Vitamin D3 Protects Against Non-Alcoholic Fatty Liver Disease in Rats by Modulating Hepatic Iron Deposition

Original Article

Marwa A. Habib[1], Maher N. Ibrahim[1], Abeer A. Khalifa[1], Dalia A. Hemead[1], Eman Ramadan Abd El Fattah[2] and Amira E. Alsemeh[2]

[1]Department of Medical Physiology, [2]Department of Human Anatomy and Embryology, Faculty of Medicine, Zagazig University, Egypt

ABSTRACT

Introduction and Aim: Increased hepatic iron deposition participate in the pathogenesis of non-alcoholic fatty liver disease (NAFLD). Hepcidin is a master iron regulator that decrease iron efflux from hepatocytes and its level in NAFLD is still controversial. 1,25-dihydroxyvitamin D3 (vitamin D3) is a potent regulator of hepcidin and its deficiency was linked with increased severity of NAFLD. Therefore, this study aimed to assess the effect of vitamin D3 on hepatic iron deposition and circulating hepcidin levels in NAFLD model induced in adult male albino rats.

Materials and Method: Sixty-four adult male rats average age five months weighing 180-200 g were distributed to eight groups. Group (1): ND (normal diet) fed for 4 weeks, Group (2): ND+VD (normal diet with vitamin D3; injected twice weekly with vitamin D3; 5 µg/kg BW) fed for 4 weeks, Group (3): HFD (high- fat diet) fed for 4 weeks, Group (4): HFD+VD (high-fat diet with vitamin D3; injected twice weekly with vitamin D3; 5 µg/kg BW) fed for 4 weeks. Group (5): ND (normal diet) fed for 12 weeks, Group (6): ND+VD (normal diet with vitamin D3; injected twice weekly with vitamin D3; 5 µg/kg BW) fed for 12 weeks, Group (7): HFD (high- fat diet) fed for 12 weeks, Group (8): HFD+VD (high-fat diet with vitamin D3; injected twice weekly with vitamin D3; 5 µg/kg BW) fed for 12 weeks. Body mass index (BMI), abdominal circumference (AC), lipid profile, and serum levels of liver enzymes, hepcidin, IL-6, iron and ferritin, hepatic levels of iron and reactive oxygen species (ROS) were measured in all groups. Histology of hepatic tissues was examined using hematoxylin & eosin, Prussian blue, and Masson's trichrome stains.

Results: Vitamin D3 induced significant reduction in BMI, AC, lipid profile parameters (except for high-density lipoprotein HDL which was increased), liver enzymes, hepcidin, IL-6, ferritin, hepatic iron and ROS., Whereas, significant increase in serum iron levels in 4 and 12W-HFD groups was noticed compared to their time-matched HFD groups. Additionally, vitamin D3 abolished the pathological changes associated with HFD in 4-week group and markedly attenuated the changes in 12-week group, and significantly diminished hepatic iron deposition and hepatic fibrosis in these groups in comparison to their time-matched HFD groups.

Conclusion: Vitamin D3 protects against HFD-induced NAFLD in adult male albino rats by suppression of hepcidin level and subsequent reduction in hepatic iron deposition that decreased oxidative stress-mediated hepatic injury and fibrosis.

Received: 21 December 2021, **Accepted:** 07 February 2022

Key Words: Fatty liver; hepcidin; high fatty diet; iron; vitamin D.

Corresponding Author: Eman Ramadan Abd El Fattah, MD, Department of Human Anatomy and Embryology, Faculty of Medicine, Zagazig University, Egypt, **Tel.**: +20 10 2224 2343, **E-mail:** emanramadan898@yahoo.com

ISSN: 1110-0559, Vol. 46, No. 2

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is regarded as one of the liver health problems worldwide that induce abnormal liver function tests[1,2,3]. NAFLD ranges from simple steatosis to non-alcoholic steato-hepatitis (NASH) that may lead to liver cirrhosis and even to hepato-cellular carcinoma[1]. Multiple insults were implicated in the pathogenesis of NAFLD, and no therapy is effective until now[4].

Increased hepatic iron deposition was implicated in the pathogenesis of NAFLD, but the mechanisms incriminated in this deposition are still unclear[5]. It was claimed to potentiate the start and progression of NAFLD by increasing oxidative stress, modifying insulin signaling and lipid absorption, and was introduced as a potential goal for treatment[6]. Iron was reported to induce toxic oxygen harm by generating oxygen free radicals through the Fenton reaction[7].

Hepcidin is a 25 amino acid-antimicrobial peptide expressed in the liver and a master iron regulator[8]. Hepcidin was reported to down regulate iron efflux from the enterocytes, macrophages and hepatocytes[9,10,11]. Pro-inflammatory cytokines were reported to up-regulate hepcidin during inflammation[12,13]. Hepcidin was suggested to have an important role in iron accumulation in NAFLD patients who showed raised serum hepcidin[14]. Some studies reported higher Hepcidin serum levels in NAFLD[15,16],while others reported that circulating hepcidin levels were associated with obesity but not with the presence of NAFLD[17].

 DOI: 10.21608/ejh.2022.112442.1618

Vitamin D circulating levels were reported to relate inversely to the severity of NAFLD[18,19]. Also, low vitamin D is associated with inflammation, insulin resistance, obesity, oxidative stress, and dyslipidemia in NAFLD[20,21]. Vitamin D is introduced as a potent regulator of hepcidin in both monocytes and hepatocytes[22]. Therefore, the current study was planned to explore the effect of 1,25-dihydroxyvitamin D3 (vitamin D3) on hepatic iron deposition and circulating hepcidin levels in high fatty diet (HFD)- induced NAFLD in adult male albino rats.

MATERIAL AND METHODS

Animal Models

After the approval of the Institution of Research Board (IRB) of Faculty of Medicine, Zagazig University, Egypt (number 4408/27-2-2018) and according to the National Institutes of Health recommendations in the guide for laboratory animals' care and use, sixty-four adult male albino rats with average age five months weighing 180-200 g were obtained from Faculty of Veterinary Medicine, Zagazig University. The rats were kept under hygienic conditions , and housed 4 animals per cage in steel wire cages (50 cm x 60 cm x 60 cm) in the house of animal of Physiology Department, Faculty of Medicine, University of Zagazig. The animals were kept in room temperature with free water access on 12 hour cycle of dark and light for 14 days in the animal house until the start of experiment. After acclimatization, the animals were distributed into eight fed groups (8 rats per group)

Group (1): ND (normal diet): rats fed normal chow diet (ND) and injected intraperitoneally (ip) with corn oil (1mL/kg BW, twice per week) for 4 weeks

Group (2): ND+VD (normal diet with vitamin D3; injected ip twice weekly with vitamin D3; 5 µg/kg BW) for 4 weeks.

Group (3): HFD (high- fat diet): rats fed HFD (containing 18% protein, 24% carbohydrate, and 58% fat and obtained from Faculty of Agriculture, Zagazig University), and injected ip with corn oil twice weekly) for 4 weeks.

Group (4): HFD+VD (high-fat diet with vitamin D3; injected twice weekly with vitamin D3; 5 µg/kg BW) fed for 4 weeks.

Group (5): ND (normal diet) for 12 weeks.

Group (6): ND+VD (normal diet with vitamin D3; injected twice weekly with vitamin D3; 5 µg/kg BW) for 12 weeks.

Group (7): HFD (high- fat diet) for 12 weeks.

Group (8): HFD+VD (high-fat diet with vitamin D3; injected twice weekly with vitamin D3; 5 µg/kg BW) for 12 weeks[23].

Anthropometric measures

At the first and last day of the experiment, the animals were weighed, nose to anus length was measured and BMI index was calculated according to the equation [body weight (g) / length2 (cm2)][24]. Abdominal circumference (AC) was measured in the largest zone of the rat's abdomen according to Gerbaix *et al*.[25].

Blood Sampling

When the experimental period ends with animals overnight fasting, samples of blood were taken from retro-orbital sinus of each rat and put in centrifuge tubes until occurrence of clotting and separation of the serum by centrifugation of blood 3000 rpm for 15 minutes. The serum was aspirated by automatic pipettes and kept in deep frozen at -20° C until tested according to Abassi *et al*.[26].

Biochemical Analysis

Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), total cholesterol (TC), triglycerides (TG), high-density lipoprotein (HDL), and hepcidin were measured using the corresponding ELISA kits (Sun Red Biotechnology, Shanghai, China) following manufacturer's protocols. IL-6 and ferritin were measured using ELISA kits (Mybiosource Co. China, Egyptian Co. for Biotechnology, Egypt, respectively) according to manufacturer's instructions. Serum iron measured by Quantichorm TM Iron Assay Kits (BioAssay System, China) as stated by manufacturer's guidelines. Low-density lipoprotein (LDL) levels were assessed using the equation: LDL=TC-HDL-TG/5 according to Friedewald *et al*.[27]. Very low-density lipoprotein (VLDL) levels were measured. using the equation: VLDL= TG/5 according to Wilson *et al*.[28].

Tissue Sampling

A small portion of hepatic tissue was excised then the homogenate was used for biochemical estimations of hepatic iron using Quantichorm TM Iron Assay Kits and hepatic reactive oxygen species (ROS) using rat ROS ELISA kits according to manufacturer's instructions according to Siqueira *et al*.[29].

Histopathological Examination

For microscopic evaluation, the liver tissues were obtained from each animal and then dissected and submerged in 10% formalin saline for two days to be treated to make 5-µm-thick paraffin sections and subsequently stained with Hematoxylin and Eosin (H&E) to view the histological features, Masson's trichrome to identify the fibers of collagen and Prussian blue for detection of iron deposition.

Histopathologic scores of NAFLD were accepted when: the degree of steatosis was as follows: (0: <5%, 1: 5%- 33% , 2 : >33% -66%, and 3: > 66%) ,. The degree of hydropic degeneration was as follows (0: no hydropic degeneration, 1: < 25%, 2: between 25% and 50%, and 3: > 50%). Portal tract affection was classified as none, mild, moderate, and severe (0–3) as follows (0: no inflammation, 1: scanty cells of inflammation in 1/3 of portal tracts,

2: increased inflammatory cells in >1/3–2/3 of portal tracts, and 3: heavily packed of inflammatory cells in >2/3 of portal tracts)[30].

