

## Effects of vitamin D3 on PPAR- $\alpha$ and its target gene NFKBIA on a Rat Model of High Carbohydrate High Fat diet induced Non Alcoholic Steatohepatitis

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### Abstract

**Objectives:** Accumulating data suggesting that Vitamin D3 could be of a potential therapeutic value for non-alcoholic fatty liver disease (NAFLD), but its underlying mechanisms are still under research. Vitamin D3 up regulates PPAR- $\alpha$  and downstream gene nuclear factor-kappa-B inhibitor subunit-alpha (NFKBIA) which may represent a novel strategy for the management of NASH. Therefore, in this study, we investigated the possible protective role of vitamin D3 on high carbohydrate high fat diet (HCHFD) animal model of NASH through PPAR- $\alpha$  and NFKBIA pathway. **Methods:** Thirty male Sprague-Dawley rats were equally divided into three groups; Group I: (negative control group) fed standard rat chow diet for 8 weeks. Group II: received HCHFD for 8 weeks. Group III: fed as group II for 8 weeks and received also, I.P administration of vitamin D3 at a dose of 5  $\mu$ g/kg twice weekly from the fourth week till the end of the study. At the end of the experiment, serum triglycerides, total cholesterol, liver enzymes, serum albumin, serum bilirubin and liver tissue oxidative stress markers (MDA, SOD and GSH) were measured. Hepatic histopathological alterations were assessed by hematoxylin and eosin, Masson's trichrome, and Oil Red O staining; the expressions of NF- $\kappa$  B p65 (ReIA) was estimated in liver tissues using immunohistochemistry and the expression of hepatic PPAR- $\alpha$  and NFKBIA genes were estimated at the level of mRNA by quantitative real-time PCR. **Results:** Nonalcoholic steatohepatitis (NASH) model was confirmed by histopathological examination as indicated by elevated NAFLD activity score (NAS) as well as elevation of serum liver enzymes, hyperlipidemia in the form elevated plasma triglycerides and total cholesterol, oxidative stress state (high MDA, low GSH and SOD) and down regulation of PPAR- $\alpha$  and NFKBIA mRNA in liver tissues which was concomitant with up regulation of inflammatory marker NF- $\kappa$  B-p65(ReIA). On the other hand, vitamin D3 ameliorated NASH by attenuation of oxidative stress (low MDA, high GSH and SOD) and up regulation of PPAR- $\alpha$  and NFKBIA genes at the level of mRNA in the liver tissues which was accompanied by decrease in hepatocyte nuclear NF- $\kappa$  B-p65 (ReIA) immunohistochemical brownish staining. **Conclusion:** Vitamin D3 attenuates NASH in HCHFD rat model by attenuation of redox state and up regulation of PPAR- $\alpha$  and its downstream gene NFKBIA in liver tissues

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### Keywords

- Vitamin D3
- HCHFD
- Nonalcoholic Steatohepatitis
- PPAR- $\alpha$
- NFKBIA.

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## INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is now considered the most common chronic liver disease all over the world, affecting 20-35% of the adults in the general population [1]. NAFLD comprises a disease spectrum ranging from simple steatosis (SS), through non-alcoholic steatohepatitis (NASH) to hepatic fibrosis, then cirrhosis and ultimately hepatocellular carcinoma (HCC) [2]. The progression from SS to NASH is an essential step in NAFLD progression and people with NASH accounts for 15-30% of NAFLD patients and is anticipated to be the main cause of liver transplantation by 2020 [3].

Much research has been directed on NAFLD pathogenesis over the previous decades, till now it is not completely understood, and no fully well-defined treatment has been established. The pathogenesis of NAFLD is as yet in view of theories, a well-accepted two hit theory by **Day and Jones**, [4] was proposed; the primary hit is liver cells triglyceride accumulation, followed by initiators of the second hit which may be either proinflammatory mediators, mitochondrial abnormality [5,6]; elevated oxidative stress condition [7] with subsequent apoptosis [8,9].

The nuclear receptors peroxisome proliferator-activated receptor alpha (PPAR- $\alpha$ ) is highly expressed in liver cells and exerts a pleiotropic effect such as modulation of fatty acid transport,  $\beta$ -oxidation and regulation of inflammatory reaction [10]. **Stienstra et al.**, [11] and **Abdelmegeed et al.**, [12] suggested that PPAR- $\alpha$

has a beneficial role against both liver steatosis and inflammatory reaction in NASH as evidenced by the increased susceptibility to NASH of PPAR- $\alpha$  knockout mice; thus PPAR- $\alpha$  activation could be an important therapeutic target in NASH.

In mammals, The NF- $\kappa$ B family comprises of five proteins, p65 or RelA, RelB, c-Rel, p50 or NF- $\kappa$ B1, and p52 or NF- $\kappa$ B2. All members form homo or heterodimers and have in common the same 300 terminal amino acid which is called Rel homology domain (RHD) [13,14], of the NF- $\kappa$ B five proteins; p65 protein is the most dominant and is present in heterodimer with p50 [15]. One of the most important controller of mammalian NF $\kappa$ B is inhibitors of NF $\kappa$ B (I $\kappa$ B $\alpha$ ); known also as Nuclear factor-kappa-B inhibitor subunit-alpha (NFKBIA); in non-stimulated cell; NFKBIA combines with the p50/p65 heterodimer, sequestering it in the cytoplasm thus hindering nuclear translocation of NF $\kappa$ B and activation of target gene transcription [16]. I $\kappa$ B $\alpha$  or NFKBIA is a target gene PPAR- $\alpha$  [17-18].

The transcription factor NF- $\kappa$ B is considered the genetic switch that regulates the genes responsible for inflammatory, immune and stress response [19]. **Siebenlist et al.**, [20] stated that it plays a pivotal role in the decision between life and death of liver cells. NF- $\kappa$ B might play a crucial role in NAFLD development and an inhibitors of NF- $\kappa$ B might be effective in NAFLD treatment [21].

Vitamin D, a well-known steroid hormone that regulates mineral metabolism, has emerged as a promising hepatoprotective agent and considerable attention to the beneficial effect of vitamin D in NAFLD treatment had been reported. Accumulating data suggested that the blood level

of 25-OH vitamin D was negatively correlated with the risk of liver insult [22, 23]. The pivotal role of vitamin D was more evidenced when reports indicating that vitamin D deficiency (VDD) in humans and animals contributes to the development of NAFLD [24]. It was found by **Manco et al.**, [25] that low levels of 25(OH) D was associated with significant increase in NAFLD Activity Score (NAS) and fibrosis in children with biopsy-diagnosed NAFLD. Several publications in recent years documenting the possible beneficial role of vitamin D in the management of NAFLD while the underlying mechanisms needs further clarification [26].

