Original Article

Prophylactic Targeting of Non-Alcoholic Fatty Liver Disease by Sirt-1 Activator (Resveratrol) and 1, 25 Dihydroxy-Vitamin D3 in Rats

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Abstract

Introduction: Non-alcoholic fatty liver disease (NAFLD) is considered to be the hepatic component of the metabolic syndrome (MS), several studies tried to prevent it. The current study aims is to study the possible effect of resveratrol (RSV), and 1, 25 Dihydroxy-vitamin D3 on NAFLD in rats model. **Methods:** Forty male albino rats were assigned into four groups, group I (normal control) that were fed normal diet (n= 10), group II (n=10) received high-fat diet(HFD) for 16 week, group III (n=10) received HFD, and RSV (30mg\kg) daily by orogastric catheter for 16 week, group IV (n=10) received HFD, and Vitamin D3 (5ug\kg body weight), intraperitoneal injection, twice per week for 16 week. **Results:** Resveratrol significantly decreases liver enzymes levels, lipid profile, increased liver superoxide dismutase (SOD), decreased liver malondialdehyde (MDA), and increased liver SIRT1 activity, good improvement in hepatic steatosis. Vitamin D3 also decreases liver function markers, lipid profile, increased liver SOD, decreased liver MDA, and increased SIRT1 activity, slight improvement in hepatic steatosis. **Conclusion:** Resveratrol may be a potential therapeutic target for treating NAFLD, and vitamin D had a hepatoprotective effect that could be mediated through antioxidant and anti-inflammatory effects.

Key words: NAFLD, NASH, Resveratrol, SIRT1, 1, 25 dihydroxy vitamin D3, oxidative stress.

Introduction

Non-alcoholic fatty liver disease (NAFLD) refers to a condition defined by ectopic fat accumulation in the form of triacylglycride (TG) in the liver. (1)

The major risk factors for NAFLD are the same as the components of the metabolic syndrome (MS): central obesity, T2DM, dyslipidemia and insulin resistance (IR). Today, NAFLD is considered to be the hepatic component of the MS. (2)

The clinical spectrum of NAFLD ranges from simple fatty change to nonalcoholic steatohepatitis (NASH), which is characterized by cytolytic changes in hepatocytes (i.e. ballooning degeneration, Mallory bodies, and lobular inflammation). Although NAFLD is mostly benign, 20–30% of the patients develop NASH. (3)

The homeostasis of fat and energy in hepatic cells is regulated by mitochondrial activities, including beta-oxidation of free fatty acids (FFAs), electron transfer and production of adenosine triphosphate (ATP), and reactive oxygen species (ROS). Mitochondrial abnormalities alter the balance between pro-oxidant and antioxidant mechanisms, leading to

the blockade of fatty acid beta-oxidation and the consequent induction of ROS production. (3)

Activation of SIRT1 could serve as an effective therapeutic approach for preventing the development of fatty liver diseases at all stages, including the onset, progression and complication, resveratrol was identified as direct SIRT1 activator for the first time. (5)

Resveratrol (RSV) (3,5,4′-trihydroxy-trans-stilbene) is a stilbenoid, a type of natural phenol, and a phytoalexin produced by several plants in response to injury or, when the plant is under attack by pathogens such as bacteria or fungi. (6)

Through the activation of AMP-activated protein kinase (AMPK), SIRT1 and alternative routes including anti-inflammatory and anti-oxidant actions, RSV may inhibit the development or progression of steatosis and steatohepatitis.(10)

Vitamin D is a fat-soluble steroid hormone involved in many functions of mineralization, cellular growth, and decreasing the risk for chronic illnesses such as diabetes, cardiovascular disease, cancer, obesity, and autoimmune disease.(11)

Aim of the Work

The aim of present work was to assess the possible protective effect of SIRT-1 activator (resveratrol) and 1, 25-dihydroxy vitamin D_3 in a rat model of non-alcoholic fatty liver disease (NAFLD).

Materials and Methods

This study was carried out on 40 male Wistar albino rats with body weight 150-200 grams. The animals were housed in standard cages, ten per cage, at room temperature, with a 12 h light-dark cycle. All procedures involving the animals were conducted in accordance with the protocol approved by the ethics Committee, Faculty of Medicine, Alexandria University.

