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Irisin contributes to the hepatoprotective effect of vitamin D in a choline deficient diet induced nonalcoholic fatty liver disease rat model

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- **Irisin**
- **vitamin D**
- **fatty liver**

Abstract

Non-alcoholic fatty liver disease (NAFLD) represents a growing cause of liver morbidity and mortality globally with no proven effective therapy yet. The aim of this research is to investigate the effect of vitamin D treatment on choline deficient (CD) diet-induced nonalcoholic fatty liver disease (NAFLD) like lesions in rats, with the possible involvement of irisin in this effect. Thirty-two rats were divided into 4 equal groups; control group, vitamin D group, CD diet group, CD diet+ vitamin D group. It was found that CD diet led to significant elevations in liver weight and index, serum liver enzymes, hepatic triglycerides (TG), malondialdehyde (MDA), inflammatory markers with significant decreases in serum 25-(OH) D3, irisin, hepatic antioxidants and peroxisome proliferator–activated receptor alpha (PPAR–α). Administration of vitamin D caused a significant improvement in all previous parameters, these results were supported by histopathological examination. It seems that vitamin D supplementation significantly reduced NAFLD induced by CD diet even in part due to irisin.

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is a worldwide disease which is now accepted as the most common cause of chronically elevated liver enzymes in both developing and developed countries owing to increasing rates of obesity and diabetes mellitus **[1, 2, 3].** It is expected to become a main indication for liver transplantation, superseding hepatitis C **[4].**

NAFLD is a term includes a wide range disease spectrum, in which triglyceride (TG) accumulation in the hepatocytes is the principal pathologic feature. This spectrum ranges from simple steatosis to more severe and progressive nonalcoholic steatohepatitis (NASH) with the former characterized by cytoplasmic TG droplets in more than 5% of hepatocytes and the later have additional hepatocellular injury, inflammatory cellular infiltration and fibrosis **[5]**.

Different theories have been postulated to explain the pathogenesis of NAFLD of these theories, two hit theory becomes the most accepted one. The first hit is represented by increased hepatocytes TG accumulation which increases the liability of the liver to second hit. This hit includes large amount of the inflammatory cytokines, oxidative stress and mitochondrial dysfunction where the liver would be more susceptible to develop necroinflammation and fibrosis leading ultimately to hepatic cirrhosis **[6]**. Fibrosis and oxidative stress were found to be the main processes in the progression of NAFLD from the start to the end **[7]**

Vitamin D plays a crucial part in human homeostasis beyond its classical hormonal role in calcium and phosphorus homeostasis which promotes bone mineralization **[8]**. Binding of vitamin D to vitamin D receptors (VDR) influences numerous signaling pathways other than its classical function including, for example, inflammation, apoptosis and oxidative stress. These findings draw attention to investigate the pleiotropic effects of vitamin D as emerging prophylactic and therapeutic strategies for diseases beyond bone metabolism **[9].**

Studies on rat, mouse and human liver specimens revealed abundant expression of VDR in Kupffer cells (KCs), sinusoidal endothelial cells, hepatic stellate cells (HSCs) and hepatocytes suggesting that the liver could be vitamin D responsive **[10].**

Irisin, a novel adipomyokine, is a cleaved product of type III fibronectin domain 5 protein (FNDC5) which is produced mainly by skeletal muscles as well as visceral and subctaneous adipose tissue **and** smaller amounts also detected in different body tissues such as liver, pancreas, brain, testes and heart **[11]**. Irisin was found to enhance browning of white adipocytes, increase body energy expenditure, improve insulin resistance, attenuate inflammation, exert antioxidant effect and stimulate endothelial cell proliferation **[12].** In addition to these metabolic effects, it was found that there is an inverse relationship between liver TG content and serum irisin levels in NAFLD **[13].**

Accumulating data suggesting that Vitamin D could be of a potential protective value for NAFLD, however its underlying mechanisms are still under research. Fortunately, vitamin D appears to share some beneficial effects with irisin concerning decrease intrahepatic lipid accumulation in NASH, in addition to the

antioxidant and anti-inflammatory effects **[14]**. Based on these data, we hypothesized that vitamin D could have a protective effect in a choline deficient diet model of NASH and that irisin might play a crucial role in the beneficial effects of vitamin D on the liver injury, inflammation, and oxidative stress. Therefore, the present study was designed to confirm the aforementioned hypothesis and to elucidate the underlying protective mechanisms in such a rat model.

