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ORIGINAL ARTICLE

Adropin is Involved in the Ameliorative Effect of Chronic Exercise in Non-alcoholic Fatty Liver Rat Model

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ABSTRACT

Background: *Non-alcoholic fatty liver disease (NAFLD) is characterized by abnormal fat metabolism in the liver due to non-alcoholic causes. Adropin is a peptide hormone with an essential role in maintaining metabolic homeostasis.* ***Methods:*** *42 male rats were divided into 2 groups, each group was subdivided into 3 subgroups: group Ia: control 4 weeks; group Ib: high fat diet (HFD) 4 weeks, group Ic: HFD 4 weeks +exercise; group IIa: control 12 weeks, group IIb: HFD 12 weeks; and group IIc: HFD 12 weeks + exercise. Serum Adropin, glucose, HOMA-IR, insulin, lipid profile, ALT, AST, TNFα, IL6, hepatic ROS, MDA, and SOD were measured. Histopathological examination, immunohistochemical study, and Mallory's trichrome staining of the liver were performed.*

Results: *significant increases in insulin resistance, lipid profile parameters, ALT, AST, TNFα, IL6, hepatic ROS, MDA, and a decrease in SOD were found in HFD groups. Histopathological examination revealed steatosis in group Ib and steatohepatitis in group IIb. These changes were associated with a decrease in serum adropin levels. Exercise in groups Ic and IIc improved these changes with a significant increase in adropin level.*

Conclusion: *HFD induced functional and structural deterioration in the liver, leading to NAFLD with a significant decrease in serum adropin level. Exercise induced a significant increase in serum adropin, which may have a role in the remission of hepatic inflammation, steatosis, and steatohepatitis.*

Keywords:
Adropin, non-alcoholic steatohepatitis, HOMA-IR, aerobic exercise

INTRODUCTION

One of the most common chronic liver disorders is non-alcoholic fatty liver disease [1]. It is strongly linked to essential obesity, hypertension, and dyslipidemia [2, 3]. Insulin resistance (IR) is considered the primary pathophysiologic mechanism initiating the gathering of triglycerides in the liver [4]. The primary insult in the pathogenesis of NAFLD is simple hepatic steatosis [3], but the succession to non-alcoholic steatohepatitis (NASH) requires the presence of extra pathophysiologic abnormalities. The outcome of reactive oxygen species (ROS) augments oxidative pressure inside hepatocytes and induces the secretion of inflammatory biomolecules, including cytokines, which mediate the succession from steatosis to steatohepatitis and finally fibrosis [5].

Adropin is "a nutrient-regulated metabolic hormone" revealed to support glucose oxidation above fatty acid oxidation in skeletal muscle, leading to a reduction in insulin resistance [6]. Altincik [7] found a reduction in adropin concentration accompanied obesity and insulin resistance, while weight loss increased the level of Adropin. Numerous studies have focused on the association of adropin with NAFLD, but there are still great discrepancies considering its level in liver diseases. Some investigators revealed an extensively lower adropin level in patients with

NAFLD than both overweight people without NAFLD and healthy controls [8, 9]. Related results were reported by Chen in mice [10]. On the other hand, other studies showed an increase in serum adropin levels in liver cirrhotic and hepatitis patients [11, 12].

Inactive life is linked to the severity of fatty liver disease irrespective of body mass, so rising physical movement through exercise can improve fatty liver disease independent of loss of weight [13, 14]. Several earlier studies that looked at the connection between exercise and adropin concentration came up with conflicting results. Some investigators found an increase in adropin levels associated with improvement of insulin resistance after 6 weeks of training [15]. Others reported a significant increase in adropin level after regular aerobic exercise training in overweight teenagers independent of body mass loss [16, 17]. On the other hand, other investigators found no effect of aerobic exercise on adropin levels [18] and Ozbay [19] detected a drop in serum adropin levels in chronic exercise. Therefore, this study was designed to examine the effect of chronic moderate bodily exercise on serum adropin level in NAFLD and the possible role of adropin in exercise mediated effects in a trial to address the previously mentioned discrepancy.

METHOD

Ethical statement

The research protocol received approval from the Institutional Review Board at Zagazig Medical Faculty, granting that the research followed instructions for lab animal use and maintenance (NIH publication No, 80-23, revised 1978) (approval number: 4562/22-4-2018).

Animals and experimental protocols

Forty-two healthy adult male albino rats of the local strain participated in the study (age "16 weeks" and weight "180- 200g "). Animals were kept in the Faculty of Medicine animal housing unit at Zagazig University. Animals had free access to water, kept in a roomy atmosphere on an ordinary lighting cycle. For initial accommodation for one week, rats were kept in animal house conditions prior to the start of the experiments [20]. The diet used in control groups was standard chow, which contained total calories of about 12.6kJ/g with the following composition; fat: 11.4%, carbohydrate: 62.8%, protein: 25.8 % [21]. HFD groups received HFD chow with a total energy content of 23.4kJ/g and the following composition; fat: 58.0%, carbohydrate: 24%, protein: 18 % according to Cha [22]. They were obtained from the Zagazig faculty of agriculture. Rats were separated randomly into two main groups. Group I: (n = 21) was then subdivided into the following subgroups (n = 7): group Ia: control 4 weeks; rats received 4 weeks of a normal chow diet, group Ib: HFD 4 weeks; rats were fed HFD for 4 weeks for the induction of simple steatosis (SS) [23], group Ic: HFD 4 weeks + exercise, in this group HFD for a duration of 4 weeks was given to rats for generation of simple steatosis. Afterward, they were subjected to a 12-week moderate exercise regimen [24]. Group II: (n=21) which was subdivided into 3 identical subgroups (n=7); group IIa: control 12 weeks; rats received a diet of normal chow for a duration of 12 weeks, group IIb: HFD 12 weeks; rats were on HFD for a duration of 12 weeks to induce NASH [23] and group IIc: HFD 12 weeks + exercise; for initiation of NASH, rats were fed HFD for a period of 12 weeks, then moderate exercise for another 12 weeks [24]. After induction of NAFLD in the exercise groups, rats continued on HFD till the end of the experiment.

Plan of swimming exercise

According to Lu [25], rats in exercise groups were primarily qualified for 15 minutes daily and period was gradually raised to 15 minutes daily until one hour of exercise per day was achieved. Swimming was finally performed one hour per day, 5 days each week, for duration of 12 consecutive weeks [26]. To avoid floating of the rats, water was mixed with a small quantity of detergent, and continuous agitation of the water was done [27]. The intensity of exercise is considered moderate if swimming is done in a continuous manner lacking floating or adding load to the rat body or tail [27]. When the exercise session was finished, a warm environment was required to keep dryness of rats.

Body mass index (BMI) and abdominal circumference (AC)

At the start and end points of the study, we estimated weight and length (nose to anus), then BMI was calculated: BMI (g/cm^2) = body weight (g) / $length^2$ (cm^2). The cutoff rate for obesity is above 0.68 g/cm^2 [28]. The waist circumference was assessed in the major region of the rat's abdomen [29].

