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Urmia University of Medical Sciences, Iran

*CORRESPONDENCE
Fatma M. Abdelhamid,
may_mokh@mans.edu.eg

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Vitamin D3 alleviates nonalcoholic fatty liver disease in rats by inhibiting hepatic oxidative stress and inflammation via the SREBP-1-c/ PPARα-NF-κB/ IR-S2 signaling pathway

Doha Reda[1], Gehad E. Elshopakey[1], Talat A. Albukhari[2], Samah J. Almehmadi[3], Bassem Refaat[3], Engy F. Risha[1], Hebatallah A. Mahgoub[4], Mohamed E. El-Boshy[1] and Fatma M. Abdelhamid[1]*

[1]Clinical Pathology Department, Faculty of Veterinary Medicine, Mansoura University, Mansoura, Egypt, [2]Department of Haematology and Immunology, Faculty of Medicine, Umm Alqura University, Makkah, Saudi Arabia, [3]Laboratory Medicine Department, Faculty of Applied Medical Sciences, Umm Al-Qura University, Makkah, Saudi Arabia, [4]Pathology Department, Faculty of Veterinary Medicine, Mansoura University, Mansoura, Egypt

Introduction: Nonalcoholic fatty liver disease (NAFLD) is a chronic disease characterized by fat deposits in liver cells, which can lead to hepatitis and fibrosis. This study attempted to explore the protective effect of vitamin D3 (VitD) against NAFLD.

Methods: Adult male albino rats were randomized into four separate groups: the negative control group was fed a standard rat chow; the positive group received a high-fat diet (20%) and 25% fructose water (NAFLD); the VitD control group was intramuscularly treated with VitD (1,000 IU/kg BW) 3 days per week for 10 weeks; and the NAFLD group was treated with VitD therapy. Biochemical and hepatic histological analyses were performed. Hepatic oxidative stress and inflammatory conditions were also studied. Hepatic expression of sterol regulatory element-binding protein 1-c (SREBP-1-c), peroxisome proliferator-activated receptor alpha (PPAR-α), and insulin receptor substrate-2 was analyzed by quantitative real-time polymerase chain reaction.

Results and discussion: The NAFLD rats exhibited elevated terminal body weight, hepatic injury markers, dyslipidemia, glucose intolerance, and insulin resistance. Moreover, the NAFLD rats had increased SREBP-1-c expression and reduced PPAR-α and IRS-2 expressions. Histological analysis showed hepatic steatosis and inflammation in the NAFLD group. In contrast, VitD administration improved the serum biochemical parameters and hepatic redox status in NAFLD rats. Also, VitD treatment ameliorated hepatic inflammation and steatosis in the NAFLD group by decreasing the expression of SREBP-1-c and increasing the expression of PPAR-α. Overall, these results suggest that VitD could have a protective effect against NAFLD and its associated complication.

KEYWORDS

hepatic steatosis, vitamin D, high-fat diet, insulin resistance, oxidative stress

1 Introduction

Nonalcoholic fatty liver disease (NAFLD) is a common disease characterized by the presence of triglyceride deposits in more than 5% of hepatocytes, in the absence of excessive alcohol consumption or other documented causes of liver damage (Rahimi and Landaverde, 2013; Shojaei Zarghani et al., 2018). It is divided into two main forms: the non-progressive form, which rarely develops into cirrhosis and is known as nonalcoholic fatty liver disease, and the progressive form, which leads to cirrhosis and hepatocellular carcinoma and is known as nonalcoholic steatohepatitis (NASH) (Loomba and Sanyal, 2013). Since NAFLD is related to obesity and insulin resistance, it is the best-known liver disease that chronically affects people of different ages (Kaufmann et al., 2021). Significant evidence suggests that excessive consumption of high-fat diets and sugar-sweetened beverages is related to the development and progression of NAFLD (Rahimi and Landaverde, 2013; Softic et al., 2016). Additionally, excessive carbohydrate and fat consumption increases the levels of blood sugar and free fatty acids, resulting in excessive neutral lipid deposition in the liver (Zivkovic et al., 2007). The severity of NAFLD can be increased by excessive fructose consumption as it promotes insulin resistance, *de novo* lipogenesis, and the development of an inflammatory, oxidative stress state (Jarukamjorn et al., 2016). Since NAFLD is a critical cause of abnormal liver enzymes, cryptogenic cirrhosis, and liver transplantation, it is important to manage and control NAFLD (Souza et al., 2012; Softic et al., 2016).

Vitamin D is a fat-soluble prohormone that can be formed in the skin after direct exposure to ultraviolet rays or taken with food (Sharifi and Amani, 2019). To achieve its biological activity, a series of sequential biochemical reactions must take place, including 25-hydroxylation in the liver and then 1-hydroxylation in the kidney (Yin et al., 2012). Classically, it has been implicated in calcium and phosphorus hemostasis, but recent studies indicate its role in managing diseases associated with inflammation and oxidative stress in both human and animal models (Sharifi and Amani, 2019). It also plays a role in improving the lipid profile (Faraji and Alizadeh, 2020). Several lines of evidence suggest that VitD can modulate liver inflammation and improve hepatic responsiveness to insulin by binding to its specific receptor in the liver (Barchetta et al., 2017). It has been demonstrated that an active form of VitD can reduce oxidative stress, generation of inflammatory factors, and hepatic fibrosis in NAFLD resulting from a high-fat diet (Liu et al., 2020). It can also alleviate fatty liver disease in an NAFLD rat model by modulating lipid metabolism and/or impeding cell senescence (Yin et al., 2012; Liu et al., 2020). Moreover, VitD intake could reduce NAFLD severity and its risk factors including dyslipidemia and obesity (Sangouni et al., 2019). Therefore, VitD supplementation may be helpful in the prevention of NAFLD by modulating some serum liver function markers, lipid profile, hepatic redox status, selective molecules involved in the inflammatory and antiinflammatory processes, and mRNA expression levels of regulatory molecules, SREBP-1-c, PPAR-α, and IRS-2 involved in lipogenesis, lipolysis, and insulin signaling, respectively.

2 Materials and methods

2.1 Materials

2.1.1 Chemicals

Cholecalciferol (vitamin D_3) was brought from Memphis Company for Pharmaceutical and Chemical Industries (Cairo, Egypt) in the form of ampoules (100,000 IU/mL).

2.1.2 Experimental animals

A total of 32 healthy male albino rats (230–250 g) of about 9 weeks of age were procured from the Animal House of Zagazig University. The rats were housed in standard polypropylene cages (four rats per cage) under appropriate conditions of temperature (25°C ± 2°C), humidity (60%–70%), and light (12-h dark/light cycles). They were provided *ad libitum* with a commercial rodent diet and plain water. The experimental protocol was approved by the Ethical Animal Research Committee of Faculty of Veterinary Medicine, Mansoura University, Egypt (Approval No. 2021; M/35).