The duration of this study was 16 weeks for all groups. Rats were divided randomly into 4 experimental groups; each of it included ten rats:

Group 1 (control group): fed on standard rat chow containing carbohydrates 51%, protein 16%, vitamins and minerals 4%, and lipids 3%, the standard diet contained 2.9 Kcal per 1g of diet for 16 week.(16)

Group 2 (NAFLD group): fed on high-fat chow from the first week, containing carbohydrate 24.55%, protein 14.47%, fat 60.98%, presenting a total of 5.28 Kcal per 1 g of diet, for 16 week.(17)

Group 3 (NAFLD+Resveratrol group): fed on high-fat chow from first week as previous group and received Resveratrol (obtained from Sigma company) 10mg daily by oral route through an orogastric catheter, Resveratrol was diluted in 1ml of distilled water for 16 week.(16)

Group 4 (NAFLD+Vitamin D3 group): fed on high-fat chow from the first week and treated with 1, 25(OH) 2D3 (obtained from Minapharm in Egypt) 5μg/kg body weight, intraperitoneal injection, twice per week from the first week to 16th week. (17)

After 16 weeks of experiment, all animals were fasted for 14 hour. Then animals were sacrificed and blood was collected into non-heparinized tubes then serum was separated by centrifugation at 3000 rpm for 15 minutes. The serum was separated and stored at -20^{0}C until biochemical parameters assay:

Serum liver enzymes; alanine aminotransferase (ALT) and aspartate aminotransferase (AST) by colorimetric activity.(18)

1- Fasting plasma total cholesterol and triglyceride (TG) levels.(19)

The liver tissues were quickly removed, washed with saline and cut into pieces. One gram of liver was homogenized with 9 volumes of phosphate buffer (0.1 M, PH7.9) and then centrifuged at 400 Xg for 15 minutes and the supernatant was stored to be used as a liver homogenate for determination for:

- Hepatic tissue level of superoxide dismutase (SOD).[20]

- Hepatic tissue level of Malondialdehyde (MDA) as an indicator of lipid peroxidation by TBARS.[20]

- Hepatic tissue level of SIRT1 by ELISA assay.[21]

The right lobe of liver from control, NAFL group and treated groups was excised and then fixed in 10% formalin solution then processed to be stained routinely with Hematoxylin and Eosin. The prepared slides were examined under light microscope using objective lens power (X100) for histological examination.

Results

Table (I): Serum levels of aspartate aminotransferase (AST)(U\L) in the different studied groups

AST (U/L)	**Group1 (Control) (n = 10)**	**Group2 (NAFLD) (n = 10)**	**Group3 (NAFLD+ Resveratrol) (n = 10)**	**Group4 (NAFLD + Vit D3) (n = 10)**	**F**	**P**
Min. – Max.	37.8 – 55	104.2 – 159.7	43.1 – 60	80.1 – 100	123.924*	<0.001*
Mean ± SD.	45.47 ± 5.34	126.35 ± 19.38	50.6 ± 5.19	92.45 ± 6.1		
$p_{control}$		<0.001*	0.716	<0.001*		
Sig. bet. grps.		p_1<0.001*,p_2<0.001*,p_3<0.001*				

H: H for **Kruskal Wallis test,** Pairwise comparison bet. each 2 groups was done using **Post Hoc Test (Dunn's for multiple comparisons test)**

P: p value for comparing between the different groups

Pcontrol: p value for comparing between **control** and each groups

p_1: p value for comparing between NAFLD and NAFLD + Resveratrol

p_2: p value for comparing between NAFLD and NAFLD + Vit D

p_3: p value for comparing between NAFLD + Resveratrol and NAFLD + Vit D

*: Statistically significant at $p \leq 0.05$

Table (II): Serum levels of alanine amino transferase (ALT) (U\L) in the different studied groups

ALT (U/L)	Group1 (Control) (n = 10)	Group2 (NAFLD) (n = 10)	Group3 (NAFLD+ Resveratrol) (n = 10)	Group4 (NAFLD + Vit D3) (n = 10)	F	P
Min. – Max.	30.1 – 50	65.6 – 95.1	50.1 – 63	51.2 – 73	45.244*	<0.001*
Mean ± SD.	40.4 ± 6	78.56 ± 10.59	55.34 ± 4.42	60.33 ± 7.16		
$p_{control}$		<0.001*	<0.001*	<0.001*		
Sig. bet. grps.		p_1<0.001*,p_2<0.001*,p_3=0.443				

H: H for **Kruskal Wallis test,** Pairwise comparison bet. each 2 groups was done using **Post Hoc Test (Dunn's for multiple comparisons test)**