Blood and liver samples collection

At end of study, cervical dislocation was done to animals under thiopental administration, and samples of blood were taken following overnight fasting. To reduce the acute effect of exercise, the final training setting was at least 2 days before scarifying the animals [24]. Blood samples were left to clot, centrifuged, and sera were collected and stored at -20 C.

After taking blood samples, laparotomy was conducted. Livers were quickly excised and cleaned completely.

Biochemical investigations

Serum adropin levels: using rat adropin ELISA enzyme-linked immunoassay kits (Sun Red Biotechnology, China, catalogue No 201-113361) according to manufacturer's instructions. Using commercially available kits, the following were estimated: Serum glucose according to Tietz [30]; serum insulin according to Temple [31]. The homeostasis model assessment of insulin resistance (HOMA-IR) was calculated: [insulin (μIU/mL) x glucose (mg/dl)/405] [32]. Serum total cholesterol (TC) levels: according to Flegg [33] and Allain [34]. Serum triglyceride (TG) levels: according to Nagele [35], Serum high density lipoprotein (HDL) levels: according to Warnick [36]. Serum low density lipoprotein (LDL) levels: LDL=TC-HDL-TG/5 according to Friedewald [37]. Serum very low density lipoprotein (VLDL) levels: VLDL= TG/5 according to Tietz [30]. Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels: according to Matsuzawa and Katunuma [38]. Serum interleukin-6 (IL-6): according to Song [39].Serum tumor necrosis factor alpha (TNF-alpha): according to Juhaz [40].

Liver Antioxidant System Evaluation:

One little section (200 mg) was sliced from each liver. After addition of saline proportionate to mass of tissue 1:9 (w/v), homogenization was done at 4˚C, the homogenate was centrifuged, and then, using the Sandwich-ELISA technique, all these parameters were estimated; hepatic tissue malondialdehyde (MDA), super oxide dismutase (SOD) as described by Satoh [41], reactive oxygen species (ROS) according to Comar [42].

Histopathological examination of the liver:

The other parts of rat livers had been immersed in a solution containing 10 present buffered formalin, then paraffin blocks were prepared and sectioned (five μm thick) and stained with hematoxylin and eosin [43]. Blindly, pathological examination was done and scored according to the Committee of NASH Pathological studies [44].

Histopathologic scores were interpreted as follows:

Steatosis was scored as (0: <5%, 1: 5%- 33%, 2: 33% -66%, 3: > 66%). Grading of hydropic degeneration: no hydropic degeneration: 0, <25%:1, 2 25% - 50%:2, > 50%: 3. Portal tract inflammation is scored as: 0 = no portal inflammation; 1 = scattering inflammatory cells in 1/3 portal tracts; 2 = increased inflammatory cells in 1/3–2/3 portal tracts; 3 = intensely stuffed inflammatory cells in more than 2/3 portal tracts.

Mallory's trichrome staining of hepatic tissue

Mallory's trichrome staining was done in the paraffin blocks to demonstrate the collagen fiber distribution around the portal triad, according to Suvarna [45].

2.9. Immuno-histochemical methods: liver sections of 5 μm thick were cut and buffered with 10-mM sodium citrate for antigen retrieval. Blocking the activity of internal peroxidase was done by using 0.9% hydrogen peroxide in absolute methanol. By addition of protein for 1 h, not related antibodies were blocked (ab64226, Abcam, Cambridge, UK). Incubation of sections was done via caspase-3 antibodies, rabbit monoclonal antibodies of IgG type antibody (Abcam, Cambridge, UK) 1:20 dilution, and rabbit monoclonal TNFα antibodies (Abcam, Cambridge, UK), 1:200 dilutions for 1 h. Sections were rinsed in phosphate buffered saline (PBS) then heated with rat IgG antibody (ab150160, Abcam, Cambridge, UK), washed in PBS and incubated with Novolink polymer (Novo-castra). Deletion of the primary antibody in the automated staining design was done for the 2 negative controls for caspase-3 and TNFα.

STATISTICAL ANALYSIS

SPSS program (version 18 for Windows) was used for processing statistical analysis (SPSS Inc., Chicago, IL, USA). Parametric data were expressed as mean ± SD, then we used the ANOVA test to compare means, followed by the student-least significant deference (LSD). Nonparametric data were recorded as median and inter quartile range (IQR) then medians were compared by Kruskall Wallis and Mann Whitney tests.

RESULTS

Biochemical results

Our results revealed a statistically significant and marked rise in BMI, AC, HOMA-IR, glucose, insulin, TC, TGs, LDL, VLDL, AST, and ALT in HFD groups (4 and 12 weeks) in opposition to control 4 weeks ($p<0.01$) and control 12 weeks ($p<0.001$) respectively. All the previous parameters were also significantly higher in HFD 12-week group (IIb) versus the HFD 4-week group (Ib) ($P<0.001$). We found a significant reduction in serum adropin and HDL in HFD 4 week and HFD 12 week groups versus control 4 week and 12 week groups, respectively, and versus each other ($P<0.001$). There was a significant rise in inflammatory markers (IL6 and TNF-α) and hepatic oxidative stress markers (MDA and ROS) in HFD 12-week group (IIb) in

opposition to control 12-week group (P<0.001). A significant decline in hepatic antioxidant marker SOD was found in the same group, comparable to the control (P<0.001) [table 1].

In exercise groups (Ic and IIc), HOMA-IR, glucose, insulin, TC, LDL, ALT, AST (P<0.001), TGs (P<0.01, p<0.001) and VLDL (P<0.05) decreased significantly compared to HFD (4 and 12 weeks), respectively. Also, serum adropin and HDL increased significantly in the same groups compared to HFD 4-week and 12-week groups, respectively (P<0.01). A significant decrease in serum IL6, TNFα (p<0.05) and hepatic tissue levels of oxidative markers MDA (P<0.001) and ROS (P<0.01) was detected in group IIc (HFD 12 weeks +exercise) versus the HFD 12 weeks' group. A significant rise in hepatic antioxidant marker SOD was established in the same group versus the HFD 12-week group (P<0.01). We found insignificant variation in BMI and AC in Ic and IIc groups (exercise groups) versus HFD 4 weeks and 12 weeks' groups, respectively (P>0.05) [table 1].

Histopathological results:

Histopathologic examinations of the liver revealed fatty penetration with a lack of necro-inflammatory lesion in the HFD 4-week group (Ib) compared to the control 4-week group (Ia) [fig. 1]. In the HFD 12-week group (IIb), foci of mixed inflammatory cell infiltration and hydropic degeneration were found versus the control 12-week group (IIa) [fig 2]. Pathologic scoring showed statistically significant variation in HFD 4 week and 12 week groups compared with control 4 week and 12 week groups, respectively, and also when compared with each other (P<0.001) [table 2]. On the other hand, exercise in groups Ic and IIc was associated with a significant decrease in histopathologic lesions and a scoring decrease comparable to HFD 4 weeks and 12 weeks (P<0.001) [table 2, figs 1&2].