2.2 Methods

2.2.1 Induction of NAFLD

To establish NAFLD, the rats received a high-fat diet (HFD) with 20% fat. The HFD was prepared according to the work of Shin et al. (2016) with some modifications (we used 19 g of butter oil and 1 g of soybean oil as a source of fat instead of corn oil and lard). In addition, 25% fructose solution was added to the drinking water for 10 weeks (about 2.5 months) (high-fat, high-fructose diet).

2.2.2 Animal treatment

After 2 weeks of acclimatization, the experimental animals were assigned to four groups (eight each), detailed as follows:

- Control group: Rats were maintained on standard chow and fructose-free water
- NAFLD group: Rats were fed an HFD and 25% fructose water
- VitD group: Rats received standard chow, fructose-free water, and VitD treatment
- NAFLD + VitD group: Rats received an HFD, 25% fructose water, and VitD treatment

Vitamin D_3 was diluted in sterile saline and then injected intramuscularly (1,000 IU/kg BW) 3 days per week throughout the experimental period as mentioned by BaSalamah et al. (2018). The body weight of each rat was taken on day 0 and then every week.

2.2.3 Collection and preparation of samples

After the 10th week, experimental rats were injected intraperitoneally with xylazine and ketamine mixture at doses of 10 mg/kg and 50 mg/kg, respectively, and retro-orbital puncture was performed to collect peripheral blood samples. The blood samples were carefully centrifuged at 1,198 × *g* for 10 min to separate the

sera. The obtained sera were then used to study some biochemical parameters. After that, hepatic tissue specimens were collected from the decapitated rats and cut into several parts. One part was used to prepare liver homogenates to assess the hepatic redox status and hepatic pro/antiinflammatory status. The protein content in the homogenates was determined, as reported by Bradford (1976). A hepatic specimen of 1 gm was maintained in RNAlater (Quigen, Germany) for gene expression studies. For histological investigation, hepatic tissue sections were formalin-fixed and then stained with hematoxylin and eosin (H&E). In addition, other sections were frozen, fixed, and stained with Oil Red O.

2.2.4 Serum biochemical analysis

Available commercial kits were utilized to evaluate the biomarkers of liver function: alanine and aspartate aminotransferases (ALT and AST, respectively) (Human Diagnostics Worldwide, Wiesbaden, Germany); alkaline phosphatase (ALP) (ELITech, Paris, France); bilirubin (total and direct) (Diamond, Cairo, Egypt); and total protein and albumin (Stanbio Laboratory, TX, United States). A Spinreact kit was used to analyze the serum glucose level, while serum insulin and 25(OH) VitD levels were quantified by enzyme-linked immunosorbent assay using the appropriate kits (Biospes, Chongqing, China, and DiaMetra SRL, Perugia, Italy, respectively). Homeostasis model assessment of insulin resistance (HOMA-IR) was detected according to the work of Matthews et al. (1985).

Different lipid parameters [triglyceride (TG), total cholesterol (TC), and high-density lipoprotein cholesterol (HDL-C)] were estimated using ready-made assay kits (Spinreact, Sant Esteve de Bas, Spain), and exceptionally very-low- and low-density lipoprotein cholesterol (VLDL-C and LDL-C) values were calculated as recorded by Friedewald et al. (1972). The biochemical serum analyses were performed following the manufacturer's guidelines and measured using a spectrophotometer (BM, Germany, 5010).

2.2.5 Assessment of hepatic redox status and serum total antioxidant capacity

Malondialdehyde (MDA) concentration and the enzymatic activities of both superoxide dismutase (SOD) and catalase (CAT), together with the reduced glutathione (GSH) level, were determined in the hepatic tissue using standard assay kits from Bio-Diagnostic Co. (Cairo, Egypt), according to the instructions in each corresponding pamphlet. Serum total antioxidant capacity (sTAC) was also estimated by using Bio-Diagnostic kits.

2.2.6 Assessment of hepatic pro- and antiinflammatory status

Specific rat ELISA kits purchased from Cusabio (Wuhan, China) were utilized for the determination of the hepatic concentration of nuclear factor kappa β (NF-κβ), whereas R&D Systems ELISA Kits (Minneapolis, MN, United States) were used to measure hepatic interleukin-10 (IL-10) levels, following the protocol in the assay kits.

2.2.7 Assessment of hepatic gene expression

RNA was first extracted from the hepatic tissue following the RNeasy Mini Kit instructions (Qiagen, Hilden, Germany). Then, cDNA was synthesized using RevertAid Reverse Transcriptase Kits from Thermo Fisher Scientific (Massachusetts, United States), following the manufacturer's instructions. Quantitative RT-PCR was performed to evaluate the relative expression levels of the following genes: sterol regulatory element-binding protein 1-c (SREBP-1-c), peroxisome proliferator-activated receptor alpha (PPAR-α), and insulin receptor substrate-2 (IRS-2). Primer sequences of SREBP-1-c (Ren et al., 2019), PPAR-α (Ding et al., 2014), and IRS-2 (Kanuri et al., 2016) are shown in Table 1. RT-PCR assay was performed using the MX3005P QPCR system. The preparation of the PCR master mix and cycling conditions was performed using the QuantiTect SYBR Green PCR Kit (Qiagen, Germany), following the manufacturer's instructions. Rat β-actin (Banni et al., 2010) was used as a housekeeping gene, and a comparative Ct method ($2^{-\Delta\Delta CT}$) was used to detect mRNA expression levels, according to the work of Livak and Schmittgen (2001).

2.2.8 Histopathological assessment of the liver

Liver sections were preserved in 10% formaldehyde solution and then immersed in paraffin. The obtained tissue was sliced into 5 μm-thick tissue blocks that were stained with hematoxylin and eosin (H&E). The obtained blocks were inspected under a light microscope, as mentioned by Kleiner et al. (2005). To assess hepatic lipid deposition, 10-μm frozen liver sections were prepared using a cryostat (LEICA CM 1800), fixed, and then, stained with ORO (Green and Kehinde, 1974). Using the XSZ-107BN microscope (China) and the Apex Minigrab (UK), the obtained sections were randomly photographed and then automatically analyzed using ImageJ (https://imagej.nih.gov/ij).