Modulation of PPAR- $\alpha$  by 1, 25 (OH)<sub>2</sub> D<sub>3</sub> was reported in diabetic rat model both in cardiac tissue [27] and liver tissue [28], while modulation of PPAR- $\alpha$  and its target gene (NFKBIA) by vitamin D<sub>3</sub> in a rat model of NASH induced by HCHFD has not been studied yet. Therefore, this work aimed to evaluate whether vitamin D could alleviate NASH model in rats induced by high carbohydrate high fat (HCHF) diet, and to clarify if modulation of genes expression of PPAR- $\alpha$  and NFKBIA by vitamin D could be a potential mechanism.

## Materials and methods

### 1. Chemicals:

#### *D-fructose:*

D-fructose was purchased from (**EL-Gomhoria Company industries, Egypt**) in the form of white powder (240 gm). A concentration of fructose 25% in drinking water was prepared every other day [29], by dissolving 25 g of fructose in a volume of 100 ml tap water in bottles, after that aluminum

foil was used to cover the bottles to avoid fructose fermentation [30], then this preparation was administered daily for eight weeks.

#### *Vitamin D<sub>3</sub> (1, 25 Di – OH cholecalciferol):*

Vitamin D<sub>3</sub> (1,25 Di OH cholecalciferol) was purchased from **Memphis (Memphis, Egypt)** as Devarol-S (2ml/amp.5mg =200.000 I.U) (diluted in 0.9% sodium chloride to get final concentration of 0.5  $\mu$ g /1 ml) [31].

## 2. Animals

#### *Experimental animals and diet:*

This study was conducted on 30 male Sprague-Dawley rats, (weight, 200-250 gm) obtained from the Mansoura Experimental Research Center (MERC), then, rats were housed 5 per cage at normal atmospheric temperature with a 12-hour light-dark cycle at Department of Physiology, Mansoura Faculty of Medicine. Animals fed rat chow and had free access to tap water for 4 weeks to be familiarized with the new environment.

The animals care was conducted according to the *Guide to the Care and Use of Laboratory Animals* (1996, published by National. Academy. Press., 2101 Constitution Ave. N.W., Washington, DC20055, and USA). All procedures of the experimental study were conducted in accordance to the guidelines of local ethical committee of Mansoura Faculty of Medicine.

#### *Experimental design and animal grouping:*

After an initial acclimatization for a period of four weeks, the rats were randomly divided into three groups of equivalent number as follow: **Group I (negative control group)** fed normal fat diet (NFD) with 15% of its calories derived from

fat and it was prepared in accordance to AIN-93G rodent diet [32] and had free access to plain water plus administration of saline (1 ml/kg) twice weekly by the I.P. route. **Group II (positive control or NASH group)** fed HCFD of which ; HFD was similar in composition to NFD, except for addition of 200 gm beef fat / kg to make percent of calories derived from fat 45% [32] and received 25% fructose-sweetened water for 8 weeks [33], plus administration of saline (1 ml/kg) twice weekly by the I.P. route .**Group III (Vitamin D3 treated NASH group)** received HCHFD as group II and after 4 weeks received treatment in the form of I.P. injection of 1,25-VD3 with a dosage of 5  $\mu$ g/kg body weight twice weekly till the end of the study.

The rats were weighed every week to adjust then the dosage of vitamin D3 according to body weight changes to maintain constant vitamin D3 dose throughout the entire experiment.

### 3. Collection of blood and tissue sampling:

One day before the end of the experiment, all rats were subjected to 12-hour overnight fasting then in the morning of the following day; all rats were weighed then under thiopental anesthesia at a dose of 75 mg/kg I.P. injection [34], blood samples were drawn by cardiac puncture then placed into a set of tubes without an anticoagulant then serum from the samples were acquired by centrifugation at 3000 x g for 20 minutes at 4°C and stored at -70 ° C until the time of analysis. . After that, the anterior abdominal wall was opened and the livers were excised and weighed then divided into two parts; one part placed in plastic cassettes and sunken in neutral buffered formalin for one day for histopathological examination, the second part was

perfused with saline and stored at liquid nitrogen for Oil red O stain, biochemical assay of markers of oxidative stress and for RT-PCR.

### 4. Biochemical analysis:

#### *Liver function tests:*

Serum ALT, AST activities, alkaline phosphatase and serum albumin were measured using (**Bio Systems S.A, Spain**) kits, and serum bilirubin was measured using (**Fortress diagnostic, UK**) kits according to manufacturer's instructions.

#### *Total cholesterol (TC) and Triacylglycerol (TAGs):*

Were measured by using enzymatic-colorimetric methods (**Centronic GmbH., Germany**).

#### *Assay of markers of oxidative stress in liver tissues:*

Parts of liver tissues of about 50 -100 mg were homogenized in 1-2 ml cold buffer (composed of 50 mM potassium phosphate, 1 mM EDTA and its pH was adjusted to be about 7.5) using mortar and pestle, after homogenization ;centrifugation occurred at a speed 4,000 rpm for duration of about 15 minutes with temperature adjusted at 4°C. then the supernatant was collected and stored at -20°C until the time of analysis ,after that colorimetric method was used to measure the level of Malondialdehyde (MDA), superoxide dismutase (SOD) and reduced glutathione (GSH) activity in the supernatant of liver homogenates in accordance to the manufacturer's instructions (Bio-Diagnostics company , Dokki , Giza, Egypt).

### *Assessment of PPAR- $\alpha$ and NFKBIA genes expression in liver tissues by real-time PCR RNA extraction and cDNA synthesis*

Total RNA was extracted from collected liver tissues (30 mg) according to the manufacturer's guidelines ( **RNeasy mini kit. , Qiagen. , Hilden., Germany**), then Nano Photometer P-330 ( **Munchen ,Germany**) was used to measure RNA concentration and quality ,after that; SensiFAST™ cDNA Synthesis Kit ( **Bioline, UK**) was used to reverse transcript one mg of RNA . Real-time PCR was done with a 20  $\mu$ L total reaction volume containing cDNA of volume 4  $\mu$ L and 1  $\mu$ L for each primer pair and a volume of 10  $\mu$ L of SYBR green ( **SensiFAST™ SYBR® N0-ROX kit; Bioline , UK**) . RT-PCR primers used in this work are listed in Table 1. Act-b was used as an endogenous control that NFKBIA and PPAR- $\alpha$  mRNA levels were adjusted as the values compared to it, PikoReal™ Real-Time PCR system ( **Finland**) was used to perform PCR.

### **5-Histopathological study**

Formalin fixed liver specimens were dehydrated using graded alcohol solutions then embedded in paraffin. The five-micron thick paraffin sections were cut and slides were stained with (i) hematoxylin and eosin for histopathological analysis (ii) Masson trichrome for assessment of fibrosis (iii) Oil Red O staining, and (iv) immunohistochemical staining of NF- $\kappa$  B p65.