Material and Methods

Diet and chemicals used:

The standard and choline deficient diet were formulated and prepared as pellets at the faculty of Agriculture, Moshtohor, Egypt. Vitamin D (Devarol-S) was purchased from Memphis, Egypt.

Animals:

This experiment included 32 male Wistar albino rats, 6-8 weeks old and weighing about 230–250 gm. After 10 days of acclimatization in the animal lab at the Medical Research Center of Benha Faculty of Medicine, they were kept in isolated cages, four in each one, under the predominant climatic conditions and controlled room temperature of 25 °C. in a 12h/12h light/dark cycle and had free access to water and food. Our study was executed according to the guidelines of the local research Ethics Committee of Faculty of Medicine, Benha University. After samples collection at the end of the experiment, rats were disposed in the incinerator of Benha University Hospital.

Experimental design:

Rats were divided randomly into equal four groups (n: 8) as follows:

Group I (control group): Given standard diet for 12 weeks.

Group II (Vitamin D group): Given standard diet and were treated with vitamin D, as 5µg/kg, intraperitoneal injection, twice weekly for 12 weeks.

Group III (choline deficient (CD) diet group): Given just CD diet for 12 weeks **[15]**, containing 58.275% corn, 21.9% soya (46%), 15% oil, 1.2% lime stone, 3% sucrose, 0.2% lysine, 0.125% common salts, 0.3% premix choline free. Both standard and CD diet were standardized by National Research Council (NRC) **[16]**

Group IV (CD diet plus Vitamin D group): Given CD diet for 12 weeks and were treated with vitamin D as 5µg/kg, intra-peritoneal injection, twice weekly during this period **[17]**. Vitamin D was dissolved in minimal volume of ethanol and diluted with saline.

At the end of the twelfth week after an overnight fasting, the animals were anesthetized with urethane (1.5 g/kg intraperitoneally) and weighed before being sacrificed. Blood samples were obtained through cardiac puncture, then abdomen was opened immediately, and the liver was removed, and its weight was recorded. Samples of blood were centrifuged at 3000 cycles (rpm) for 15 minutes and the supernatant was kept at (–20ºC) until used in determination of liver functions, vitamin D and irisin levels. The liver was divided into two parts, the first part was stored at (−80°C) for hepatic estimation of TG contents, oxidative stress markers (reduced glutathione (GSH), superoxide dismutase (SOD) and malondialdehyde (MDA)), inflammatory markers (tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6)), peroxisome proliferator-activated receptor alpha (PPAR-α). The other one was kept in formaldehyde to be stained with Hematoxylin and

Eosin (H&E) for histopathological assessment of NAFLD lesion.

Liver index % calculation:

Liver index % was determined as follow: (liver weight (LW)/ body weight (BW) x 100) **[18]**.

Assessment of hepatic function:

Serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), total bilirubin and albumin were estimated according to instructions of the manufacturer utilizing commercial kits obtained from the Egyptian organization of biotechnology, Egypt.

Assessment of TG and oxidative stress in hepatic tissue:

Part of the stored liver samples were cut and homogenized utilizing a Mixer Mill MM 400 (Retsch, Germany) for TG and oxidative stress markers (GSH, SOD and MDA**)** assessment according to the manufacturer's protocol using the commercial kits provided by (Jiancheng Bioengineering Institute, Nanjing, China) and (Biodiagnostic, Egypt) respectively.

Assessment of inflammatory markers and PPAR–α in hepatic tissue:

Inflammatory markers (TNF-αand IL-6) and PPAR–α were measured according to manufacturers' instructions using ELISA kits (Abcam, USA) and (Cusabio Biotech Co., China) respectively.

Measurement of serum vitamin D and irisin:

Serum levels of 25-(OH) D_3 was estimated utilizing rat's 25-OH VD ELISA kits (MyBioSource, USA). Serum irisin was measured according to manufacturers' instructions using ELISA kits (My BioSource, USA).