Immuno-histochemical examination of liver:

Against monoclonal antibodies of TNF-alpha and Caspase-3 in all groups to clarify the localization of positive cells in all experimental groups. The immuno-positive reaction was localized in the cytoplasm of affected hepatocytes in the control groups for 4 weeks, and for 12 weeks of normal diet **(Fig4. 4a, 4d) and (Fig5. 5a,5d),** the liver tissue revealed negative immunoreactions against monoclonal antibodies to TNFα and Caspase-3. However, in the HFD 4-week group **(Fig. 4b, 5b),** few TNFα and few caspase-3 immune-positive hepatocytes were detected, which revealed a non-significant difference from the 4-week control group. Group Ic (HFD 4 weeks +exercise) revealed negative immunoreactions for TNFα **(Fig. 4c)** and caspase-3 **(Fig. 5c)**. However, the HFD 12-week group exhibited abundant TNFα **(Fig. 4e)** and caspase-3 **(Fig. 5e)** immuno-positive hepatocytes, which revealed a significant increase compared to control (12-week) group. On the contrary, group IIc (HFD 12 weeks +exercise) revealed few abundant TNFα and caspase-3 immuno-positive hepatocytes **(Fig.4F, 5f respectively)** that showed a significant decline in comparison to the HFD 12-weeks group.

We subsequently assessed the relationship between serum adropin and different measured variables in HFD 4 weeks and 12 weeks and exercise groups (Ic and IIc) [table 3]. Serum adropin showed a significant negative correlation with ALT, AST, TC, TGs, LDL, VLDL, glucose, insulin, the HOMA-IR index, IL6, TNF, hepatic tissue MDA, and ROS (P<0.05). However, there was a significant positive correlation between adropin and serum levels of HDL and hepatic tissue SOD (P<0.05). In addition, serum adropin had a significant negative association with histopathological scoring of steatosis, portal tract inflammatory cells, and hydropic degeneration of hepatocytes (P<0.05) [table 3].

Table 1: Measured biochemical parameters in all studied groups

Groups Parameters mean ± SD	Group Ia Control 4weeks	Group Ib HFD 4weeks	Group Ic HFD 4weeks +exercise	Group IIa Control 12weeks	Group IIb HFD 12weeks	Group IIc HFD 12weeks + exercise
Final BMI (g/cm^2)	0.49±0.07	0.75±0.07[a]	0.67±0.075[a]	0.61±0.07[a,b]	0.89±0.08[a,b,c,d]	0.80±0.16[a,c,d]
Final AC (cm)	17.57±1.02	21±1.9[a]	20.01±0.07[a]	18.01±0.66[b,c]	24.5±1.7[a,b,c,d]	23.86±1.95[a,b,c,d]
Serum ALT (U\L)	29.3±7.98	72.3±19.39[a]	33.71±8.67[b]	30.21±7.75[b]	131.39±12.36[a,b,c,d]	85.0±11.698[a,b,c,d,e]
Serum AST (U\L)	101.20±30.99	175.38±13.37[a]	107.14±29.4123[b]	103.28±31.1[b]	228.8±14.01[a,b,c,d]	174.38±12.31[a,c,d,e]
Serum TC (mg/dl)	90.0±8.34	185.7±13.31[a]	96.43±9.07[b]	94.14±8.35[b]	244.71±28.4[a,b,c,d]	140.57±7.52[a,b,c,d,e]
Serum TG	65.86±13.06	90.86±6.87[a]	76.86±5.27[b]	70.86±12.99[b,c]	131.0±7.39[a,b,c,d]	104.43±7.99[a,c,d,e]

Groups Parameters mean ± SD	Group Ia Control 4weeks	Group Ib HFD 4weeks	Group Ic HFD 4weeks +exercise	Group IIa Control 12weeks	Group IIb HFD 12weeks	Group IIc HFD 12weeks + exercise
(mg/dl)						
Serum HDL (mg/dl)	40.78±5.98	28.57±3.26[a]	36.36±3.10[b]	38.43±5.53[b]	20.28±5.65[a,b,c,d]	29.26±3.78[a,c,d,e]
Serum LDL (mg/dl)	36.04±11.5	136.97±15.28[a]	44.7.28±4.87[b]	41.54±11.5[b,c]	197.23±32.5[a,b,c,d]	90.41±2.14[a,b,c,d,e]
Serum VLDL (mg/dl)	13.2±2.6	18.17±1.37[a]	15.37±1.1[b]	14.17±2.59[b]	26.2±1.48[a,b,c,d]	20.9±1.6[a,c,d,e]
Serum glucose(mg/dl)	77.14±14.03	111±17,57 [a]	84.4±11.24 [b]	78.7±13.99 [b]	145±10.80[a,b,c,d]	105.19±11.23[a,c,d,e]
Serum insulin(µIU\ml)	17.23±2.07	22.43±1.90 [a]	18.571±2.29 [b]	18.43±2.37 [b]	26.143±3.72[a,b,c,d]	22.71±1.98[a,c,d,e]
HOMA-IR	3.37±0.99	6.18±0.79 [a]	3.9±0.99 [b]	3.6±0.57 [b]	9.44±2.04[a,b,c,d]	5,87±1.11[a,c,d,e]
Serum IL-6 (pg/ml)	45.57±3.7	47.57±4.35	47.43±3.8	46.29±3.95	65.28±3.49[a,b,c,d]	55.14±3.48[a,b,c,d,e]
Serum TNF(pg/ml)	54.57±3.735	54.285±3.49	57.29±3.07	55.86±3.7	76.0±4.3[a,b,c,d]	67.86±1.95[a,b,c,d,e]
Hepatic MDA (µg/g tissue)	35.86±3.8	39.77±3.76	38.18±3.51	38.59±4.34	65.56±4.62[a,b,c,d]	46.09±3.59[a,b,c,d,e]
Hepatic ROS (nmol/mg)	1.33±0.08	1.37±0.07	1.34±0.06	1.36±0.08	2.01±0.28[a,b,c,d]	1.63±0.17[a,b,,c,d,e]
Hepatic SOD (µg/g tissue)	10.95±0.76	10.13±0.73	10.44±0.7	10.91±0.76	6.5±1.08[a,b,c,d]	8.94±0.76[a,b,c,d,e]
Serum adropin(ng/ml)	33.28±4.27	24.27±1.65[a]	32±4.32[b]	32.14±4.06[b]	19±2.16[a,b,c,d]	25.28±3.49[a,c,d,e]

*Significant: p<0.05, [a] significant versus control 4weeks group, [b] significant versus HFD 4weeks group, [c] significant versus HFD 4weeks+exercise group, [d] significant versus control 12weeks group, [e] significant versus HFD 12weeks group.

Table 2: Scoring of hepatic histopathologic lesions in all studied groups

Parameter	Group Ia Control(4weeks)	Group Ib HFD(4weeks)	Group Ic HFD(4weeks) +exercise	Group IIa Control(12weeks)	Group IIb HFD(12weeks)	Group IIc HFD(12weeks) +exercise
	Median (IQR)	Median (IQR)	Median (IQR)	Median (IQR)	Median (IQR)	Median (IQR)
Steatosis	0 (0)	2 (1-3) [a]	1 (1 – 2) [a,b]	0(0)[b,c]	2 (2-3)[a,b,c,d]	2 (1-2)[a,c,d,e]
Hydropic degeneration	0 (0)	1 (1-3) [a]	2 (0-2) [a,b]	0 (0)[b,c]	3 (2-3)[a,b,c,d]	1 (1-2)[a,c,d,e]
Portal tract inflammation	0 (0)	2 (1-3) [a]	1 (0-1)[a,b]	0 (0)[b,c]	2 (2-3)[a,b,c,d]	1 (0-2)[a,b,c,d,e]

[a] significant versus control 4weeks group, [b] significant versus HFD 4weeks group, [c] significant versus HFD 4weeks+exercise group, [d] significant versus control 12weeks group, [e] significant versus HFD 12weeks group.

