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Histologic, Metabolic, and Inflammatory Changes in the Liver of High-fat Diet-induced Obese Rats before and after Vitamin D Administration

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ABSTRACT

The current study aimed to investigate the effects of vitamin D administration on the markers of inflammation and metabolic damages in the liver of high-fat diet-induced obese rats.

Forty male Wistar rats were divided into two groups of control receiving a normal diet (ND) and intervention receiving a high-fat diet (HFD). After 16 weeks, each group was divided into two groups including ND, ND + vitamin D, HFD, and HFD + vitamin D. Vitamin D was administered by oral gavage for five weeks at the dose of 500 IU/kg. Hepatic MCP-1, TGF-β, and NF-ϰB levels, serum liver enzymes, and serum lipids, and histological and structural changes in the liver were determined.

Vitamin D administration significantly reduced the monocyte chemoattractant protein (MCP)-1 concentrations in the HFD + vitamin D group compared with the HFD group and reduced liver Transforming growth factor beta (TGF-β) levels in both vitamin D-treated groups ($p<0.05$). Moreover, a significant reduction in the serum levels of aspartate amino transferase (AST) and alanine amino transferase (ALT) in vitamin D treated groups was identified ($p<0.05$). A significant improvement in lipids and a pronounced improvement in the markers of liver histology damage including fat accumulation, aggregation of inflammatory cells, pre-apoptotic changes, hepatic sinusoidal dilatation, and necrotic pyknosis in the Kupffer cells were also identified.

Our results demonstrated that vitamin D has potential effects in ameliorating the inflammatory, metabolic, and histologic changes in the liver of these animals.

Keywords: Inflammation; Liver histology; Obesity; Vitamin D

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INTRODUCTION

Obesity, a main health concern of the 21st century, is characterized by excessive fat accumulation in the

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adipose and non-adipose tissues. It is associated with a cluster of chronic metabolic disorders.[1] Obesity is also considered a chronic low-grade inflammatory disorder because of the increased production of pro-inflammatory molecules including interleukins, and tumor necrosis factor (TNF)-α by adipose tissue.[2] However, recent studies have reported that numerous other organs including hepatocytes also are able to produce these cytokines related to medical comorbidities of obesity. These inflammatory cytokines include TNF-α, Interleukin 1 beta (IL-1β), Interleukin 6 (IL-6), Transforming growth factor beta 1 (TGF-β), monocyte chemoattractant protein (MCP), proteins of nuclear factor (NF)-κB pathway, and numerous cytokines involved in liver damage and that of other organs.[3]

The liver is a target organ for most of these inflammatory cytokines. From the pathological point of view, several key inflammatory mediators play an important role in the pathogenesis of obesity-induced liver damage, and blockage of their expression is targeted in the therapeutic approaches of these abnormalities. Among them, TGF-β as the potent activator of fibroblasts is well known to induce myofibroblastic activation, collagen deposition and wound contraction. TGF-β is a key mediator of fibrosis in the liver tissue and has been shown to contribute to pro-fibrotic remodeling in the hepatic tissue, inducing hepatic failure. TGF-β is a central molecule in numerous liver abnormalities. It leads to trans-differentiation of hepatic stellate cells into myofibroblasts by its fibrogenic action and is also an important negative regulator of proliferation and an inducer of apoptosis.[4] Silencing TGF-β gene expression is mainly targeted as a novel anti-fibrotic therapeutic in the liver failure. Moreover, TGF-β and MCP-1 act in a close relationship with each other in the pathogenesis of hepatic failure. MCP-1 is a potent inducer of TGF-β production by injured hepatocytes and also enhances the fibrogenic potential of mature macrophages by inducing TGF-β and stimulating collagen synthesis; while TGF-β exerts potent pro-fibrotic actions by inducing myofibroblast activation and stimulating the synthesis of various extracellular matrix proteins.[5] MCP-1 plays a key role in the pathophysiological processes of liver injury and hepatic failure. It is involved in the attraction of mononuclear cells in the liver fibrosis process and its strong secretion contributes to the leukocytes infiltration into the hepatic stellate cells. Anti- MCP-1 gene therapy has been proposed as a therapeutic approach against hepatic fibrosis.[6]