Real-time PCR analyses for mRNA expression of hepatic PPAR–α:

The other part of the stored liver samples was cut and homogenized by Mixer Mill MM400 (Retsch, Germany) to separate mRNA. Total RNA was separated from 25 mg tissue utilizing RNA purification kits of Jena Bioscience Germany. The amount of PPAR–α mRNA was determined with ABI Prism 7900HT quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) (Applied Biosystems, Foster City, CA). The primer sequences used were: PPAR–α 5′-GTCAGCTGCCCTGCTGTCCC -3′(forward); 3′-CGAAAGAAGCCCTTGCAGCC -5′ (reverse) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), endogenous control, 5′-TGTTCCAGTATGACTCTACC -3′(forward); 3′-TCACCCCATTTGATGTTAGC -5′(reverse). Complementary DNA (cDNA) was synthesized from equal amounts of mRNA with reverse transcription using G-storm thermal cycler (England). For real-time PCR, 0.4 μM of every primer and 20 ng cDNA were utilized in a reaction volume of 25 μL containing SYBR Green PCR Master Mix (Applied Biosystems Inc., USA). According to the RQ manager program ABI SDS software (ABI 7900), the data appeared as a sigmoid-shaped amplification plots and arranged in a way that the number of cycles were put against fluorescence when linear scale is used. The Threshold Cycle (CT) was assisted as a calculation tool for starting template amount of each sample. As the samples of both control and treated groups were used as calibrators, the expression levels were set to be one. As the relative quantities of PPAR–α gene is normalized against GAPDH, changes of gene fold expression

were determined using the equation of 2-ΔΔCT **[19]**.

Histopathological examination:

For light microscopy examination, liver specimens were fixed in 10% buffered formalin, embedded in paraffin and then thin sections (4 μm) were cut, using a microtome, from the paraffin blocks for hematoxylin-eosin (HE) staining. Liver specimens were blindly examined using the NASH Clinical Research Histological scoring which includes steatosis, lobular inflammation, and hepatocellular ballooning **[20]**.

Statistical analysis:

The statistical analysis was conducted using the Software, Statistical Package for Social Science, (SPSS Inc. Released 2009- PASW Statistics for Windows Version 21.0. Chicago: SPSS Inc.) The collected data were summarized in terms of mean ± Standard Deviation (SD). One-way Analysis of Variance (ANOVA; F) test was used to compare differences in the mean value between studied groups when appropriate. Post-Hoc multiple comparison tests using the LSD method were used to test differences in pairs. The Mann-Whitney test (z) and the Kruskal Wallis test were used to compare two and more than two non-parametric data. Statistical significance was accepted at P value <0.05.

Results

Changes in liver function, BW, LW, liver index and liver TG in different studied groups (table 1):

In CD diet group, significant increases in serum aspartate transferase (AST), alanine transferase (ALT), alkaline phosphatase (ALP), LW, liver index and liver TG were detected when compared with control and vitamin D groups. As for albumin, total bilirubin levels and BW, they showed no statistically significant differences between all studied groups. Treatment with vitamin D resulted in a significant decrease in serum AST, ALT, ALP, LW, liver index and liver TG in CD diet+ vitamin D group compared with CD diet group.

Changes in liver oxidative status, antioxidative status and inflammatory markers levels in different studied groups (table 2):

Liver homogenates of CD diet group showed significant increases in MDA, TNF-α and IL-6 levels that were accompanied with significant decreases in GSH activity levels and SOD contents when compared with control and vitamin D groups. On the other side, treatment with vitamin D significantly decreased MDA, TNF-α and IL-6 levels while increased GSH activity levels and SOD contents in liver homogenates of CD diet+ vitamin D group when compared with CD diet group.

Changes in serum 25-(OH) D_3, irisin, hepatic PPAR–α levels and PPAR–α gene mRNA expressions (Log 10 relative units) in different experimental groups (table 3):

In CD diet group, significant decreases in serum levels of vitamin D, irisin in addition to hepatic PPAR-α and its gene expression were detected when compared with control and vitamin D groups. Vitamin D treatment induced significant increases in serum levels of vitamin D, irisin in addition to hepatic PPAR-α and its gene expression in CD diet+ vitamin D group when compared with CD diet group.

NAFLD score by H & E of hepatocytes in different experimental groups (Table 4), Fig. 1(A,B,C,D):

CD diet group showed severe macrovesicular steatosis Fig.1(C). The NAFLD score was significantly higher in CD diet group compared with control group Fig. 1(A) and vitamin D group Fig. 1(B). Treatment with vitamin D in CD diet + vitamin D group as in Fig. 1(D) showed mild hydropic degeneration (ballooning) and the NAFLD score was significantly lower than CD diet group.

