver were determined. Then, cell senescence was also measured by using senescence-associated β-galactosidase (SAβ-gal) staining.
Results: It was also found that active VD increased the concentration of VD in serum and VDR in liver of NAFLD rats, and alleviated hepatic fibrosis. Besides, treatment of $1,25(OH)_2D_3$ at 1 μg/kg, 5 μg/kg or 10 μg/kg reduced oxidative stress and inflammation, inhibited the p53-p21 signaling pathway and consequent cell senescence. Furthermore, treatment of $1,25(OH)_2D_3$ at a dosage of 5 μg/kg made the most impact on these factors.
Conclusion: Collectively, the evidences from this study demonstrated that active VD could alleviate the development of NAFLD through blocking the p53-p21 signaling pathway, which provided a novel nutritional therapeutic insight for NAFLD.
© 2019 Elsevier Masson SAS. All rights reserved.

Abbreviations: NAFLD, Non-alcoholic fatty liver disease; VD, Vitamin D; ALT, Alanine aminotransferase; TBARS, Thiobarbituric acid-reactive substance; MDA, Malonaldehyde; TAOC, Total antioxidant capacity; TNF, Tumor necrosis factor.

* Corresponding author. Department of Clinical Nutrition, the Children's Hospital, Zhejiang University School of Medicine, National Clinical Research Center for Child Health, No. 3333, Binsheng road, Binjiang District, 310052 Hangzhou, Zhejiang Province, P.R. China.
E-mail address: maming73@zju.edu.cn (M. Ma).

https://doi.org/10.1016/j.clinre.2019.10.007
2210-7401/© 2019 Elsevier Masson SAS. All rights reserved.

Please cite this article in press as: Ma M, et al. Active vitamin D impedes the progression of non-alcoholic fatty liver disease by inhibiting cell senescence in a rat model. Clin Res Hepatol Gastroenterol (2019), https://doi.org/10.1016/j.clinre.2019.10.007

Introduction

Remarkably, the overall prevalence of non-alcoholic fatty liver disease (NAFLD) in children has reached approximately 10%, including up to 17% in teenagers and 40—70% among obese children [1]. NAFLD can also result in various complications like cirrhosis, steatohepatitis, fibrosis, advanced liver failure and hepatocellular cancer, all of which contribute to worse prognoses [2]. As a type of fatty liver disease, NAFLD is usually correlated with several metabolic comorbidities such as diabetes, dyslipidemia and obesity, and the two known extremes of NAFLD include: non-alcoholic fatty liver (NAFL) and non-alcoholic steatohepatitis (NASH) [3,4]. The risk factors of NAFLD comprise of diabetes, obesity, high-carbohydrate diet and older age [5]. Currently, the general treatments for NAFLD are weight loss through healthy diet and exercise, and pharmacological options such as vitamin E, pioglitazone and pentoxifylline shows tentative efficiency, which also indicated that novel and more effective treatment is to be explored [6]. Interestingly, vitamin D (VD) has been previously demonstrated to be correlated with NAFLD, which caught our attention [7].

VD is one of the vitamins that are usually deficient in obese children and adolescents [8]. Recently, the therapeutic status of VD has been uplifted since being regarded as a crucial agent in immunoregulation and anti-fibrosis [9]. Furthermore, the importance of VD in the occurrence and pathogenesis of liver-related disease has been proved in several former studies. For instance, VD was found to display function in fatty liver- and alcoholic liver-related diseases [10], and the lack of 25-hydroxyvitamin D [25(OH)D], one form of VD, exhibited correlations with unfavorable prognosis in alcoholic liver disease patients [11]. Also, 1,25-dihydroxyvitamin D3[1,25$(OH)_2D_3$], one form of active VD, has been involved in various cell activities such as cell growth, apoptosis and immunoregulation [12]. In addition, 1,25$(OH)_2D_3$ has been demonstrated to diminish oxidative stress in high-fat diet-induced NAFLD, decrease the generation of inflammatory factors, inhibit the cell senescence of vascular smooth muscle cells (VSMCs), and suppresses the liver fibrosis, indicating promising nutrition supplemental therapeutic insight for liver disease [13—16]. Additionally, 1,25$(OH)_2D_3$ suppresses proliferation of gastric cancer cells through vitamin D receptor (VDR) and mutant p53 interaction by regulating p21/CDK2 [17]. Moreover, VD also has been reported to improve cell growth of adipose-derived stem cells by regulating the p53-p21 signaling pathway, suggesting that pathway modulation with VD may offer some new insight with NAFLD as well [18]. In former researches, the p53-p21 signaling pathway has been proved to participate in several biological processes including cell senescence. For example, caveolin-1 regulated the nucleus pulposus cells senescence induced by oxidative stress via the p53-p21 signaling pathway [19], and silenced B-myb enhanced senescence of vascular endothelial cells through ROS-regulated p53-p21 signaling pathway [20]. From the aforementioned findings, we hypothesized that active VD could protect against NAFLD by regulating oxidative stress, cell senescence and inflammation via mediation of the p53-p21 signaling pathway. Therefore, the current study aims to demonstrate the mechanism that active VD inhibited NAFLD by repressing cell senescence, thus providing a novel nutritional therapeutic insight for NAFLD.

Methods and materials

Ethics statement

All animal experiments were carried out in accordance with the principles and procedures of Guide for the Care and Use of Laboratory Animal by the National Institutes of Health and under a protocol approved by the Laboratory Animal Care and Use Committee of the Zhejiang University. All efforts were made to minimize of the suffering of the included animals.

Animal treatment

The male Sprague-Dawley rats of specific pathogen free (SPF) grade (aged 8 weeks) were purchased from Zhejiang University Laboratory Animal Center (Hangzhou, China). The rats were fed in SPF animal laboratory individually under a 12:12-h light/dark cycle in a room with constant temperature of 21 °C ± 3 °C with a humidity of 60—65% and had free access to food and water. The rats were randomly divided into following 5 groups with 10 in each group: normal group (rats were fed with the standard diet), NAFLD group (rats were fed with the high-fat diet containing 72% standard diet, 20% lard, 2% cholesterol, 5% egg yolk powder and 1% bile salt, and then intraperitoneally injected with corn oil at 1 mL/kg from the 4th week [2 times per week]), NAFLD + VD1 group (NAFLD rats were intraperitoneally injected with 1,25$[OH]_2D_3$ at 1 μg/kg from the 4th week [2 times per week]), NAFLD + VD5 group (NAFLD rats were intraperitoneally injected with 1,25$[OH]_2D_3$ at 5 μg/kg from the 4th week [2 times per week]), and the NAFLD + VD10 group (NAFLD rats were intraperitoneally injected with 1,25$[OH]_2D_3$ at 10 μg/kg from the 4th weeks [2 times per week]). The serum of rats was collected at the 4th, 8th, and 12th week, respectively. At the end of the 12th weeks, the rats were euthanized following 12—13 h of fasting food and water. Next, the body weight, body length and liver wet weight of rats were measured, and the rat liver index (%) = liver wet weight/the body weight × 100% was calculated. The blood and liver tissues were collected for serum and liver histopathologic detection.

Hematoxylin-eosin (HE) staining

The tissue sections were dewaxed, hydrated, stained with hematoxylin (PT001, Shanghai Bogoo Biotechnology Co., Ltd., Shanghai, China) at room temperature for 10 min, and differentiated by 1% hydrochloric ethanol for 30 s. Afterwards, the sections were stained with eosin (0001-H, Beijing CinHuaLvYuan Science and Technology Ltd., Beijing, China) at room temperature for 1 min and then dehydrated with gradient alcohol (70%, 80%, 90%, 95% and 100%, each concentration for 1 min). Next, the sections were cleared with xylene carbonate for 2 min and with xylene I and II (GD-RY1215-12, Shanghai Guduo Biotechnology Co., Ltd, Shanghai, China) two times respectively (each time for

Please cite this article in press as: Ma M, et al. Active vitamin D impedes the progression of non-alcoholic fatty liver disease by inhibiting cell senescence in a rat model. Clin Res Hepatol Gastroenterol (2019), https://doi.org/10.1016/j.clinre.2019.10.007

1 min), mounted by neutral balsam, photographed under a light microscope to observe the morphologic changes of the liver tissues (400 ×). Each experiment was repeated 3 times.