Nuclear factor- kB (NF-kB) is a transcriptional regulator of genes involved in immunity and inflammatory response. Recent attention has been focused on the pathophysiological role of NF-κB in the liver abnormalities.[7] NF-κB has been proposed as a key regulator of inflammation and cell death, in the development of hepatocellular injury, fibrosis, and hepatocellular death;[8] however, it acts as a two-edged sword and its inhibition may not only exert beneficial effects but also negatively impact hepatocyte viability, especially when NF-κB inhibition is pronounced. Therefore, it is important to find an appropriate therapeutic approach or a drug that either exerts only a moderate effect on NF-κB activity or can be specifically delivered into non-parenchymal cells. It will be essential to avoid the increase in liver injury associated with the complete NF-κB blockade in the hepatocytes.[9]

Therefore, developing therapeutic strategies and targeting these inflammatory molecules would reduce the obesity-associated hepatic abnormalities. Vitamin D, as an endogenous hormone, has been classically well-known for its role in human bone homeostasis; however, recent studies have identified a much broader spectrum of its activity.[10] Although the role of vitamin D in liver disease is not well known, several studies have demonstrated the anti-inflammatory and anti-fibrotic roles of this vitamin and its potential benefits in the natural history of chronic liver diseases, such as chronic hepatitis C and non-alcoholic fatty liver disease (NAFLD).[11] One study addressed the association of vitamin D deficiency with the degree of liver function, fibrosis, and infectious complications in the liver and suggested its use as a prognostic index and a diagnostic tool in liver abnormalities.[12] A population-based study on 2,649 individuals carried out by Skaaby et al showed that low serum vitamin D was associated with a higher risk of both fatal and non-fatal liver diseases with a hazard ratio of 0.88 (95% confidence interval, 0.79-0.99).[13] In another cross-sectional study involving 6,055 health check-up subjects, low serum vitamin D status was associated with higher severity of NAFLD.[14] However, considering our review of the literature, evaluating the therapeutic role of vitamin D in the health of liver abnormalities is a neglected factor. In the current study, we aimed to evaluate the potential role of

vitamin D administration in the histologic, metabolic, and inflammatory changes in the liver of high-fat diet-induced obese rats.

MATERIALS AND METHODS

The design of the current study has been explained in our previous reports.[15-16] The protocol has been approved by the ethics committee of Tabriz University of Medical Sciences (N: TBZMED.REC.1396.58443). Therefore, the baseline characteristics of animals and study procedures are reported here briefly.

Animals, Diets, and Experimental Procedures

Forty male Wistar rats (weighed 200-220 grams) were purchased from the Animal Care and Resource Center, Pasteur Institute (Karaj, Iran). After a week of acclimatization and feeding a standard laboratory chow diet, rats were randomly assigned into two groups (n=20, each group): either a normal diet (ND) or high-fat diet (HFD). ND included 10% fat, 30% protein, and 60% carbohydrate, and HFD included 59% fat, 11% protein, and 30% carbohydrate.[17] After four months, the groups were randomly assigned into two subgroups including ND, ND+vitamin D, HFD, and HFD+vitamin D, supplemented with vitamin D or Migliol (Sigma Aldrich, USA) 500 IU/kg/d administered by oral gavage alongside their prior diets for five weeks. Moreover, body weight was weekly measured by a scale (PAND Industries, px3000, 5 kg±1g), while food intake was monitored three times a week. Seven days after vitamin D supplementation, rats' daily food intake was recorded in a metabolic cage until sacrifice. Briefly, five rats from each group were housed per cage and the amount of remaining food from the past 24 h was weighed every day.

Preparation of Blood and Liver Samples

After overnight fasting, the rats were anesthetized with Ketamin (6.6mg/kg) and Xylazine (0.3mg/kg) intraperitoneally. Blood samples were obtained by cardiac puncture and centrifuged at 10000 g at 4°C for 20 min; sera were separated and stored in an ultra-low temp freezer (Jal Tajhiz Production, Iran) at -80°C until assay. Finally, following the decapitation of all rats, their livers were removed and the hemisphere was collected and immediately stored at -80°C until further use.