Statistical analysis

The statistical analysis was done using SPSS program (version 18) (SPSS Inc. Chicago, IL, USA). The data obtained in this study were presented as mean ± SD. One-way Analysis of Variance (ANOVA) (followed by LSD post hoc test and Tukey's post hoc test) was used to relate statistical differences between the groups. Pearson's correlation was used to analyze correlations between % areas of hepatic iron deposition and hepatic fibrosis in all studied groups. The histopathologic scores of NAFLD were evaluated by Kruskal-Wallis test followed by Mann-Whitney test to state difference between groups. *P value* <0.05 was considered statistically significant for all performed statistical tests.

RESULTS

Table 1 showed the results of anthropometric, serum and hepatic parameters in all studied groups. As regard the anthropometric parameters, the final BMI and AC showed a significant time-dependent increase in 4W and 12W-HFD groups when compared with their time-matched ND groups and a significant decrease in 4W and 12W-HFD+VD groups when compared with their time-matched HFD groups. No significant difference was found between 4W-HFD+VD and 4W-ND groups. However, a significant increase was found in 12W-HFD+VD group when compared with 12W-ND group.

As regard the serum biochemical parameters, the levels of ALT, AST, TC, TG, LDL, VLDL, hepcidin, IL-6, ferritin were significantly and progressively increased in 4W and 12W-HFD groups in comparison to their time-matched ND groups and significantly decreased in 4W and 12W-HFD +VD groups when compared with their time-matched HFD groups. Also, serum hepcidin levels were significantly decreased in 4W and 12W-ND+VD groups when compared with their time-matched ND groups. All parameters showed insignificant difference in 4W-HFD+VD when compared with 4W-ND groups, however, they showed a significant increase in 12W-HFD+VD group when related with 12W-ND group except for AST and ferritin which showed insignificant difference.

However, serum levels of HDL and iron showed a significant progressive decrease in 4W and 12W-HFD groups in comparison to their time-matched ND groups and a significant increase in 4W and 12W-HFD +VD groups when compared with their time-matched HFD groups. Also, iron serum levels were significantly increased in 4W and 12W-ND+VD groups when compared with their time-matched ND groups. No significant difference in serum levels of HDL and iron was found between 4W-HFD+VD and 4W-ND groups. However, a significant decrease was found in 12W-HFD+VD group when compared with 12W-ND group.

As regard the Hepatic parameters, hepatic iron and ROS levels were significantly increased in 4W and 12W-HFD groups in comparison to their time-matched ND groups and significantly decreased in 4W and 12W-HFD +VD groups when compared with their time-matched HFD groups. Also, they were significantly decreased in 4W and 12W-ND+VD groups when compared with their time-matched ND groups. No significant difference in hepatic iron and ROS levels was found between 4W-HFD+VD and 4W-ND groups. However, a significant increase was found in 12W-HFD+VD group when compared with 12W-ND group.

Histological assessment of the liver

Light microscopic examination of liver tissue from all experimental groups were seen in figures 1-6. The hepatic tissue from the 4W-ND (Figures 1a,b) and 4W-ND+VD (Figures 1c,d) groups revealed normal structure of hepatocytes, it was arranged in cords around the central vein and separated by blood sinusoids. Hepatocytes had rounded vesicular nuclei and acidophilic cytoplasm whereas 4W-HFD group showed fat deposition in hepatocytes (Figures 2a,b). In 4W-HFD+VD group hepatic sections exhibited retained normal structure of hepatocytes, with less fat deposition and displayed a similar form to the control group (Figures 2c,d).

The hepatic tissue from 12W-ND (Figures 3a,b) and 12W-ND+VD (Figures 3c,d) groups showed the same normal structure as in 4W-ND group. 12W-HFD group displayed degenerative alterations in hepatocytes along with fat droplet deposition and infiltration of inflammatory cells (Figures 4a,b). 12W-HFD+VD group displayed a similar form to 4W-HFD+VD group with less fat deposition and inflammatory cells (Figures 4c,d).

Table 2 shows histopathological scoring of NAFLD in all studied groups. Administration of vitamin D3 along with HFD in 4 and 12 week groups induced a significant decrease in the grades of fat deposition in hepatocyte, hydropic degeneration and portal tract inflammation in comparison to the corresponding HFD groups.

Figure 5 shows Prussian blue stain results in all studied groups. No iron deposition was found in 4W-ND, 4W-ND+VD, 12W-ND and 12W-ND+VD groups (Figures 5a,b,e,f) respectively. Apparent significant increase in iron deposition was detected mainly inside blood sinusoid in 4W-HFD group (Figure 5c) as compared to 4W-ND group. In contrary, the 4W-HFD+VD group showed significant decrease in iron deposition inside the blood sinusoids as compared to the 4W-HFD group (Figure 5d). As regard the 12W-HFD group, there was obvious increase in iron deposition mainly in blood sinusoids which revealed a significant increase compared to both 12W-ND and 4W-HFD groups (Figure 5g). However, the 12W-HFD+VD group revealed a significant decrease in iron deposition in comparison to the 12W-HFD group, but still revealed a significant increase when compared to the 12W-ND group

(Figure 5h). These outcomes were confirmed statistically by measuring the % area of iron deposition in liver tissues (Figure 5i).

Figure 6 shows Masson's trichrome stain results in all studied groups. Delicate collagen fibers were found around the portal triad in 4W-ND, 4W-ND+VD, 12W-ND and 12W-ND+VD groups (Figures 6a,b,e,f) respectively. The 4W-HFD group displayed apparent increase in collagen fibers around the portal triad that exhibited a significant increase compared to 4W-ND group (Figure 6c). In contrary, the 4W-HFD+VD group showed significant decrease in collagen fibers around the portal triad as compared to the 4W-HFD group (Figure 6d). As regard the 12W-HFD group, there was obvious increase in distribution of fibers of collagen around the portal triad where it revealed a significant increase compared to both 12W-ND and 4W-HFD groups (Figure 6g). However, the 12W-HFD+VD group revealed significant decrease in collagen fibers in comparison to the 12W-HFD group, but revealed a significant increase when compared to the 12W-ND group (Figure 6h). These results were confirmed statistically by measuring the % area of the collagen fibers in liver tissues (Figure 6i).

Table 3 shows correlation between % area of hepatic iron deposition indicated by Prussian blue stain and % area of hepatic fibrosis indicated by Masson's trichrome stain in all studied groups. A significant positive correlation was found between hepatic iron deposition and hepatic fibrosis in 4W-HFD, 4W-HFD+VD, 12W-ND+VD, 12W-HFD and 12W-HFD+VD groups.

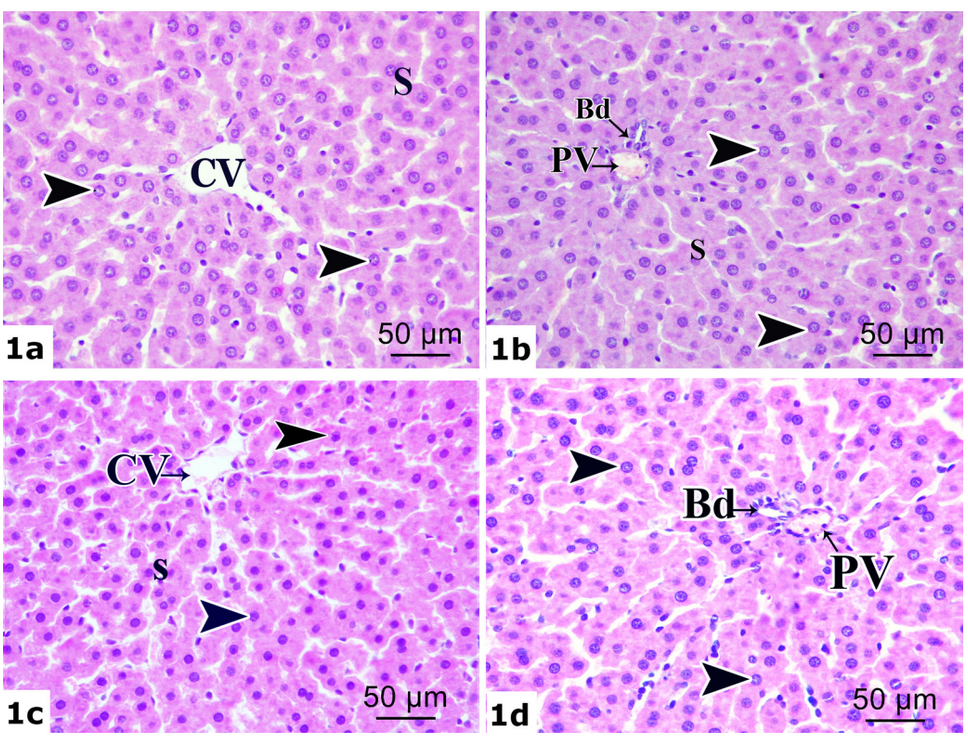

Fig.1: Photomicrographs of H&E-stained rat liver tissues of 4W- normal diet groups: 4W-ND (a, b), 4W-ND+VD (c, d). Fig.1 (a, c) show normal structure of the liver with firmly arranged cords of polygonal hepatocytes having rounded vesicular nuclei and acidophilic cytoplasm (arrowheads) radiating from the central vein (CV) and separated by blood sinusoids (S). Fig.1 (b, d) show the portal area containing the portal vein (PV) and the bile duct (Bd) surrounded by normal hepatocytes (arrowheads) and separated by blood sinusoids (S). Scale bar= 50 µm, x400