#### *Oil Red O staining:*

Liver tissues, which were frozen in optimum cutting temperature compound, were cut at thickness of 5  $\mu$ m, then mounted on slides and left for 1-2 hours to dry then 10% formalin was used to

fix these sections for a duration of 10 min , after that they were rinsed with PBS (PH 7.4), at that point slide were left till dry, after that the slides were set in 100% propylene glycol for term of 2 min then stained in 0.5% Oil Red O solution for around 30 min, after that they were transferred to an propylene glycol solution of conc. of 85% for 1 min. then rinsed in distilled water for 2 times, and lastly hematoxylin was used as a counter stain.

#### *Scoring of NASH:*

Histological examination was performed using NAFLD activity score (NAS) which is the total score resulting from the sum of individual scores of steatosis (0–3), lobular inflammation (0–2), and hepatocellular ballooning (0–2). NASH was diagnosed if rats have total score of  $\geq 5$  [35].

#### *Immunohistochemical staining and assessment for NF- $\kappa$ B p65:*

Serial sections (5  $\mu$ m) were deparaffinized, rehydration and antigen retrieval was performed in the **PT Link (Dako PT100)**, the then slides were processed **utilizing** an automated staining protocol on the **Autostainer Link 48 (Dako AS480)** using NF- $\kappa$ B/p65 Ab-1 (Cat. #RB-9034-R7, Neomarkers, Labvision, Fremont, California, USA).

#### *Semi-quantification of positive cells by immunohistochemistry*

The number of NF- $\kappa$  B p65 positive cells was counted in 30 random high-power fields the NF- $\kappa$ B- p65 **positive cells** were calculated by counting manually the cells with NF- $\kappa$ B-positive nuclei [36].

## 6- Statistical analysis:

SPSS version 16.0 was used for statistical analysis. One way analysis of variance (ANOVA) for quantitative data with Tukey's post hoc analysis was used for multiple group comparisons. Mean  $\pm$

standard deviation (mean $\pm$ SD) was used for quantitative data. Pearson's rank test was performed to determine correlation between all study cases together (not group by group). A probability level  $p$  of  $\leq 0.05$  indicates a significant statistical difference.

**Table (1): Sequences of primers for Quantitative RT-PCR**

Gene	GeneBank Accession No.	Forward sequence	Reverse sequence	Expected product size(bp)
<b>NfkBIA</b>	NM_001105720.2	CCCAA GTACCCGGATA CAGC	GGGCAACTCATCTTCCGTGA	1583
<b>PPAR-<math>\alpha</math></b>	NM_013196.1	TCCTCTGGTTGTCCTTGA	CAGTCTGGCTCGCCTCTAA	2022
<b>Act-b</b>	NM_031144.3	CACCATGTACCCAGGCATTG	CACACAGAGTACTTGCGCTC	1293

PPar- $\alpha$ , Rattus norvegicus peroxisome proliferator activated receptor alpha, NfkBIA, Rattus norvegicus nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha, Act-b, Rattus norvegicus actin, beta

## Results

*Effect of 1,25-VD3 on body and liver weight , serum triglycerides and total cholesterol and liver function tests :*

Changes in body and liver weight among all groups are shown in Table 2; NASH group fed HCHFD had marked obesity as shown by significant increase in body weight and also significant increase in liver weight when compared to negative control group and these changes in body and liver weight were partially but significantly lowered with 1, 25-VD3 treatment but their values were still significantly higher than negative control group.

Serum triglycerides and cholesterol for all studied groups are shown in Table 2; NASH group showed hyperlipidemia in the form of significant elevation of serum triglycerides, total cholesterol, when compared to negative control group ( $p < 0.0001$ ) and these metabolic disturbances were partially but

significantly lowered with 1, 25-VD3 treatment but their values were still significantly higher than negative control group.

Liver function tests are shown in (Table 2); showing significant elevation of serum liver enzymes (ALT, AST and alkaline phosphatase) in NASH group when compared to negative control group and the group treatment with 1,25-VD3 exhibited significant reduction of liver enzymes but still higher than negative control group. Compared with the negative control group, there was significant elevation of serum bilirubin in NASH group and this level was decreased significantly with 1,25-VD3 treatment ; however in all groups; serum bilirubin was still within normal range, moreover ,no significant differences were observed for serum albumin levels in all studied groups ( $P > 0.05$ ).

#### *Effects of 1,25-VD3 on liver tissue oxidative stress markers :*

The alterations of oxidative stress markers in liver tissue are shown in Table 3 , showing significant increase in hepatic MDA level with concomitant significant depletion in antioxidant system as depicted by decline in GSH and sodium oxide dismutase (SOD) level in NASH group compared to negative control group ( $p < 0.01$ ). On the other hand 1,25-VD3 treated groups partially corrected this oxidative stress by increasing hepatic level of GSH and SOD accompanied by significant decrease in MDA concentration as compared to NASH group ( $p < 0.01$ ).

#### *Effects of 1,25-VD3 on liver histology and NAFLD score:*

As illustrated in Fig.1 ,H&E-stained sections; negative control group displayed intact lobular architecture with cord like arrangement of hepatocytes (Fig.1 A1) , with no obvious hepatic steatosis , hepatocellular ballooning and minor foci of inflammatory cells (Fig.1 A2) , However, in group HCHFD rats ,there was abnormal structure of hepatic lobule (Fig.1 B1),marked macro-vesicular steatosis ,ballooning and mild to moderate lobular inflammation (Fig.1 B2);their NAS scores were significantly elevated in NASH group ( $6.1 \pm 0.56$ ) while treatment with vitamin D3 decreased markedly steatosis ,ballooning and inflammation (Fig.1 C1 and C2) with significant decrease in mean NAS scores to ( $2.8 \pm 0.78$ ).

As regard to Oil Red O staining, there were small scattered lipid droplets ( $< 5\%$ ) in few liver cells of negative control group (Fig.1 A5). In contrast, liver cells of NASH group were of different sizes

and full of lipid droplets (Fig.1 B5), while vitamin D treated group shows diffuse distribution but fewer lipid droplets of hepatocytes when compared with NASH group (Fig.1 C5).

As illustrated in Fig. 1; (**Masson's trichrome staining**), there was no obvious fibrosis (indicated by blue coloration) in negative control group (Fig.1 A3). While, some rats showed fibrosis around sinusoidal spaces and in the portal area with bridging fibrosis in group HCHFD (Fig.2 B3), the group receiving vitamin D treatment has lower level of fibrosis than that of HCHFD group (Fig.1 C3).

#### *Effect of 1,25-VD3 on expression of hepatic NF- $\kappa$ B p65:*

Immunohistochemistry staining of NF- $\kappa$  B p65 are shown in (Fig1 A4, B4 and C4). indicating up-regulation of NFK- $\beta$  p65 in NASH group in the form of obvious brownish nuclear staining and increased brownish cytoplasmic staining when compared with negative control group which showed weakly positive brownish staining in liver tissue and nearly no nuclear staining and treatment with vitamin D3 significantly down regulated this hepatic NF- $\kappa$  B p65 protein expression as evidenced by decrease in nuclear staining but that was accompanied by an increase in brownish cytoplasmic staining.