Liver Histological Assessments

The livers were fixed in 10% formalin and then embedded in paraffin. Liver samples were sectioned serially with 50 μm intervals and 5 μm thicknesses. Sections of liver were stained with hematoxylin and eosin (H & E) and studied with a light microscope. The 50 μm interval was chosen based on the size of the liver lobules. Pathological features in this study have categorized to mild, moderate and severe stage according to previous studies based on scoring.[18,19]

ELISA

Before and after vitamin D supplementation, serum measurement was made to determine initial and terminal vitamin D levels by individual enzyme-linked immunosorbent assay kit (ELISA) (Eastbiopharm, Zhejiang, China), according to the manufacturer's instructions. Liver tissues were homogenized in phosphate buffered saline (PBS) and centrifuged at 10000 g at 4°C for 20 min, and clear supernatants were collected and the total protein concentration was measured by protein assay kit (Pars Azmun, Tehran, Karaj, Iran). MCP-1, TGF-β, and NF-κB concentrations in the supernatants of the liver tissue were also determined using routine ELISA kits (Hangzhou Eastbiopharm, Zhejiang, China). Sera were also extracted from the blood samples for biochemical assays including alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), γ-glutamyl transferase (GGT), low density lipoprotein cholesterol (LDL-C) total cholesterol (TC), triglyceride (TG), and high density lipoprotein cholesterol (HDL-C). Laboratory assessments were performed by Abbott ALCYON TM 300 autoanalyzer using commercial ELISA kits (Pars-Azmoon, Tehran, Iran).

Statistical Analysis

All statistical analyses were performed using SPSS software, version 16.0. Kolmogorov–Smirnov test was performed for normality of the distributions of variables. Data are expressed as mean±SD. The data were analyzed using one-way analysis of variance (ANOVA) followed by post hoc Tukey test and paired sample t-test for comparisons between multiple groups and two groups, respectively. Repeated measures test was also used wherever needed. $p<0.05$ was considered as statistically significant.

Table 1. One-Way ANOVA results of the effect of vitamin D on biochemical parameters in high-fat diet-induced obesity

Groups	ND	ND +Vitamin D	HFD	HFD + Vitamin D	P†
AST (U/L)	32.00 ±25.52	11.40 ± 6.50	39.60 ± 18.67	25.80±16.93	0.001
ALT (U/L)	82.40±12.75	66.00 ±14.83	74.40 ± 15.45	55.20 ± 19.37	0.049
ALP (U/L)	211.42±56.44	198.14 ± 32.25	192.84± 25.17	191.72 ± 22.24	0.83
GGT (U/L)	22.52± 10.49	17.50 ± 8.16	27.54 ± 5.59	27.52± 3.45	0.14
LDL-C (mg/dl)	27.82 ± 5.88	19.56 ± 5.39	23.96 ± 5.81	19.04 ± 4.63	0.05
HDL-C (mg/dl)	30.08 ±7.54	27.64 ± 7.16	50.56 ± 5.02	70.32 ± 3.28	0.79
TG (mg/dl)	41.60 ± 5.01	40.56 ± 5.02	54.02 ± 10.51	46.14 ±5.32	0.043
TC (mg/dl)	74.02± 3.15	70.32 ± 3.28	73.38 ±7.68	65.00 ± 2.47	0.034

ND, normal diet; HFD, high fat diet; AST, aspartate amino transferase; ALT, alanine amino transfersae; ALP, alkaline phosphatase; GGT, gamma glutamyl transferase; LDL-C, low density lipoprotein cholesterol; HDL-C, high density lipoprotein cholesterol; TG, triglyceride; TC, total cholesterol; Data are expressed as means ± SD. Statistical differences between groups were assessed by one-way ANOVA followed by *Tukey's* test for *Post Hoc* analysis. The †P value indicated intergroup differences. $P < 0.05$ was considered as statistically significant.

RESULTS

Summarized therapeutic actions of vitamin D against high-fat diet-induced changes in the liver and serum lipids are presented in the graphical abstract.

Changes in Serum Vitamin D Concentrations, Food Intake, and Body Weight in Treatment Groups

The results of this section have been reported in our previous publications.[16, 17] Briefly, during vitamin D supplementation, the food intake of the HFD+vitamin D group significantly decreased compared with the ND group (p= 0.008). In the ND+vitamin D group versus the ND group, food intake was not significantly decreased (p=0.54). Accordingly, the baseline body weights were similar among the different groups. However, there was a significant difference in body weights of all treated groups at the end of the study (p=0.001). Vitamin D supplementation reduced weight in the HFD+ vitamin D group (p=0.02), but no significant difference was observed between the ND group and the ND + vitamin D group (p=0.11). As expected, vitamin D administration led to a marked increase in serum vitamin D concentrations in the ND + vitamin D and HFD + vitamin D groups (p=0.001); whereas, serum vitamin D concentrations reduced in the HFD and ND groups.