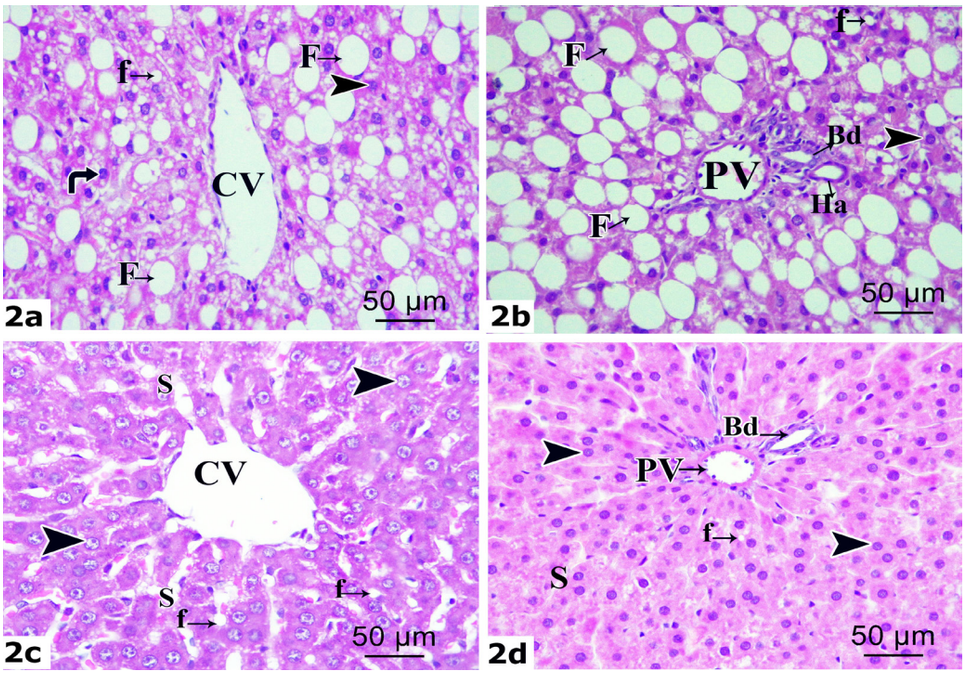

Fig. 2: Photomicrographs of H&E-stained rat liver tissues of 4W- high fat diet groups: 4W-HFD (a, b) and 4W-HFD+VD (c, d): Fig.2 a shows interrupted liver structure around slightly dilated and congested central vein (CV). Most hepatocytes demonstrate macrovesicular (F) and microvesicular (f) fat droplets. Few hepatocytes show hydropic degeneration with vacuolated cytoplasm (curved arrow) and some normal hepatocytes can be observed (arrowhead). Fig.2 b shows the portal triad, portal vain (PV), hepatic artery (Ha) and bile duct (Bd). Most hepatocytes demonstrate macrovesicular (F) and microvesicular (f) fat droplets and some normal hepatocytes can be observed (arrowhead). Fig.2 c shows nearly normal appearance with slightly dilated central vein (CV). Most hepatocytes exhibit rounded vesicular nuclei and acidophilic cytoplasm (arrowhead) and separated by slightly dilated blood sinusoids (S). Minimal microvesicular fat drplets (f) can be noticed. Fig.2 d shows the portal triad □portal vein (PV), hepatic artery (Ha) and bile duct (Bd)□ is surrounded by normal hepatocytes (arrowheads) and well-organized blood sinusoids (S). A few dispersed hepatocytes containing minute vacuoles (f) can be observed. Scale bar= 50 µm, x400

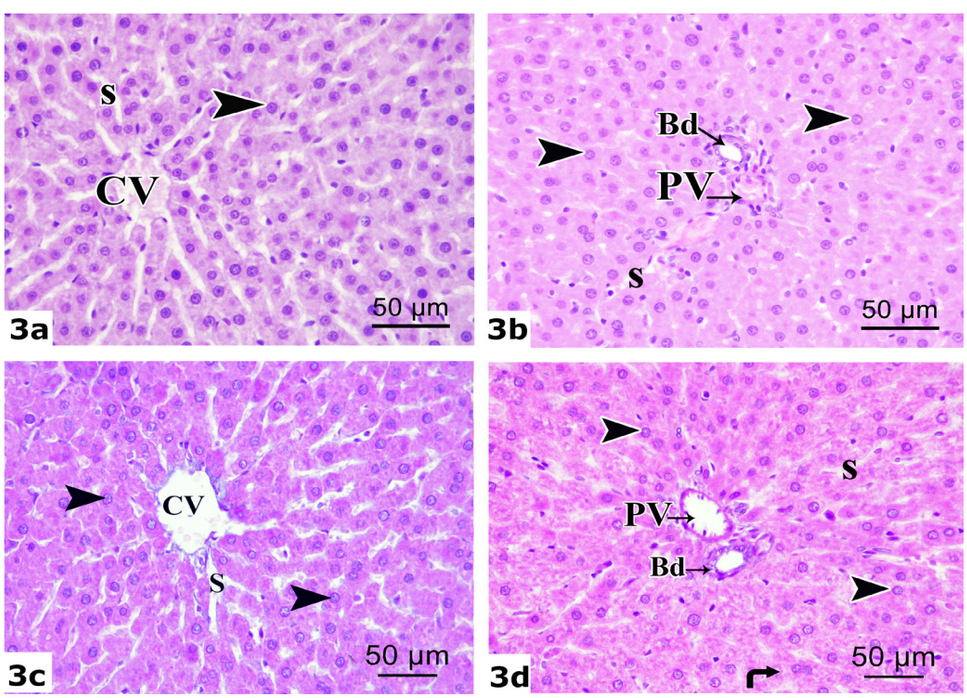

Fig. 3: Photomicrographs of H&E-stained rat liver tissues of 12W- normal diet groups: 12W-ND (a, b), 12W-ND+VD (c, b). Fig. 3 (a, c) show normal structure of the liver with firmly arranged cords of polygonal hepatocytes with rounded vesicular nuclei and acidophilic cytoplasm (arrowheads) radiating from the central vein (CV) and separated by blood sinusoids (S). Fig.3 (b, d) show the portal area containing the portal vein (PV) and bile duct (Bd) surrounded by normal hepatocytes (arrowheads) and blood sinusoids (S). Scale bar= 50 µm, x400

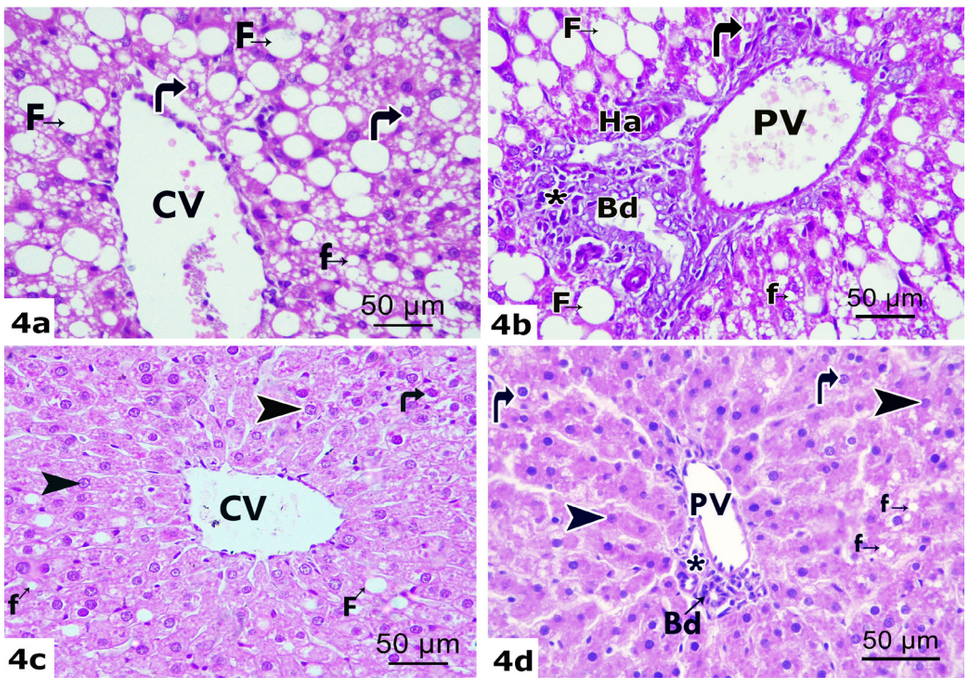

Fig.4: Photomicrographs of H&E-stained rat liver tissues of 12W- high fat diet groups: 12W-HFD (a, b) and 12W-HFD+VD (c, d). Fig.4 a shows disrupted liver structure around dilated central vein (CV). Most hepatocytes have vacuolated cytoplasm with hydropic degeneration (curved arrow). Macrovesicular (F) and branch of hepatic artery (Ha) and microvesicular (f) fat droplets can be observed. Fig.4 b shows proliferation of the bile ducts (Bd) with mononuclear cell infiltrations (star). Most hepatocytes have vacuolated cytoplasm with hydropic degeneration (curved arrow). Macrovesicular (F) and microvesicular (f) fat droplets can be observed. Fig.4 c shows most hepatocytes with rounded vesicular nuclei and acidophilic cytoplasm) but few hepatocytes have vacuolated cytoplasm with dark nuclei (curved arrow) radiating from slightly dilated central vein (CV). Minimal macrovesicular (F) and microvesicular (f) fat droplets can be noticed. Fig.4 d shows some normal hepatocytes with rounded vesicular nuclei and acidophilic cytoplasm (arrowhead) and other hepatocytes have vacuolated cytoplasm with dark nuclei (curved arrow). Slightly dilated portal vein (PV) and bile duct (Bd) surrounded by few mononuclear cell infiltrations (star) can be observed. Minimal microvesicular fat droplets (f) can be noticed. Scale bar= 50 µm, x400