Table 3: Correlation between serum adropin level and measured parameters in HFD and exercise groups.

parameters	r
ALT	**-0.77***
AST	**-0.88***
TC	**-0.85***
TGs	**-0.85***
HDL	**+0.77***
LDL	**-0.64***
VLDL	**-0.85***
Glucose	**-0.83***

parameters	r
Insulin	**-0.78***
HOMA-IR	**-0.81***
IL6	**-0.72***
TNF-α	**-0.71***
MDA	**-0.75***
SOD	**+0.62***
ROS	**-0.72***
BMI	**0.07**
AC	**0.05**
Steatosis	**-0.73***
Hydropic degeneration	**-0.78***
Portal tract inflammation	**-0.75***

* significant correlation (p<0, 05)

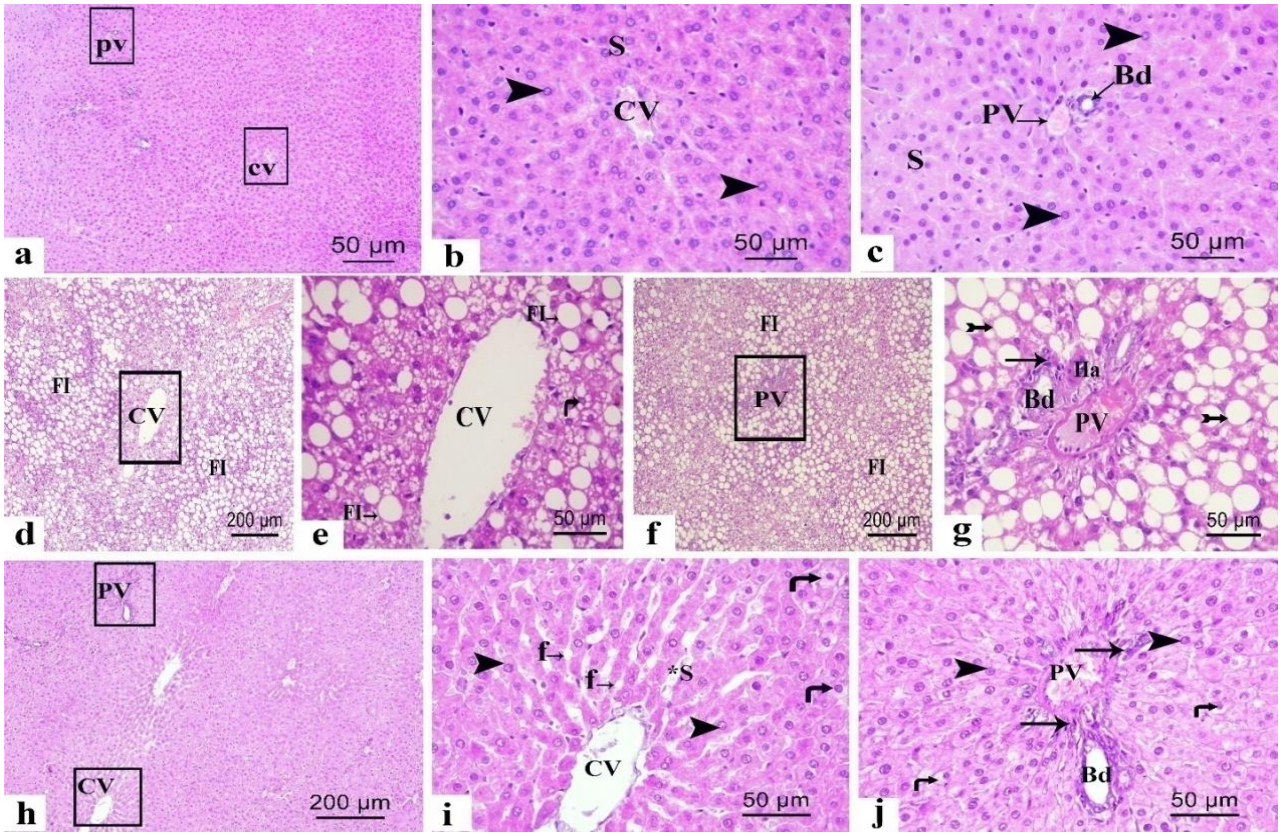

Figure 1: Showing photomicrographs of H&E-stained rat liver tissues of 4weeks studied groups: **control 4weeks a, b, c: a)** viewing normal architecture of liver lobules. **b)** Higher magnification of the boxed area **(a)** showing typical design of the liver with hepatocytes have rounded vesicular nuclei and acidophilic cytoplasm (**arrow head**) initiate from the central vein **(CV)** and separated by normal blood sinusoids **(S)**. **c)** Another photomicrograph viewing the portal vein **(PV)** and the bile duct **(Bd)** surrounding by normal hepatocyte and separated by normal blood sinusoids **(S). HFD 4weeks d,f)** moderate and random steatosis **(FI)** involves some hepatic lobules. **e)** Higher magnification of the boxed area in **Fig. d** showing disrupted liver style around slightly dilated and packed central vein **(CV).** Most hepatocytes demonstrate macro vesicular statosis **(FI)** and microvesicularsteatosis **(f)**. Few hepatocyte show hydropic gegeneration with vaculated cytoplasm **(curved arrow)** and some normal hepatocytes can be observed **(arrow head). g)** Higher magnification of the boxed area in **Fig. f** showing the portal triad, portal vein (PV), hepatic artery (Ha), bile duct (Bd). Most hepatocytes demonstrate macro vesicular steatosis **(FI)** and microvesicularsteatosis **(f)** and some normal hepatocytes can be observed (arrow head). Few cellular

infilterations can b seen around the portal vein **(arrow). HFD 4weeks +exercise, h)** shows nearly normal appearance with slightly dilated central and portal veins. **i)** Higher magnification of the upper boxed area in **Fig. h** shows normal architecture of the liver with rounded vesicular nuclei and acidophilic cytoplasm (**arrow head**) initiate from dilated central vein **(CV)** and separated by little expanded blood sinusoids **(S). j)** Higher magnification of the lower boxed area **in Fig. h** shows the slightly dilated and thickened portal vein **(PV)** and bile duct **(Bd)** surrounding by normal hepatocyte (arrow head) and separated by well-organized blood sinusoids **(S).** A few scattered hepatic cells have vacuolated cytoplasm **(curved arrow)**. **(a, d, f, h) Scale bar= 200 µm, x100; (b, c, e, g, i, j) Scale bar= 50 µm, x400.**