Immunohistochemistry

The tissue sections were dewaxed, dehydrated by gradient alcohol, immersed in 3% methanol H_2O_2 for 20 min, and repaired by antigen repair solution in a water-bath. Next, the sections were cooled down under tap water, incubated with normal goat serum blocking solution (C-0005, Shanghai Haoran Bio Technologies Co., Ltd., Shanghai, China) at room temperature for 20 min and incubated with rabbit antibody to β-gal (ab4761, 1: 200; Abcam Inc., Cambridge, UK) at 4 °C overnight. The next day, the sections were incubated with secondary antibody, goat anti-rabbit antibody to immunoglobulin G (IgG; ab9361, 1: 1000, Abcam Inc., Cambridge, UK) at 37 °C for 20 min, and with horse radish peroxidase (HRP)-labeled streptomycin avidin protein working solution (0343-10000U, Imunbio, Beijing, China) at 37 °C for 20 min. Then, the sections were developed with diaminobenzidine (DAB; ST033, Whiga Biosmart Co., Ltd., Guangzhou, Guangdong, China), counterstained with hematoxylin (PT001, Shanghai Bogoo Biotechnology Co., Ltd., Shanghai, China) for 1 min, and returned to blue by 1% ammonia. The sections were dehydrated with gradient alcohol, cleared with xylene and mounted with neutral balsam. Lastly, the sections were observed and photographed under a microscope, with 5 high-power fields randomly selected in each section and 100 cells counted in each field. Each experiment was repeated 3 times.

Masson staining

Paraffin-embedded sections were heated at 65 °C for 3 h, dewaxed, dehydrated and immersed in 10% trichloroacetic acid solution and 10% potassium bichromate solution for 40 min, respectively. Next, sections were stained with hematoxylin (PT001, Shanghai Bogoo Biotechnology Co., Ltd., Shanghai, China) for 8 min, immersed in the mixed solution of 1% ponceau (HL12202, Shanghai Haling Biotechnology Co., Ltd., Shanghai, China) and 1% fuchsin (HPBIO-SJ820, Shanghai HePeng Biotechnology Co., Ltd., Shanghai, China) for 40 min, and then successively added with 1% glacial acetic acid and 1% molybdic acid liquid to terminate the reaction. Then, sections were added with the mixture of 1% brilliant green, 1% phosphomolybdic acid and running water to terminate the reaction, dehydrated, mounted with transparent neutral balsam and placed under an optical microscope to observe the morphologic changes of the musculi multifidus. With 5 high-power fields randomly observed in each section (200 ×), it could be found that musculature was presented in red, nucleus in purple and collagenous fiber in blue. Each experiment was repeated 3 times. Each iamge was evaluated according to semi-quantitative scoring standard: 1 score, not seen; 2 scores, occasional; 3 scores, scattered visible; 4 scores, more common; 5 scores, large pieces of fusion.

Culture of primary hepatocytes and cell suspension preparation

The primary hepatocytes were obtained by Seglen's two-step collagenase perfusion from rats after fasting for 14 h. The liver was perfused with calcium-free perfusion solution I at 30 mL/min for 15 min and with perfusion solution II containing collagenase at 20 mL/min for 7 min, then washed two times with ice-cold Hanks' buffered salt solution and centrifuged at 60 × g for 3 min at 4 °C to harvest primary hepatocytes. The cells were seeded in a cell culture plate coated with type I collagen. Finally, the cells were centrifuged with Dulbecco's modified Eagle's medium (DMEM) and centrifuged at 50 × g for 3 min at 4 °C. The cells were cultured at 37 °C under 5% CO_2 with DMEM containing 5% fetal bovine serum (FBS) and 100 U/mL penicillin/streptomycin. After perfusion, the hepatocyte suspension was first passed through a 100 meshes stainless steel filter to remove connective tissues and cell masses, centrifuged at 300 rpm for 5 min. The cell suspension was then filtered by a 200 meshes stainless steel filter, and then centrifuged with 300 rpm for 3 min. The above procedure was repeated two times. The precipitated hepatocytes were dispersed evenly with 50 mL hepatocytes cleaning solution, added to a pre-prepared discontinuous density gradient of Percoll and centrifuged at 500 rpm for 5 min. The hepatocytes were suspended because the density was close to the Percoll solution, the tissue fragments were precipitated at the bottom, while the hepatic non-parenchymal cells, broken hepatocytes and red blood cells were drifting at the top. The hepatocytes were dispersed into cell suspension with hepatocyte culture medium containing 10% FBS.

Senescence-associated β-galactosidase (SAβ-gal) staining

The hepatocytes were cultured with the cell culture medium in a 6-well plate. After the cell culture medium was discarded, the hepatocytes were fixed with 1 mL SAβ-gal staining fixative at the room temperature for 20 min, rinsed 3 times with phosphate buffer saline (PBS) (each time for 3 min), and then incubated with 1 mL staining working solution at 37 °C overnight. Cells were observed and counted under a microscope with blue cells deemed as senescent cells. Each experiment was repeated 3 times.

Flow cytometry

The prepared hepatocyte suspension was treated with blueberry anthocyanins and hydrogen peroxide, and then incubated with dichlorofluorescein-diacetate (DCF-DA; Sigma-Aldrich Chemical Company, St Louis, MO, USA). Upon entering into the cells, DCF-DA could be oxidized by reactive oxygen species (ROS) and then generate into fluorescigenic 2', 7'-DCF52. Subsequently, a flow cytometer (BD FACSCalibur, Becton, Dickinson and Company, Franklin L., New Jersey, USA) was applied in order to examine the fluorescent level of DCF, with negative control (NC) and positive control set.

Please cite this article in press as: Ma M, et al. Active vitamin D impedes the progression of non-alcoholic fatty liver disease by inhibiting cell senescence in a rat model. Clin Res Hepatol Gastroenterol (2019), https://doi.org/10.1016/j.clinre.2019.10.007

2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) method

The protein concentration of liver homogenate was determined, and the ABTS working solution was prepared and store at room temperature for 24 h in the dark. Each detection well of the 96-well plate was added with 200 uL of ABTS working solution. Meanwhile, the blank well was added with 10 uL of PBS, while the standard curve detection well was added with 10 uL of Trolox, and the sample detection well was added with 10 uL of sample. After being completely mixed, the plate was incubated at room temperature for 5 min. Then, A734 was determined using an excitation wavelength of 740 nm, and then the total antioxidant capacity (TAOC) value was calculated according to the standard curve, as expressed by the ratio of the TAOC concentration of samples to the protein concentration of samples.

Biochemical analysis

The total cholesterol (TC), triglyceride (TG), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels in serum samples were detected using a biochemistry analyzer (7020, HITACHI, Tokyo, Japan).

Enzyme-linked immunoassay (Elisa)

The serum collected from rats in each group at the 12th week was centrifuged at 3000 rpm for 5-10 min with the supernatant collected for detection. The serum 25-hydroxyvitamin D (25-OH-D) antibody levels were determined in strict accordance with the instructions of human 25-OH-D Elisa kit (JKSJ-2537, Shanghai gelatins Biotech Co., Ltd., Shanghai, China). The liver homogenate of rats in each group was centrifuged at 3000 rpm for 5 min with the supernatant collected for detection. Then, the contents of interleukin-6 (IL-6), IL-2, tumor necrosis factor (TNF)-α, thiobarbituric acid-reactive substance (TBARS) and malonaldehyde (MDA) were determined respectively using the IL-6 kit (JLC1236), IL-2 kit (JLC1706), TNF-α kit (LC2062), TBARS kit (JLC1752) (all provided by Shanghai gelatins Biotech Co., Ltd., Shanghai, China) and MDA kit (A003-1, Nanjing JianCheng Bioengineering Institute, Nanjing, Jiangsu, China).