Table (1): Changes in liver function, BW, LW, liver index % and liver TG in different studied groups:

Variable		**Control group** (n =8)	**Vitamin D group** (n =8)	**CD diet group** (n = 8)	**CD diet + Vit. D group** (n =8)
AST (U/L)	**Mean ±SD**	46.73 ± 6.52	46.84±6.58	95.56±12.67*	56.56±2.18*#
ALT (U/L)	**Mean ±SD**	51.79 ± 6.03	51.43±5.74	81.76±9.32*	60.59±2.36*#
ALP (U/L)	**Mean ±SD**	134.83±0.29	134.89±0.56	156.16±0.45*	136.71±3.56*#
Albumin (g/dl)	**Mean ±SD**	3.83± 0.26	3.92±0.47	4.05 ± 0.32	3.88±0.32
Total Bilirubin (mg/dl)	**Mean ±SD**	0.92 ± 0.15	0.93±0.22	1.02±0.21	1±0.24
BW (g)	**Mean ±SD**	249.48±10.70	246.29±8.57	247.8±5.44	253.42±5.59
LW (g)	**Mean ±SD**	11.99 ± 0.76	12.27±0.89	16.36±1.11*	13.83±0.48*#
Liver index %	**Mean ±SD**	4.83±0.29	5.01±0.42	6.26±0.46*	5.46±0.20*#
Liver TG (mg/g protein)	**Mean ±SD**	6.04±0.61	6.36±0.76	28.93±0.63*	15.61±0.64*#

AST, aspartate transferase; ALT, alanine transferase; ALP, alkaline phosphatase, BW, body weight; LW, liver weight; TG, triglycerides.

* Significant differences compared to control group; # Significant differences compared to CD diet group.

Table (2): Changes in liver oxidative status, antioxidative status and inflammatory markers levels in different studied groups:

Variable		**Control group** (n =8)	**Vitamin D group** (n =8)	**CD diet group** (n = 8)	**CD diet + Vit. D group** (n =8)
MDA (umol/g protein)	**Mean ±SD**	76.73 ± 6.52	76.84±6.58	145.56±12.67*	86.56±2.18*#
GSH (mmol/g protein)	**Mean ±SD**	8.01±0.42	7.83±0.29	4.46±0.20*	7.16±0.45*#
SOD (mmol/g protein)	**Mean ±SD**	10.09±0.44	9.9±0.30	6.53±0.28*	9.24±0.43*#
TNF-α (pg/mg protein)	**Mean ±SD**	42.63±4.69	46.75±5.15	57.38±5.40*	44.38±3.74*#
IL-6 (pg/mg protein)	**Mean ±SD**	65.38±3.99	68.13±13.89	103.75±18.87*	63.38±1.77*#

MDA, malondialdehyde; GSH, reduced glutathione; SOD, superoxide dismutase, TNF-α, tumor necrosis factor alpha; IL-6, interleukin-6.

* Significant differences compared to control group; # Significant differences compared to CD diet group.

Table (3): Changes in serum 25-(OH) vitamin D, irisin, hepatic PPAR–α levels and PPAR–α gene mRNA expressions (Log 10 relative units) in different experimental groups:

Variable		Control group (n =8)	Vitamin D group (n =8)	CD diet group (n = 8)	CD diet + Vit. D group (n =8)
25-(OH) vitamin D (ng/ml)	**Mean ±SD**	64.19 ± 13.10	60±5.58	27.44±1.95*	47.25±5.20*#
Irisin (ng/ml)	**Mean ±SD**	10.13±3.68	9.75±6.18	3.13±2.70*	8.50±2.20*#
PPAR–α (ng/mg protein)	**Mean ±SD**	3.06±0.42	2.63±0.91	0.60±0.58*	2.75±0.53*#
PPAR–α gene expressions	**Mean ±SD**	5.01±0.42	4.83±0.29	1.46±0.20*	4.16±0.45*#

PPAR–α, peroxisome proliferator activated receptor alpha.
* Significant differences compared to control group; # Significant differences compared to CD diet group.

Table (4): NAFLD score by H&E of hepatocytes in different experimental groups:

Variable		Control group (n =8)	Vitamin D group (n =8)	CD diet group (n = 8)	CD diet + Vit. D group (n =8)
Steatosis	**Mean ±SD**	00.00±0.00	00.00±0.00	2.88±0.35*	1.5±0.53*#
Lobular inflammation	**Mean ±SD**	00.00±0.00	00.00±0.00	2.75±0.71*	1.13±0.35*#
Hepatocellular ballooning	**Mean ±SD**	00.00±0.00	00.00±0.00	1.88±0.35*	0.88±0.35*#

* Significant differences compared to control group; # Significant differences compared to CD diet group.