P: p value for comparing between the different groups

Pcontrol: p value for comparing between **control** and each groups

p_1: p value for comparing between NAFLD and NAFLD + Resveratrol

p_2: p value for comparing between NAFLD and NAFLD + Vit D

p_3: p value for comparing between NAFLD + Resveratrol and NAFLD + Vit D

*: Statistically significant at $p \leq 0.05$

Table (III): Serum level of triglycerides (TG) (mg\dl) in the different studied groups

TG (mg/dl)	Control (n = 10)	NAFLD (n = 10)	NAFLD+ Resveratrol (n = 10)	NAFLD + Vit D (n = 10)	F	P
Min. – Max.	112.7–198	189–246.1	160.1–191	169.0 – 205.5	14.788*	<0.001*
Mean ± SD.	160.18±25.58	214.11±19.74	179.69±11.96	186.68±12.65		
$p_{control}$		<0.001*	0.100	0.014*		
Sig. bet. grps.		p_1=0.001*,p_2=0.010*,p_3=0.829				

F: **F for ANOVA test**, Pairwise comparison bet. each 2 groups was done using **Post Hoc Test (Tukey)**

P: p value for comparing between the different groups

Pcontrol: p value for comparing between **control** and each group

p_1: p value for comparing between NAFLD and NAFLD + Resveratrol

p_2: p value for comparing between NAFLD and NAFLD + Vit D

p_3: p value for comparing between NAFLD + Resveratrol and NAFLD + Vit D

*: Statistically significant at $p \leq 0.05$

Table (IV): Serum levels of cholesterol (mg\dl) in the different studied groups

Cholesterol (mg/dl)	Control (n = 10)	NAFLD (n = 10)	NAFLD+ Resveratrol (n = 10)	NAFLD + Vit D (n = 10)	F	P
Min. – Max.	103.5 – 150.2	170.3 – 209.7	109.9 – 185.0	147.0 – 190.0	26.544*	<0.001*
Mean ± SD.	125.9 ± 16.18	188.7 ± 11.69	144.5 ± 22.29	162.61±13.52		
$p_{control}$		<0.001*	0.072	<0.001*		
Sig. bet. grps.		p_1<0.001*,p_2=0.006*,p_3=0.083				

F: **F for ANOVA test**, Pairwise comparison bet. each 2 groups was done using **Post Hoc Test (Tukey)**

P: p value for comparing between the different groups

Pcontrol: p value for comparing between **control** and each group

p_1: p value for comparing between NAFLD and NAFLD + Resveratrol

p_2: p value for comparing between NAFLD and NAFLD + Vit D

p_3: p value for comparing between NAFLD + Resveratrol and NAFLD + Vit D

*: Statistically significant at $p \leq 0.05$

Table (V): Hepatic tissue level of superoxide dismutase (SOD) (u\gm protein)

Liver level of SOD (u\gm protein)	Control (n = 10)	NAFLD (n = 10)	NAFLD+ Resveratrol (n = 10)	NAFLD + Vit D (n = 10)	F	P
Min. – Max.	19.20 – 34.70	12.30 – 19.20	15.10 – 33.0	16.20 – 27.70	18.238*	<0.001*
Mean ± SD.	28.27±4.62	14.89±2.23	26.10±6.29	21.07±3.43		
$p_{control}$		<0.001*	0.691	0.004*		
Sig. bet. grps.		p_1<0.001*,p_2=0.017*,p_3=0.068				

F: **F for ANOVA test**, Pairwise comparison bet. each 2 groups was done using **Post Hoc Test (Tukey)**

P: p value for comparing between the different groups

Pcontrol: p value for comparing between **control** and each group

p_1: p value for comparing between NAFLD and NAFLD + Resveratrol

p_2: p value for comparing between NAFLD and NAFLD + Vit D

p_3: p value for comparing between NAFLD + Resveratrol and NAFLD + Vit D

*: Statistically significant at $p \leq 0.05$

Table (VI): Hepatic tissue level of manoldehyde (MDA) (nmol\gm tissue)

Liver level of MDA (nmol/gm tissue)	Control (n = 10)	NAFLD (n = 10)	NAFLD+ Resveratrol (n = 10)	NAFLD + Vit D (n = 10)	F	P
Min. – Max.	8.6 – 16.9	13.5 – 40	12 – 21.8	13.0 – 32.90	14.117*	<0.001*
Mean ± SD.	12.89 ± 3.1	29.34 ± 9.69	16.9 ± 3.51	24.57 ± 6.31		
$p_{control}$		<0.001*	0.485	0.001*		
Sig. bet. grps.		p_1<0.001*,p_2=0.334,p_3=0.044*				