Western blot analysis

The liver tissues at the 12th week were lysed by protein lysis buffer at 4 °C for 30 min, centrifuged at 12,000 r/min for 20 min at 4 °C. Next, the supernatant was extracted, and the protein concentration was determined using a bicinchoninic acid (BCA) kit (20201ES76, Yeasen Company, Shanghai, China). Then, the protein was separated with polyacrylamide gel electrophoresis (PAGE) and transferred onto a polyvinylidene fluoride (PVDF) membrane using the wet-transferring method. The membrane was blocked with 5% bovine serum albumin (BSA) at room temperature for 1 h, and incubated with diluted primary antibody, rabbit anti-rat antibodies against VDR (ab109234, 1: 1000), p53 (ab131442, 1: 1000), p21 (ab109199, 1: 1000) and cysteine-rich 61 (CYR61 or CCN1; ab24448, 1: 500) at 4 °C overnight. The next day, the membrane was incubated with HRP-labeled goat anti-rabbit antibody against IgG (ab205718, 1: 20000) at room temperature for 1 h. All aforementioned antibodies were purchased from Abcam Inc. (Cambridge, UK). After that, the membrane was developed and analyzed by the ImageJ 1.48u software (National Institutes of Health, Bethesda, MA, USA) with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) used as internal reference. The relative protein expression was expressed as the gray value of the ratio of protein to GAPDH. Each experiment was repeated 3 times.

Statistical analysis

Statistical analyses were processed using the SPSS 21.0 statistical software (IBM Corp., Armonk, NY, USA). Measurement data were expressed as mean $\pm$ standard deviation. All data were tested for normal distribution and homogeneity of variance were tested. If conforming to normal distribution and homogeneity of variance, data among multiple groups were compared by one-way analysis of variance (Anova) or repeated measure Anova, and the pairwise comparison within group was analyzed by post hoc test. Data with skewed distribution or heterogeneity of variance were analyzed by rank-sum test. A value of $P < 0.05$ was considered to be statistically significant.

Results

Active VD alleviates the progression of NAFLD in rats

Firstly, we measured the body weight, liver wet weight and liver index of rats in each group. As shown in Table 1, the rats in the NAFLD group exhibited higher liver index and liver wet weight compared to the normal group ($P < 0.05$), while there were no significant differences in terms of body weight and body length. NAFLD rats treated with $1,25(OH)_2D_3$ at 1 μg/kg, 5 μg/kg and 10 μg/kg presented with obviously decreased liver index and liver wet weight, which were especially lower in NAFLD rats injected with 5 μg/kg $1,25(OH)_2D_3$ than NAFLD rats injected with 1 μg/kg and 10 μg/kg ($P < 0.05$).

Subsequently, serum indicators of rats in each group were compared, and the results are presented in Table 2. At the 4th, 8th, and 12th weeks after successful model establishment, the rats in the NAFLD group exhibited increased levels of ALT, AST, TG and TC in serum compared to those in the normal group ($P < 0.05$). As the time of 1, $25(OH)_2D_3$ treatment expanded, the levels of ALT, AST, TG and TC in serum were found to exhibit a decrease in NAFLD rats injected with $1,25(OH)_2D_3$ at 1 μg/kg, 5 μg/kg and 10 μg/kg. Notably, the levels of ALT, AST, TG and TC in serum were lower in NAFLD rats injected with 5 μg/kg of $1,25(OH)_2D_3$ compared to those injected with 1 μg/kg and 10 μg/kg $1,25(OH)_2D_3$ ($P < 0.05$).

Then, HE staining was conducted in order to observe the morphologic changes of the liver tissues in each group. The normal group exhibited no degeneration or necrosis of hepatocytes and no inflammatory cells appeared in portal area. Compared with the normal group, the NAFLD group

Please cite this article in press as: Ma M, et al. Active vitamin D impedes the progression of non-alcoholic fatty liver disease by inhibiting cell senescence in a rat model. Clin Res Hepatol Gastroenterol (2019), https://doi.org/10.1016/j.clinre.2019.10.007

Table 1 The effect of 1, 25$(OH)_2D_3$ treatment on the body length, body weight, liver wet weight and liver index of rats in each group.

Group	Body length (mm)	Body weight (g)	Liver wet weight (g)	Liver index (%)
Normal	205.92 ± 4.35	338.67 ± 3.12	13.12 ± 0.48	3.87 ± 0.15
NAFLD	210.18 ± 5.24	334.58 ± 6.16	20.20 ± 0.66[a]	6.04 ± 0.24[a]
NAFLD + VD1	202.76 ± 3.69	335.55 ± 5.04	14.82 ± 1.67[b]	4.42 ± 0.49[b]
NAFLD + VD5	208.24 ± 4.88	337.17 ± 4.08	13.14 ± 0.58[b,c]	3.89 ± 0.17[b,c]
NAFLD + VD10	206.83 ± 4.05	336.40 ± 9.50	14.96 ± 1.36[b]	4.45 ± 0.45[b]

Liver index (%): liver wet weight/body weight × 100%. The data above were measurement data, which were expressed as mean ± standard deviation.
[a] $P < 0.05$ compared with the normal group.
[b] $P < 0.05$ compared with the NAFLD group.
[c] $P < 0.05$ compared with the NAFLD + VD1 and NAFLD + VD10 groups. $n = 10$.

Table 2 Comparison of serum indicators of rats in each group.

Group	Normal	NAFLD	NAFLD + VD1	NAFLD + VD5	NAFLD + VD10
ALT (U/L)					
4w	42.43 ± 2.44	59.01 ± 4.23[a]	49.11 ± 4.67[b]	42.86 ± 4.71[b,c]	48.67 ± 4.10[b]
8w	46.20 ± 4.58	68.43 ± 4.33[a]	53.34 ± 4.36[b]	46.58 ± 3.30[b,c]	52.45 ± 5.67[b]
12w	42.80 ± 1.51	82.58 ± 6.44[a]	50.34 ± 3.44[b]	43.04 ± 3.46[b,c]	49.22 ± 5.34[b]
AST (U/L)					
4w	112.56 ± 5.36	150.64 ± 6.54[a]	124.45 ± 5.33[b]	113.94 ± 6.95[b,c]	123.34 ± 5.03[b]
8w	119.39 ± 8.39	169.54 ± 6.45[a]	133.43 ± 8.11[b]	120.61 ± 6.71[b,c]	130.35 ± 7.67[b]
12w	111.06 ± 5.44	187.78 ± 6.77[a]	125.34 ± 7.45[b]	112.55 ± 9.67[b,c]	124.52 ± 8.38[b]
TG (mmol/L)					
4w	0.98 ± 0.07	1.19 ± 0.07[a]	1.10 ± 0.07[b]	0.99 ± 0.05[b,c]	1.09 ± 0.06[b]
8w	0.93 ± 0.07	2.43 ± 0.08[a]	1.08 ± 0.06[b]	0.95 ± 0.08[b,c]	1.06 ± 0.08[b]
12w	0.94 ± 0.09	2.78 ± 0.08[a]	1.06 ± 0.06[b]	0.96 ± 0.05[b,c]	1.05 ± 0.06[b]
TC (mmol/L)					
4w	3.31 ± 0.20	4.14 ± 0.14[a]	3.72 ± 0.12[b]	3.39 ± 0.20[b,c]	3.71 ± 0.11[b]
8w	3.88 ± 0.13	6.54 ± 0.15[a]	4.12 ± 0.11[b]	3.90 ± 0.17[b,c]	4.10 ± 0.17[b]
12w	3.53 ± 0.19	6.78 ± 0.12[a]	3.84 ± 0.15[b]	3.62 ± 0.11[b,c]	3.81 ± 0.09[b]

ALT: alanine aminotransferase; AST: aspartate aminotransferase; TG: triglyceride; TC: total cholesterol. The data above were measurement data, which were expressed as mean ± standard deviation.
[a] $P < 0.05$ compared with the normal group.
[b] $P < 0.05$ compared with the NAFLD group.
[c] $P < 0.05$ compared with the NAFLD + VD1 and NAFLD + VD10 groups. $n = 10$.

showed obviously increased steatotic hepatocytes, which presented as the mixed steatosis of large and small vesicles, increased ballooning degenerated hepatocytes and visible focal necrosis. Comparatively, the treatment of 1,25$(OH)_2D_3$ at 1 μg/kg, 5 μg/kg and 10 μg/kg decreased the steatotic hepatocytes, ballooning degenerated hepatocytes and focal necrosis (Fig. 1A).