Vitamin D Administration, MCP-1, TGF-β, and NF-kβ Concentrations in the Liver Tissues of Treatment Groups

The comparison of MCP-1, TGF-β, and NF-kβ concentrations in the liver tissues of rats is presented in Figure 1. MCP-1 concentrations in the HFD group was significantly higher compared with that in the other groups (p= 0.02). In other words, vitamin D administration significantly reduced the MCP-1 concentrations in the HFD + vitamin D group compared with the HFD group. No change was seen in the liver MCP-1 concentrations in the ND + vitamin D group compared with the ND group. Vitamin D administration significantly reduced TGF-β concentrations in the liver tissue of HFD + vitamin D group compared with the HFD group (p= 0.005) and in the ND + vitamin D group compared with the ND group (p= 0.04). No significant difference was identified in the liver NF-kβ concentrations in the treatment groups.

Vitamin D Administration and Serum AST, ALT, ALP, γGT Concentrations and Serum Lipids in Treatment Groups

The comparison of liver enzymes and serum lipids of treatment groups are presented in Table 1. Vitamin D administration significantly reduced AST and ALT concentrations in the treatment groups (p< 0.05). No significant change was observed in the ALP or γ GT concentrations. Serum LDL concentration after vitamin D administration significantly reduced in the ND + vitamin D and HFD + vitamin D groups compared with the ND and HFD groups (p< 0.05). Serum TG and TC levels also reduced in the HFD+ vitamin D group compared with the HFD group (p< 0.05). No significant change was observed in the serum HDL level.