F: **F for ANOVA test**, Pairwise comparison bet. each 2 groups was done using **Post Hoc Test (Tukey)**

P: p value for comparing between the different groups

Pcontrol: p value for comparing between **control** and each group

p_1: p value for comparing between NAFLD and NAFLD + Resveratrol

p_2: p value for comparing between NAFLD and NAFLD + Vit D

p_3: p value for comparing between NAFLD + Resveratrol and NAFLD + Vit D

*: Statistically significant at $p \leq 0.05$

Table (VII): Hepatic tissue level of SIRT1 (lu\ml) in the different studied groups

Hepatic tissue level of SIRT1 (Iu/ml)	Group1 (Control) (n = 10)	Group2 (NAFLD) (n = 10)	Group3 (NAFLD+ Resveratrol) (n = 10)	Group4 (NAFLD + Vit D3) (n = 10)	F	p
Min. – Max.	2.6 – 6.3	0.8 – 4.2	2.50 – 5.50	2.5 – 5.1	4.452*	0.009*
Mean ± SD.	4.15 ± 1.3	2.55 ± 1.13	4.05 ± 0.98	3.74 ± 0.97		
$p_{control}$		0.013*	0.997	0.839		
Sig. bet. grps.		p_1=0.022*,p_2=0.093,p_3=0.922				

F: **F for ANOVA test**, Pairwise comparison bet. each 2 groups was done using **Post Hoc Test (Tukey)**

P: p value for comparing between the different groups

Pcontrol: p value for comparing between **control** and each group

p_1: p value for comparing between NAFLD and NAFLD + Resveratrol

p_2: p value for comparing between NAFLD and NAFLD + Vit D

p_3: p value for comparing between NAFLD + Resveratrol and NAFLD + Vit D

*: Statistically significant at $p \leq 0.05$

Histological results

Light microscopic examination of liver sections from the control group (Group 1) showed normal liver architecture (Figures 1).

Liver examination of NAFLD group showed, disturbed liver architecture. Bridging fibrosis between portal tracts, Hepatocytes revealed microvesicular, macrovesicular steatosis and hepatocellular ballooning. (Figure 2).

Liver sections from Resveratrol group, showed a good hepatic architecture. Most hepatocytes appeared normal except for few with rarefied cytoplasm, macrovesicular and microvesicular steatosis. (Figure 3).

Liver sections from vitamin D group, showed disturbed liver architecture. Hepatocytes showed microsteatosis, macrovesicular steatosis, hepatocellular ballooning and rarefied cytoplasm. Mallory's bodies were seen within hepatocytes. (Figure 4).

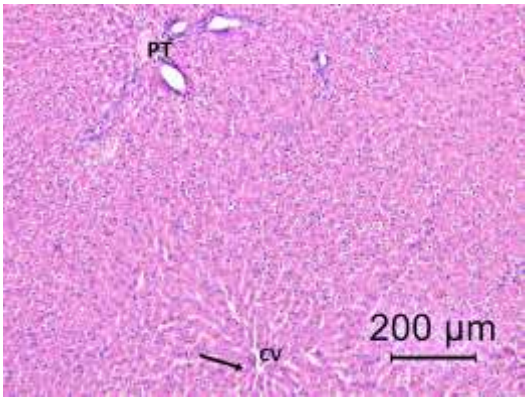

Figure (1): A photomicrograph of rat liver of control group, showing normal liver architecture; cords of hepatocytes (thin black arrow) are seen radiating from the central vein (CV) and separated by blood sinusoids. Notice the portal tract (PT) at the periphery of the hepatic lobule enclosed by a very little amount of connective tissue.

(H&E stain, Mic. Mag. × 100)

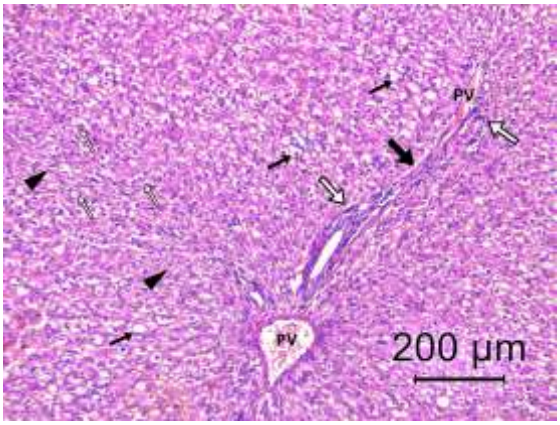

Figure (2): A photomicrograph of a rat liver of NAFLD group, showing bridging fibrosis between portal tracts (thick black arrow).Hepatocytes showing macrovesicular steatosis (black arrow head), hepatocellular ballooning (thin black arrow) and Mallory's bodies (thin white arrow). Notice periportal cellular infiltration (thick white arrow).