Additionally, Masson staining was employed to observe the liver fibrosis in tissues from each group. It could be observed in Fig. 1B that the normal group exhibited no fibrous tissue formation in the hepatic lobule, while the NAFLD group showed increased blue collagenous fiber infiltration. Importantly, injection with 1,25$(OH)_2D_3$ at 1 μg/kg, 5 μg/kg and 10 μg/kg reduced blue collagenous fiber infiltration in NAFLD rats.

Moreover, Elisa was applied to examine the expression patterns of 1,25$(OH)_2D_3$ in serum of rats. It was revealed that the expression of 1,25$(OH)_2D_3$ in serum was lower in the NAFLD group than that in the normal group ($P < 0.05$). Comparatively, the NAFLD + VD1, NAFLD + VD5 and NAFLD + VD10 groups presented with increased expressions of 1,25$(OH)_2D_3$ in serum relative to the NAFLD group ($P < 0.05$). Remarkably, the expression of 1,25$(OH)_2D_3$ in serum was higher in the NAFLD + VD5 group than in the NAFLD + VD1 and NAFLD + VD10 groups ($P < 0.05$; Fig. 1C).

Furthermore, Western blot analysis was employed to detect the expression of VDR in liver in each group. Data presented in Fig. 1D revealed that the NAFLD group exhibited significantly decreased expression of VDR in liver relative to the normal group ($P < 0.05$). After treatment of 1,25$(OH)_2D_3$ at 1 μg/kg, 5 μg/kg and 10 μg/kg, the expression of VDR was increased in liver, especially was high after the treatment of 1,25$(OH)_2D_3$ at a dosage of 5 ug/kg.

Please cite this article in press as: Ma M, et al. Active vitamin D impedes the progression of non-alcoholic fatty liver disease by inhibiting cell senescence in a rat model. Clin Res Hepatol Gastroenterol (2019), https://doi.org/10.1016/j.clinre.2019.10.007

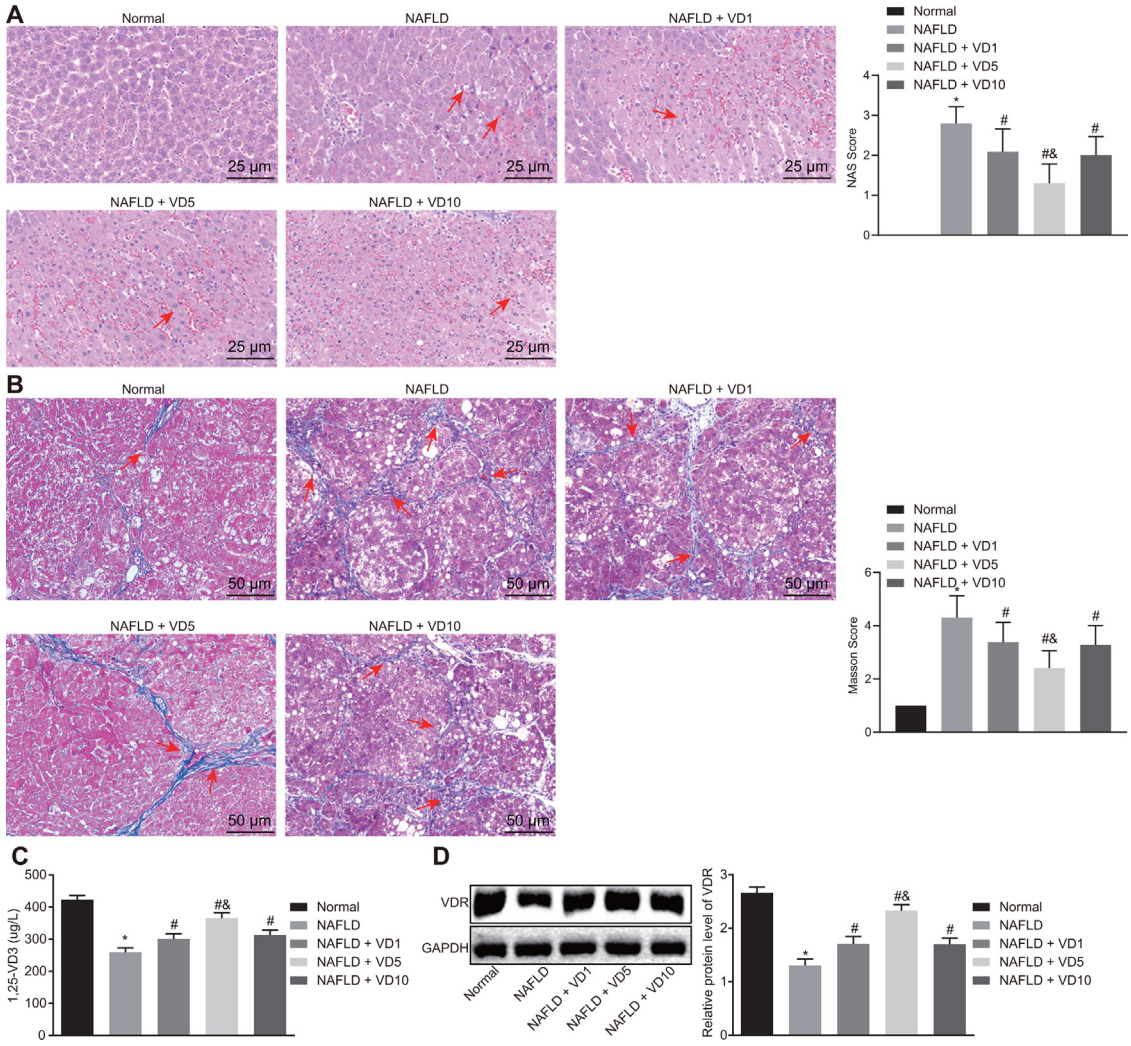

Figure 1 The progression NAFLD is ameliorated by active VD in rats. A. The pathological changes of liver of in each group examined by HE staining (× 400). B. The liver fibrosis in each group examined by Masson staining (× 200). C. The expression of $1,25(OH)_2D_3$ in serum of each group measured by Elisa. D. The expression of VDR in liver in each group examined by western blot analysis. *: $P < 0.05$ compared with the normal group; #: $P < 0.05$ compared with the NAFLD group; &: $P < 0.05$ compared with the NAFLD + VD1 and NAFLD + VD10 groups. All data were measurement data, which were expressed as mean ± standard deviation and analyzed by one-way analysis of variance, followed by post hoc test. $n = 10$. NAFLD: non-alcoholic fatty liver disease; VD: vitamin D; HE: hematoxylin-eosin; TG: triglyceride; TC: total cholesterol; Elisa: enzyme-linked immunoassay; VDR: vitamin D receptor.

All the aforementioned findings led us to the conclusion that the NAFLD rat models were successfully established, $1,25(OH)_2D_3$ treatment could impede the progression of NAFLD, and $1,25(OH)_2D_3$ treatment at a dosage of 5 μg/kg exhibits the best therapeutic efficiency.

Active VD reduces oxidative stress

Elisa, ABTS method and flow cytometry were employed in order to measure the contents of MDA and TBARS in liver, the TAOC of liver and the level of ROS, respectively. The contents of MDA, TBARS and ROS level were found to be higher, while the content of TAOC was lower in the NAFLD group compared to the normal group (all $P < 0.05$), but opposite trends were noted after treatment of $1,25(OH)_2D_3$ at 1 μg/kg, 5 μg/kg and 10 μg/kg, and were strikingly obvious after being treated with 5 μg/kg dosage of $1,25(OH)_2D_3$ (Fig. 2A—D). Therefore, it could be concluded that active VD could ameliorate NAFLD through suppressing oxidative stress.