2.2.9 Data analysis

The outputs were given as means ± SEM. The Statistical Package of Social Services, version 22 (SPSS 22), was used to perform all statistics using ANOVA, followed by Duncan's multiple-range test to perform the comparisons between the tested groups. Statistical significance was displayed at $p < 0.05$. Positive ORO-stained areas were evaluated by ANOVA, followed by Tukey's test for group comparison (p-value ≤ 0.05), using GraphPad Prism for macOS, version 9.2.0 (283).

3 Results

3.1 Effect of vitamin D treatment on body weight

Figure 1 shows that the initial body weights were similar in all the experimental groups. However, a remarkable increase was noted in the terminal body weights of the NAFLD rats compared to the control rats ($p < 0.05$). No substantial variation was observed in the body weight of the NAFLD + VitD compared with the NAFLD group.

3.2 Vitamin D treatment improved serum biochemical parameters in NAFLD rats

As shown in Table 2, NAFLD rats showed marked ($p < 0.05$) elevation in the serum ALT, AST, and ALP activities compared to

TABLE 1 Forward and reverse primers for SREBP-1-c, PPAR-α, and IRS-2.

Rat β-actin	F: 5′ -TCCTCCTGAGCGCAAGTACTCT-3′
	R: 5′-GCTCAGTAACAGTCCGCCTAGAA- 3′
SREBP-1-c	F: 5′-AGGAGGCCATCTTGTTGCTT- 3′
	R: 5′-GTTTTGACCCTTAGGGCAGC- 3′
PPAR-α	F: 5′-TCTGTGGGCTCACTGTTC- 3′
	R: 5′-AGGGCTCATCCTGTCTTTG- 3′
IRS-2	F: 5′-GAAGCGGCTAAGTCTCATGG- 3′
	R: 5′-GACGGTGGTGGTAGAGGAAA- 3′

SREBP-1-c, sterol regulatory element-binding protein 1-c; ***PPAR-α,*** peroxisome proliferator-activated receptor alpha; ***IRS-2,*** insulin receptor substrate.

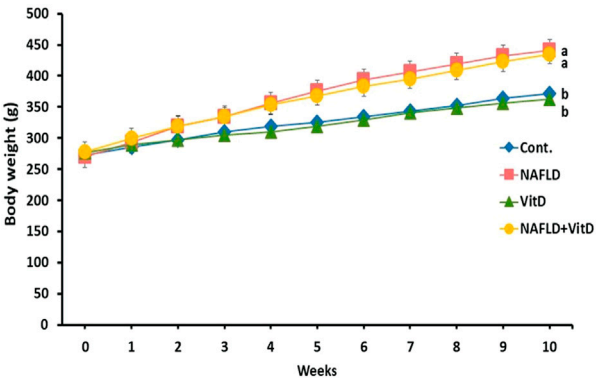

FIGURE 1
Changes in the body weight (g) of experimental groups during the study period. Data are expressed as means ± SEM, in which those with different superscripts differed significantly ($p < 0.05$). a is used for the high value, while b is used for the lower one. *Cont.*, control; *NAFLD,* nonalcoholic fatty liver disease; and *VitD*, vitamin D.

the control rats. Conversely, VitD administration in rats with NAFLD markedly ($p < 0.05$) normalized ALT and ALP serum activities and caused a substantial ($p < 0.05$) decrease in serum AST activity but did not return it to its normal value, suggesting that VitD has hepatoprotective properties. Additionally, the total and direct bilirubin concentrations were pronouncedly ($p < 0.05$) increased in the NAFLD group (Table 2), whereas the total protein and globulin levels were pronouncedly ($p < 0.05$) decreased as opposed to the control rats (Table 2). VitD had no significant ($p > 0.05$) effect on either bilirubin or proteinogram serum results in the NAFLD + VitD group versus NAFLD rats.

Serum glucose and insulin levels, together with HOMA-IR index, were sustainably ($p < 0.05$) increased, while the serum 25 (OH)VitD level was decreased ($p < 0.05$) significantly in the NAFLD group compared the control group, suggesting disorders of glucose metabolism and a state of insulin resistance. However, VitD treatment markedly ($p < 0.05$) attenuated the high-fat and fructose diet (HFFD)-mediated increase in glucose, insulin, and HOMA-IR values and significantly ($p < 0.05$) improved the serum 25(OH)VitD level in the NAFLD+ VitD group compared to the untreated NAFLD rats (Table 2).

3.3 Vitamin D treatment improved the serum lipid profile in NAFLD rats

Table 2 shows that rats in the NAFLD group had significantly ($p < 0.05$) higher TG, TC, VLDL-C, and LDL-C serum levels, while the HDL-C serum level significantly decreased compared with the control rats, indicating impaired lipid homeostasis. Serum levels of TG and VLDL-C notably ($p < 0.05$) decreased, while TC and LDL-C levels almost returned to their basal values, and the HDL-C serum concentration was significantly increased in the NAFLD + VitD group by intramuscular VitD treatment as opposed to the NAFLD group.

3.4 Vitamin D improved hepatic antioxidant defense status and serum total antioxidant capacity in NAFLD rats

As shown in Table 3, NAFLD rats exhibited an imbalance in hepatic redox status, as evidenced by a substantial ($p < 0.05$) increment in the hepatic MDA concentration and marked decreases in sTAC, hepatic SOD, GSH, and CAT concentrations compared to the control group. In contrast, VitD effectivity decreased the MDA hepatic concentration and increased hepatic SOD and GSH concentrations in the NAFLD + VitD group compared to the NAFLD group.

3.5 Vitamin D treatment modulated the hepatic pro- and antiinflammatory status in NAFLD rats

A significant ($p < 0.05$) increase in the NF-κB level and a sustainable ($p < 0.05$) decrease in the IL-10 level were observed in the liver of NAFLD rats with respect to the control group, indicative of hepatic inflammation. As expected, vitamin D treatment reduced hepatic inflammation and ameliorated the antiinflammatory status as indicated in the NAFLD + VitD group versus the untreated NAFLD group (Table 4).

TABLE 2 Impact of VitD on alterations in the serum liver injury indices, proteinogram, glucose hemostasis parameters, 25(OH)VitD, and lipid profile of NAFLD rats.