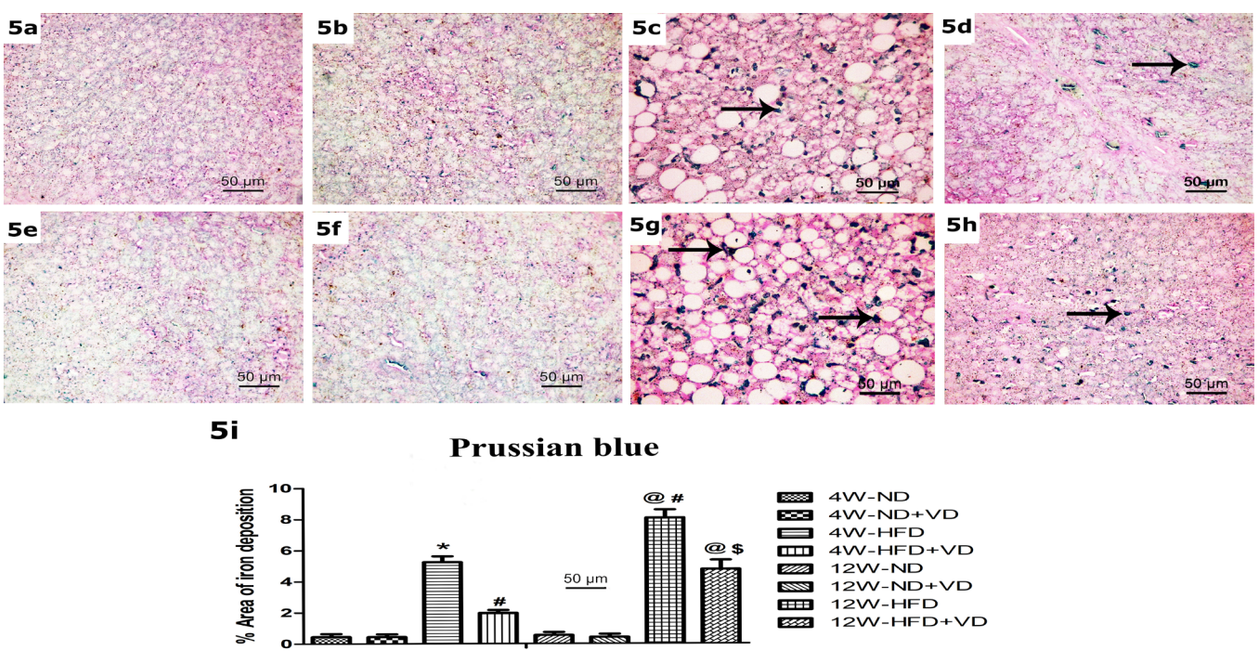

Fig.5: Prussian blue stained liver sections in different experimental groups: a) 4W-ND, b) 4W-ND+VD, c) 4W-HFD, d) 4W-HFD+VD, e) 12W-ND f), 12W-ND+VD, g) 12W-HFD and h) 12W-HFD+VD. Arrows indicates increase blue staining of iron deposition inside the blood sinusoid in 4W-HFD and 12W-HFD compared to 4W-ND and 12W-ND groups and decrease in iron deposition in 4W-HFD+VD and 12W-HFD+VD compared to 4W-HFD and 12W-HFD. i) Bar chart demonstrating the analysis of the % area of iron deposition (x400) of the different experimental groups.* Significant difference compared to the 4W-ND, P< 0.05. # Significant difference compared to the 4W-HFD group, P< 0.05. @ Significant difference compared to the 12W-ND group, $ Significant difference compared to the 12W-HFD group, P< 0.05. blue, x 50 µm, x400.

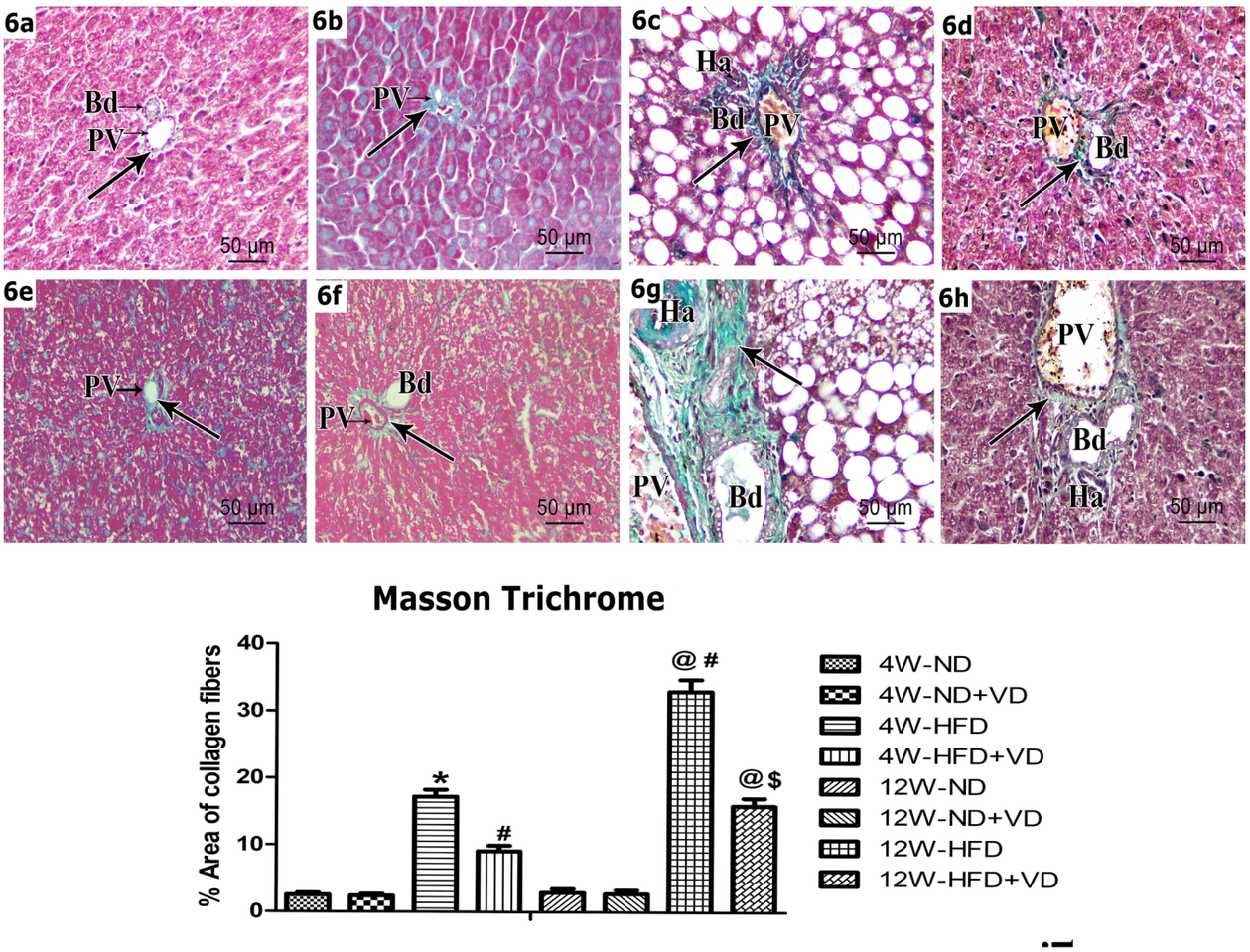

Fig.6: Masson's trichrome stained liver sections in different experimental groups: a) 4W-ND group, b) 4W-ND+VD, c) 4W-HFD, d) 4W-HFD+VD, e) 12W-ND, f) 12W-ND+VD, g) 12W-HFD and h) 12W-HFD+VD. Arrows indicates green staining of the collagen fibers around the portal triad (portal vein (PV), bile duct (Bd), hepatic artery (Ha) which increases in 4W-HFD and 12W-HFD compared to 4W-ND and 12W-ND groups and decreases in 4W-HFD+VD and 12W-HFD+VD compared to 4W-HFD and 12W-HFD. i) Bar chart demonstrating the analysis of the % area of collagen fibers (x400) of the different experimental groups. * Significant difference compared to the 4W-ND, P< 0.05. # Significant difference compared to the 4W-HFD group, P< 0.05. @ Significant difference compared to the 12W-ND group, $ Significant difference compared to the 12W-HFD group, P< 0.05. Masson trichome, x 50 µm, x400.

Table 1: Measured parameters in all studied groups (mean ± SD)

Groups / Parameters	4W-ND	4W-ND +VD	4W-HFD	4W-HFD +VD	12W-ND	12W-ND+VD	12W-HFD	12W-HFD+VD
Final BMI (g/cm2)	0.51±0.07	0.48±0.02	0.72±0.09^{a,b}	0.54±0.06^{b,c}	0.63±0.02^{a,b,c,d}	0.63±0.02^{b,c}	0.84±0.02^{a,b,c,d,e,f}	0.72±0.02^{a,b,d,e,f,g}
Final AC (cm)	17.5±0.9	16.6±1.1	23.3±1.1^{a,b}	18.2±1.1^{b,c}	21.1±0.6^{a,b,c,d}	20.3±0.5^{a,b,c,d}	24.6±0.6^{a,b,c,d,e,f}	22.4±0.5^{a,b,d,e,f,g}
Serum ALT (U\L)	38.75±1.49	36.25±1.48	99.37±7.29^{a,b}	42.0±3.7^{b,c}	39.25±1.04^{c}	37.62±1.06^{c}	162.50±7.07^{a,b,c,d,e,f}	104.00±8.71^{a,b,d,e,f,g}
Serum AST (U\L)	145±8.1	141.25±7.4	163.12±7.5^{a,b}	150.63±6.23^{b,c}	145.63±7.8^{c}	141.50±4.8^{c,d}	193.50±13.2^{a,b,c,d,e,f}	151.75±5.6^{b,c,f,g}
Serum TC (mg/dl)	73.6±3.3	70.6±1.7	123.2±5.9^{a,b}	76.6±1.7^{b,c}	76.5±2.6^{b,c}	75.5±3.8^{b,c}	163.1±5.2^{a,b,c,d,e,f}	111.4±5.9^{a,b,c,d,e,f,g}
Serum TG (mg/dl)	44.2±4.4	41.4±5.3	79.5±4.0^{a,b}	46.50±5.37^{c}	49.1±1.8^{b,c}	49.0±2.4^{b,c}	110.7±10.1^{a,b,c,d,e,f}	71±9.1^{a,b,c,d,e,f,g}
Serum HDL (mg/dl)	42.5±1.7	43.7±1.6	33.9±3.1^{a,b}	41.62±2.3^{b,c}	42.3±1.5^{c}	43.1±0.8^{c}	26.3±1.8^{a,b,c,d,e,f}	33.1±2.3^{a,b,d,e,f,g}
Serum LDL (mg/dl)	21.3±2.6	20.6±1.6	73.5±7.9^{a,b}	25.7±2.3^{c}	24.4±3.0^{c}	22.6±3.9^{c}	113.6±5.1^{a,b,c,d,e,f}	64.1±6.1^{a,b,c,d,e,f,g}
Serum VLDL (mg/dl)	8.9±0.9	8.3±1.1	15.9±0.8^{a,b}	9.3±1.1^{c}	9.8±0.4^{c}	9.8±0.5^{c}	22.1±2.0^{a,b,c,d,e,f}	14.2±1.8^{a,b,c,d,e,f,g}
Serum Hepcidin (ng/ml)	41.9±5.1	22.26±1.7^{a}	196.17±27.9^{a,b}	52.1±9.7^{b,c}	49.32±7.4^{b,c}	25.20±4.2^{c,d,e}	361.21±32.8^{a,b,c,d,e,f}	194.47±31.2^{a,b,d,e,f,g}
Serum IL-6 (pg/ml)	45.1±4.8	41.5±4.5	73.7±9.9^{a,b}	48.5±4.0^{c}	47.9±5.4^{c}	44.6±4.7^{c}	206.6±12.3^{a,b,c,d,e,f}	89.1±9.5^{a,b,c,d,e,f,g}
Serum Iron (µg/dl)	132.3±16.2	154.6±13.7^{a}	101.3±8.3^{a,b}	122.5±9.6^{b,c}	133.2±16.9^{b,c}	153.3±13.2^{a,c,d,e}	64.5±6.9^{a,b,c,d,e,f}	93.7±8.7^{a,b,d,e,f,g}
Serum Ferritin (ng/ml)	185.6±6.7	180.0±7.0	209.7±11.3^{a,b}	190.5±6.5^{c}	184.4±7.1^{c}	173.3±6.5^{c,d}	223.6±10.4^{a,b,c,d,e,f}	182.3±7.9^{e,g}
Hepatic Iron (µg/g tissue)	73.9±2.5	68.3±2.6^{a}	87.0±3.0^{a,b}	76.0±1.9^{b,c}	77.0±2.4^{b,c}	69.0±4.9^{a,c,d,e}	139.1±5.4^{a,b,c,d,e,f}	99.3±6.5^{a,b,c,d,e,f,g}
Hepatic ROS (nmol/mg)	1.4±0.05	1.2±0.02^{a}	1.8±0.04^{a,b}	1.4±0.05^{b,c}	1.4±0.04^{b,c}	1.3±0.03^{a,c,d,e}	2.4±0.2^{a,b,c,d,e,f}	1.6±0.1^{a,b,c,d,e,f,g}