Active VD inhibits the p53-p21 signaling pathway and cell senescence

Immunohistochemistry and SAβ-gal staining were conducted to examine the positive rates of β-gal and SAβ-gal, respectively. As depicted in Fig. 3A and B, compared with the normal group, the NAFLD group exhibited significantly increased positive rates of β-gal and SAβ-gal (all $P < 0.05$). Relative to the NAFLD group, the NAFLD + VD1, NAFLD + VD5 and NAFLD + VD10 groups all exhibited significantly decreased positive rates of β-gal and SAβ-gal (all $P < 0.05$), which were especially lower in the NAFLD + VD5

Please cite this article in press as: Ma M, et al. Active vitamin D impedes the progression of non-alcoholic fatty liver disease by inhibiting cell senescence in a rat model. Clin Res Hepatol Gastroenterol (2019), https://doi.org/10.1016/j.clinre.2019.10.007

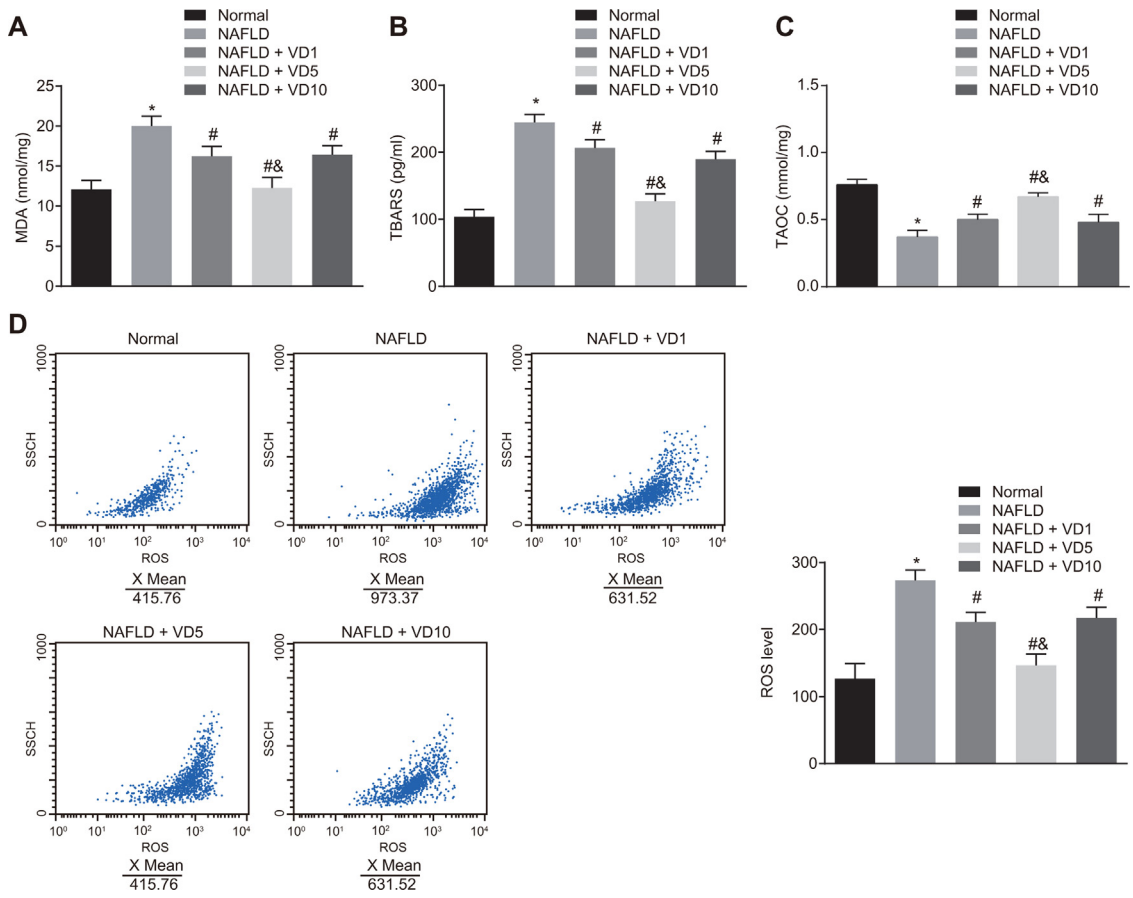

Figure 2 Active VD inhibits the oxidative stress. A. The content of MDA in liver of each group examined by Elisa. B. The content of TBARS in liver of each group examined by Elisa. C. The TAOC in liver of each group detected by ABTS method. D. The level of ROS in each group detected by flow cytometry. *: $P < 0.05$ compared with the normal group; #: $P < 0.05$ compared with the NAFLD group; &: $P < 0.05$ compared with the NAFLD + VD1 and NAFLD + VD10 groups. All data were measurement data, which were expressed as mean ± standard deviation and analyzed by one-way analysis of variance, followed by post hoc test. $n = 10$. NAFLD: non-alcoholic fatty liver disease; VD: vitamin D; Elisa: enzyme-linked immunoassay; TBARS: thiobarbituric acid-reactive substance; TAOC: total antioxidant capacity; ABTS, 2: 2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid).

group than that in the NAFLD + VD1 and NAFLD + VD10 groups (all $P < 0.05$).

Subsequently, Western blot analysis and Elisa were used to examine the expression of p53, p21 and CCN1 and the levels of IL-2, IL-6 and TNF-α in liver, respectively. The expression of p53, p21 and CCN1 and the levels of IL-2, IL-6 and TNF-α were noted to be significantly increased in the NAFLD group compared with the normal group (all $P < 0.05$). After treatment of $1,25(OH)_2D_3$ at 1 μg/kg, 5 μg/kg and 10 μg/kg, the expression of p53, p21 and CCN1 and the levels of IL-2, IL-6 and TNF-α were decreased, and were the most apparently reduced after being treated with 5 μg/kg dosage of $1,25(OH)_2D_3$ (Fig. 3C—D).

From the findings above, we demonstrated that active VD could alleviate the development of NAFLD by suppressing cell senescence via disrupting the p53-p21 signaling pathway.

Discussion

NAFLD is a metabolic syndrome characterized by the presence of liver fat accumulation of excessive hepatocytes in the absence of significant alcohol intake [21]. Common treatments for NAFLD comprise of lifestyle changes, medication and operative therapy; however, more effective therapeutic regimens for NAFLD are still required for optimal treatment [22]. Interestingly, VD deficiency is correlated with NAFLD as well as the disease severity, and a previous study indicated VD supplementation as a potentially effective therapy for NAFLD [9]. Thus, the current study hypothesized that active VD could affect the progression of NAFLD. Ultimately, our findings demonstrated that certain dose of $1,25(OH)_2D_3$, a form of VD, could attenuate oxidative stress and inhibit cell senescence as well as inflammatory response by suppressing the p53-p21 signaling pathway, thus delaying the development of NAFLD.