Items	Groups			
	Control	NAFLD	VitD	NAFLD+ VitD
***ALT* (*U/L*)**	44.80 ± 2.15^{b}	59.20 ± 3.02^{a}	45.20 ± 1.59^{b}	50 ± 2.43^{b}
***AST* (*U/L*)**	53.80 ± 2.31^{c}	76.80 ± 2.21^{a}	56.60 ± 1.11^{c}	62.70 ± 0.86^{b}
***ALP* (*U/L*)**	333.14 ± 18.73^{b}	424.74 ± 14.77^{a}	316.88 ± 12.40^{b}	342.40 ± 13.42^{b}
***Total bilirubin* (*mg/dL*)**	0.22 ± 0.19^{b}	0.40 ± 0.02^{a}	0.25 ± 0.03^{b}	0.38 ± 0.03^{a}
***Direct bilirubin* (*mg/dL*)**	0.13 ± 0.03^{b}	0.28 ± 0.02^{a}	0.13 ± 0.02^{b}	0.23 ± 0.02^{a}
***Indirect bilirubin* (*mg/dL*)**	0.09 ± 0.02^{a}	0.12 ± 0.01^{a}	0.12 ± 0.03^{a}	0.15 ± 0.02^{a}
***Total protein* (*g/dL*)**	9.41 ± 0.20^{a}	7.72 ± 0.06^{b}	9.49 ± 0.23^{a}	8.06 ± 0.15^{b}
***Albumin* (*g/dL*)**	4.73 ± 0.26^{a}	4.39 ± 0.10^{a}	4.33 ± 0.25^{a}	4.59 ± 0.10^{a}
***Globulin* (*g/dL*)**	4.68 ± 0.12^{a}	3.33 ± 0.12^{b}	5.17 ± 0.42^{a}	3.47 ± 0.18^{b}
***Glucose* (*mg/dL*)**	94.55 ± 3.71^{c}	141.49 ± 5.01^{a}	97.45 ± 1.20^{c}	115.87 ± 3.64^{b}
***Insulin* (*μIU/mL*)**	5.37 ± 0.12^{c}	8.20 ± 0.17^{a}	5.40 ± 0.06^{c}	6.53 ± 0.58^{b}
HOMA-IR	1.21 ± 0.04^{c}	2.82 ± 0.05^{a}	1.27 ± 0.03^{c}	1.93 ± 0.13^{b}
***25(OH)VitD* (*ng/mL*)**	36.27 ± 1.07^{c}	16.05 ± 0.35^{d}	150.00 ± 7.22^{a}	112.50 ± 7.22^{b}
***TG* (*mg/dL*)**	182.78 ± 4.88^{c}	262.06 ± 9.51^{a}	177.49 ± 6.53^{c}	231.70 ± 5.50^{b}
***TC* (*mg/dL*)**	182.99 ± 2.46^{b}	199.20 ± 3.34^{a}	182.59 ± 4.27^{b}	186.60 ± 1.29^{b}
***VLDL-C* (*mg/dL*)**	36.55 ± 0.98^{c}	52.41 ± 1.23^{a}	35.49 ± 1.31^{c}	46.34 ± 1.10^{b}
***LDL-C* (*mg/dL*)**	70.44 ± 4.80^{b}	86.54 ± 4.79^{a}	70.60 ± 5.50^{b}	70.66 ± 5.08^{b}
***HDL-c* (*mg/dL*)**	76.00 ± 1.76^{a}	60.25 ± 1.24^{c}	76.50 ± 1.96^{a}	69.60 ± 3.41^{b}

Data for (n = 8) are presented as means ± SEM, in which those with different superscripts differed significantly ($p < 0.05$). a is used for the high value, b and c are used for intermediate values between a and d, and d is used for the lower value. *Cont*., control; *NAFLD,* nonalcoholic fatty liver disease; *VitD*, vitamin D; ***ALT***, alanine aminotransferase; ***AST***, aspartate aminotransferase; ***ALP,*** alkaline phosphatase; ***HOMA-IR***: homeostatic model assessment for insulin resistance; ***TG,*** triglyceride; ***TC,*** total cholesterol; ***VLDL-C,*** very-low-density lipoprotein cholesterol; ***LDL-C,*** low-density lipoprotein cholesterol; ***HDL-C,*** high-density lipoprotein cholesterol.

TABLE 3 Impact of VitD on alterations in the hepatic redox system and serum total antioxidant capacity of NAFLD rats.

Items	Groups			
	Control	NAFLD	VitD	NAFLD+ VitD
***MDA* (*nmol/g tissue*)**	54.58 ± 2.85^{b}	76.40 ± 2.48^{a}	48.62 ± 3.11^{b}	52.49 ± 1.32^{b}
***SOD* (*U/g.tissue*)**	478.68 ± 14.54^{a}	384.86 ± 11.70^{c}	480.26 ± 8.44^{a}	436.36 ± 11.58^{b}
***GSH* (*mg/g.tissue*)**	5.56 ± 0.29^{a}	3.30 ± 0.20^{c}	5.57 ± 0.15^{a}	4.61 ± 0.16^{b}
***CAT* (*U/g.tissue*)**	1.93 ± 0.01^{a}	1.75 ± 0.03b	1.90 ± 0.01^{a}	1.82 ± 0.03^{b}
***sTAC* (*mM/L*)**	1.17 ± 0.15^{a}	0.47 ± 0.03^{b}	1.05 ± 0.05^{a}	0.53 ± 0.06^{b}

Data for (n = 8) are presented as means ± SEM, in which those with different superscripts differed significantly ($p < 0.05$). a is used for the high value, b and c are used for intermediate values between a and d, and d is used for the lower value. *Cont*., control; *NAFLD,* nonalcoholic fatty liver disease; *VitD*, vitamin D; ***MDA,*** malondialdehyde; ***SOD,*** superoxide dismutase; **CAT,** catalase; ***GSH,*** reduced glutathione; ***sTAC,*** serum total antioxidant capacity.

3.6 Vitamin D improved gene expression in the liver of NAFLD rats

Hepatic gene expression analysis shown in Figure 2 revealed that the hepatic mRNA expression of SREBP-1-c was markedly ($p < 0.05$) upregulated, while hepatic PPAR-α and IRS-2 mRNA expressions levels were markedly ($p < 0.05$) downregulated in the NAFLD rats relative to the control group. Moreover, VitD treatment significantly ($p < 0.05$) abolished the HFFD-mediated downregulation of SREBP-1-c and significantly promoted the mRNA expression of PPAR-α and IRS-2 in the liver of the NAFLD + VitD group versus the NAFLD group.

TABLE 4 Impact of Vit D on changes in the hepatic inflammatory status of NAFLD rats.

Items	Groups			
	Control	NAFLD	VitD	NAFLD+ VitD
***NF*-κβ (*pg/g protein*)**	40.00 ± 2.52^{c}	474.67 ± 16.38^{a}	48.67 ± 4.91^{c}	115.33 ± 9.06^{b}
***IL-10* (*pg/g protein*)**	143.00 ± 1.53^{a}	74.00 ± 6.35^{c}	137.64 ± 4.09^{a}	121.33 ± 3.76^{b}

Data for (n = 8) are presented as means ± SEM, in which those with different superscripts differed significantly ($p < 0.05$). a is used for the high value, b and c are used for intermediate values between a and d, and d is used for the lower value. *Cont*., control; *NAFLD*, nonalcoholic fatty liver disease; *VitD*, vitamin D; ***NF*-κβ**, nuclear factor kappa β; ***IL-10*,** interleukin-10.