W: week; ND: normal diet; HFD: high-fat diet; VD: vitamin D3; AC: abdominal circumference; ROS: reactive oxygen species. a: significant versus 4W-ND group, b: significant versus 4W-ND+VD group, c: significant versus 4W-HFD group, d: significant versus 4W-HFD+VD group, e: significant versus 12W-ND group, f: significant versus 12W-ND+VD group, g: significant versus 12W-HFD group.

Table 2: Histopathological scoring of NAFLD in all studied groups (Median± IQ range)

	4W-ND	4W-ND +VD	4W-HFD	4W-HFD +VD	12W-ND	12W-ND+VD	12W-HFD	12W-HFD+VD	Chi-square
Steatosis	0 (0-0)	0 (0-0)	2.5 (1-3)^{a,b}	0(0-2)^{c}	0(0-0)^{c}	0(0-0)^{c}	3 (2-3)^{a,b,d,e,f}	1 (0-2)^{a,b,c,e,f,g}	p<0.001
Hydropic Degeneration	0 (0-0)	0 (0-0)	1 (1-2)^{a,b}	0(0-1)^{c}	0 (0-0)^{c}	0 (0-0)^{c}	2.5 (1-3)^{a,b,c,d,e,f}	1 (0-2)^{a,b,e,f,g}	p<0.001
Portal Tract inflammation	0 (0-0)	0 (0-0)	1 (1-2)^{a,b}	0(0-1)^{c}	0 (0-0)^{c}	0 (0-0)^{c}	2 (1-3)^{a,b,c,d,e,f}	1 (0-2)^{a,b,e,f,g}	p<0.001

W: week; ND: normal diet; HFD: high-fat diet; VD: vitamin D3. a: significant versus 4W-ND group, b: significant versus 4W-ND+VD group, c: significant versus 4W-HFD group, d: significant versus 4W-HFD+VD group, e: significant versus 12W-ND group, f: significant versus 12W-ND+VD group, g: significant versus 12W-HFD group.

Table 3: Correlation between % area of hepatic iron deposition and hepatic fibrosis in all studied groups

Groups	R	*P value*
4W-ND	0.572	*P*>0.05 (0.138)
4W-ND+VD	0.552	*P*>0.05 (0.156)
4W-HFD	0.779	*P*<0.05 (0.023)
4W-HFD+VD	0,808	*P*<0.05 (0.015)
12W-ND	0.383	*P*>0.05 (0.349)
12W-ND+VD	0.740	*P*<0.05 (0.036)
12W-HFD	0.857	*P*<0.01 (0.007)
12W-HFD+VD	0.885	v<0.01 (0.003)

W: week; ND: normal diet; HFD: high-fat diet; VD: vitamin D3

DISCUSSION

The risk of developing NAFLD has been correlated to the excessive fat intake among animals and humans[31]. Also, hepatic fat accumulation was considered the first mark in the pathogenesis of the NAFLD[32]. In the present study, the final body weight, AC, BMI, serum levels of TC, TG, LDL and VLDL showed a significant and time-dependent increase in the 4 W and 12W-HFD groups accompanied by a significant decrease in HDL level in the same groups. These results which indicate that obesity was successfully induced by HFD are consistent with those of several previous studies on HFD-fed rats[23,33,34]. Moreover, the results of liver histopathology demonstrated fat deposition in hepatocytes of 4W-HFD group indicating hepatic steatosis, while, degenerative changes were found in hepatocytes of 12W-HFD group accompanied by inflammatory cell infiltration indicating NASH[23].

Hepatocytes arranged in disordered manner with losing of liver architecture. It showed vacuolated cytoplasm with hydropic degeneration 4W and 12W-HFD groups. These results are inconsistence of Jahn *et al*.[35] who added that there was hepatocellular ballooning with marked inflammatory cell infiltration around hepatocytes and diffuse infiltration around the portal vein in high fat sugar diet fed rats compared to low fat diet fed rats .

A concomitant significant and time-dependent increase in liver enzymes was found in HFD groups. Consistent with these results, several studies reported the occurrence of hepatic steatosis and steatohepatitis in HFD-fed rats after 4 and 12 weeks respectively[33,36,37].

It was reported that pro-inflammatory cytokines are integrated in the pathogenesis of NAFLD[38]. Also, NAFLD was presented as a chronic inflammatory condition that involve both systemic and intrahepatic inflammation[11]. Consistent with these studies, the current study revealed a significant and progressive rise in serum levels of IL-6 in 4W and 12W-HFD groups. This study revealed a significant time-dependent increase in serum hepcidin levels in 4 W and 12W-HFD groups. This finding is consistent with other studies that revealed elevated hepcidin levels in NAFLD[14,39,40]. This increase may be explained by the inflammatory condition that accompanied NAFLD induced by HFD and was confirmed by increased serum IL-6 levels. Consistent with this explanation, some studies demonstrated increased hepcidin levels in obesity and other chronic inflammatory conditions[41,42]. Also, hepcidin was reported to be induced by systemic or intrahepatic inflammation[11]. Moreover, hepcidin was found to be up controlled by pro-inflammatory cytokines including IL-6, IL-1 and TNF-α[5]. In contrast to the previous studies, the study by Lu *et al*.[43] reported that lack of hepcidin expression might cause early development of fibrosis in NAFLD following HFD. Also, other studies found that circulating hepcidin levels were associated with the presence of obesity rather than NAFLD[14,44].

Increased hepatic iron deposition was found in NAFLD, but the mechanisms incriminated in this deposition are still unclear[5]. Valenti *et al*.[45] linked hepatocellular iron deposition with the severity of liver fibrosis. In line with these reports, the existing study had a significant and time-dependent increase in hepatic iron level in 4W and 12W-HFD groups. This finding was confirmed by the results of Prussian blue stain of hepatic tissue which revealed significant increase in iron deposition mainly inside blood sinusoid in 4W and 12W-HFD groups. Moreover, the results of Masson's trichrome stain of hepatic tissue showed significant increase in fibers of collagen around the portal triad in 4W and 12W-HFD groups. Also, a significant positive correlation was found between the % area of hepatic iron deposition and hepatic fibrosis. These findings of Masson's trichrome stain are consistent with those of Zhu *et al*.[23]. The present study suggested that elevated hepcidin levels may be incriminated in inducing increased hepatic iron deposition.

In support of this hypothesis, the study by Pietrangelo[46] reported that hepcidin induced by inflammatory cytokines in NAFLD might cause hepatic iron deposition. Moreover, increased hepatic hepcidin level was reported to be associated with iron retention in hepatic macrophages[47,48].

Oxidative stress was reported to have a crucial role in the development and advancement of NAFLD through inducing changes in insulin sensitivity and modulation of inflammation[49]. Excess iron caused damage to cells from oxidative stress due to generation of ROS[50]. Nelson *et al*.[5] reported that elevated hepatic deposition of iron induced generation of ROS that increased hepatic oxidative stress and inflammation, resulting in liver injury, steatohepatitis and fibrosis. Consistent with these studies, the present study showed a significant increase in hepatic ROS level in HFD groups which can be attributed to the increased hepatic iron established in these groups.

This study showed an increase in a serum ferritin levels significantly in HFD groups when matched with control groups which may be attributed to increased hepatic iron overload found in these groups. In accordance with this finding, Britton *et al*.[6] and Ryan *et al*.[51] reported that iron could cause hyperferritinemia either by direct release of ferritin or cytokine-¬mediated stimulation of ferritin release by other cells, and thus ferritin levels reflect liver iron in NAFLD. Also, hyperferritinaemia was associated with obesity and insulin resistance in NAFLD and serum ferritin was considered a biomarker of body iron stores[23,52]. Moreover, hyperferritinemia was observed in one-third of patients with non-alcoholic fatty liver disease (NAFLD) and Metabolic Syndrome[14]. In contrast, the study by Bugianesi *et al*.[53] demonstrated that serum ferritin level was not associated with hepatic iron concentration in NAFLD, but was considered a marker of severe histologic damage., Kim *et al*.[54] correlated ferritin levels with the severity of NAFLD in absence or presence of hepatic iron deposition. The present study also found a significant decrease in serum iron levels in HFD groups

when compared with control groups. This decrease may be explained by elevation of serum hepcidin level in HFD groups that caused iron retention inducing decrease in serum iron. Consistent with our finding, Ganz[55] found that the increased hepcidin levels induced iron sequestration within reticuloendothelial system resulting in decrease iron available for erythropoiesis and hemoglobin synthesis. Also, Ryan *et al*.[51] reported that serum iron was lower in NAFLD patients where iron is sequestered in macrophages.