Initially, the results obtained from the current study demonstrated that active VD treatment reduced the fibrosis, steatosis and necrosis of hepatocytes of rats with NAFLD. Importantly, it was revealed that active VD could ameliorate oxidative stress in NAFLD, illustrated by increased VDR but decreased expression of TBARS, MDA, TAOC and ROS. VD, as an agent for inhibiting fibrosis, has been widely studied in nontumorous chronic diseases, and the role of

Please cite this article in press as: Ma M, et al. Active vitamin D impedes the progression of non-alcoholic fatty liver disease by inhibiting cell senescence in a rat model. Clin Res Hepatol Gastroenterol (2019), https://doi.org/10.1016/j.clinre.2019.10.007

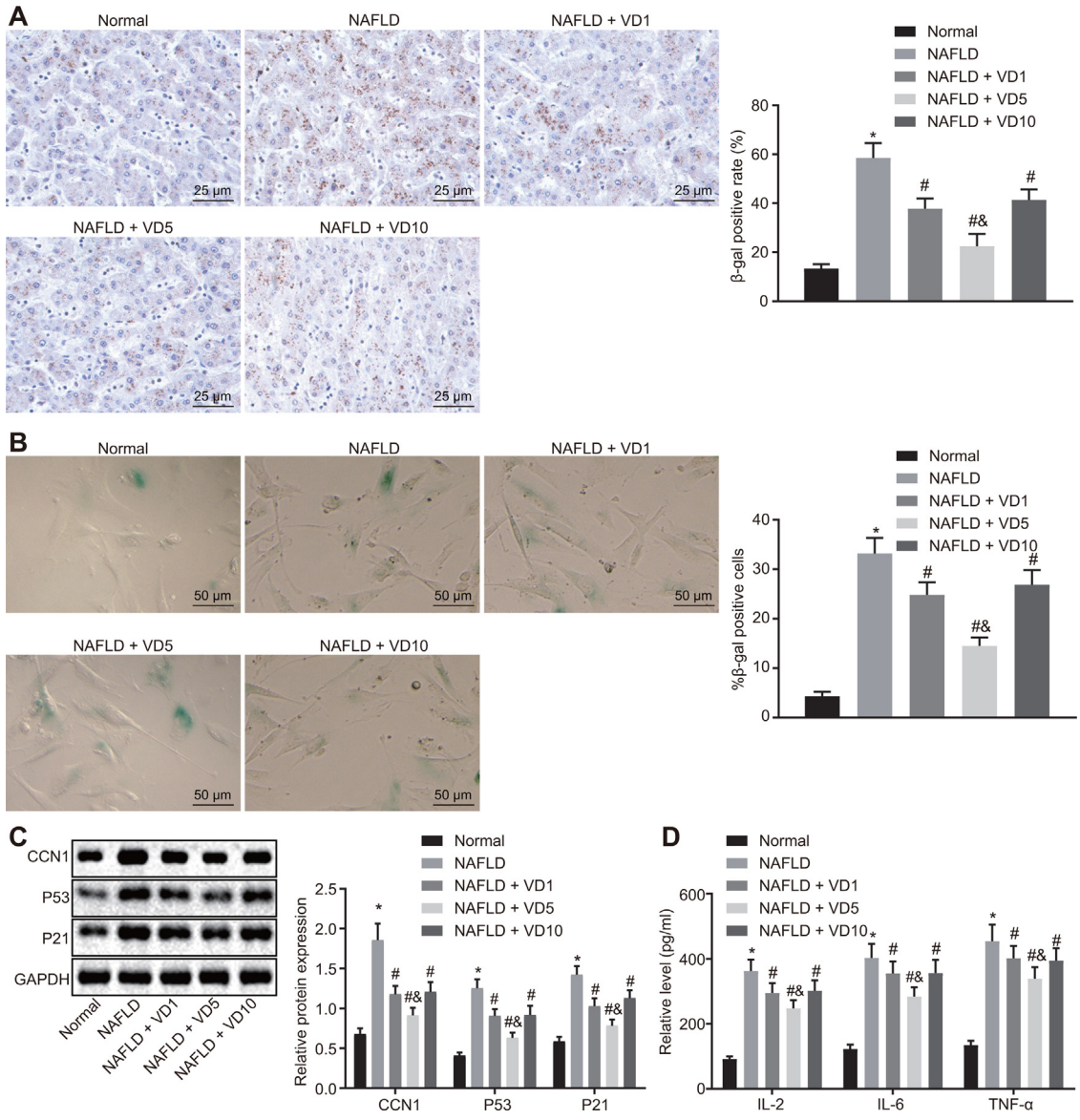

Figure 3 Active VD suppresses cell senescence by blocking the p53-p21 signaling pathway. A. The positive rate of β-gal in each group examined by immunohistochemistry (× 400). B. The positive rate of SAβ-gal in each group examined by SAβ-gal staining (× 400). C. The expression of p53, p21 and CCN1 in liver of each group examined by western blot analysis. D. The levels of IL-2, IL-6 and TNF-α in liver of each group detected by Elisa. *: $P < 0.05$ compared with the normal group; #: $P < 0.05$ compared with the NAFLD group; &: $P < 0.05$ compared with the NAFLD + VD1 and NAFLD + VD10 groups. All data were measurement data, which were expressed as mean ± standard deviation and analyzed by one-way analysis of variance, followed by post hoc test. $n = 10$. NAFLD: non-alcoholic fatty liver disease; VD: vitamin D; SAβ-gal: senescence-associated β-galactosidase; IL-2: interleukin-2; IL-6: interleukin-6; TNF-α: tumor necrosis factor; Elisa: enzyme-linked immunoassay.

$1,25(OH)_2D_3$ in liver disease has been explored, which then offered some new insights [23]. In line with our findings, a previous study also highlighted that VD_3 exert its effect on decreasing the metabolism and oxidative stress in the liver of rats with streptozotocin-induced diabetes [24]. Moreover, 25(OH)D, the active VD, has been proved to profoundly reduce the expression of TBARS, thus alleviating oxidative stress in atherosclerosis [25]. In addition, VDR serves as the means for VD to exhibit an immunoregulator role in several diseases, and calcitriol, the active form of VD_3, could inhibit ROS-induced cellular damage, thus preventing the oxidative stress-induced cell death in nonmalignant prostate cells [26]. VD also contributes to decreased ROS in different lung diseases [27]. Moreover, VD was previously found to decrease the level of MDA in serum and liver enzymes and then reduced oxidative stress in NAFLD [13]. The aforementioned findings are consistent with our results that active VD impedes the progression of NAFLD by alleviating oxidative stress. However, El-Sherbiny et al. proposed that VD nanoemulsion has more prominent hepatoprotective effect against high-fat diet (HFD)-induced liver injury in comparison with conventional oral VD, thereby

Please cite this article in press as: Ma M, et al. Active vitamin D impedes the progression of non-alcoholic fatty liver disease by inhibiting cell senescence in a rat model. Clin Res Hepatol Gastroenterol (2019), https://doi.org/10.1016/j.clinre.2019.10.007

representing a more efficient formulation [28], which needs further probing. Others studies also found that $1,25(OH)_2D_3$ protects against HFD-induced rat model of NAFLD by inducing the nuclear translocation of nuclear factor-erythroid-2-related factor 2[13], decreasing toll-like receptors [29] or repressing sirtuin[30].