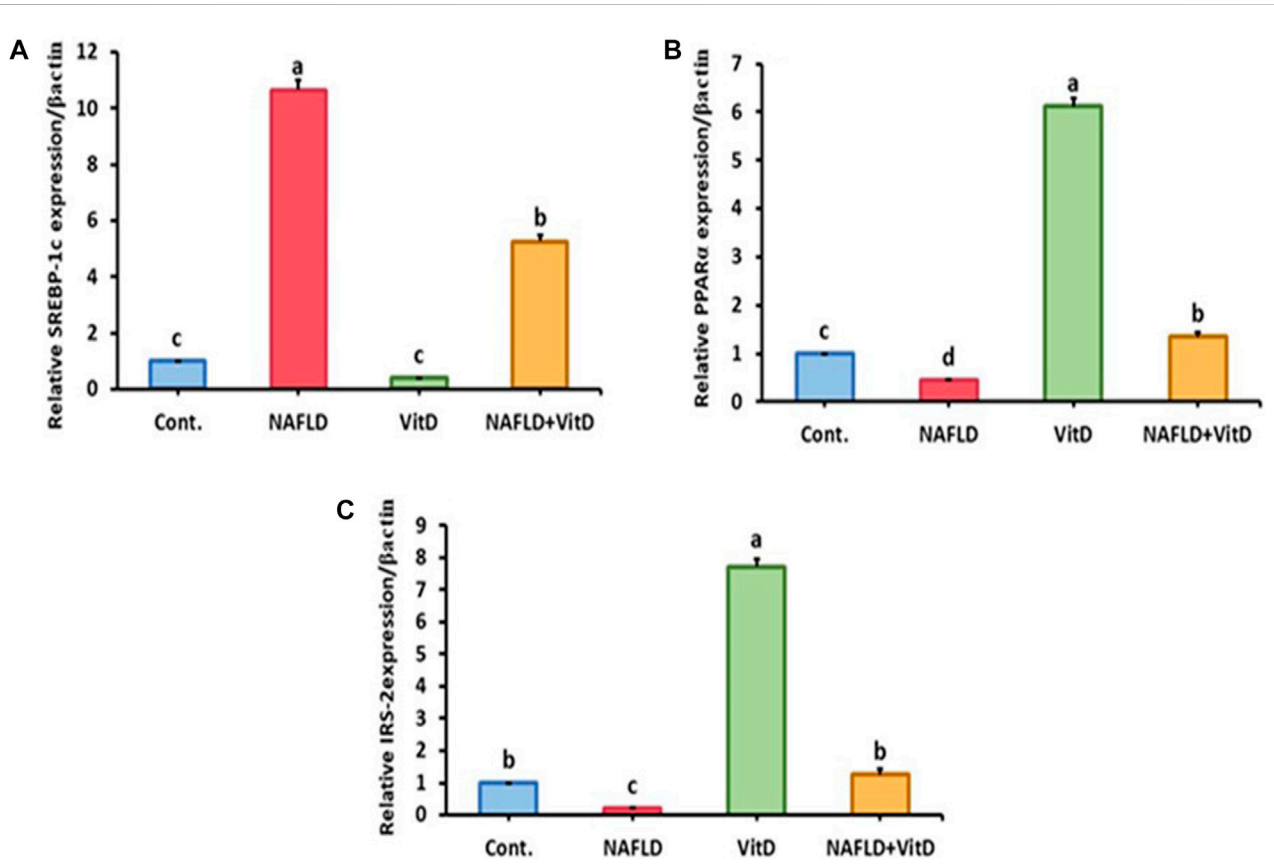

FIGURE 2
Impact of VitD on the hepatic mRNA expression levels of NAFLD rats. **(A)** SREBP-1-c, **(B)** PPAR-α, and **(C)** IRS-2. Data are shown as means ± SEM, in which those with different superscripts differed significantly ($p < 0.05$). a is used for the high value, b and c are used for intermediate values between a and d, and d is used for the lower value. *Cont*., control; *NAFLD*, nonalcoholic fatty liver disease; and *VitD*, vitamin D. *SREBP-1-c*, sterol regulatory element-binding protein 1-c; *PPAR-α*, peroxisome proliferator-activated receptor alpha; and *RS-2*, insulin receptor substrate-2.

3.7 Vitamin D ameliorated the hepatic histopathological changes observed in NAFLD rats

Histopathological analysis of the hepatic sections revealed that the livers of the control and VitD-treated rats had normal tissue architecture without any detected pathological changes (Figures 3A, E). On the contrary, Table 5 shows that the liver of the NAFLD rats showed parenchyma with intracytoplasmic fat vacuoles and a centrally located nucleus indicating microvesicular fatty changes. The hepatic parenchyma also showed ballooning degeneration and lobular inflammation as opposed to the negative control group (Figures 3B, C). In addition, the liver of the NAFLD rats showed periportal microgranuloma, which can be defined as aggregates of epithelioid cells and other inflammatory cells, including lymphocytes. On the other hand, VitD effectively ameliorated these histopathological abnormalities in the NAFLD + VitD group, as evidenced by a decrease in the observed hepatic microvesicular fatty changes and inflammatory infiltrates when compared to the NAFLD group (Figures 3F, G).

Figure 4 shows photomicrographs of ORO-stained hepatic sections. Histology of the liver was normal in the control and