#### *Effect of 1,25-VD3 on mRNA expression of PPAR- $\alpha$ and NFKBIA in liver tissue:*

Group fed HCHFD were found to have significant decrease in mRNA expression of PPAR- $\alpha$  (Fig.2) and NFKBIA (Fig.3) as compared to negative control group ( $p < 0.0001$ ). Meanwhile; the 1,25-

VD3 treatment group showed significant up regulation, of mRNA expression of PPAR- $\alpha$  and NFKBIA ( $p < 0.0001$ ) when compared to HCHFD group.

**Table 2:** Effects of treatment with Vitamin D on body weight, liver weight, serum triglycerides, total cholesterol and some liver function tests

Groups	Group I n= 10	Group II n= 10	Group III n= 10	One way ANOVA
Variables				p-value
Body weight (gm)	414.2 $\pm$ 13.62	507.7 $\pm$ 17.86 <sup>a</sup>	453.1 $\pm$ 18.04 <sup>a, b</sup>	<0.0001*
Liver weight (gm)	11.0 $\pm$ 1.23	13.93 $\pm$ 0.67 <sup>a</sup>	12.6 $\pm$ 0.92 <sup>a, b</sup>	<0.0001*
Serum triglyceride levels (mg/dl)	54.22 $\pm$ 8.63	119.54 $\pm$ 8.58 <sup>a</sup>	75.6 $\pm$ 15.30 <sup>a, b</sup>	<0.0001*
Serum total cholesterol levels (mg/dl)	50.51 $\pm$ 14.30	111.07 $\pm$ 16.71 <sup>a</sup>	72.594 $\pm$ 8.39 <sup>a, b</sup>	<0.0001*
Serum AST(IU/L)	76.53 $\pm$ 6.46	234.9 $\pm$ 55.81 <sup>a</sup>	114.14 $\pm$ 12.08 <sup>b</sup>	<0.0001*
Serum ALT(IU/L)	51.31 $\pm$ 7.60	101.03 $\pm$ 16.20 <sup>a</sup>	66.66 $\pm$ 11.95 <sup>a, b</sup>	<0.0001*
Serum Alkaline phosphatase (IU/L)	149.96 $\pm$ 29.28	343.15 $\pm$ 49.95 <sup>a</sup>	238.07 $\pm$ 64.36 <sup>a, b</sup>	<0.0001*
Serum albumin levels (mg/dl)	3.01 $\pm$ 0.33	4.03 $\pm$ 0.65 <sup>a</sup>	4.02 $\pm$ 0.55 <sup>a</sup>	<0.0001*
Serum total bilirubin levels (mg/dl)	0.24 $\pm$ 0.01	0.36 $\pm$ 0.04 <sup>a</sup>	0.30 $\pm$ 0.06 <sup>a, b</sup>	<0.0001*

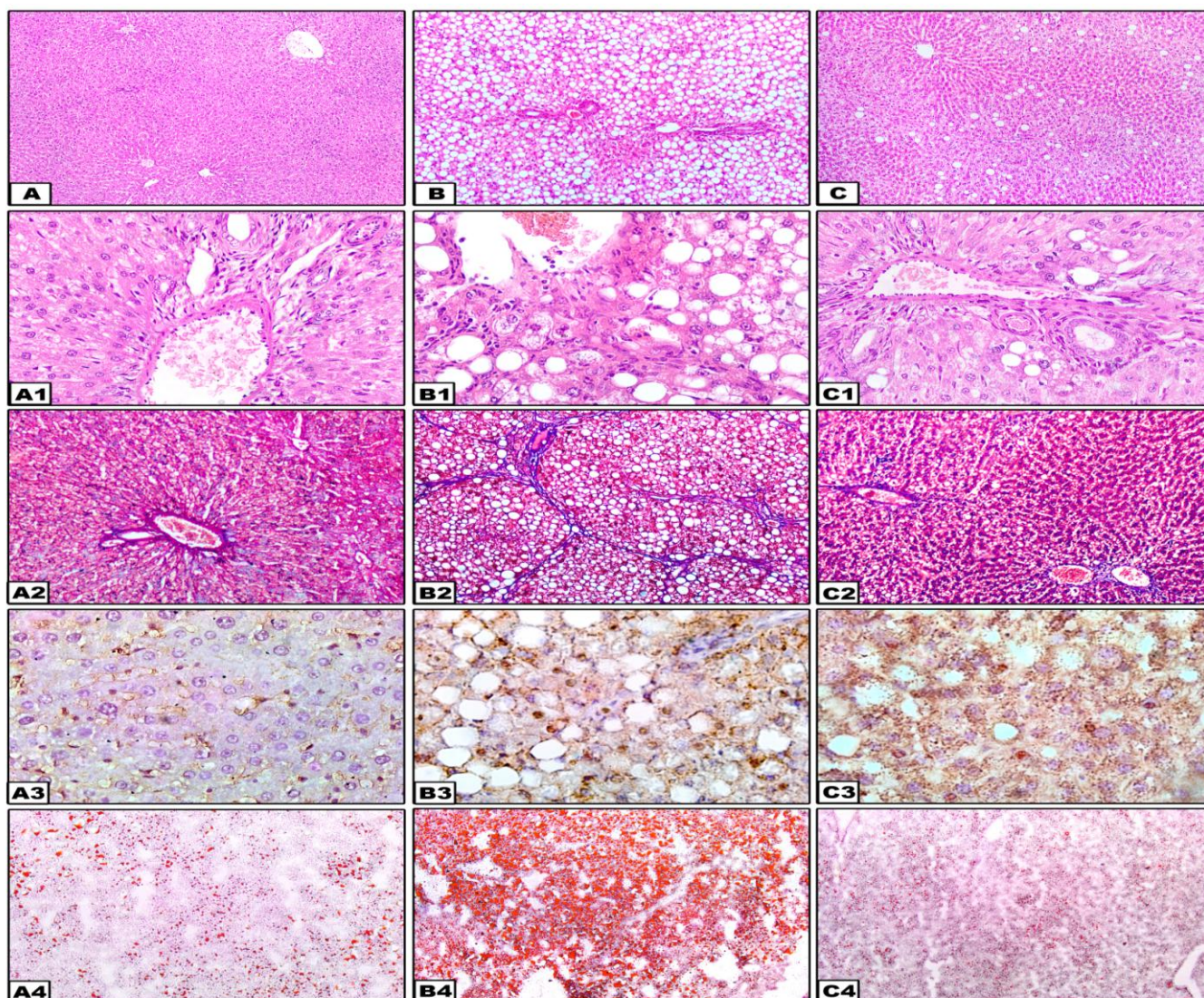
Values are expressed as mean  $\pm$  SD. \*P was considered significant at <0.05; a Significance vs control group , b Significance vs NASH induced group using One way ANOVA followed by Tukey's post hoc test for multiple comparison. ALT alanine aminotransferase, AST aspartate aminotransferase.