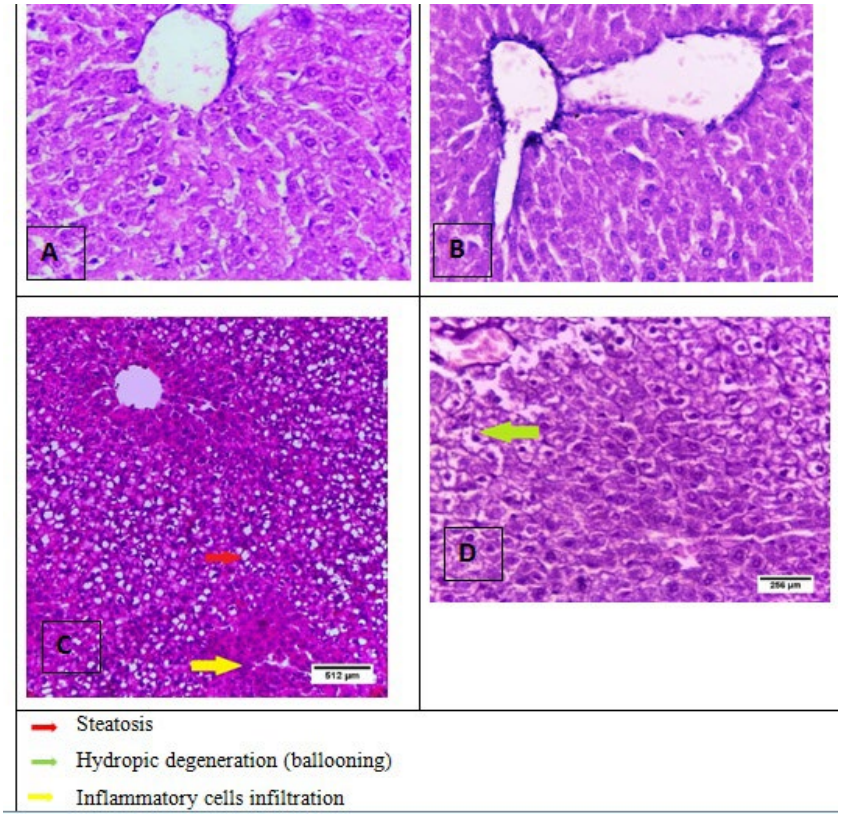

Figure 1 (A, B, C, D): Group I (A) (X 200); group II (B) (X 200) showing preserved hepatic architecture with central vein and radially arranged hepatocytes with patent sinusoids in between; group III (C) (X 100) showing intense hepatocytes macrovesicular steatosis involving > 70% of the hepatic lobule and inflammatory cells infiltrations; group IV (D) (X 200) showing mild hydropic degeneration (ballooning) in the form of mottled cytoplasm, few scattered cells showing cytoplasmic clear vacuoles.

Discussion

Although NAFLD has become an important health problem issue that threatens the world due to its extensive spread and serious sequelae, its etiological components that determine disease sequences still remain not clear **[21]**. Accordingly, effective measures for NAFLD detection and treatment should be developed. CD diet was chosen in the present study as it initiates a rapid onset similar histological picture resembling human fatty liver **[22].**

The current study demonstrated that CD diet administration for 12 weeks caused histopathological NAFLD in the form of steatosis and inflammatory cellular infiltration. This result is in agreement with **Hussien et al. [1]** and **Han et al. [15]** who revealed that CD diet intake in white rats led to typical histopathological NAFLD features. Also, serum liver biomarkers ALT, AST and ALP were higher when compared with control group. Concerning the hypothesis supposed to clarify the mechanism by which CD diet can cause fatty liver, **Santos et al. [23]** and **Van Herck et al. [24]** demonstrated that choline is a basic component for phosphatidylcholine synthesis, which is an important element for very low-density lipoprotein (VLDL) particles synthesis, so CD diet stimulates hepatic TG aggregation as VLDL is accounted for their efflux from the liver.