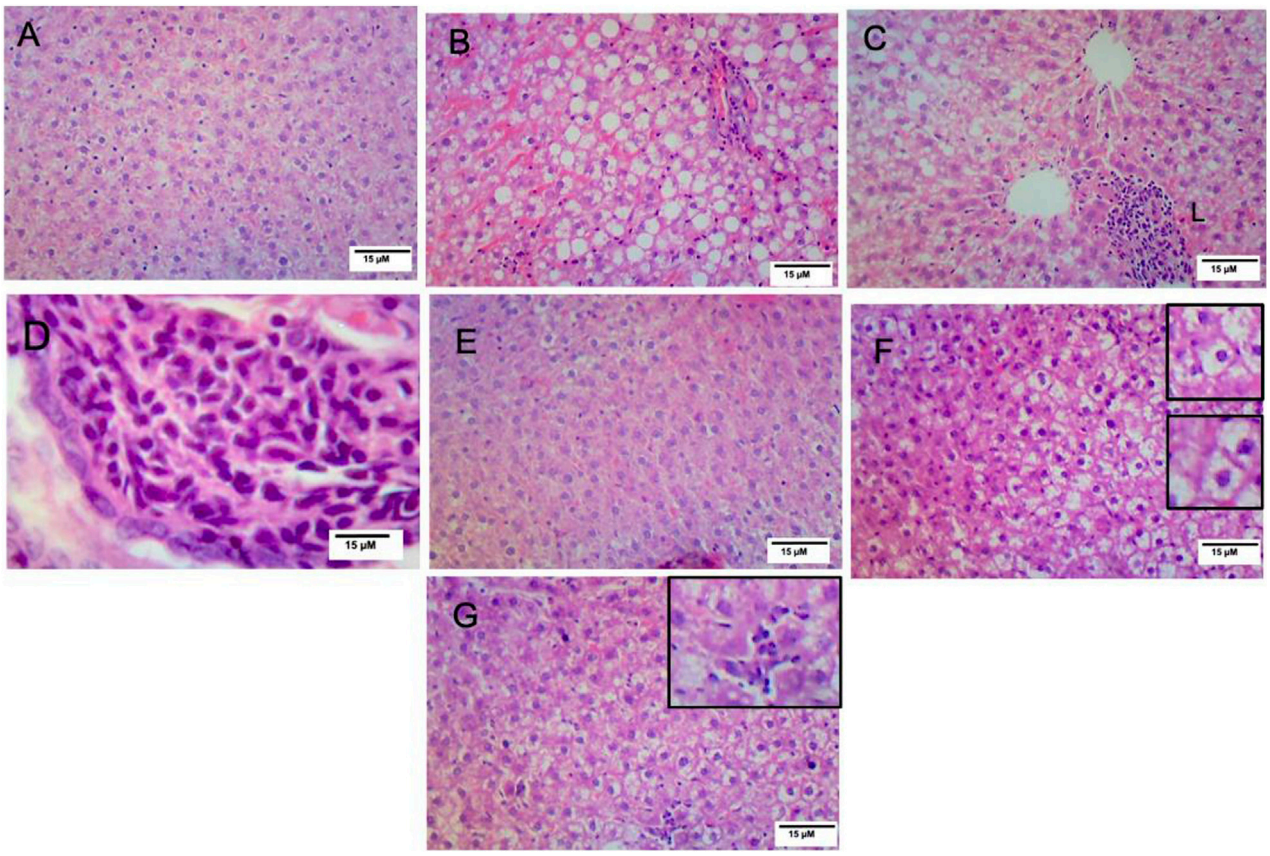

FIGURE 3
Effect of VitD on hepatic histopathological alterations in NAFLD rats (H&E staining). **(A,E)** Micrographs of the control group and vitamin D-treated group showing normal liver histology (10x). **(B)** Micrograph of the NAFLD group showing microvesicular steatosis and ballooning degeneration (10x). **(C)** Micrograph of the NAFLD group showing lobular inflammation consisting mainly of polymorphonuclear cell infiltration (10x). **(D)** Micrograph of the NAFLD group showing periportal microgranuloma (40x). **(F)** Micrograph of the NAFLD + VitD group showing decreased microvascular steatosis (bottom inset) and hepatocyte ballooning injury (top inset) (10x) compared to the NAFLD group. **(G)** Micrograph of the NAFLD + VitD group showing mild lobular inflammation (inset) (10x).

VitD groups (Figures 4A, C), respectively. In the NAFLD group, the liver showed widespread lipid droplets of varied sizes occupying the cytoplasm of hepatocytes (Figure 4B). As expected, the liver of the NAFLD + VitD group had a fewer number of lipid droplets than the NAFLD group (Figures 4D, E).

4 Discussion

Nonalcoholic fatty liver disease is a common liver disorder that can range from asymptomatic steatosis to inflammation and fibrosis, which may lead to hepatic cirrhosis and carcinoma (Katsiki et al., 2016). Routine treatments for NAFLD include lifestyle adjustment and surgical therapy; however, the most effective therapeutic regimens for NAFLD are still under study (Liu et al., 2020). Nowadays, the various functions of VitD are known due to its immunomodulatory, antiinflammatory, and insulin-sensitizing properties (Barchetta et al., 2020). Therefore, VitD could be effectively used as a therapy for NAFLD (Eliades and Spyrou, 2015). From this point of view, this study was conducted to explore the possible protective effects of VitD administration against NAFLD caused by a high-fat and fructose diet (HFFD).

In the current study, the HFFD caused a marked increase in the body weight of rats, which may be attributed to the ability of fructose to promote leptin resistance and serum ghrelin levels (Muriel et al., 2021). Vitamin D could not modulate the increase in body weight induced by the HFFD that was previously recorded in rats fed an obesogenic diet and treated with vitamin D (Mazzone et al., 2018; Shojaei Zarghani et al., 2018; Al-Badarein and Ahmad, 2021).

Evaluation of the serum activity of ALT is considered a valuable tool for the detection of NAFLD. In our study, liver function biomarkers, including ALT, AST, ALP, and total and direct bilirubin, were markedly increased in the NAFLD group, indicating hepatocellular damage. This may be due to the lipogenic potential of fructose as it increases hepatic *de novo* lipogenesis (DNL) at multiple levels, leading to intrahepatic fat accumulation (Mouzaki and Allard, 2012; Charrez et al., 2015; Jin and Vos, 2015; Softic et al., 2016). This was confirmed histopathologically in our work with H&E and ORO staining. Administration of VitD had a hepatoprotective effect, as detected by lowering the elevated liver enzymes activities (Han et al., 2015;

TABLE 5 Hepatic scoring and histopathological changes in all experimental groups.

Item	Definition	Score	Control	NAFLD	VitD	NAFLD + VitD
Steatosis grade	Low- to medium-power evaluation of parenchymal involvement by steatosis					
	<5%	0	+		+	
	5%–33%	1				+
	>33%–66%	2		+		
	“66%>	3				
Location	Predominant distribution pattern					
	Zone 3 or not present	0	+		+	
	Zone 1	1				
	A zonal	2				
	Panacinar	3		+		+
Microvesicular steatosis	Contiguous patches					
	Not present	0	+		+	
	Present	1		+		+
Fibrosis stage	None	0	+	+	+	+
Lobular inflammation	Overall assessment of all inflammatory foci					
	No foci	0	+		+	
	<2 foci per 200X field	1				+
	2–4 foci per 200X field	2		+		
	>4 foci per 200X field	3				
Portal inflammation	Assessed from low magnification					
	None to minimal	0	+		+	+
	Greater than minimal	1		+		
Microgranulomas	Small aggregates of macrophages					
	Absent	0	+		+	
	Present	1		+		+
Liver cell injury Ballooning	None	0	+		+	
	Few balloon cells	1				
	Many cells/prominent ballooning	2		+		+
Acidophil bodies	None to rare	0	+	+	+	+
	Many	1				
Pigmented macrophages	None to rare	0	+	+	+	+
	Many	1				
Megamitochondria	None to rare	0	+		+	
	Many	1		+		+
Other findings **Mallory’s hyaline**	Visible on routine stains					
	None to rare	0	+		+	
	Many	1		+		+

(Continued on following page)

TABLE 5 (*Continued*) Hepatic scoring and histopathological changes in all experimental groups.