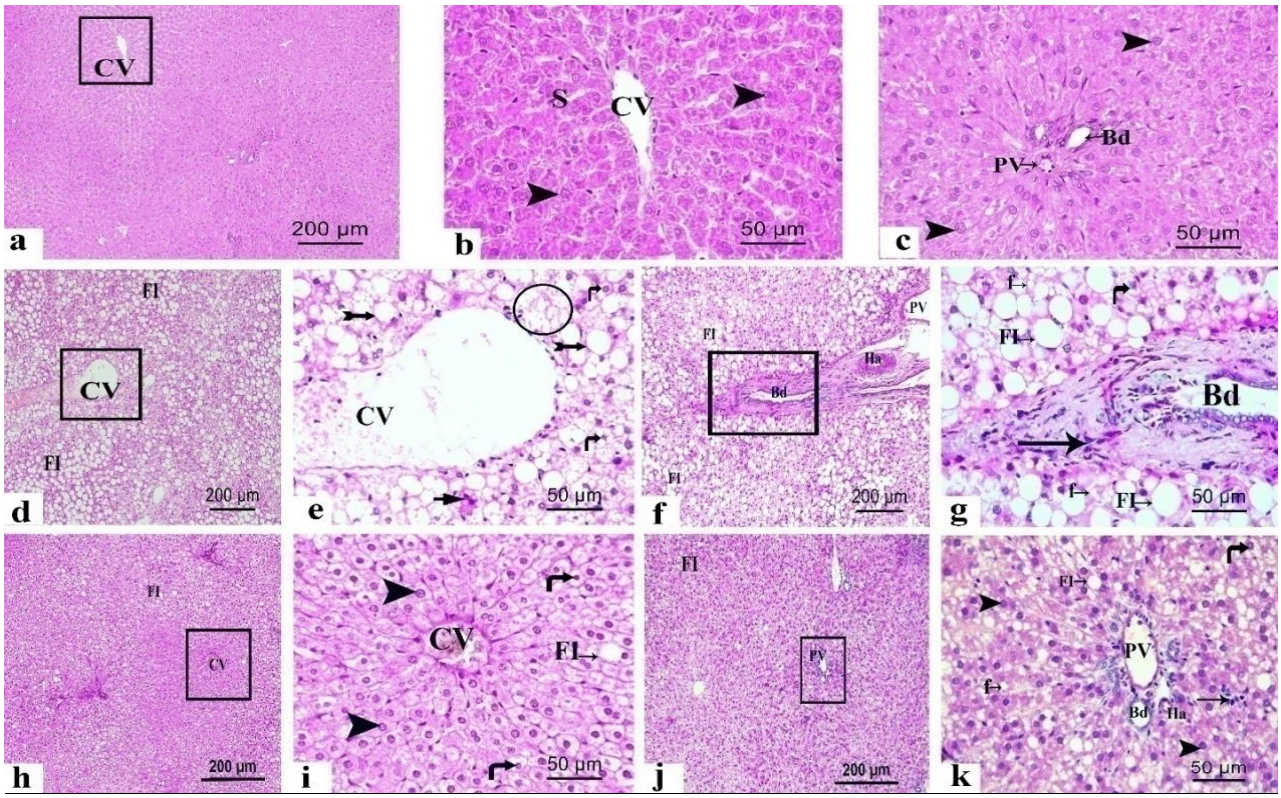

Figure 2: Representative photomicrographs of H&E-stained rat liver tissues of 12weeks studied groups. **Control 12 weeks a, b, c: a)** viewing normal design of liver lobules. **b)** Higher magnification of the boxed area showing apparently normal style of the liver with hepatocytes have rounded vesicular nuclei and acidophilic cytoplasm (**arrow head**) initiate from the central vein **(CV)** and separated by normal blood sinusoids **(S)**. **c)** Another photomicrograph showing the portal vein **(PV)** and the bile duct **(Bd)** surrounding by normal hepatocyte (arrow head) and separated by normal blood sinusoids **(S).** Rat's liver of **HFD 12weeks d, f)** Intense and random steatosis (FI) in most hepatic lobules around both central (CV) and portal veins (PV). Notice thickening of bile duct **(Bd) and hepatic artery (Ha). e)** Higher magnification of the boxed area in **Fig. d** showing disrupted liver architecture around dilated central vein **(CV).** Most hepatocytes have vacuolated cytoplasm with hydropic degeneration **(curved arrow).** Necrotic foci **(circle**) and macro vesicular **(FI)** and microvesicular **(f)** steatosis can be observed. **g)** Higher magnification of the boxed area in **Fig. f** showing thickened dilated bile duct **(Bd)** with mononuclear cell infiltrations (arrow)**.** Most hepatocytes have vacuolated cytoplasm with hydropic degeneration **(curved arrow)** and macro vesicular **(FI)** and microvesicular (f) steatosis can be observed in rat liver of **HFD 12weeks+exercise, h, j)** showing partial restoring of the normal appearance with slightly dilated central (CV) and slightly dilate portal veins (PV) with minimal macro vesicular steatosis **(FI)**. **i)** Higher magnification of the boxed area **(h)** shows some hepatocytes with rounded vesicular nuclei and acidophilic cytoplasm (**arrow head**) but others hepatocyte has vacuolated cytoplasm with dark nuclei **(curved arrow)** radiating from slightly dilated central vein **(CV).** Minimal macro vesicular steatosis **(FI)** can be noticed. **k)** Higher magnification of the boxed area **(j)** shows some hepatocytes are normal with rounded vesicular nuclei and acidophilic cytoplasm but others hepatocytes have vacuolated cytoplasm with dark nuclei **(curved arrow).** Slightly dilated portal vein **(PV)** and bile duct **(Bd) (S)** surrounded by few mononuclear cell infiltrations (arrow) can be observed**.** Micro (f) and macro vesicular steatosis **(FI)** can be noticed (**a, d, f, h, j) Scale bar= 200 µm, x100; (b,c,e,g,i,k) Scale bar= 50 µm, x400**