(H&E stain, Mic. Mag. × 100)

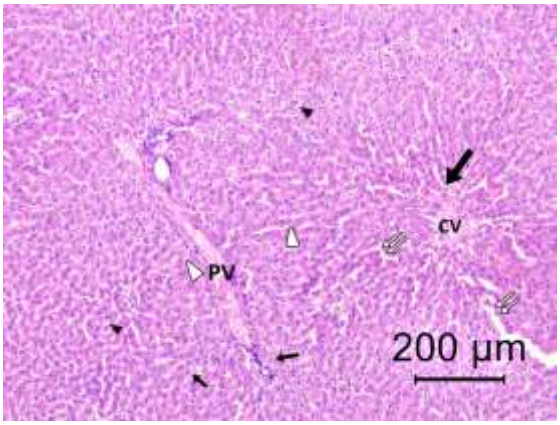

Figure (3) A photomicrograph of a rat liver from Resveratol group, showing good liver architecture; where cords of hepatocytes (thick black arrow) are radiating from central vein (CV). Some blood sinusoids are dilated (double thin white arrow). Microvesicular steatosis (white arrow head), and rarefied cytoplasm (thin black arrow).

(H&E stain, Mic. Mag. × 100)

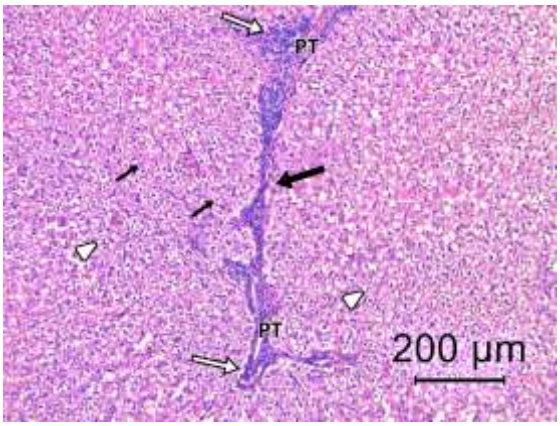

Figure (4): A photomicrograph of a rat liver from Vitamin D group, showing disturbed liver architecture. Hepatocytes show macrovesicular steatosis (white arrow head) and rarefied cytoplasm (thin black arrow). Bridging fibrosis (thick black arrow). Notice periportal inflammatory cellular infiltration (thick white arrow).

(H&E stain, Mic. Mag. × 100)

Discussion

Non-alcoholic fatty liver disease (NAFLD) is the most common form of chronic liver diseases. Two thirds of the patients are asymptomatic. It is characterized by the accumulation of triglycerides in the liver and spans a histological spectrum of liver disease, ranging from simple steatosis to steatohepatitis, fibrosis and rarely to cirrhosis or even hepatocellular carcinoma.[22]

The current work showed a significant increase in serum liver function markers (AST, ALT) in NAFLD group when compared to control rats. This elevation is attributed to lipid deposition in liver causing damage of hepatocytes. [23] Moreover, these changes coincide with the histopathological changes of hepatic tissues observed in the present work which showed loss of the normal liver architecture.

Moreover, rats on HFD received resveratrol caused significant decrease in liver enzymes (AST, ALT). Ren et al, [24] reported that elevated serum level of ALT is a marker of inflammation and oxidative stress and AST is also related to oxidative stress. Therefore, the study may be attributed to the anti-inflammatory and antioxidant effect of RSV. [25]

In the present study, rats received preventive vitamin D3 decrease liver enzymes, this is may be attributed to antioxidant effect of vitamin D3,[27] similar results was found by Zhu et al,[19] as they found that both serum ALT and AST, in rats received vitamin D3 were considerably reduced compared to those in NAFLD.

On contrary, Chachay et al, [28] reported that levels of alanine and aspartate aminotransferases increased significantly in resveratrol group. Hariri et al,[15] showed that no significant effect of Vitamin D on liver enzymes except one which revealed that Vitamin D together with calcium carbonate can reduce liver enzymes.