The present data revealed non-significant increment in the serum albumin and bilirubin levels in CD diet fed rats when compared with control ones. These data are in accordance with previous studies which revealed that bilirubin and albumin levels are mostly not changed in NAFLD and just rise at the late stage of the disease when cirrhosis occurs **[25, 26, 27]**. The results of our investigation additionally revealed that there were significant increments in liver weight, liver index and TG contents with a non-significant difference concerning BW, in CD diet rats when compared with control ones. These data are in agreement with **Hussien et al. [1]** who found that CD diet administration resulted in decreased TG secretion out of the liver, as VLDL, and hence, leading to their aggregation in the liver without affection of the enzymes responsible for de novo lipogenesis. So, there is increment in LW without increment in BW.

The current results revealed significant higher MDA levels (a lipid peroxidation marker) with lower antioxidant enzymes levels (GSH and SOD) in liver homogenates of CD diet group. These went parallel with former studies which declared that fatty liver was associated with a significantly low GSH and SOD hepatic activity as well as high MDA levels **[1, 23, 28, 29]**. Oxidative stress is believed to be a significant contributor to NAFLD pathogenesis and its progression, moreover, there are many evidences of the beneficial outcomes of several antioxidants' classes on amelioration of NASH development **[30]**. In support with these findings, **Santos et al. [23]** demonstrated that reduction of choline, as a component of the mitochondrial membrane, causes a critical change in this organelle that led to changes in mitochondrial electron transport chain (ETC) with excess reactive oxygen species (ROS) production. **Hussien et al. [1]** also reported that activation of Kupffer cells and other inflammatory cells in NASH produces ROS by the activity of nicotinamide adenine dinucleotide phosphate oxidase. These ROS can oxidize polyunsaturated fats found in the membranes of cell and its

organelles producing lipid peroxidation metabolites like MDA **[31]**.

Our present data revealed significant increases in hepatic inflammatory markers (TNFα and IL-6) in CD fed rats when compared with control ones. In agreement with our findings, previous results found significant increases in these inflammatory markers in NAFLD cases indicating their major role in disease pathogenesis and progression **[32, 33]**. **Faheem and El-naga [34]** reported that increased ROS in NAFLD activates nuclear factor kappa B (NF-κB), a transcription factor, which in turn upregulates the expression of many pro-inflammatory cytokines mainly (TNFα, IL-6).

As for serum 25-(OH) D_3, our data demonstrated that there was a significant reduction in serum 25-(OH) D_3 in rats given CD diet when compared with controls. Several researchers found that a lower level of serum 25-(OH) D_3 is related to the presence of NAFLD and its severity **[17, 35]**. In support of this result, **Cordeiro et al. [36]** detected low serum 25-(OH) D_3 in NASH patients diagnosed by the gold standard investigation of NASH, which is liver biopsy. Additionally, **Han et al. [15]** found a strong inverse relationship between NAFLD and 25-(OH) D_3 levels. This could be explained by the role of the liver as a key organ in vitamin D activation by transforming vitamin D_3 into 25-(OH) D_3, so affection of this organ may explain why vitamin D was seen deficient in most liver disorders **[36].** There are also various studies showing that liver tissue of those who had low level of vitamin D had increased levels of hepatic mRNA inflammatory markers such as resistin, IL-4, IL-6 and TNFα **[37]**. These results suggest that vitamin D deficiency may play a role in NASH pathogenesis through stimulation of an inflammatory reaction cascade **[38]**. Interestingly, it seems that vitamin D deficiency should be considered as a consequence of liver disease, and its deficiency causes or worsens it. As several studies demonstrated that a lower level of serum 25-(OH) D_3 is related to the incidence and progression of NASH **[17, 35].**

In the present study, we found a significant decrease in serum irisin level in CD fed rats when compared with controls. This finding was supported by previous studies conducted by **Mo et al. [13]** and **El-Kattawy and Ashour [39]** who revealed that there is inverse relationship between liver TG contents and circulating irisin level. On the other hand, **Hu et al. [12]** reported that there was a temporary mild increment in the serum level of irisin earlier during NAFLD and this could be as a compensatory mechanism, but with the progression of the disease, irisin resistance occurred and it could not be able to increase anymore. Irisin level may be related to the degree of NAFLD severity, it is higher in mild cases while it decreases in moderate and more sever ones. Furthermore, this conflict concerning irisin level in NAFLD might be due to differences in age, sex or even race of studied cases.

Our current study demonstrated that low serum vitamin D and irisin levels in CD diet group were accompanied by decrease hepatic PPAR–α expression, a main regulator of hepatic fatty acids β-oxidation. In accordance of our result, **Karimi-Sales et al. [40]** and **Pydyn et al. [41]** showed that NAFLD patients had decreased levels of hepatic PPAR–α expression.