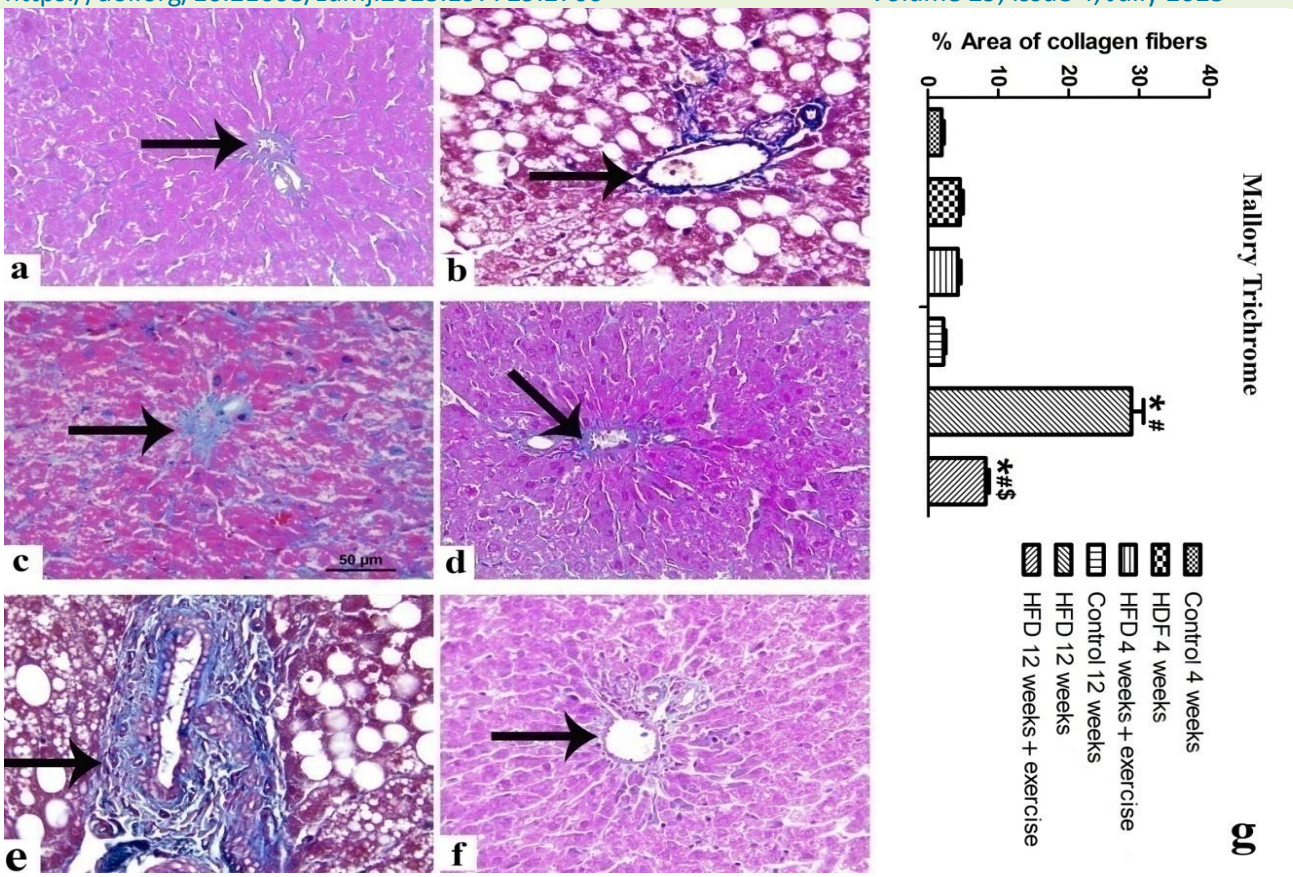

Figure 3: Mallory's trichrome stained liver sections in different experimental groups, **a)** control 4weeks, **b)** HFD 4weeks, **c)** HFD 4weeks+ exercise, **d)** control 12weeks, **e)** HFD 12weeks **f)** HFD 12weeks +exercise. Arrow indicated the blue staining of the collagen fibers around the portal triad **g)** Bar chart demonstrating the analysis of the area % of collagen fibers (x400) of the dissimilar trial groups. The statistical analysis was done by using one-way ANOVA, then by Tukey's post hoc test. Standards are represented as the mean ± SD (n=7). ***:** Significant variability compared to HFD 4weeks group (P<0.05). **#:** Significant variation compared to control 12weeks group, P<0.05. **$:** Significant variation compared to HFD 12weeks group, P<0.05. **Mallory trichrome, x 50 µm, x400.**

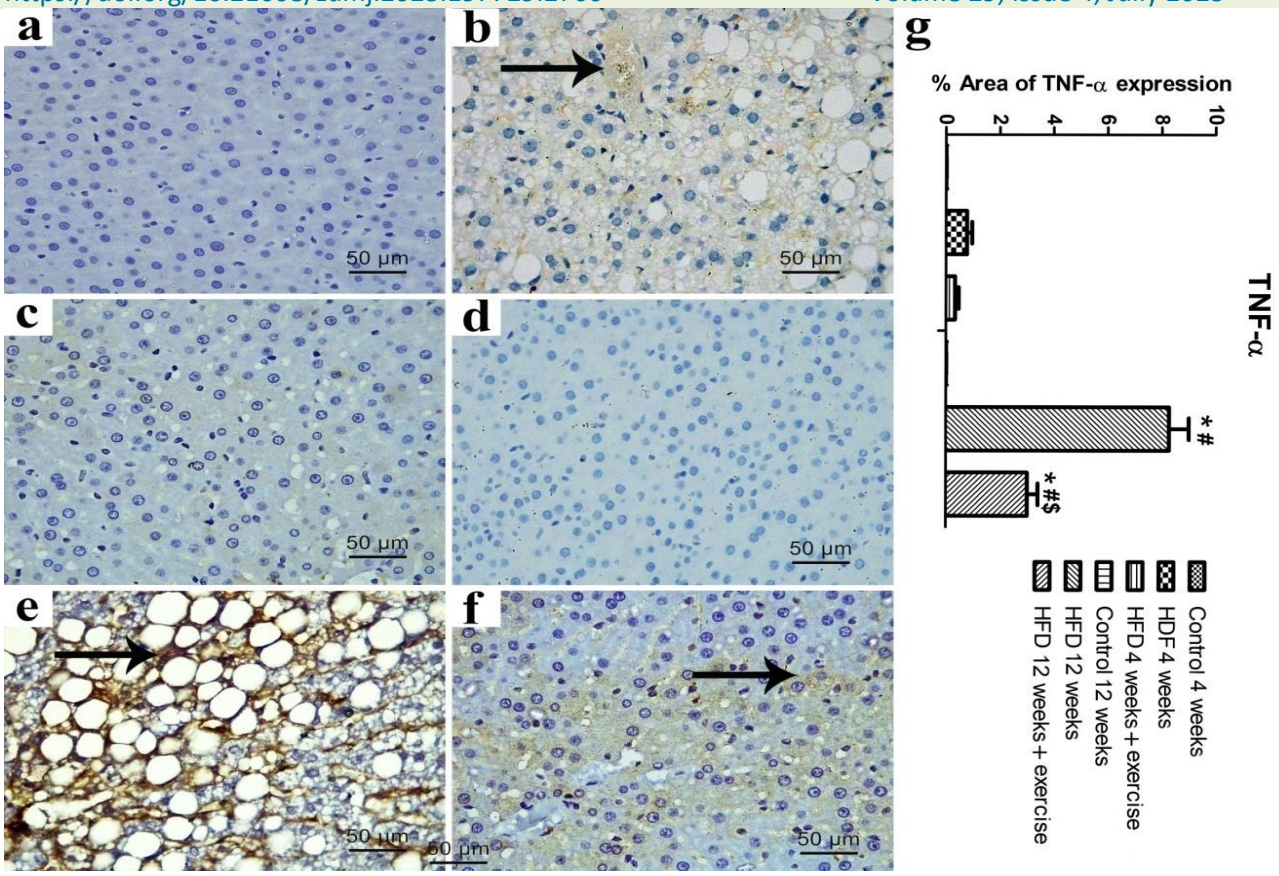

Figure 4: Representative images showing TNF-alpha immune-positive cells expression, **(a)** control 4weeks, **(b)** HFD 4weeks, **(c)** HFD 4weeks + exercise, **(d**) control 12weeks, **(e)** HFD 12weeks, **(f)** HFD 12weeks + exercise. Arrows signifying dark brown staining of immune-positive hepatocytes. **(g)** Chart showing the quantitative analysis of the % area of TNFα immune-positive reaction. Statistical analysis was done using one-way ANOVA, then by Tukey's post hoc test. Principles are represented as the mean ± SD (n= 7). ***** Significant variation in comparison to HFD 4weeks group, P<0.05. **#:** Significant variation compared to control 12weeks group, P< 0.05. **$:** Significant variation in relation to HFD 12weeks group, P<0.05. **Scale bar = 50 µm, x40.**