Item	Definition	Score	Control	NAFLD	VitD	NAFLD + VitD
Glycogenated nuclei	Contiguous patches					
	None to rare	0	+	+	+	+
	Many	1				

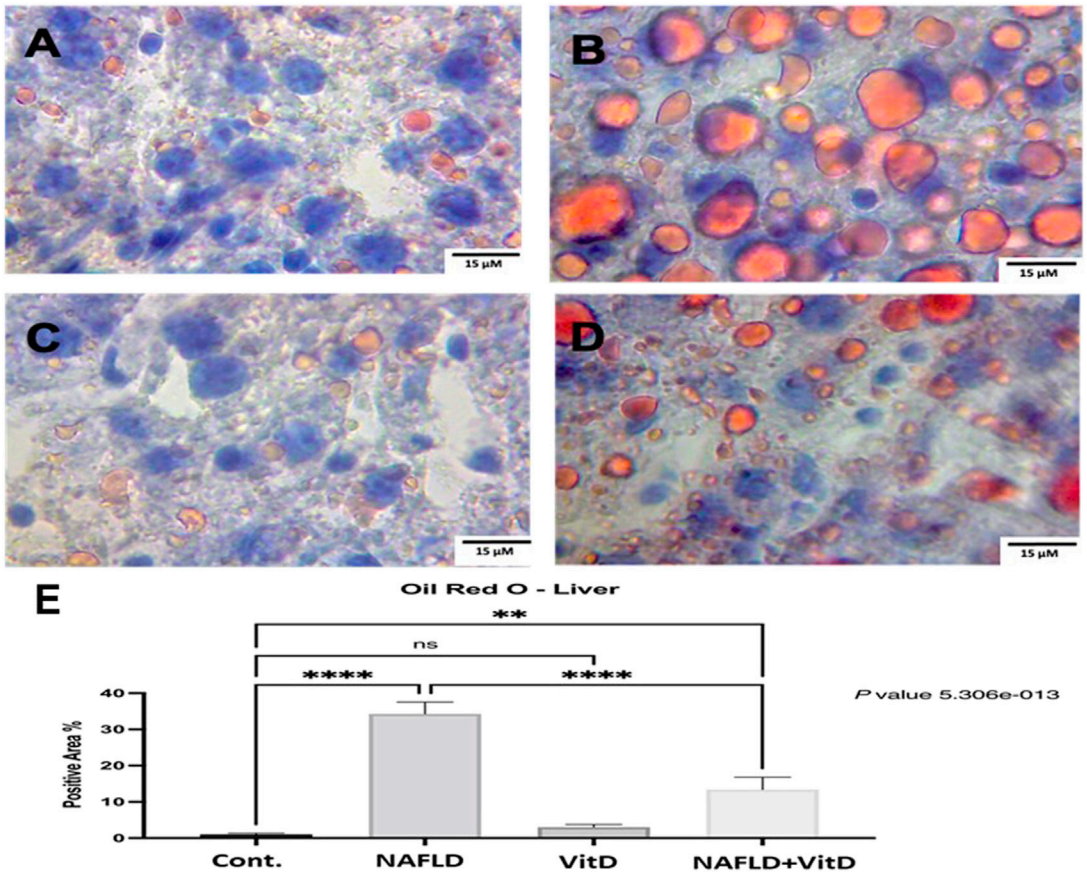

FIGURE 4
Photomicrographs of Oil Red O-stained hepatic sections (40x). **(A,C)** Cont. group and vitamin D-treated group: showing normal liver histology. **(B)** NAFLD group: showing widespread lipid droplets of variable size occupying the cytoplasm of hepatocytes. **(D)** NAFLD + VitD group: showing decreased lipid deposits compared with the NAFLD group. **(E)** Mean positive area percentage ±SEM in each group. Data were analyzed using one-way ANOVA, followed by Tukey's test for group comparison. ns = not significant. * = p value ≤ 0.05. ** = p value ≤ 0.01. *** = p value ≤ 0.001. **** = p value ≤ 0.0001.

Zhu et al., 2017), which was confirmed histopathologically by a significant reduction in the hepatic lipid burden. The same findings were reported previously by Shojaei Zarghani et al. (2018) in rats fed an HFFD and treated with a vitamin D and calcium combination. The prevention of intrahepatic fat accumulation and the reduction in hepatic cholesterol content may be the cause of the reduction in the hepatic lipid burden (Mazzone et al., 2018; Shojaei Zarghani et al., 2018). Hence, VitD has been reported to downregulate SREBP-1-c and stimulate PPAR-α (Yin et al., 2012).

In the current study, the NAFLD group showed glucose intolerance and impaired insulin sensitivity, which is consistent with the findings obtained by Geetha et al. (2014). This may be a result of high fructose consumption, as it has been reported that fructose-1-phosphate activates mitogen-activated protein kinase, which phosphorylates insulin receptor substrate 1 at the serine residue, leading to hepatic insulin resistance (IR) (Lim et al., 2010; Chang et al., 2014). In addition, fructose itself promotes forkhead box protein O1 synthesis in the liver, leading to an increase in gluconeogenesis, which, together with IR, leads to hyperglycemia and glucose intolerance (Mouzaki and Allard, 2012). In contrast, VitD administration improved glucose hemostasis in the NAFLD group (Sergeev and Song, 2014). This

improvement could be attributed to the ability of calcitriol to activate AMPK via the calcium/calmodulin protein kinase beta pathway, leading to attenuating gluconeogenesis and promoting glycolysis (Leung, 2016; Szymczak-Pajor et al., 2020). Additionally, Sung et al. (2012) claimed that VitD can promote insulin action either directly by stimulating insulin receptor expression or indirectly through the regulation of Ca^{++} levels.