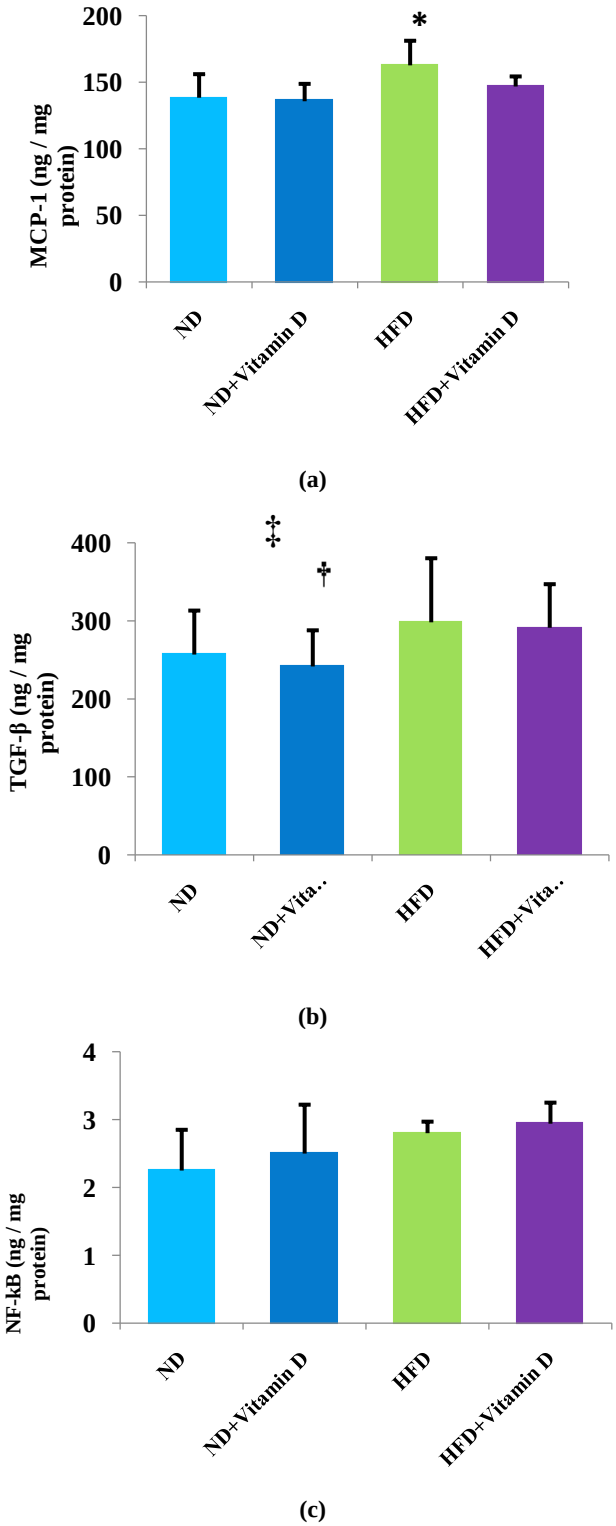

Figure 1. Effects of vitamin D administration on transforming growth factor beta (TGF-β) (A), monocyte chemoattractant protein (MCP)-1 (B) and nuclear factor kappa (NF-K) β (C) concentrations in the liver tissue of rats. The* Significant difference between HFD and other treatment groups (p= 0.02), The † Significant difference between ND and ND + vitamin D group (p= 0.005), The ‡ Significant difference between HFD and HFD + vitamin D group (p= 0.04).