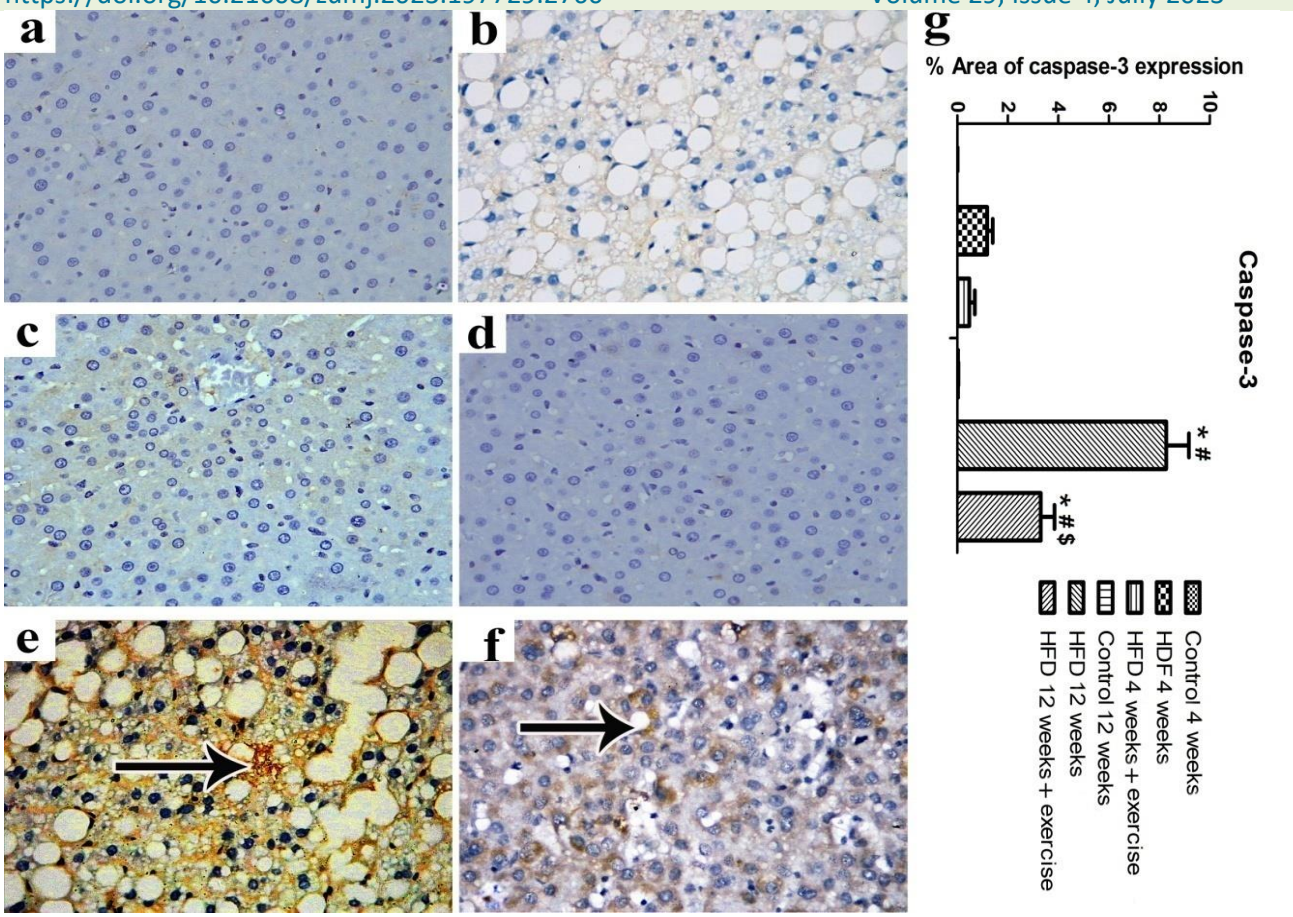

Figure 5: Representative descriptions viewing caspase-3 immune-positive cells expression, **(a)** control 4weeks, **(b)** HFD 4weeks, **(c)** HFD 4weeks+ exercise**, (d)** control 12weeks, **(e)** HFD 12weeks, **(f)** HFD 12weeks + exercise. Arrows signifying dark brown staining of immune-positive apoptotic hepatocyte. **(g)** Charts showing the quantitative analysis of the % area of caspase immune-positive reaction. Statistical analysis was done using one-way ANOVA, then by Tukey's post hoc test. Standards are represented as the mean ± SD (n= 7). ***:** Significant disparity in relation to HFD 4weeks group, P<0.05. **#:** Significant disparity in relation to 12weeks control group, P<0.05. **$:** Significant variation in relation to HFD 12weeks group, P<0.05. **Scale bar = 50 µm, x40.**

DISCUSSION

Our results declared a progressive statistically significant decrease in adropin level in all HFD groups for 4 weeks (SS) and 12 weeks (NASH) versus control groups for 4 and 12 weeks, respectively. The decrease was also significant when comparing the NASH group with the SS group. Our results matched previous reports that demonstrated a decline in adropin in NAFLD patients [46, 9]. A similar finding was reported by Chen [10], who demonstrated that, mice with adropin deficiency had enhanced hepatic steatosis as well as NASH, while synthetic adropin administration attenuated NASH development in mice. Conversely, St-Ong [47] reported that serum adropin levels were increased in subjects on diets rich in fat but low in carbohydrates. The above mentioned study showed the effect of acute intake of a high fat diet, contrary to the chronic intake shown in our study. Furthermore, other investigators reported an increase in serum adropin levels in cirrhotic alcoholic fatty liver patients, suggesting a different modulation of adropin in liver diseases. The small number of patients enrolled in that study is another possible limitation [11].

This progressive decrease in serum adropin in HFD groups can be partially attributed to dyslipidemia, lipid profile parameters (TC, TG, LDL, and VLDL) in HFD groups showed a marked increase with a negative correlation to adropin. Ghoshal [48] found that excess cholesterol suppresses hepatic expression of Enho mRNA, leading to lower adropin production in mice fed a high-fat diet. Also, HFD was reported to induce a significant increase in nuclear liver X receptor (LXR) in the NAFLD rat model [49], which could be a cause of the decrease in Enho mRNA expression with a subsequent decrease in serum adropin level in HFD groups. This theory

was confirmed by the in vivo study of Kumar [50], who found that treatment of mice with the LXR agonist GW3965, induced a reduction in hepatic Enho mRNA expression, leading to a progressive decrease in adropin level [51]. Also, a role for miR-29 was detected in the suppression of hepatic adropin expression in obesity. MiR-29 is a special type of microRNA that is up-regulated in different rodent obesity models. The increased miR29b leads to increased degradation of Enho mRNA [52] and a decrease in adropin level. Hung [52] also, found that this suppression of Enho gene expression was affected at the level of transcription and mRNA stability. It is worth mentioning that the miR29 family also plays a vital role in the regulation of hepatic lipid metabolism [53] and insulin sensitivity [54], so it might provide a link between adropin deficiency and metabolic disturbance in obesity.