The connection between vitamin D deficiency, obesity and severity of NAFLD has been investigated. Cordeiro *et al*.[56] reported increased prevalence of vitamin D deficiency in obese individuals, that worsens with progression of NAFLD. Moreover, Dasarathy *et al*.[19] linked hypovitaminosis D with the severity of NAFLD. Consistent with this, the present study showed that vitamin D3 significantly reduced serum levels of TC, TG, LDL, VLDL and liver enzymes, but significantly increased serum levels of HDL in 4 W and 12W-HFD groups in comparison to their time-matched HFD groups. Parallel to these changes, the histopathological examination of liver tissue revealed that vitamin D3 abolished the pathological changes of NAFLD induced by HFD in 4W group, nearly retained the normal architecture of hepatocytes, and markedly attenuated the changes induced by HFD in 12W group. This finding showed similar result with the study by Zhu *et al*.[23] who reported that lipid profile, liver enzymes and histopathological changes of NAFLD were markedly reduced in animals administrated with vitamin D along with HFD in comparison to HFD groups.

Vit.D3 co-adminisration showed a decrease cytoplasmic vaculation and fat deposition in hepatocytes in 4W and 12W-HFD groups , these data are inconsistence with Bingul etal.[57] who observed that there was neither microvesicular steatosis nor hepatocyte ballooning in alchoholic and non alchoholic fatty liver diseases .

Vit. D3 treatment diminished hepatic ROS formation, lipid and protein oxidation products and inflammation in the liver together with histopathological improvements as improvement of hepatic architecture, decrease in the grades of fat deposition in hepatocyte, hydropic degeneration and portal tract inflammation in comparison to the corresponding HFD groups. These results are in accordance with Bingul etal.[57] who stated that, there was improvement of the biochemical and histopathological results in alchoholic and non alchoholic fatty liver diseases.

The present study also demonstrated that vitamin D3 produced significant reduction in BMI and AC. This finding is constant with the study by Farhangi *et al*.[21] which demonstrated that vitamin D reduced food intake and weight gain in HFD-induced obese rats. In contrast to our results, the studies by Wamberg *et al*.[58] and Mason *et al*.[59] showed that treatment with vitamin D had no effect on body weight reduction. Also, Sharifi *et al*.[60] found that vitamin D did not change the serum levels of hepatic enzymes in adults with NAFLD. In addition, Tabrizi *et al*.[61] reported that vitamin D did not affect lipid profiles in patients with NAFLD. The reason for this may be attributed to the species difference as these studies were performed on human patients.

Vitamin D was reported to reduce the production of inflammatory cytokines and induce anti- inflammatory response[62,63]. In line with these reports, the present study found that vitamin D3 significantly decreased serum IL-6 levels in 4 W and 12W-HFD groups when compared with their time-matched HFD groups. Consistent with this finding, Bikle[64] found that vitamin D reduced the expression of inflammatory biomarkers in adults with NAFLD.

Vitamin D was presented as a potent regulator of hepcidin trough suppression of hepcidin in monocytes and hepatocytes[22]. Additionally, it was reported that high-dose vitamin D induced alterations of the hepcidin-ferroportin axis in monocytes through decreasing hepcidin while increasing ferroportin in the presence of an inflammatory stimulus and led to a reduction of pro-hepcidin cytokine, IL-6 and IL-1b, therefore facilitating iron efflux during inflammation[63]. Based on these findings, the current study investigated the vitamin D3 effect on serum hepcidin level and found that vitamin D3 significantly reduced serum hepcidin levels in 4 and 12W-HFD-fed groups when compared with their time-matched HFD groups. This effect may be explained by the anti-inflammatory effect of vitamin D3 through decreasing the level of IL-6. This explanation is supported with the studies by Baeke *et al*.[65] and Nemeth[66] which reported that vitamin D inhibits the production of inflammatory cytokines which directly induce hepcidin production. In contrast to these studies, Atkinson *et al*.[67] reported insignificant change in serum IL-6 and hepcidin levels after cholecalciferol treatment in children with chronic kidney disease. This distinction may be explained by the different underlying pathological condition and/or the species difference. The present study also found that vitamin D3 significantly reduced serum hepcidin level in normal diet groups. This finding led us to suggest that vitamin D3 has a direct effect on hepcidin level independent of inflammatory cytokines. This finding is consistent with the study by Smith *et al*.[68] which revealed that vitamin D3decreased plasma hepcidin significantly in healthy adults, in absence of inflammatory conditions.

Vitamin D is a well-documented suppressor of oxidative stress[69,70]. Farhangi *et al*.[21] reported a reduction in levels of oxidative stress indices after vitamin D intake. In agreement with these studies, the present study revealed that vitamin D3 significantly reduced hepatic ROS level in 4 and 12W-HFD-fed groups when compared with their time-matched HFD groups. This anti-oxidant effect of vitamin D3 may be explained by the suppressive action of vitamin D3 on serum hepcidin level which negatively regulate iron leading to increase in serum iron and decrease hepatic iron. The diminished hepatic iron is suggested to decrease the production of hepatic ROS level and thus

protect the liver against its oxidative injury. To confirm this hypothesis, the hepatic iron, serum iron and serum ferritin were measured in vitamin D3-treated groups. The present results demonstrated a significant reduction in hepatic iron and serum ferritin, while, a significant increase in serum iron in comparison to HFD groups. These findings were further confirmed by the results of Prussian blue staining of hepatic tissue which showed that vitamin D3 significantly diminished iron deposition in 4 and 12W-HFD-fed groups compared with their time-matched HFD groups. This reduction in hepatic iron deposition was accompanied by significant reduction in hepatic fibrosis indicated by decrease of % area of collagen fibers in the hepatic tissues stained with Masson's trichrome stain. Consistent with the present findings, Dunn *et al*.[71] reported that vitamin D decreased hepatic iron and serum ferritin through its suppressive effect on hepcidin. Additionally, the study by Zhu *et al*.[23] demonstrated a reduction in the level of fibrosis in hepatic tissues of vitamin D-treated HFD group in comparison to HFD groups. In contrast to the previous studies, Braithwaite *et al*.[72] reported that vitamin D3 had no effect on hepcidin or other markers of iron status and inflammation in pregnancy. This contradiction may be explained by presence of other implying factors such as hormonal changes occurring in pregnancy.

The present study found that vitamin D3 significantly increased serum iron level in 4 Wand 12W-ND groups when compared with their time-matched ND groups. This effect may be explained by the direct effect of vitamin D3 on hepcidin which was found to be increased in the same groups. Consistent with this finding. Bacchetta *et al*.[22] demonstrated that vitamin D might directly affect serum hepcidin level through vitamin D response element on the HAMP gene of hepcidin. Furthermore, the study by Smith *et al*.[68] showed that high-dose vitamin D3 supplementation significantly reduced circulating hepcidin concentrations after one week among healthy adults in absence of inflammatory conditions.

CONCLUSION

The current study found that administration of vitamin D3 abolished the NAFLD changes associated with HFD in 4 weeks group, and markedly attenuated the changes associated with HFD in 12 weeks group. The protective effect of vitamin D3 against progression of NAFLD may be mediated by suppression of hepcidin level and subsequent reduction in hepatic iron deposition that decrease oxidative stress-mediated hepatic injury and fibrosis.

ABBREVIATIONS

non-alcoholic fatty liver disease **(NAFLD)**, normal diet **(ND)**, high-fat diet **(HFD)**, VD **(vitamin D3)**, abdominal circumference **(AC)**, body mass index **(BMI)**, reactive oxygen species **(ROS)**, non-alcoholic steato-hepatitis **(NASH)**, Institution of Research Board **(IRB)**, intraperitoneally **(ip)**, Serum alanine aminotransferase **(ALT)**, aspartate aminotransferase **(AST)**, total cholesterol **(TC)**, triglycerides **(TG)**, high-density lipoprotein **(HDL)**, Very low-density lipoprotein **(VLDL)**.

CONFLICT OF INTERESTS

There are no conflicts of interest.

REFERENCES

1. Oh, M.K., Winn, J. and Poordad, F. (2008). Review article: diagnosis and treatment of nonalcoholic fatty liver disease. Aliment. Pharmacol. Ther., 28, 503–522.
2. Law, K. and Brunt, E.M.(2010). Nonalcoholic fatty liver disease. Clin. Liver Disease, 14, 591–604.
3. Rinella, M.E. (2015) Nonalcoholic fatty liver disease a systematic review. JAMA – J. Am. Med. Assoc., 313(22), 2263-2273.
4. Cimini, F.A., Barchetta, I., Carotti, S., Morini, S. and Cavallo, M.G. (2019). Overview of studies of the vitamin D/vitamin D receptor system in the development of non-alcoholic fatty liver disease. World J. Gastrointest. Pathophysiol., 10, 11-16.
5. Nelson, J.E, Klintworth, H. and Kowdley, K.V. (2012). Iron Metabolism in Nonalcoholic Fatty Liver Disease. Curr. Gastroenterol. Rep. 14, 8–16.
6. Britton, L.J., Subramaniam, V.N. and Crawford, D.H.G. (2016) Iron and non-alcoholic fatty liver disease. World J. Gastroenterol., 22 (36), 8112-8122.
7. González-Domínguez, Á, Visiedo-García, F.M., Domínguez-Riscart, J., González-Domínguez, R., Mateos, R.M., and Lechuga-Sancho, A.M. (2020). Iron Metabolism in Obesity and Metabolic Syndrome. Int J Mol Sci., 21(15), 5529-5556.
8. Park, C.H., Valore, E.V., Waring, A.J. and Ganz, T. (2001) Hepcidin, a urinary antimicrobial peptide synthesized in the liver. J. Biol. Chem., 276, 7806-7810.
9. Nemeth, E., Rivera, S., Gabayan, V., Keller, C., Taudorf, S., Pedersen, B.K., *et al*.. (2004) IL-6 mediates hypoferremia of inflammation by inducing the synthesis of the iron regulatory hormone hepcidin. J. Clin. Invest. , 113, 1271-1276.
10. De Domenico, I., Ward, D.M., Langelier, C., Vaughn, M.B., Nemeth, E., Sundquist, W.I., *et al*. .(2007). The molecular mechanism of hepcidin-mediated ferroprotein down-regulation. Mol. Biol. Cell., 18, 2569–2578.
11. Corradini, E and Pietrangelo, A. (2012). Iron and steatohepatitis. J. Gastroenterol. Hepatol., 27(2), 42–46.
12. Lee, P., Peng, H., Gelbart, T., Wang, L. and Beutler, E. (2005). Regulation of hepcidin transcription by interleukin-1 and interleukin-6. Proc. Natl. Acad. Sci. USA., 102, 1906–1910.
13. Wrighting, D.M. and Andrews, N.C. (2006). Interleukin-6 induces hepcidin expression through STAT3. Blood. , 108: 3204–3213.