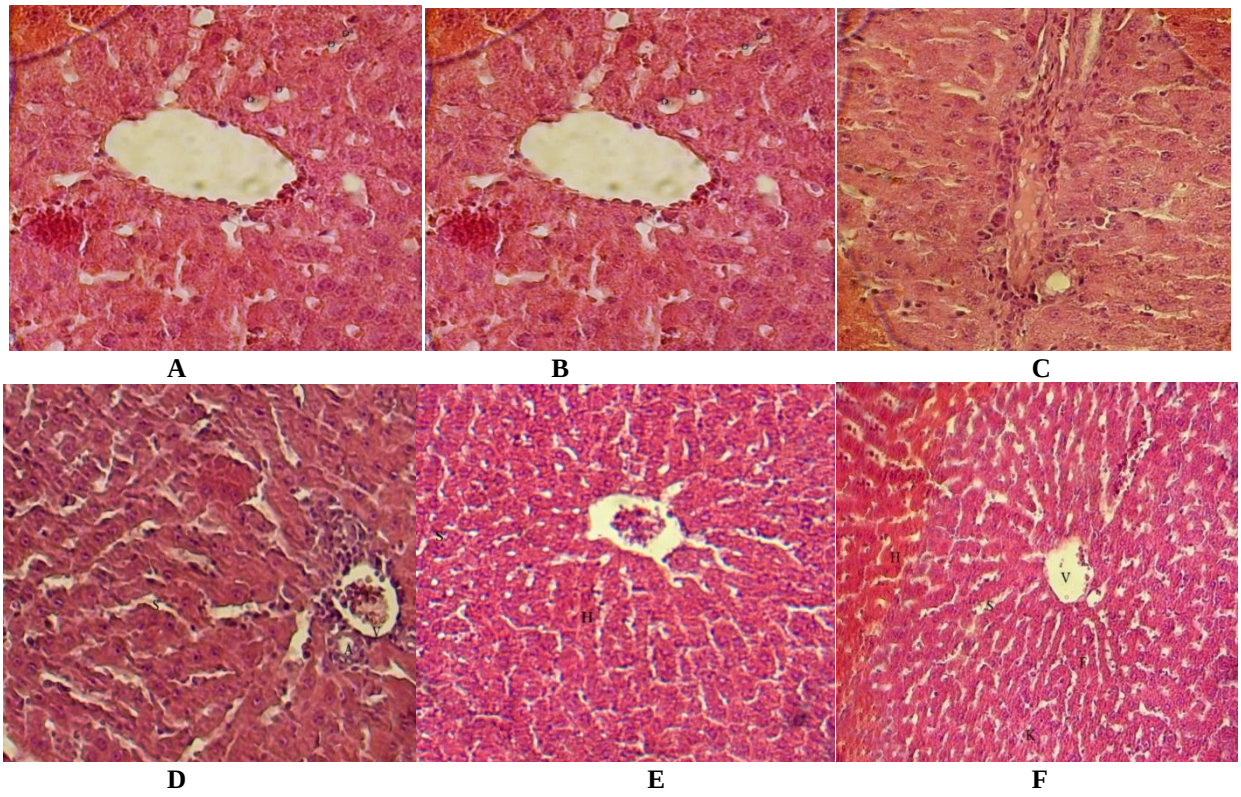

Figure 2. Histopathological evaluation of rat livers in a study of vitamin D effect in obesity. Liver sections were stained with hematoxylin-eosin (H & E). Micrographs are representative pictures with magnification 400 X. A, Liver section of the high fat diet (HFD) group. Fat droplets in the photomicrographs are apparent. Mononuclear inflammatory cells and lymphocytes infiltration. Hepatocyte necrosis, nucleus and cytoplasm damage in the sinusoid covering cells. Hepatocytes cytoplasm are filled with small vacuoles which were uniform in size and smaller than the centrally located nucleus as a sign of pre-apoptosis. Vascular dilation (D) of sinusoids and fat accumulation is present. The necrotic pyknosis in the nucleus is a sign of liver fibrosis. B, Liver section of HFD group, in this photomicrograph, the interstitial fibrosis in the hepatocytes and sinusoids, inflammatory cells accumulation (L) in the connective tissue of hepatic lobules, dilation (D) of hepatic sinusoids and pyknosis of the Kupffer cells' nucleus are shown. C. Liver section of normal diet (ND) + vitamin D group, in this photomicrograph, the central lobular vein (V) alongside with the hepatic sinusoids (S) is presented. Normal hepatocytes (H) with euchromatin nucleus and healthy appearance of Kupffer (K) cells are also shown. D. Liver section of HFD + vitamin D group. Portal vein (V), hepatic artery (A) and sinusoids (S). In this photomicrograph, fat droplets and fat infiltration in the cytoplasm of hepatocytes are significantly lower compared with the HFD group. Reduced sinusoidal damage and necrosis due to high-fat diet have been shown compared with the HFD group. More of the hepatocytes have a normal nucleus and nucleolus. However, there is a mild accumulation of inflammatory cells in the liver. E. Liver section of HFD + vitamin D group. In this micrograph, normal hepatocytes (H) with a normal nucleus and nucleolus are shown. Normal appearance of Kupffer cells, reduced fat accumulation in the hepatocytes, reduced inflammation and reduced dilation of sinusoids are other features of the liver tissue of this group. F. Liver section of ND group central vein (C), hepatocytes (H), fat droplets (F), Kupffer cells (K), sinusoids (S). In this photomicrograph, normal hepatocytes with the euchromatin nucleus and nucleolus are apparent. Central lobular vein alongside liver sinusoids with normal appearance is also presented.

Table 2. Structural changes in the livers of high fat diet induced obese rats after vitamin D administration

Group	ND	ND +vitamin D	HFD	HFD + vitamin D
Fibrosis				
None	1	4	1	4
Mild	1	1	1	1
Moderate	0	0	2	1
Severe	4	0	1	0
Bile ducts hyperplasia				
None	2	5	1	5
Mild	0	0	1	1
Moderate	0	0	2	0
Severe	4	0	1	0
Hyperemia				
None	2	5	0	5
Mild	0	0	2	1
Moderate	0	0	2	0
Severe	4	0	1	0
Hemorrhage				
None	0	5	0	5
Mild	0	0	2	1
Moderate	0	0	2	0
Severe	6	0	1	0
Fatty infiltration				
None	2	4	0	4
Mild	1	1	1	1
Moderate	0	0	2	1
Severe	3	0	2	0
Hepatitis				
None	1	5	0	5
Mild	0	0	1	1
Moderate	0	0	3	0
Severe	5	0	1	0
Necrosis				
None	1	4	1	5
Mild	1	1	1	1
Moderate	0	0	2	0
Severe	4	0	1	0

HFD, high fat diet; ND, normal diet; A minimum of 10 fields for each liver slide was examined and assigned for the severity of changes (n = 6 for ND, HFD and HFD + vitamin D and n = 5 for ND + vitamin D).

Vitamin D Administration, Structural and Histological Changes in the Livers of Treatment Groups

Histological examination of the rat livers is shown in Table 2 and Figure 2. The normal structure of liver tissue in the ND and ND + vitamin D groups are shown in Figure 1 C, F. As shown in this figure, the liver tissue including normal hepatocytes with the normal euchromatin nucleus and nucleolus and central lobular vein alongside liver sinusoids are in their normal appearance. Whereas, in the HFD group (Figure 1, A and B), the liver injury and disrupted healthy appearance of liver is apparent; these hepatic injuries included fat accumulation, mononuclear inflammatory

cells, lymphocytes infiltration, hepatocyte necrosis, nucleus and cytoplasm damage in the sinusoidal cells, pre-apoptotic changes, hepatic sinusoidal dilatation, and necrotic pyknosis in the Kupffer cells. Vitamin D administration in the HFD + vitamin D group (Figure 1, D and E) led to reduced lipid accumulation, reduced sinusoidal damage and necrosis, and mild accumulation of inflammatory cells.