Fortunately, our results proved that vitamin D treatment, in CD diet + vitamin D group, significantly reduced ALT, AST and ALP levels, liver weight and index, hepatic TG, oxidative stress and inflammatory status of the liver, in addition to increase serum irisin, 25-(OH) D_3 and hepatic PPAR–α expression when compared with CD diet untreated rats. This was accompanied by histopathological improvement of hepatic structure.

These data are in accordance with those conducted by **Han et al. [15]** and **Karatayli et al. [42]** who reported that vitamin D hepatoprotective effect against NAFLD was based on improvement of liver enzymes, liver weight and index, in addition to restoration of hepatic architecture on histopathological examination.

We assumed that the beneficial effect of vitamin D against NAFLD was mediated, in a part, through irisin and to prove this, circulating irisin level was measured. We found that there was a significant decrease in serum irisin level in CD diet group, which expressed hypovitaminosis D, and this decrease was corrected upon vitamin D treatment. This result was in agreement with **Raafat and Abulmeaty [43]** and **Nadimi et al. [44]** who found that low serum vitamin D was accompanied by decreased circulating irisin level. Increased serum irisin after administration of vitamin D could be explained by the interaction between VDR and peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC- 1α), a transcriptional factor that upregulates FNDC5 expression, the precursor of irisin **[44].** When vitamin D binds to VDR, it increases PGC-1α expression which in turn leads to increase circulating irisin level. Additionally, vitamin D has been shown to activate p38/MAPK (mitogen-activated protein kinase) in muscle. PGC-1α and irisin are regulated by the activation of p38/MAPK **[45].**

The hepatoprotective role of irisin in ameliorating hepatic fat accumulation in NAFLD cases was documented in earlier studies which reported that irisin can protect against hepatic fat deposition by enhancement of PPAR–α expression and secretion, a master key which regulates fat oxidation **[13, 46]**. **Shiri-Sverdlov et al. [47]** found that PPAR–α increases TG hydrolysis and high-density lipoprotein (HDL) synthesis, leading to the decrease of hepatic lipid accumulation.

Mazur-Biały et al. [48] revealed the anti-inflammatory property of irisin, proved by decrease proinflammatory markers (TNF-α, IL-6), in lipopolysaccharide-activated adipocytes through shifting of macrophage state from proinflammatory (M1) to anti-inflammatory (M2) ones. It also can inhibit one of hepatic lipogenic protein arginine methyl transferase 3 (PRMT3) **[49].**

Regarding the antioxidant role of irisin, many previous studies proved its antioxidant effect. **Bi et al. [50]** found that irisin reduced MDA level while increased SOD and GSH levels in experimental model of liver ischemia reperfusion injury. This is due to the ability of irisin to increase the expression of uncoupling protein 2 (UCP-2) which leads to enhancement of mitochondrial biogenesis and alleviating oxidative stress **[13].** Moreover, irisin can upregulate the expression of many antioxidant enzymes like catalase and SOD, on the other hand, it decreases ROS production **[51].**

Although there are many former studies that describe the protective effect of vitamin D against NAFLD **[52]**, fortunately our results may be the first to elucidate the involvement of irisin in the hepatoprotective effect of vitamin D against CD-induced NAFLD.

In conclusion, our study demonstrated that CD diet model produces NALFD histologically close to the human NAFLD, including hepatic fat accumulation, liver inflammation and oxidative stress. Concomitant administration of vitamin D to CD diet fed rats showed a protective effect against NAFLD. This protective effect could be in part due to the capability of vitamin D to stimulate irisin secretion, in addition to its antioxidant and anti-inflammatory effects. Our study may be the first to demonstrate the significant role of irisin in the hepatoprotective effect achieved by vitamin D.

Limitations of the study:

We did not analyze the effect of CD diet for longer time periods. Another limitation, we did not examine the effect of CD diet on mitochondrial B-oxidation which would allow to verify the possible positive influence of vitamin D on mitochondrial utilization of fatty acids in NAFLD rat model.

Recommendations:

Studying other molecular mechanisms underlying vitamin D mediated protection against NAFLD. Further studies on the possible curative effect of vitamin D against NAFLD lesion.

Conflict of interest:

No conflict of interest was declared by the authors.

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