Furthermore, we uncovered that active VD suppressed cell senescence and reduced inflammation of NAFLD through inhibition of the p53-p21 signaling pathway, evidenced by down-regulated positive expressions of SAβ-gal, and diminished levels of IL-2, IL-6, TNF-α, p21, p53 and CCN1. Interestingly, CCN1, a protein linked to the senescence of hepatic stellate cells, was found to be remarkably lower in Otsuka Long-Evans Tokushima fatty rats relative to Long-Evans Tokushima Otsuka rats [31]. CCN1 expression is overexpressed in response to liver injuries and has the potency to repress liver fibrogenesis and potentiate fibrosis regression [32]. More specifically, CCN1 could further induce the generation of ROS, resulting in dose-dependent cell senescence [33], which was in line with the results of the present study that CCN1 was enhanced in NAFLD rats and hindered following active VD treatments. Upregulated Saβ-gal, a typical marker applied for senescence discrimination, was found to lead to the phonotypical features of senescence cells [34]. In a prior study, VD was found to repress the expression of SAβ-gal and thus inhibit the senescence of VSMCs and VDR-treated VSMCs [15]. Another study also highlighted the involvement of $1,25(OH)_2VD_3$ in various processes such as immunoregulation and suppression of apoptosis [35]. Moreover, administration of $1,25(OH)_2VD_3$ (calcitriol) inhibited hepatic lipogenesis and inflammation of non-alcoholic steatohepatitis (NASH) [36]. Similarly, VD has been revealed to suppresses inflammation through down-regulating the levels of TNF-α and IL-6 in the ischemia-reperfusion injury, which is crucial in liver dysfunction [37]. In addition, the anti-inflammatory effect of VD has been detected in cells of inflammatory bowel disease [12]. Trovato et al. believed that VD restriction promote inflammatory response and decreases the expression of IGF-1 in the liver, contributing to worsening the fat-induced symptoms [38]. Also, restoration of systemic and VD deficiency with chronic VD treatment could effectively diminish TNF-α-modulated immunological abnormalities correlated to the intestinal/adipose tissues and hepatic steatosis in NASH rats [39]. In both liver and adipose tissues, diminished expression of 70-kDa family of heat shock, a protein associated with cell senescence, was identified to be correlated with insulin resistance and with NAFLD progression [40]. The vital role of the tumor suppressor p53 in the initiation and preservation of cell senescence as well as the stimulative function of p21, as a downstream gene of p53, in p53-induced senescence have been previously described [41]. It was established that liver regeneration could be impaired in p21 transgenic mice [42]. Additionally, p53 has been implicated to influence the activities of potential anti-cancer factors including $1,25(OH)_2D$ [43]. Another study revealed that the mammary cells treated with $1,25(OH)_2D_3$ exhibited lower expression of p21 [44]. Also, the p53-p21 signaling pathway inhibited oxidative stress and senescence of congenital dyskeratosis cells with insufficient telomerase [45]. Meanwhile, the p53-p21 signaling pathway also participated in the senescence of bone marrow-derived mesenchymal stem cells from patients with systemic lupus erythematosus [46]. Hence, these results and findings highlight the suppressive role of VD in cell senescence and inflammation by blocking the p53-p21 signaling pathway in NAFLD.

In conclusion, the key findings of the current study demonstrated the inhibitory effect of active VD, $1,25(OH)_2D_3$, on the progression of NAFLD by repressing oxidative stress, cell senescence and inflammation through the inhibition of the p53-p21 signaling pathway. This study might offer some insights for future therapy for NAFLD. However, the number of samples in present might be a limitation, and the correlation between active VD and p53-p21 required to be further elucidated by more investigations. It also remains necessary to identify the side-effects of $1,25(OH)_2D_3$ to efficiently incorporate therapeutic use. Nevertheless, the unique role of active VD highlighted in the current study still serves as the basis for novel strategies for a potential nutritional approach to treating NAFLD.

Availability of data and materials

The datasets generated/analyzed during the current study are available.

Funding

This study was supported by Research Fund of Zhejiang Major Medical and Health Science and Technology & National Ministry of Health (WKJ-ZJ-1804)and Education Department Fund of Zhejiang Province (Y201635973).

Contribution of authors

Ming Ma, Qi Long and Fei Chen designed the study. Ting Zhang and Wenqiao Wang collated the data, carried out data analyses and produced the initial draft of the manuscript. Ming Ma, Qi Long and Fei Chen contributed to drafting the manuscript. All authors have read and approved the final submitted manuscript.

Disclosure of interest

The authors declare that they have no competing interest.

Acknowledgements

The authors would like to express their deepest appreciation to Professor Junfen Fu, Dr. Xuelian, Zhou, and Dr. Wei Li from Children's Hospital, Zhejiang University School of Medicine who provided assistance to research and experiment and Dr. Mayila Maimaiti from College of Public Health, Zhejiang University School of Medicine who provided guidance to animal experiments and statistical analysis.

References

[1] Clemente MG, Mandato C, Poeta M, Vajro P. Pediatric non-alcoholic fatty liver disease: recent solutions, unresolved