DISCUSSION

In the current study, significant beneficial effects of vitamin D in reducing the levels of the inflammatory parameters including MCP-1, TGF-β concentrations, reducing liver enzymes and serum lipids, and improving liver histological features were demonstrated. In a previous study by Abdelkader et al[20] higher MCP-1 concentrations were introduced as an early predictor of spontaneous bacterial peritonitis, and vitamin D deficiency was considered as a trigger of a liver abnormality. Another study revealed that vitamin D down-regulated lipopolysaccharide-induced MCP-1 expression; the suppressive effect of vitamin D on MCP-1 was mediated via the vitamin D receptor.[21] In agreement with our results, in a study by Ning et al[22] the administration of vitamin D to diabetic rats caused significant lower expression of hepatic MCP-1. The authors concluded that the effectiveness of 1, 25 (OH) 2D3 in attenuating the increase of NF-κ B, MCP-1, ICAM-1, and TGF-β1 in the liver of diabetic rats suggests that 1, 25 (OH) 2 D3 might serve as an anti-inflammatory agent for diabetes mellitus-induced liver disease. In the current study, vitamin D had no significant effect on the liver NF-κB concentrations in none of the vitamin D-treated groups. Although NF-κB has been well- known for its inflammatory potential, and several studies have demonstrated the role of cross-talk between NF-κB and STAT 3 pathways in the pathogenesis of hepatocellular carcinoma and liver abnormalities,[23] there are serious concerns about this issue. It has been previously suggested by Luedde T et al that NF-κB acts as a two-edged sword and inhibition of NF-κB may not only exert beneficial effects but also negatively impact hepatocyte viability, especially when NF-κB inhibition is pronounced. They have also suggested that finding appropriate targets or identifying drugs that either exerts only a moderate effect on NF-κB activity or can be specifically delivered to non-parenchymal cells is essential to avoid the increase in liver injury associated with complete NF-κB blockade in the hepatocytes.[9] Considering the hepatoprotective role of NF-κB against TNF-α-induced apoptosis in the liver and its action in wound healing as previously established,[24] the beneficial effects of vitamin D in the current study, indicated by reduced MCP-1 and TGF-β concentrations whilst no change in NF-κB concentrations, could be a valuable finding, suggesting it as a good therapeutic vehicle.

Vitamin D also reduced the hepatic TGF-β concentrations in the HFD + vitamin D and ND + vitamin D groups. Similar findings were also observed in the previous reports. It has been shown that vitamin D counteracts fibrogenic TGF-β signaling in the human hepatic stellate cells both receptor-dependently and –independently.[25] Another study revealed that vitamin D deficiency promoted liver tumor growth in transforming growth factor-β/Smad3-deficient mice through Wnt and Toll-like receptor 7 pathway modulation.[26] Similarly, the marked improvement in the liver histologic features in the current study was in agreement with that in previous studies; low serum vitamin D concentrations was correlated with liver fibrosis and steatosis.[27] Yet, another study by Lucaci et al found similar results, showing the association of vitamin D deficiency with a high activity grade and stage of liver fibrosis in the patients with acute viral hepatitis of liver fibrosis.[28] In the current study, it was also demonstrated that vitamin D modified the altered lipid abnormalities and reduced serum LDL, TG, and TC concentrations in the high-fat diet-induced obese rats. In fact, dyslipidemia is a very common feature of obesity and a high-fat diet is a useful method to induce a model of dyslipidemia in animals or human.[29,30] Therapeutic roles of vitamin D in the modification of lipid abnormalities have been previously confirmed in several studies.[31,32] Further studies would clarify the strong beneficial role of vitamin D, this steroid hormone, in the treatment of numerous obesity-related disorders. The current study has several limitations; the expression pattern of inflammatory cytokines of the liver was better to be measured. Moreover, the initial measurements of inflammatory cytokines were not possible because of the possibility of animals' drop-out. However IHC staining is very important in histological evaluation, lack of IHC staining also was another limitation of this study.

The results of this study demonstrated potential beneficial effects of vitamin D in improving the liver biochemical, inflammatory, and histological markers in

the high-fat diet-induced obese rats. Therefore, confirming the current findings with further animal and human studies would guarantee the usefulness of this vitamin as a therapeutic approach in obesity-induced liver abnormalities.

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