14. Rametta, R., Fracanzani, A.L., Fargion, S. and Dongiovanni, P. (2020). Dysmetabolic Hyperferritinemia and Dysmetabolic Iron Overload Syndrome (DIOS): Two Related Conditions or Different Entities? Curr. Pharm. Des., 26(10), 1025-1035.
15. Senates, E., Yilmaz, Y., Colak, Y., Ozturk, O., Altunoz, M.E., Kurt, R., *et al*.. (2011). Serum Levels of Hepcidin in Patients with Biopsy Proven Nonalcoholic Fatty Liver Disease. Metab. Syndr. Relat. Disord., 9(4), 287–290.
16. Datz, C., Muller, E. and Minerva, E.A. (2017) iron overload and non-alcoholic fatty liver disease. endocrinologica., 42(2), 173-183.
17. Auguet, T., Aragonès, G., Sabench, F., Berlanga, A., MartõÂnez, S., Del Castillo, D., *et al*. 2017) Hepcidin in morbidly obese women with nonalcoholic fatty liver disease. PLoS ONE, 12(10), p187065. (Number of pages)
18. Eliades, M., Spyrou, E., Agrawal, N., Lazo, M., Brancati, F.L., Potter, J.J., *et al*. . (2013). Meta-analysis: vitamin D and non-alcoholic fatty liver disease. Aliment. Pharmacol. Ther., 38(3), 246-254.
19. Dasarathy, J., Periyalwar, P., Allampati, S., Bhinder, V., Hawkins, C., Brandt, P., *et al*. (2014). Hypovitaminosis D is associated with increased whole body fat mass and greater severity of non-alcoholic fatty liver disease. Liver Int., 34(6), 118-127.
20. Mutt, S.J., Hyppönen, E., Saarnio, J., Järvelin, M.R. and Herzig, K.H.(2014) Vitamin D and adipose tissue-more than storage. Front. Physiol., 5, p228.
21. Farhangi, M.A., Mesgari-Abbasi, M., Hajiluian, G., Nameni, G. and Shahabi, P. (2017) Adipose tissue inflammation and oxidative stress: the ameliorative effects of vitamin D. Inflammation, 40(5), 1688-1697.
22. Bacchetta, J., Zaritsky, J.J., Sea, J.L., Chun, R.F., Lisse, T.S., Zavala, K., *et al* (2014) Suppression of Iron-Regulatory Hepcidin by Vitamin D. J. Am. Soc. of Nephrol., 25(3), 564-572.
23. Zhu, C., Liu, Y., Wang, H., Wang, P., Qu, H., Wang, B., *et al*. . (2017) Active form of vitamin D ameliorates non-alcoholic fatty liver disease by alleviating oxidative stress in a high-fat diet rat model. Endocrine J., 64(7), 663-673.
24. Novelli, E., Diniz, Y., Galhardi, C., Ebaid, G., Rodrigues, H., Mani, F., *et al*. (2007) Anthropometrical parameters and markers of obesity in rats Laboratory Animals Ltd. Lab. Anim., 41, 111–119.
25. Gerbaix, M., Metz, L., Ringot, E. and Courteix, D. (2010). Visceral fat mass determination in rodent: validation of dual energy X-ray absorptiometry and anthropometric techniques in fat and lean rats. Lipids Health Dis., 9, P140.
26. Abbasi, M.H., Fatima, S., Naz, N., Ihtzaz A. Malik, I.A., Sheik,, N. (2013). Effect of Nerium oleander (N.O.) Leaves Extract on Serum hepcidin, Total Iron, and Infiltration of ED1 Positive Cells in Albino Rat. BioMed Research International, Volume 2013, Article ID 125671, 8 pages
27. Friedewald, W.T., Levy, R.I. and Fredrickson, D.S. (1972) Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. Clin. Chem., 18: 499-502.
28. Wilson PWF, Loren A, Zech b, Richard E, Gregg b, Ernst J, *et al*. (1985) Estimation of VLDL cholesterol in hyperlipidemia. J. Clinica. Chimico. Act., 151, 285:291.
29. Siqueira, I. R., Fochesatto, C., Torres, I. L. S., Dalmaz, C., Netto, C. A. (2005). Aging affects oxidative state in hippocampus, hypothalamus and adrenal glands of Wistar rats. Life Sci., 78, 271–278.
30. Kleiner, D.E., Brunt, E.M., Van Natta, M., Behling, C., Contos, M.J., Cummings, O.W., *et al*. (2005). Design and validation of a histological scoring system for nonalcoholic fatty liver disease. Hepatology, 41, 1313-1321.
31. Yki-Jarvinen, H. (2014). Non-alcoholic fatty liver disease as a cause and a consequence of metabolic syndrome, Lancet Diabetes Endocrinol., 2 (11), 901-910.
32. Levene, A.P. and Goldin, R.D. (2012) The epidemiology, pathogenesis and histopathology of fatty liver disease. Histopathology, 61, 141–152.
33. Asalah, A.K.h, Alsayed, M.A., Abd Al-Aleem, D.I. and El Malkey, N.F. (2014). Alcoholic Fatty Liver Disease: Correlation with Metabolic and Haemostatic Parameters. Basic Sci. Med., 3(4), 69-84.
34. Eisinger, K., Liebisch, G., Schmitz, G., Aslanidis, C., Krautbauer, A. and Buechler, C. (2014). Lipidomic Analysis of Serum from High Fat Diet Induced Obese Mice. Int. J. Mol. Sci., 15 (2), 2991–3002.
35. Jahn, D., Dorbath,D., Kircher, S., Nier, A., Bergheim, I., etal.(2019). Beneficial Effects of Vitamin D Treatment in an Obese Mouse Model of Non-Alcoholic Steatohepatitis. Nutrients, 11, 77
36. Svegliati-Baroni, G., Candelaresi, C., Saccomanno, S., Ferretti, G., Bachetti, T., Marzioni, M., *et al*. (2006) Gastrointestinal, Hepatobiliary and Pancreatic Pathology. A Model of Insulin Resistance and Nonalcoholic Steatohepatitis in Rats. Am. J. Pathol., 169(3), 846-860
37. Zhang, X., Yang, J., Guo, Y., Ye, H., Yu, C., Xu, C., *et al* (2010). Functional Proteomic Analysis of Nonalcoholic Fatty Liver Disease in Rat Models: Enoyl–Coenzyme A Hydratase Down-Regulation Exacerbates Hepatic Steatosis. Hepatology, 51(4), 1190-1199.

38. Tilg, H. (2010). The role of cytokines in non-alcoholic fatty liver disease. Dig. Dis., 28 (1), 179-185.

39. Haap, M., Machann, J., von Friedeburg, C., Schick, F., Stefan, N., Schwenzer, N.F., *et al*. . (2011) Insulin Sensitivity and Liver Fat: Role of Iron Load. J. Clin. Endocrinol. Metab., 96, 958–961.

40. Chen W, Wang X, Huang, LI and Liu BO. (2016). Hepcidin in non-alcoholic fatty liver disease regulated by the TLR4/NF-κB signaling pathway. Exp. Ther. Med., 11(1), 73-76.

41. Dao MC and Meydani SN. (2013). Iron biology, immunology, aging, and obesity: four fields connected by the small peptide hormone hepcidin. Adv. Nutr., 4, 602–617.

42. Demircioğlu, F., Görünmez, G., Dağıstan, E., Göksügür, S.B., Bekdaş, M., Tosun, M., *et al*. . (2014). Serum hepcidin levels and iron metabolism in obese children with and without fatty liver: case–control study. Eur. J. Pediatr., 173(7), 947-951.

43. Lu, S., Bennett, R.G., Kharbanda, K.K. and Harrison-Findik, D.D. (2016). Lack of hepcidin expression attenuates steatosis and causes fibrosis in the liver. World J. Hepatol., 8(4), 211-225.

44. Vuppalanchi, R., Troutt, J.S., Konrad, R.J., Ghabril, M., Saxena, R., Bell, L.N., *et al* (2013). Serum hepcidin levels are associated with obesity but not liver disease. Obesity (Silver Spring), 22(3), 836-41.

45. Valenti, L., Fracanzani, A.L., Bugianesi, E., Dongiovanni, P., Galmozzi, E., Vanni, E., *et al*. . (2010) HFE genotype, parenchymal iron accumulation, and liver fibrosis in patients with nonalcoholic fatty liver disease. Gastroenterology, 138, 905–912.

46. Pietrangelo, A. (2011) Hepcidin in human iron disorders: therapeutic implications. J. Hepatol., 54, 173–181.

47. Ganz, T. (2005). Hepcidin-a regulator of intestinal iron absorption and iron recycling by macrophages. Best Pract. Research Clin. Haematol., 18, 171-182.

48. Cohen, L.A., Gutierrez, L., Weiss, A., Leichtmann-Bardoogo, Y., Zhang, D.L., Crooks, D.R., *et al*. (2010). Serum ferritin is derived primarily from macrophages through a nonclassical secretory pathway. Blood, 116, 1574-1584.