Since NAFLD is associated with lipid disorders, serum lipid profile parameters were investigated. The NAFLD group showed elevations in the serum concentration of TG, TC, VLDL-C, and LDL-C but lower serum HDL-C concentrations. This impairment may be contributed to increase DNL induced by fructose consumption, leading to the formation of fatty acids to produce hepatic TG. The increased hepatic lipid content leads to a decrease in intracellular apo-B degradation and increased production and secretion of VLDL-C (Stanhope and Havel, 2010). On the other hand, the decreased HDL blood level was contributed to the decreased post-heparin plasma LPL/HL ratio and the increased VLDL levels (Mooradian et al., 2008). Concerning the impact of VitD on lipid profile parameters, it ameliorated lipid levels in the serum, which may refer to its ability to promote calcium absorption from the intestine. This calcium forms insoluble soap with fatty acids, especially saturated fatty acids, resulting in increased fecal fat excretion and a decrease in its digestibility (Christensen et al., 2009; Subih et al., 2018). It may also increase lipoprotein lipase enzymes, promote the formation of high-density lipoprotein cholesterol particles, and regulate serum apo-lipoprotein A-1 levels (Hassan et al., 2020; Melguizo-Rodríguez et al., 2021). Our result was previously reported by Farhangi et al. (2017)and El-Sherbiny et al. (2018). In contrast, Shojaei Zarghani et al. (2018) reported insignificant changes in the serum level of TG, TC, and LDL in groups fed with an HFFD and treated with a vitamin D and calcium combination.

Hepatic redox imbalance has been known to be involved in the development and progression of NAFLD from simple steatosis to a more severe form (Spahis et al., 2016). Our data indicated that NAFLD rats had increased hepatic MDA and decreased hepatic antioxidant enzymes and GSH, which was previously reported by Nasri et al. (2015). This may be due to increased superoxide and hydrogen peroxide ions from HFFD feeding (Jarukamjorn et al., 2016). In addition, the antioxidant potential of cells decreases when the excess fructose activates DNL, leading to a reduction in NADPH (Charrez et al., 2015). In agreement with Zhu et al. (2017), our data demonstrated that VitD improved hepatic redox status. The antioxidant effect of VitD may be associated with the inhibition of NADPH oxidase and the enhancement of nuclear factor erythroid 2-related factor 2 nuclear translocation (Nakai et al., 2013; Zhu et al., 2017). Additionally, VitD enhances the synthesis of metal ion protein carriers. These metal ions are critical for the action of various enzymes such as antioxidant enzymes (Hassan et al., 2020).

Our data revealed that NAFLD rats showed an increase in hepatic NF-κB and a decline in the IL-10 levels. There are many potential cellular mechanisms leading to activating inflammatory signaling in NAFLD as a high-caloric diet activates the IKK/NF-κB pathway in adipocytes, hepatocytes, and associated macrophages. IKK activation leads to NF-κB translocation and increased expression of numerous markers and potential mediators of inflammation (Pradhan, 2007). Therefore, the activation of the NF-κB signaling pathway as the most enriched pathway was associated with hepatic inflammation and resulted in an increase in NF-κB expression in NAFLD (Sangouni et al., 2019; Zhao et al., 2022). Meanwhile, the decreased IL-10 levels are linked to T-helper-2 malfunctions (Cano Barquilla et al., 2014). Our findings were previously recorded by Theodoro et al. (2021). On the contrary, VitD administration reduced hepatic inflammation, which was confirmed in our histopathological results by decreasing inflammatory cell infiltration. This could be due to the ability of calcitriol to interfere with NF-κB via increasing the expression of the inhibitory protein (IκB) in peripheral blood mononuclear cells and reducing the nuclear translocation of the NF-κB subunit p65 (Krishnan and Feldman, 2011; Chen et al., 2020). Moreover, the IL-10 hepatic level was significantly enhanced upon VitD treatment, which is in agreement with a prior report (Refaat et al., 2021). This could be attributed to enhancing T-helper-2 cell differentiation and overproduction of antiinflammatory cytokines such as IL-10 after VitD administration (Bishop et al., 2021; Sharma et al., 2021).

To further confirm the hepatic lipid burden in our NAFLD model with a decrease in its oxidation, we performed qualitative real-time PCR for analyzing the hepatic expression of lipid metabolic genes including SREBP-1-c and PPAR-α. The results revealed disturbances in the expression of both genes in the NAFLD group including an increase in SREBP-1-c expression and a decrease in PPAR-α expression, which was previously observed by Wang et al. (2022). The explanation for the observed changes in the hepatic SREBP-1-c and PPAR-α mRNA levels is provided by Nagai et al. (2002), who observed that fructose can stimulate SREBP-1-c but suppress PPAR-α expression levels in rat liver. VitD administration resulted in reverse changes in both genes' expression in the liver of the NAFLD group. This may be related to the ability of VitD to interfere with the activation of SREBP-1-c by enhancing the ubiquitin-mediated degradation of the SREBP cleavage-activating protein (Asano et al., 2017). Similar results were previously observed in male rats fed with an HFD and treated with calcitriol at doses 5 μg/kg B.W, I/P, and 5 ng/g B.W, I/M, twice per week, respectively (Yin et al., 2012; Kong et al., 2014).

The impairment of the insulin signal and insulin resistance was further confirmed in our NAFLD model by a significant decrease in the hepatic expression of IRS-2. This may refer to the ability of SREBP-1-c to suppress IRS-2 promoter activity by competing with its transactivator (Ide et al., 2004). Several studies confirmed that hepatic IRS-2 mRNA expression was downregulated in HFD-fed rodent models (Xing et al., 2011; Qiu et al., 2015; Yang et al., 2019). Contrariwise, vitamin D administration enhanced hepatic IRS-2 expression in NAFLD rats. Our result is in parallel with that obtained by Szymczak-Pajor et al. (2020), who speculated that the active form of VitD enhances the transcriptional activation of the IR gene, which improves insulin signaling.

5 Conclusion

This study clarified that an HFFD led to a substantial increase in body weight and hepatic injury indices, along with disorders in glucose hemostasis and lipid metabolism. In addition, hepatic inflammation and oxidative damage occurred, which led to the development and progression of NAFLD. We also concluded that VitD may protect against HFFD-induced NAFLD through its antioxidant and antiinflammatory effects. In addition, VitD had ameliorative effects on glucose hemostasis and lipid profile.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material; further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was reviewed and approved by the Ethical Animal Research Committee of Faculty of Veterinary Medicine, Mansoura University, Egypt (Approval No. 2021 M/35).

Author contributions

DR: methodology, formal analysis, data curation, and writing—original draft, review, and editing. GE, ER, and FA: conceptualization, validation, visualization, and editing—final draft and supervision. HM, TA, SA, BR, and ME-B: methodology, data curation and analysis, and writing and reviewing. FA: final reviewing and preparing the manuscript for publication. All authors read and approved the final manuscript. All authors listed made a substantial, direct, and intellectual contribution to the work and approved it for publication. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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