**Table 3:** Effects of treatment with Vitamin D on liver tissue oxidative stress markers PPAR- $\alpha$  and NFKBIA mRNA expression and NAFLD activity score:

Groups	Group I n= 10	Group II n= 10	Group III n= 10	One way ANOVA
Variables				p-value
Liver tissue MDA (nmol/mg protein/ml)	86.82 $\pm$ 10.76	279.21 $\pm$ 25.73 <sup>a</sup>	148.84 $\pm$ 53.77 <sup>a, b</sup>	<0.0001*
Liver tissue SOD Activity units/mg protein	649.76 $\pm$ 38.01	344.18 $\pm$ 72.59 <sup>a</sup>	547.06 $\pm$ 79.26 <sup>a, b</sup>	<0.0001*
Liver tissue GSH (mg/ g liver tissue)	90.63 $\pm$ 10.45	35.15 $\pm$ 24.25 <sup>a</sup>	78.14 $\pm$ 12.58 <sup>b</sup>	<0.0001*
DNA-binding activity of liver NFKBIA	1.0 $\pm$ 0.00	0.26 $\pm$ 0.03 <sup>a</sup>	0.63 $\pm$ 0.06 <sup>a, b</sup>	<0.0001*
DNA-binding activity of liver PPAR $\alpha$	1.0 $\pm$ 0.00	0.441 $\pm$ 0.05 <sup>a</sup>	0.72 $\pm$ 0.07 <sup>a, b</sup>	<0.0001*
NAFLD score	0.2 $\pm$ 0.4	6.1 $\pm$ 0.56 <sup>a</sup>	2.8 $\pm$ 0.78 <sup>a, b</sup>	<0.0001*

Values are expressed as mean  $\pm$  SD. \*P was considered significant at <0.05; a Significance vs control group , b Significance vs NASH induced group using One way ANOVA followed by Tukey's post hoc test for multiple comparison. MDA; malondialdehyde, GSH; glutathione, SOD;superoxide dismutase, PPAR $\alpha$ ; peroxisome proliferator-activated receptor alpha ,NFKBIA; nuclear factor-kappa-B inhibitor subunit-alpha



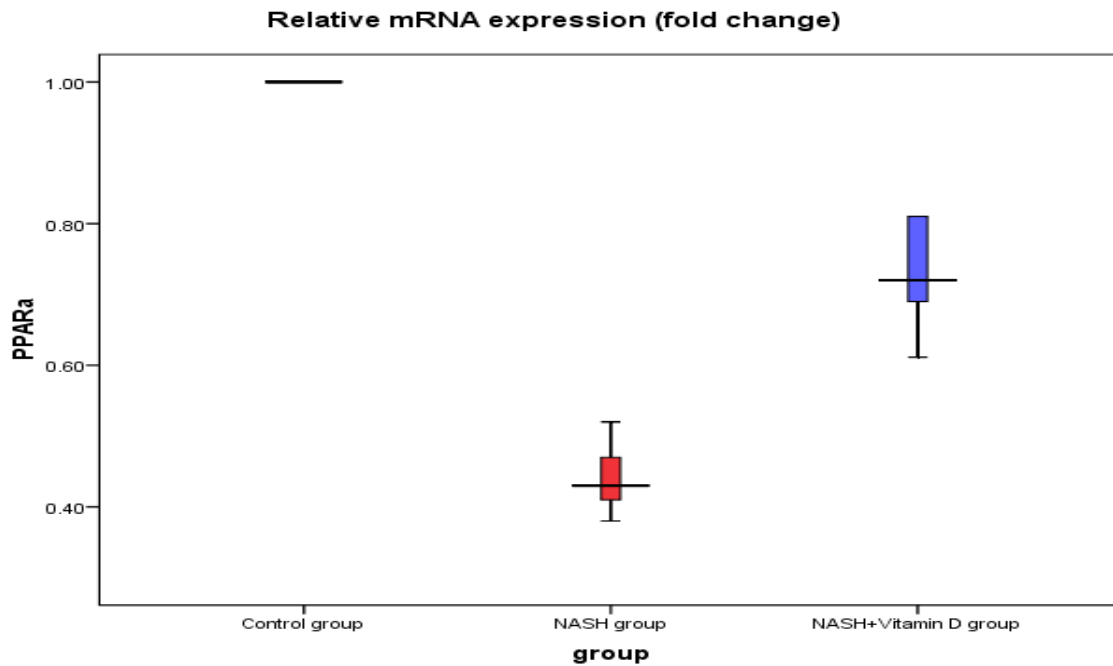


**Fig. 1 Effects of vitamin d3 therapy on NASH:** Photo micrograph of (A) negative control group (B) NASH group (C)Vitamin D3 treated group ;(100×). Sections stained with hematoxylin and eosin (1), (400×) Sections stained with hematoxylin and eosin (2), Masson trichrome stain (3),Immunohistochemistry of NFKβ-p65 (4)Oil red O staining (5).-Negative control group; showed intact hepatic lobule with cord like cell arrangement (A1) no steatosis, ballooning or inflammation (A2) slight or no fibrosis by masson trichrome (A3) small scattered lipid droplets (< 5%) in hepatocyte by Oil O red stain (A5),while NASH group showed distorted hepatic lobule (B1) marked macrovesicular and micro vesicular steatosis ,ballooning with mild to moderate lobular inflammation (B2) perivenular fibrosis with formation of fibrous septa by masson trichrome (B3) marked increase in size and distribution of lipid droplets inside hepatocytes by Oil red O stain (B5),Vitamin D3 treated group showed some preservation of hepatic lobule (C1) significant decrease in steatosis ,ballooning with mild lobular inflammation (C2) perivenular fibrosis without formation of fibrous septa by masson trichrome (C3) diffuse distribution but fewer lipid droplets of hepatocytes by Oil red O stain (C5).-Immunohistochemical staining of NFK-β; NASH group showed nuclear staining and increased brownish cytoplasmic staining (B4) when compared with negative control group which showed weakly positive brownish staining and nearly no nuclear staining (A4) and treatment with vitamin D3 significantly decreases this brownish staining of nuclei (C4).