In current study, there was a significant elevation of serum TG, and cholesterol in NAFLD group when compared to control group. These alterations were described by study done by Meryem et al,[29] as they reported that HFD induces lipid and lipoproteins metabolic disorders, associated with abnormal expression of pathway enzymes lipid storage (lipoprotein lipase (LPL))and lipid mobilization enzyme [hormone-sensitive lipase (HSL)].

Administration of RSV to rats significantly improve serum TG, and cholesterol, Shang J et al,[30] observed that RSV improve in these parameters via AMPK activation and dowenregulation of SREBP-1c and FAS expressions, thus preventing lipid synthesis.

Also, administration of vitamin D3 to rats decreases serum TG, and cholesterol, this is may be attributed the vitamin D3 mediated reduction of serum triglycerides to the increases in serum calcium which enhancing intestinal calcium absorption. [32] Several mechanisms are suggested to explain the effect of calcium on lipids, including its reducing role in fatty acid absorption via the formation of insoluble calcium-fatty complexes in the gut. [33]

Regarding to lipid peroxidation and antioxidant, oxidative stress, beta-oxidation of fatty acids and the resultant oxidants such as ROS are among the important mechanisms in the development of NAFLD, especially for NASH. High hepatic MDA levels and lower SOD levels in NAFLD group, in agreement with other studies. [34]

The antioxidant effects of RSV were demonstrated in our study that RSV leads to enhanced levels of SOD, and reduced level of MDA, which was attributed to the important mechanism, is the complex interaction of SIRT1 with FOXO transcription factors which leads to production of ROS-detoxifying enzymes, including SOD, and catalase. [35]The current results were supported by Bujanda et al. [20]

Administration of vitamin D significantly decreased MDA tissue level compared to NAFLD group. Zhu et al, [19] hypothesized that vitamin D would enhance antioxidant capacity by modulating Nuclear factor-erythroid-2-

related factor 2 (Nrf2) to combat oxidative stress.

As regard the results of the hepatic levels of SIRT1: The data of the present work showed that SIRT1 expression was significantly decreased in the NAFLD group compared with the control group. Previous study, they found that SIRT1 values were significantly lower in fat liver infiltration, although they have no definitive explanation for this expression.[36] This is agreement with study conducted by Mariani et al, [37] showing that the fatty liver infiltration had lower amount of SIRT1 level compared with no NAFLD affected group.

In addition, the present study revealed that RSV supplementation a significantly increased in SIRT1 levels. These finding is running in parallel with a previous study, RSV administration apparently restored SIRT1 levels in liver of HFD fed mice, simultaneously RSV suppressed the expression of the indicated LD associated genes, leading to suppression of hepatic lipid content, so RSV decrease expression of LD associated genes in liver and hepatocytes through the SIRT1-mediated signaling pathway.[38]

The present study given 1,25(OH)3D increases SIRT1 activity .These results suggest that vitamin D might promote fat mobilization and hence decrease intracellular fat accumulation and increase lipolysis, concurrently with an increase of activity in SIRT1.[40]

Supporting these findings Chang et al,[41] revealed that 1,25(OH)3D increased SIRT1 expression and activity, and proposes SIRT1 activation is closely related to the activation of a steroid hormone receptor, vitamin D receptor (VDR), which mediates 1,25(OH)3D-induced genomic changes.

Finally, the result of the present study was confirmed by the histological examination, the HFD diet feeding induced loss of the normal liver architecture. Bridging fibrosis between portal tracts, and lobular inflammatory cellular infiltrates, macro, micro steatosis were noticed. The hepatic steatosis induced by increasing the FFA load imposed on the liver and reducing fatty acid-β oxidation. [49, 50] Also these changes are in agreement with Sampey et al [51]

Administration of resveratrol show most hepatocytes appeared normal except for few with rarefied cytoplasm, macrovesicular and microvesicular steatosis, no fibrosis was noticed.

The current result supported by Zhou et al[38] showed administration of resveratrol strongly attenuated hepatic steatosis this is attributed to that resveratrol suppress the expression of the indicated lipid droplet(LD) associated genes in liver by SIRT1 activity and upregulating SIRT1 expression.

Administration of vitamin D showed a slight improvement when compared to HFD group which was agreement with Barcheta et al [52]. The protective effects of vitamin D may be related to a reduction in oxidative stress and an increase in antioxidant capacity.

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