49. Chen, Z., Tian, R., She, Z., Cai, J. and Li, H. (2020). Role of oxidative stress in the pathogenesis of nonalcoholic fatty liver Disease. Free Radic. Biol. Med., 152,116–141.

50. Babitt, J.L. and Lin, H.Y. (2011). The molecular pathogenesis of hereditary hemochromatosis. Semin. Liver Dis., 31, 280-292.

51. Ryan, J.D., Armitage, A.E., Cobbold, J.F., Banerjee, R., Borsani, O., Dongiovanni, P., *et al*. (2017). Hepatic iron is the major determinant of serum ferritin in NAFLD patients. Liver Int., 38, 164–173.

52. Fernandez-Real, J.M. and Manco, M. (2014). Effects of iron overload on chronic metabolic diseases. Lancet. Diab. Endocrinol. , 2, 513-526.

53. Bugianesi, E., Manzini, P., D'Antico, S., Vanni, E., Longo, F., Leone, N., *et al*. (2004). Relative contribution of iron burden, HFE mutations, and insulin resistance to fibrosis in nonalcoholic fatty liver. Hepatology, 39, 179–187.

54. Kim, C.W., Chang, Y., Sung, E., Shin, H. and Ryu, S. (2012) .Serum ferritin levels predict incident non-alcoholic fatty liver disease in healthy Korean men. Metabolism, 61, 1182-1188.

55. Ganz, T. (2013). Systemic iron homeostasis. Physiol Rev., 93, 1721–1741.

56. Cordeiro, A., Pereira, S., Saboya, C.J. and Ramalho, A. (2017). Relationship between Nonalcoholic Fatty Liver Disease and Vitamin D Nutritional Status in Extreme Obesity. Can. J. Gastroenterol. Hepatol., p9456897.

57. Bingul , I., Aydin , F., Kucukgergin , C., Dogan- Ekici , I., Semra Dogru –Abbasoglu, S., etal. (2021). The effect of 1,25-dihydroxyvitamin D3 on liver damage, oxidative stress, and advanced glycation end products in experimental nonalcoholic- and alcoholic- fatty liver disease.

58. Turk J Med Sci., 51 (3), 1500–1511.Wamberg, L., Kampmann, U., Stødkilde-Jørgensen, H., Rejnmark, L., Pedersen, S.B. and Richelsen, B. (2013). Effects of vitamin D supplementation on body fat accumulation, inflammation, and metabolic risk factors in obese adults with low vitamin D levels—Results from a randomized trial. Eur. J. Intern. Med., 24, 644–649

59. Mason, C., Xiao, L., Imayama, I., Duggan, C., Wang, C.Y., Korde, L. *et al* (2014). Vitamin D3 supplementation during weight loss: A double-blind randomized controlled trial123. Am. J. Clin. Nutr., 99, 1015–1025.

60. Sharifi, N., Amani, R., Hajiani, E. and Cheraghian, B. (2014). Does vitamin D improve liver enzymes, oxidative stress, and inflammatory biomarkers in adults with nonalcoholic fatty liver disease? A randomized clinical trial. Endocrine, 47(1), 70-80.

61. Tabrizi, R., Moosazadeh, M., Lankarani, K.B., Akbarim, M., Heydari, S.T., Kolahdooz, F., *et al*. (2017).The effects of vitamin D supplementation on metabolic profiles and liver function in patients with non-alcoholic fatty liver disease: A systematic review and meta-analysis of randomized controlled trials. Diabetes Metab. Syndr., 11, 975–982.

62. Yin, K. and Agrawal, D.K. (2014). Vitamin D and inflammatory diseases. J. Inflamm. Res., 7, 69–87.

63. Zughaier, S.M., Jessica, A., Alvarez, J.A., Sloan, J.H., Konrad, R.J. and Tangpricha, V. (2014). The role of vitamin D in regulating the iron-hepcidin- axis in monocytes. J. Clin. Transl. Endocrinol., 1, 19-25.

64. Bikle, D. (2014). Nonclassic Actions of Vitamin D. J. Clin. Endocrinol. Metab., 94(1), 26–34.

65. Baeke, F., Gysemans, C., Korf, H. and Mathieu, C. (2010). Vitamin D insufficiency: implications for the immune system. Pediatr. Nephrol., 25,1597–1606.

66. Nemeth, E. (2010). Targeting the hepcidin–axis in the diagnosis and treatment of anemias. Adv. Hematol., (2), 750643.

67. Atkinson, M.A., Juraschek, S.P., Bertenthal. M.S., Detrick, B., Susan, L., Furth, S.L., *et al*. (2016). Pilot study of the effect of cholecalciferol supplementation on hepcidin in children with chronic kidney disease: Results of the D-fense Trial. Pediatr. Nephrol., 32(5), 859-868.

68. Smith, E.M., Alvarez, J.A., Kearns, M.D., Hao, L., Sloan, J,H., Konrad, R.J., (2017). High-dose vitamin D3 reduces circulating hepcidin concentrations: A pilot, randomized, double-blind, placebo-controlled trial in healthy adults. Clin. Nutr., 36(4), 980-985.

69. Hamden, K., Carreau, S., Jamoussi, K., Miladi, S., Lajmi, S., Aloulou, D., Ayadi, F. and Elfeki, A. (2009). 1Alpha, 25 dihydroxyvitamin D3: therapeutic and preventive effects against oxidative stress, hepatic, pancreatic and renal injury in alloxan-induced diabetes in rats. J. Nutr. Sci. Vitaminol. (Tokyo), 55, 215-222.

70. Bhat, M. and Ismail, A. (2015). Vitamin D treatment protects against and reverses oxidative stress induced muscle proteolysis. J. Steroid Biochem. Mol. Biol., 152, 171-179.

71. Dunn, L.L., Suryo Rahmanto, Y. and Richardson, D.R. (2007). Iron uptake and metabolism in the new millennium. Trends. Cell Biol., 17, 93–100.

72. Braithwaite, V.S., Sarah, R., Crozierm S, R, D'Angelo, S., Ann Prentice, A., Cooper, C., *et al*. MAVIDOS, Trial Group. (2019). The Effect of Vitamin D Supplementation on Hepcidin, Iron Status, and Inflammation in Pregnant Women in the United Kingdom. Nutrients, 11(190),2-11.

الملخص العربى

فيتامين د٣ يحمى ضد مرض الكبد الدهنى الغير كحولى فى ذكور الجرذان البيضاء البالغة بتعديل ترسيب الحديد الكبدى

مروة عبد العزيز حبيب[١]، ماهر نجيب ابراهيم[١]، عبير البيومي خليفه[١]، داليا عاطف حامد[١]، ايمان رمضان عبد الفتاح[٢]، اميرة ابراهيم السمح[٢]

[١]قسم الفسيولوجي، [٢]قسم التشريح وعلم الاجنة، كلية الطب البشرى، جامعة الزقازيق، مصر

الخلفية: تساهم زياده ترسيب الحديد في الكبد في التسبب في تطور مرض الكبد الدهني الغيركحولي. تعتبر ماده الهيبسيدين منظم للحديد حيث يقلل من تدفقه من خلايا الكبد. يعتبر فيتامين د٣ منظم فعال للهيبسيدين وقد ارتبط نقصه بزياده شده حدوث مرض الكبد الدهني الغير كحولي.

الهدف من العمل: تهدف هذه الدراسه إلى تقييم تأثير فيتامين د٣ على ترسب الحديد في الكبد ومستويات الهيبسيدين المنتشرة في نموذج مرض الكبد الدهني الغير كحولي المستحث في ذكور الجرذان البيضاء البالغة.

المواد والطريقة: تم تقسيم ٦٤ من ذكور الجرذان البالغة إلى مجموعات تغذية كل منها ٤ و ١٢ أسبوعًا. تم تقسيم كلاهما إلى٤ مجموعات ١-نظام غذائي عادي ،٢- نظام غذائي عادي مع اعطاءحقن فيتامين د٣مرتين اسبوعيا بنسبه ٥ ميكروغرام / كجم من وزن الجسم) ،٣- نظام غذائي عالي الدهون، و٤- نظام غذائي عالي الدهون مع اعطاءحقن فيتامين د٣مرتين اسبوعيابنسبه ٥ ميكروغرام / كجم من وزن الجسم. تم قياس مؤشر كتلة الجسم ومحيط البطن ومستويات الدهون ومستويات نسبه انزيمات الكبد والهيبسيدين و انترلوكين٦ ونسبه الحديد في الكبد والفيريتين وأنواع الأكسجين التفاعلية في جميع المجموعات. تم فحص أنسجة الكبد باستخدام صبغه الهيماتوكسيلين والأيوزين والأزرق البروسي وصبغه ماسون ثلاثي الألوان.

النتائج: تسبب فيتامين د٣ في انخفاض ذو دلالة احصائية في مؤشر كتلة الجسم ، ومقاييس محيط البطن ، ومقاييس الدهون (باستثناء HDL الذي تم زيادته) ، وإنزيمات الكبد ، والهيبسيدين ، و انترلوكين٦ ، والفيريتين ، والحديد الكبدي ، و وأنواع الأكسجين التفاعلية ، بينما أدى إلى زيادة ذو دلالة احصائية في مستويات الحديد في الدم في ٤ و ١٢ اسبوع في مجموعتي التغذيه عاليه الدهون. بالإضافة إلى ذلك ، اظهرت النتائج الهستولوجية مقدرة فيتامين د٣ على الغاء التغيرات المرضية المرتبطة بمجموعه ذات التغذيه عاليه الدهون في مجموعة لمدة ٤ أسابيع وخفف بشكل ملحوظ التغييرات في مجموعة ١٢ أسبوعًا ، وقلل بشكل ذو دلالة احصائية فى ترسيب الحديد الكبدي والتليف الكبدي في هذه المجموعات مقارنة بمجموعات التغذيه عاليه الدهون التي لم تاخد فيتامين د٣ المتطابقة مع الوقت.

الخلاصة: يحمي فيتامين د٣من مرض الكبد الدهني الغير كحولي الناجم عن التغذيه عاليه الدهون في ذكورالجرذان البيضاء البالغة عن طريق قمع مستوى الهيبسيدين والتخفيض اللاحق في ترسب الحديد الكبدي الذي يقلل من الإصابة والتليف الكبدي الناتج عن الإجهاد التأكسدي.