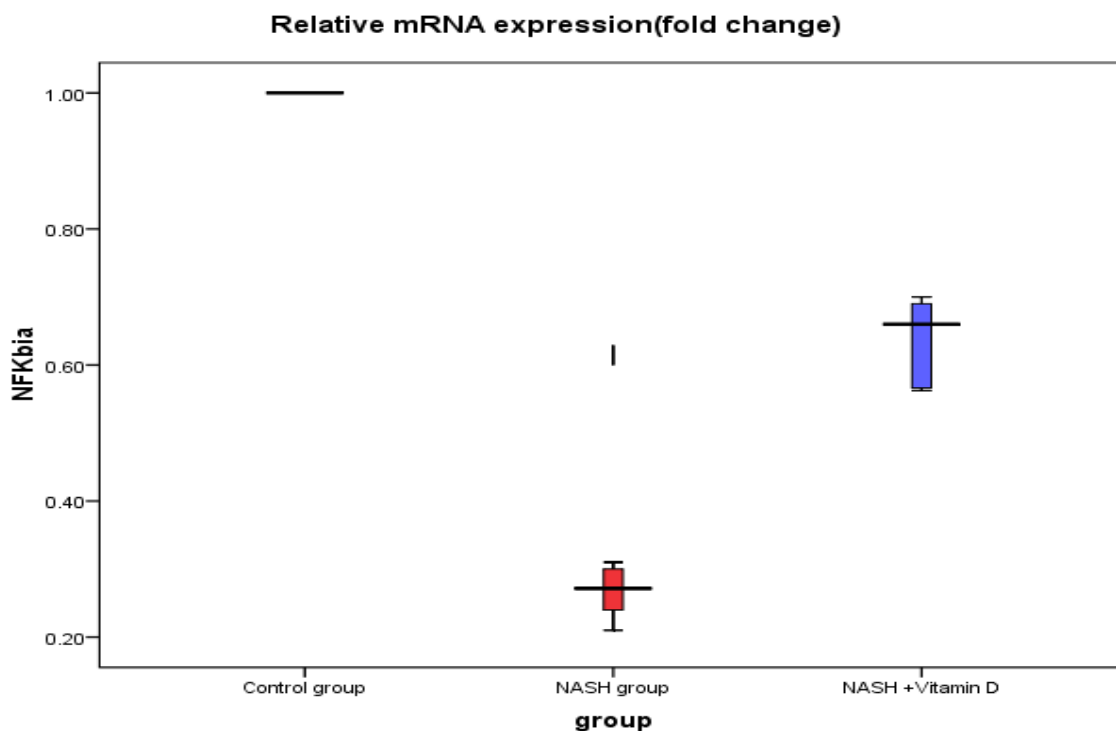
*As regard correlation between NAS score as independent variable and PPAR-α or NFKBIA as dependent variable table (4) and figures (4,5 and 6) show the following:*

There was negative correlation between NAS score and PPAR-α or NFKBIA ( $P < 0.001$ ). On the

other hand, there was positive correlation between PPAR-α level and NFKBIA in liver tissue ( $P < 0.001$ ).



**Fig. 2.** Liver tissue PPAR- $\alpha$  levels. Values are expressed as mean  $\pm$  SD. \*P was considered significant at  $<0.05$ ; a significant as compared to the control group, b significant as compared to NASH group using One way ANOVA followed by Tukey's post hoc test for multiple comparison.

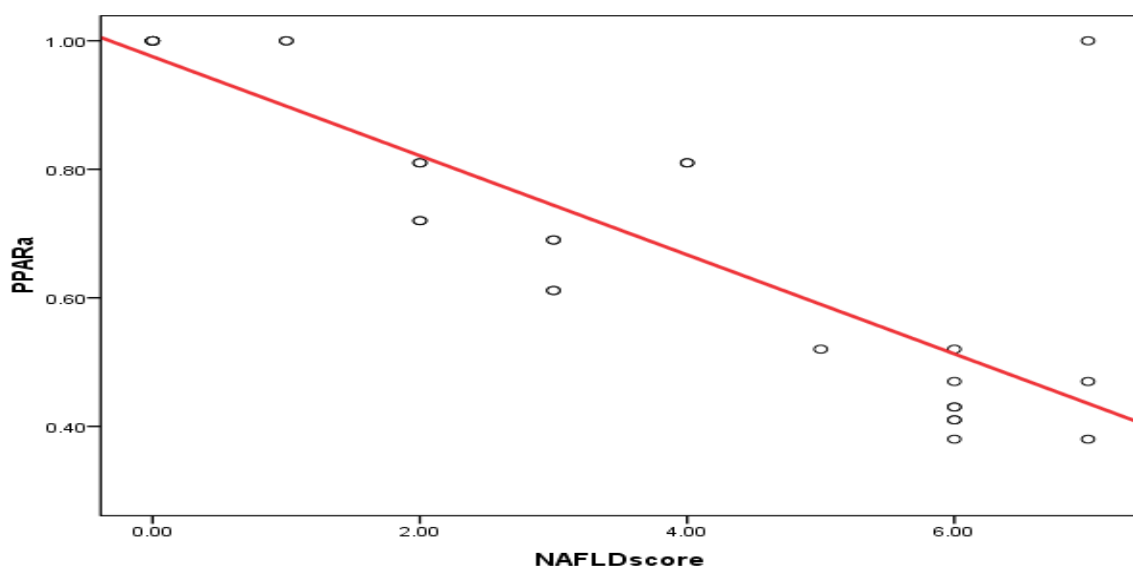


**Fig. 3.** NFKBIA mRNA relative expression. Values are expressed as mean  $\pm$  SD. \*P was considered significant at  $<0.05$ ; a significant as compared to the control group, b significant as compared to NASH group using One way ANOVA followed by Tukey's post hoc test for multiple comparison.

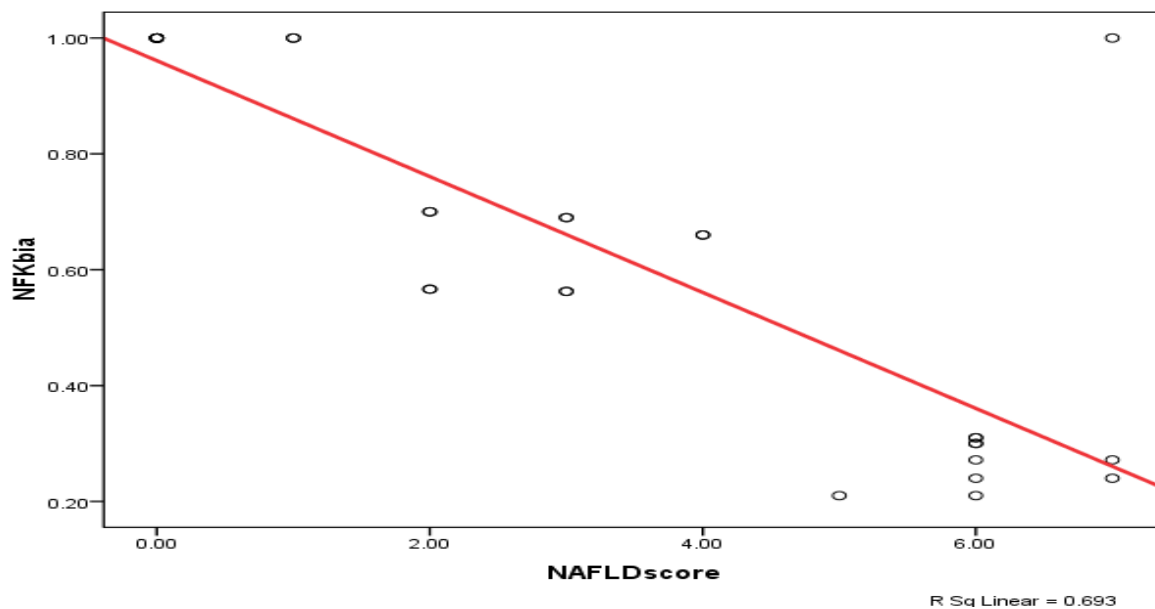
**Table (4):** Pearson correlation between NAS score as independent variable and PPAR- $\alpha$  or NFKBIA as dependent variable:

NAS score	PPAR- $\alpha$		NFKBIA	
	R	P value	R	P value
	-0.808	$P < 0.001$	-0.797	$P < 0.001$
PPAR- $\alpha$			0.934	$P < 0.001$

Pearson correlation (significance at  $p \leq 0.05$ ). r = reflection coefficient. NAS =NAFLD activity score. PPAR- $\alpha$  = peroxisome proliferator-activated receptors alpha. NFKBIA = nuclear factor-kappa-B inhibitor subunit-alpha



**Fig. 4.** Pearson correlation between NAFLD score and relative mRNA expression of. PPAR- $\alpha$



**Fig. 5.** Pearson correlation between NAFLD score and relative mRNA expression of NFKBIA.

**Discussion:**

NAFLD is the commonest cause of chronic liver disease, constituting a major risk factor that could

progress to cirrhosis, liver failure and hepatocellular carcinoma [37, 38, 39]. The disease is so dangerous that the National Institutes of Health refers to it as a “silent disease” [40].

Therefore, there is an unmet need to choose an appropriate animal model of NASH and to fully clarify the underlying mechanisms of NASH and consequently develop new approach for treatment or intervention of disease progression.

In the present, the HCHFD was chosen to understand the potential beneficial mechanisms of vitamin D3 on NASH associated changes, because the addition of the high fructose content to a high fat diet had been reported to reproduce all the features of NASH [41]. Fructose can promote de novo lipogenesis, contributing to first hit and also promote inflammation through endogenous toxins of fructose metabolites thus contributing also to the second hit of the pathogenesis of NASH [42].

Moreover, these animals displayed a NASH like phenotype similar to human NASH with significant increase in hepatic reactive oxygen species (ROS) [43], Furthermore, a study by **Ishimoto et al.**, [44] showed that with mice administration of sucrose with high-fat foods leads to a synergistic effect that may induce liver inflammation and fibrogenesis.

In the current study, rats fed HCHFD for eight weeks showed significant hyperlipidemia in the form of elevated serum triglycerides and total cholesterol, elevation of liver enzymes, but with no significant difference in serum albumin or bilirubin; Furthermore, histological examination of liver showed typical changes found in NAFLD in the form of marked steatosis, ballooning and foci of inflammatory infiltrate with average NAS score of  $(6.1 \pm 0.56)$  with fibrosis in the some rats however fibrosis not rich significant level, these histological changes were associated with significant elevation of hepatic oxidative stress

parameters, increased NF- $\kappa$ B-p65 expression and decreased expression of PPAR $\alpha$  and NFKBIA, Alteration in biochemical parameters combined with pathologic features of the liver, indicates that the experimental preparation of NASH rat model using HCHFD in the present work was successful.

Accumulation of lipids inside hepatocytes is considered as the 'first hit' in the 'two-hit theory' assumed by **Day and Jones**, [4]. In the present study, steatosis was confirmed by histopathological examination under light microscope either by H&E showing Marked macro vesicular and micro vesicular steatosis or Oil O red stain showing significant increase in distribution of hepatocyte which were full of lipid droplet of different sizes, in addition to these histopathological changes there were concomitant hyperlipidemia in NASH-induced group in the form of significant increase in serum triglycerides and total cholesterol.

Lipid accumulation in the hepatocytes in our work could be attributed to two mechanisms; (i) excessive fatty acids uptake which is achieved in present work through high fat diet and de novo lipogenesis in liver [45] which is achieved also in the present work through fructose consumption [41]; fructose metabolism in the liver increases cholesterol biosynthesis [46] and activates enzyme that catalyzes the final step in TG synthesis [47]. (ii) The other mechanism of steatosis is impaired beta fatty acid oxidation [12]. PPAR- $\alpha$  is the nuclear which is the nuclear receptors responsible for hepatic lipid metabolism and fatty acid oxidation [48].

In the current work, assessment of PPAR- $\alpha$  gene expression using RT-PCR revealed that there was

down regulation of PPAR- $\alpha$  in liver tissue of NASH group as compared to negative control group, this finding is in agreement with many investigators ; Mandard et al ., [49] found that there was significant decrease in the expression of hepatic PPAR- $\alpha$  in NAFLD . Ning et al ., [28] found also, down regulation of hepatic PPAR- $\alpha$  in a NASH model of diabetic rat .Treatment with vitamin D3 in the present work resulted in partial correction of hyperlipidemia as shown by significant decrease in serum triglycerides and cholesterol as well as decrease lipid accumulation in the hepatocytes as assessed by histopathological examination.

We assumed that this effect is mediated through PPAR- $\alpha$  pathway and to prove this RT-PCR for mRNA of PPAR- $\alpha$  was done ; we found that there was significant down regulation of hepatic expression of PPAR- $\alpha$  mRNA in the liver cells of NASH group and this down regulation was reversed in the vitamin D3 treated group .This unique role of PPAR- $\alpha$  in correction of lipid abnormality in NASH had been proved by many investigators ; Ning et al., [28] mentioned that there was correction of lipid abnormalities as well as hepatic steatosis by vitamin D3 through increase hepatic PPAR- $\alpha$  gene expression .Also Yin et al.,[26] stated that 1,25(OH)2D3 has a significantly lower serum lipid levels ,decreasing hepatic lipid uptake, hence attenuating steatosis .These above mentioned finding support the therapeutic effect of vitamin D3 through PPAR- $\alpha$  pathway in correction of hyperlipidemia and amelioration of hepatic steatosis which is the first hit in NASH pathogenesis