issues, and future research directions. World J Gastroenterol 2016;22(36):8078—93.
[2] Kenneally S, Sier JH, Moore JB. Efficacy of dietary and physical activity intervention in non-alcoholic fatty liver disease: a systematic review. BMJ Open Gastroenterol 2017;4(1):e000139.
[3] Chalasani N, Younossi Z, Lavine JE, Charlton M, Cusi K, Rinella M, et al. The diagnosis and management of nonalcoholic fatty liver disease: practice guidance from the American Association for the Study of Liver Diseases. Hepatology 2018;67(1):328—57.
[4] Huang K, Jiang YJ, Fu JF, Liang JF, Zhu H, Zhu ZW, et al. The Relationship between Serum 25-Hydroxyvitamin D and Glucose Homeostasis in Obese Children and Adolescents in Zhejiang, China. Endocr Pract 2015;21(10):1117—24.
[5] Iser D, Ryan M. Fatty liver disease — a practical guide for GPs. Aust Fam Physician 2013;42(7):444—7.
[6] Rinella ME, Sanyal AJ. Management of NAFLD: a stage-based approach. Nat Rev Gastroenterol Hepatol 2016;13(4):196—205.
[7] Barchetta I, Angelico F, Del Ben M, Baroni MG, Pozzilli P, Morini S, et al. Strong association between non alcoholic fatty liver disease (NAFLD) and low 25(OH) vitamin D levels in an adult population with normal serum liver enzymes. BMC Med 2011;9:(85).
[8] Vanderschueren D, Pye SR, O'Neill TW, Lee DM, Jans I, Billen J, et al. Active vitamin D (1,25-dihydroxyvitamin D) and bone health in middle-aged and elderly men: the European Male Aging Study (EMAS). J Clin Endocrinol Metab 2013;98(3):995—1005.
[9] Eliades M, Spyrou E. Vitamin D: a new player in non-alcoholic fatty liver disease? World J Gastroenterol 2015;21(6):1718—27.
[10] Han YP, Kong M, Zheng S, Ren Y, Zhu L, Shi H, et al. Vitamin D in liver diseases: from mechanisms to clinical trials. J Gastroenterol Hepatol 2013;28(1):49—55.
[11] Trepo E, Ouziel R, Pradat P, Momozawa Y, Quertinmont E, Gervy C, et al. Marked 25-hydroxyvitamin D deficiency is associated with poor prognosis in patients with alcoholic liver disease. J Hepatol 2013;59(2):344—50.
[12] Ryz NR, Patterson SJ, Zhang Y, Ma C, Huang T, Bhinder G, et al. Active vitamin D (1,25-dihydroxyvitamin D3) increases host susceptibility to citrobacter rodentium by suppressing mucosal Th17 responses. Am J Physiol Gastrointest Liver Physiol 2012;303(12):G1299—311.
[13] Zhu CG, Liu YX, Wang H, Wang BP, Qu HQ, Wang BL, et al. Active form of vitamin D ameliorates non-alcoholic fatty liver disease by alleviating oxidative stress in a high-fat diet rat model. Endocr J 2017;64(7):663—73.
[14] Sharifi N, Amani R, Hajiani E, Cheraghian B. Does vitamin D improve liver enzymes, oxidative stress, and inflammatory biomarkers in adults with non-alcoholic fatty liver disease? A randomized clinical trial. Endocrine 2014;47(1):70—80.
[15] Andres V. Vitamin D puts the brakes on angiotensin II-induced oxidative stress and vascular smooth muscle cell senescence. Atherosclerosis 2014;236(2):444—7.
[16] Muzaffar R, Kudva G, Nguyen NC, Osman MM. Incidental diagnosis of thrombus within an aneurysm on 18F-FDG PET/CT: frequency in 926 patients. J Nucl Med 2011;52(9):1408—11.
[17] Li M, Li L, Zhang L, Hu W, Shen J, Xiao Z, et al. 1,25-Dihydroxyvitamin D3 suppresses gastric cancer cell growth through VDR- and mutant p53-mediated induction of p21. Life Sci 2017;179:88—97.
[18] Zhang P, Li J, Qi Y, Zou Y, Liu L, Tang X, et al. Vitamin C promotes the proliferation of human adipose-derived stem cells via p53-p21 pathway. Organogenesis 2016;12(3):143—51.
[19] Ding L, Zeng Q, Wu J, Li D, Wang H, Lu W, et al. Caveolin1 regulates oxidative stressinduced senescence in nucleus pulposus cells primarily via the p53/p21 signaling pathway in vitro. Mol Med Rep 2017;16(6):9521—7.
[20] Zhou Z, Yin Y, Chang Q, Sun G, Lin J, Dai Y. Downregulation of B-myb promotes senescence via the ROS-mediated p53/p21 pathway, in vascular endothelial cells. Cell Prolif 2017;50(2), http://dx.doi.org/10.1111/cpr.12319.
[21] Sherif ZA, Saeed A, Ghavimi S, Nouraie SM, Laiyemo AO, Brim H, et al. Global epidemiology of nonalcoholic fatty liver disease and perspectives on US minority populations. Dig Dis Sci 2016;61(5):1214—25.
[22] Lebovics E, Rubin J. Non-alcoholic fatty liver disease (NAFLD): why you should care, when you should worry, what you should do. Diabetes Metab Res Rev 2011;27(5):419—24.
[23] Shany S, Sigal-Batikoff I, Lamprecht S. Vitamin D and myofibroblasts in fibrosis and cancer: at cross-purposes with TGF-beta/SMAD Signaling. Anticancer Res 2016;36(12):6225—34.
[24] George N, Kumar TP, Antony S, Jayanarayanan S, Paulose CS. Effect of vitamin D3 in reducing metabolic and oxidative stress in the liver of streptozotocin-induced diabetic rats. Br J Nutr 2012;108(8):1410—8.
[25] Tarcin O, Yavuz DG, Ozben B, Telli A, Ogunc AV, Yuksel M, et al. Effect of vitamin D deficiency and replacement on endothelial function in asymptomatic subjects. J Clin Endocrinol Metab 2009;94(10):4023—30.
[26] vinh quoc Lu'o'ng K, Nguyen LT. The beneficial role of vitamin D in obesity: possible genetic and cell signaling mechanisms. Nutr J 2013;12(89), http://dx.doi.org/10.1186/1475-2891-12-89.
[27] Luong KV, Nguyen LT. Beneficial role of vitamin D3 in the prevention of certain respiratory diseases. Ther Adv Respir Dis 2013;7(6):327—50.
[28] El-Sherbiny M, Eldosoky M, El-Shafey M, Othman G, Elkattawy HA, Bedir T, et al. Vitamin D nanoemulsion enhances hepatoprotective effect of conventional vitamin D in rats fed with a high-fat diet. Chem Biol Interact 2018;288:65—75.
[29] Roth CL, Elfers CT, Figlewicz DP, Melhorn SJ, Morton GJ, Hoofnagle A, et al. Vitamin D deficiency in obese rats exacerbates nonalcoholic fatty liver disease and increases hepatic resistin and Toll-like receptor activation. Hepatology 2012;55(4):1103—11.
[30] Wu T, Liu YH, Fu YC, Liu XM, Zhou XH. Direct evidence of sirtuin downregulation in the liver of non-alcoholic fatty liver disease patients. Ann Clin Lab Sci 2014;44(4):410—8.
[31] Linden MA, Sheldon RD, Meers GM, Ortinau LC, Morris EM, Booth FW, et al. Aerobic exercise training in the treatment of non-alcoholic fatty liver disease related fibrosis. J Physiol 2016;594(18):5271—84.
[32] Kim KH, Chen CC, Monzon RI, Lau LF. Matricellular protein CCN1 promotes regression of liver fibrosis through induction of cellular senescence in hepatic myofibroblasts. Mol Cell Biol 2013;33(10):2078—90.
[33] Borkham-Kamphorst E, Schaffrath C, Van de Leur E, Haas U, Tihaa L, Meurer SK, et al. The anti-fibrotic effects of CCN1/CYR61 in primary portal myofibroblasts are mediated through induction of reactive oxygen species resulting in cellular senescence, apoptosis and attenuated TGF-beta signaling. Biochim Biophys Acta 2014;1843(5):902—14.
[34] Momtaz S, Baeeri M, Rahimifard M, Haghi-Aminjan H, Hassani S, Abdollahi M. Manipulation of molecular pathways and senescence hallmarks by natural compounds in fibroblast cells. J Cell Biochem 2019;120(4):6209—22.
[35] Zhu T, Liu TJ, Shi YY, Zhao Q. Vitamin D/VDR signaling pathway ameliorates 2,4,6-trinitrobenzene sulfonic acid-induced colitis by inhibiting intestinal epithelial apoptosis. Int J Mol Med 2015;35(5):1213—8.
[36] Kong M, Zhu L, Bai L, Zhang X, Chen Y, Liu S, et al. Vitamin D deficiency promotes nonalcoholic steatohepatitis through impaired enterohepatic circulation in animal model. Am J Physiol Gastrointest Liver Physiol 2014;307(9):G883—93.
[37] Seif AA, Abdelwahed DM. Vitamin D ameliorates hepatic ischemic/reperfusion injury in rats. J Physiol Biochem 2014;70(3):659—66.

Please cite this article in press as: Ma M, et al. Active vitamin D impedes the progression of non-alcoholic fatty liver disease by inhibiting cell senescence in a rat model. Clin Res Hepatol Gastroenterol (2019), https://doi.org/10.1016/j.clinre.2019.10.007

[38] Trovato FM, Castrogiovanni P, Szychlinska MA, Purrello F, Musumeci G. Early effects of high-fat diet, extra-virgin olive oil and vitamin D in a sedentary rat model of non-alcoholic fatty liver disease. Histol Histopathol 2018;33(11):1201—13.
[39] Su YB, Li TH, Huang CC, Tsai HC, Huang SF, Hsieh YC, et al. Chronic calcitriol supplementation improves the inflammatory profiles of circulating monocytes and the associated intestinal/adipose tissue alteration in a diet-induced steatohepatitis rat model. PLoS One 2018;13(4):e0194867.
[40] Di Naso FC, Porto RR, Fillmann HS, Maggioni L, Padoin AV, Ramos RJ, et al. Obesity depresses the anti-inflammatory HSP70 pathway, contributing to NAFLD progression. Obesity (Silver Spring) 2015;23(1):120—9.
[41] Kim YY, Jee HJ, Um JH, Kim YM, Bae SS, Yun J. Cooperation between p21 and Akt is required for p53-dependent cellular senescence. Aging Cell 2017;16(5):1094—103.
[42] Torbenson M, Yang SQ, Liu HZ, Huang J, Gage W, Diehl AM. STAT-3 overexpression and p21 up-regulation accompany impaired regeneration of fatty livers. Am J Pathol 2002;161(1):155—61.
[43] Abu El Maaty MA, Strassburger W, Qaiser T, Dabiri Y, Wolfl S. Differences in p53 status significantly influence the cellular response and cell survival to 1,25-dihydroxyvitamin D3-metformin cotreatment in colorectal cancer cells. Mol Carcinog 2017;56(11):2486—98.
[44] Maund SL, Shi L, Cramer SD. A role for interleukin-1 alpha in the 1,25 dihydroxyvitamin D3 response in mammary epithelial cells. PLoS One 2013;8(11):e81367.
[45] Westin ER, Aykin-Burns N, Buckingham EM, Spitz DR, Goldman FD, Klingelhutz AJ. The p53/p21(WAF/CIP) pathway mediates oxidative stress and senescence in dyskeratosis congenita cells with telomerase insufficiency. Antioxid Redox Signal 2011;14(6):985—97.
[46] Gu Z, Jiang J, Tan W, Xia Y, Cao H, Meng Y, et al. p53/p21 Pathway involved in mediating cellular senescence of bone marrow-derived mesenchymal stem cells from systemic lupus erythematosus patients. Clin Dev Immunol 2013;2013:(134243).

Please cite this article in press as: Ma M, et al. Active vitamin D impedes the progression of non-alcoholic fatty liver disease by inhibiting cell senescence in a rat model. Clin Res Hepatol Gastroenterol (2019), https://doi.org/10.1016/j.clinre.2019.10.007