The observed decrease in adropin could also be a consequence of the inflammatory reaction and oxidative stress associated with insulin resistance, which is a feature of NAFLD. In the current study, there was a significant rise in serum (IL6, TNFα), hepatic immune staining of TNFα and the apoptotic marker caspase-3, hepatic tissue (MDA, ROS) levels, and a significant progressive decline in hepatic tissue SOD in NASH group (IIb), when compared with both simple steatosis group (Ib) and its control group (IIa). As well, we found a significant negative association between adropin level and both serum IL6 and TNF levels as well as hepatic tissue MDA& ROS levels, while there was a significant positive association between SOD and adropin level in the same group. These results are in accordance with those of some researchers who found a negative association between serum adropin and IL6 levels and supposed that inflammation was the underlying cause of the decline in adropin levels in patients with liver disease [55]. Some studies reported that excess fat accumulation in hepatocytes was linked to increased oxidative strain, inflammation, and apoptosis of hepatic tissue [56], initiating a reduction in the adropin level.

Besides, the study of Jasazwili [57] found that adropin inhibits discrimination of pre-adipocytes into adult adipocytes by reducing lipid gathering and the appearance of adipogenic genes in 3T3-L1 cells. Thus, adropin can reduce macrophage infiltration by decreasing fat accumulation and, hence, improving inflammation. Therefore, it could be fulfilled that, dyslipidemia is linked with IR as a result of high fat diet induced lowering of serum adropin level, leading to more IR and hyperlipidemia, creating a viscous circle with more lowering of serum adropin, thereby exacerbating simple steatosis (SS) and mediating its progression to inflammation (NASH).

Exercise training in groups Ic (HFD 4 weeks + exercise) and group IIc (HFD 12 weeks + exercise) induced a significant rise in adropin levels associated with improved metabolic parameters in the form of a decline in TC, TGs, LDL, and VLDL and a rise in HDL levels. A decrease in insulin resistance was detected by reduced insulin, blood glucose, and HOMA-IR. Hepatic cell injury was also alleviated in these groups versus HFD 4 weeks and HFD 12 weeks, respectively, as detected by the significant diminishment in AST, ALT, hepatic immune staining of caspase-3, and the significant decline in collagen fibers around the portal tirade, together with improved pathologic features and histopathologic scoring. Conflicting findings were previously recorded concerning the relationship between exercise and adropin. Parallel to our data, Zhang [17] showed that, 12 weeks of aerobic exercise in obese adolescents significantly increased adropin levels independent of body weight loss. Fujie [16] found that serum adropin levels increased after regular aerobic exercise performance. On the other hand, the acute effect of exercise in overweight women was studied by Alizadeh [18], and they found no effect on adropin level. Hieda [58] also found no significant difference in plasma adropin levels following short-term aerobic exercise in male Zucker. In the study of Ozbay [19], different results were obtained when exercise was performed indoors or outdoors. The indoor group showed no acute or chronic effects on adropin levels, while the outdoor group showed a drop in adropin levels during chronic exercise, but no change was detected after single aerobic exercise. From previous studies, it seems that, species differences, type of exercise, duration, and even environment and climate can affect adropin expression.

The increase in adropin seen in exercise groups (Ic and IIc) may be related to a reduction in hepatic cellular stress due to the positive role of exercise on NAFLD. Exercise training improves lipid metabolism, reduces fat, and alleviates steatosis in liver tissue [59], which is in context with our results. The elevation in hepatic mitochondrial oxidation markers was also found following exercise training, which activates adenosine monophosphate-activated protein kinase (AMPK) with an increase in lipid oxidation [60].

Exercise training was also reported to decrease the expression of miR-29 in skeletal muscles. In 2017, Massart [61] found that, 5 days of swimming exercise training in rodents induced a reduction in miR-29a and miR-29c expression in gastrocnemius muscle and in humans; they found a decrease in miR-29c in vastus lateralis muscle after 14 days of exercise training. This decrease in miR-29 could be a cause of the elevation in adropin levels in groups Ic and IIc, as miR-29 was reported to increase the degradation of Enho mRNA, as previously mentioned.

Insulin resistance was considered the motivating force in NASH. So, improving insulin resistance through exercise, as was found in our study, is one of the proposed ways by which physical exercise improve NASH [62]. The increase in adropin by exercise could be a cause of this improvement, as adropin is frequently reported to ameliorate IR and enhance glucose tolerance [6].

In our study, exercise induced a reduction in serum IL6 and TNFα and hepatic tissue immune expression of TNFα in group IIc (HFD 12 weeks + exercise) versus group IIb (HFD 12 weeks), and these levels were negatively correlated with Adropin. Such findings agree with those of Van der Windt et al [63]. In contrast, Houghton [59] showed no effect of 12 weeks of exercise on inflammatory markers in NASH patients. An anti-inflammatory effect for adropin was previously suggested as adropin induced a decrease in the pancreatic expression of TNF-α and IL-6 in diabetic rats [64]. Additionally, group IIc showed a statistically significant reduction in MDA and ROS oxidative stress indicators and an increase in SOD. There is evidence that exercise reduces oxidative damage while increasing liver antioxidant enzymes like SOD1 and SOD2, catalase, and glutathione peroxidase [65]. The rise in adropin seen by the effect of exercise might play a role in this improvement, as Chen [10] demonstrate that adropin can elicit an antioxidant response through activation of the Nrf2 pathway, which may alleviate the progress of non-alcoholic steatohepatitis.

We also found that, aerobic training did not induce a clinically significant weight loss in groups Ic & IIc proving that exercise alone might independently diminish steatosis in the liver. This result matched Hashida [66], who observed that the improvement in steatosis was independent of BMI. In contrast, Takahashi [67] stated a significant positive connection between changes in liver fat and changes in BMI. Despite the absence of overall weight loss in several studies, a decline in hepatic fat content was achieved; supporting the thought that exercise has a direct consequence on the liver [15].

CONCLUSION

HFD produced extensive biochemical, functional, and histological alterations in the liver, leading to NAFLD associated with a significant decline in serum adropin level. This lowering of serum adropin levels may play a vital role in the progression of steatosis and mediating its progression to inflammation (NASH). The elevated adropin level caused by exercise may have a role in remission of hepatic inflammation, steatosis, and steatohepatitis. The improvement in insulin resistance, lipid profile, and reduction in inflammatory and oxidative stress mediators and their correlation with adropin suggest an anti-inflammatory and anti-oxidative role for adropin, and therefore it could be used not only as a biomarker but also as a treatment for NAFD. Future investigations involving in vivo adropin administration are required to determine whether the increase in adropin level in exercise groups was a consequence or a cause of this improvement.

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None of the authors reported any potential conflict of interest

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