Interestingly, our data about liver enzymes in the current work revealed that, there was elevation of AST (more than 3 folds) and ALT (nearly 2 folds) and alkaline phosphatase (2 folds), indicating that there was a state of hepatic injury [50] . The use of liver enzymes to diagnose severity of NASH is a matter of controversy, Marchesini et al.,[51] and Sorbi et al , [52] mentioned that there is increased levels of transaminases in NAFLD , with ratio of ALT to AST more than one and this classical ratio can be used to distinguish between steatosis of NAFLD and alcoholic fatty liver disease (AFLD) , with the latter having by a high AST:ALT ratio; but as NAFLD progress from hepatic steatosis to NASH and hepatic fibrosis ; AST exceeds ALT resulting in increase in ratio AST :ALT ,and this is consistent with the finding of our work . Browning et al., [53] stated that the elevated aminotransferases are not sensitive for detection of hepatic steatosis and it may be normal in patients with advanced fibrosis and cirrhosis .Furthermore; Calvaruso and Craxi, [54] stated that normal liver enzymes could exist with underlying hepatic insult and Harrison et al., [55] attributed the difference of basal ALT/AST levels to variation of mouse strains, gender, age, and dietary condition during the experiment such diet ingredient , percent of fat, and duration of experiment

According to the two-hit theory, the transition from simple steatosis to NASH requires the contributors to the second hit such as oxidative stress associated with lipid peroxidation and mitochondrial dysfunction [7] and proinflammatory mediators [5].

In line with previous studies [56, 57] we found that livers of NASH group had a significant increase in MDA (marker of increased lipid peroxidation) and significant decrease in SOD, and GSH which are considered free radical scavengers; these findings indicate that there was a condition of oxidative stress and provide good proof for the role of this oxidative stress condition in the pathophysiology of NASH. Also, it has been suggested that ROS in that mitochondrial ROS deplete hepatic antioxidants allowing more accumulation of ROS [58]. In the present study, subsequent treatment with vitamin D3 significantly ameliorated oxidative stress as evidenced by lowering MDA level and increased GSH and SOD levels relative to NASH group. The antioxidant effect of vitamin D3 had been proved in various experimental models [59, 60, and 61]. Also, **Saggese et al.**, [59] confirmed this effect in diabetic rat through reduction of lipid peroxidation and enhancement of free radical scavengers such as glutathione peroxidase and catalase, besides the antioxidant effect of Vitamin D3 may be also mediated through PPAR- $\alpha$  up regulation as mentioned by **Toyama et al.**, [62]. Who found that PPAR- $\alpha$  directly upregulated catalase expression, thus alleviating hepatocytes oxidative stress state in a rat model of fibrosis due to thioacetamide.

As regard the effect of Vitamin D3 on oxidative stress in NASH model, **Roth et al.**, [63] stated that NAFLD progression after a high-fat diet was exacerbated by deficiency of vitamin D, through the activation of toll-like receptors, leading to more hepatic inflammation accompanied by increase in oxidative stress parameters. Moreover,

in a randomized clinical trial; vitamin D treatment of adults with NAFLD resulted in reduced serum MDA, liver enzymes, oxidative stress, and reduced the expression of inflammatory biomarkers [64]. Therefore, it may be concluded that beneficial effect of vitamin D3 is at partially mediated through its antioxidant property as a result of PPAR- $\alpha$  up regulation.

The transcription factor NF- $\kappa$  B is responsible for inflammatory, immune and stress response [19]. NF- $\kappa$  B might play crucial role throughout the course of NAFLD [21]. Normally in non-stimulated cells; I $\kappa$ B $\alpha$  (NFKBIA) inhibitory proteins interacts with NF- $\kappa$  B, sequestering it in the cytoplasm and preventing its translocation into the nucleus; thus hindering activation of NF- $\kappa$  B target gene transcription. In response to cell stimulation, I $\kappa$ B $\alpha$  undergoes degradation, allowing translocation of NF- $\kappa$  B into the nucleus and activation of target genes [65].

We hypothesize that vitamin D has anti-inflammatory effect through regulation NF- $\kappa$  B-p65 expression and to prove this assumption; immunohistochemistry of NF- $\kappa$  B-p65 was done in the present work; which revealed marked increase in nuclear NF- $\kappa$  B-p65 immunohistochemical nuclear brownish staining in hepatocytes in NASH group when compared to negative control group and this indicates increased nuclear NF- $\kappa$  B translocation with subsequently activation of downstream genes and treatment with vitamin D3 decreased this nuclear staining but unexpectedly, this was accompanied by increased granular cytoplasmic staining in hepatocytes. This effect of vitamin D3 on NF- $\kappa$  B-p65 expression could be

explained by **Cohen-Lahav et al.**, [66]., who found that vitamin D had no effect on total protein or mRNA levels of NF- $\kappa$  B -p65 but did decrease its nuclear translocation with concomitant increase cytoplasmic NF $\kappa$ B-p65 levels .

In current work to investigate the underlying mechanisms of inhibition of NF- $\kappa$  B; two parameters were assessed through RT-PCR technique , NFKBIA and PPAR  $-\alpha$  mRNA expression; it revealed that in NASH group there was down regulation of both genes and this down regulation was reversed by Vitamin D3 treatment .This inhibition of NF- $\kappa$  B by Vitamin D3 could be attributed to two mechanism ;first up regulation of NFKBIA with subsequent dimerization with NF- $\kappa$  B in cytoplasmic and prevention of nuclear translocation .This finding is in accordance with **Cohen-Lahav et al .**, [66] ,who mentioned that 1,25(OH)2D3 was shown to up-regulate the inhibitor of NF $\kappa$ B (I $\kappa$ B- $\alpha$ ) by increasing mRNA stability and decreasing I $\kappa$ B - $\alpha$  phosphorylation .The second mechanism is PPAR $\alpha$  up regulation by vitamin D3 which by turn up regulates downstream gene NFKBIA, leading to subsequent inhibition of NF- $\kappa$  B nuclear translocation ,this finding is in agreement with **Larter et al.**, [67] who stated that treatment with Wy 14 643 (a PPAR- $\alpha$  agonist) in diabetic mice with NASH, upregulates NFKBIA and decreased nuclear levels of NF- $\kappa$  B p65 which resulted in decrease in mRNA of TNF- $\alpha$ , NCF2 and MCP1 resulting in decrease in the numbers of neutrophils and macrophages. As a limitation of this study; there was no assessment of Vitamin D3 serum level among all experimental groups ;also, vitamin D3 protective effect against fibrosis could not be

assessed because there was fibrosis but not occur in all rat and so no significant difference among all groups ; this could be attributed to short duration of study. So ,we suggest future studies with longer duration to assess effect on fibrosis and if there is long term complications of vitamin D3 as a potential therapeutic agent for NAFLD .

### Conclusion:

In summary, the major finding of our study is that HCHFD model of NASH is a perfect model and provides valuable insight into NAFLD pathogenesis and that Vitamin D3 has a beneficial effect against NASH development rat at different levels of the two hit theory, through PPAR $\alpha$  and NFKBIA up regulation which suppress NF- $\kappa$  B signaling pathway; all of these support the protective role of Vitamin D3 in amelioration NASH pathogenic changes.

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