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Effect Of Aerobic Exercise on Endothelial Cell Function and Vascular Endothelial Growth Factor Expression In Nonalcoholic Fatty Liver Rat Model

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ABSTRACT

This work aimed to assess presence of angiogenesis in nonalcoholic fatty liver disease and to evaluate the possible role of exercise on endothelial function, as anti-inflammatory, anti-oxidative and anti-angiogenic therapy in preventing the progression of NAFLD in rats. Rats were separated into 2 main groups, G I which was subdivided into 3 subgroups, GI a: 4weeks control, GI b: 4weeks HFD, G I c: 4weeks HFD +exercise; GII which was subdivided into 3 subgroups, GII a: 12weeks control, GII b: 12weeks HFD and GII c: 12 weeks HFD +exercise. Blood glucose, serum insulin, HOMA-IR, lipid profile, CRP, SOD, MDA and ROS were measured. Moreover, histopathological and immunohistochemical studies (VEGF, iNOS) were examined. The results showed that 4 and 12weeks HFD induced simple steatosis and non-alcoholic steatohepatitis in GI b and II b, respectively associated with significant decrease in serum and hepatic TNOX which was more obvious in GII b. Significant improvement in endothelial hepatic function, metabolic, inflammatory, oxidative stress and histopathological parameters was found in GI c and II c. It can be concluded that the impairment in endothelial function and and expression of VEGF in HFD groups can be improve by physical exercise suggesting abeneficial role for exercise on hepatic endothelial function.

I. INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) purely included simple steatosis (SS) without inflammation and it's progressive form, nonalcoholic steatohepatitis (NASH) with inflammation and hepatocellular injury with or without fibrosis ***(Buzzetti et al., 2016 and Younossi et al., 2019).*** Pathogenesis of NAFLD is multifactorial, two hypotheses were well recognized, the first one involved disturbance of fatty acid metabolism that led to hepatic fatty infiltration. The second one may be oxidative or metabolic stress as mitochondrial dysfunction, endoplasmic reticulum stress and increased proinflammatory cytokines leading to subsequent inflammation and fibrosis ***(Marra et al., 2018 and Rezk et al., 2019).***

The liver endothelium is mainly formed of liver sinusoidal endothelial cells (LSECs), which have a fenestrate organized into sieves that regulate the transport of macromolecules across the sinusoid ***(Poisson et al., 2017 and Zapotoczny et al., 2018).*** Many studies proved that mice fed a high-fat diet had insulin resistance in their LSECs which lead to impairment of insulin-dependent vasodilatation. Also, nitric oxide consuming enzyme (NOX1) highly expressed in their LSECs ***(Pasarín et al., 2011; Pasarín et al., 2012 and Pasarín et al., 2017)***.

Capillarization of LSEC appears early in the course of NAFLD because tissue damage which occurred from both fat accumulation and lipo toxicity results in reduced sinusoidal perfusion and changes in sinusoidal architecture ***(Cogger et al., 2016; Papageorgiou et al., 2017; Hammoutene and Rautou, 2019 and Lei et al., 2021).***

Progression of simple steatosis to steatohepatitis is accompanied by adhesion of leukocytes to the sinusoidal endothelium followed by infiltration of leukocytes within liver parenchyma to form inflammatory foci. This chronic inflammation and capillarized LSECs cause up-regulation of vascular endothelial growth factor (VEGF) and its receptors which are expressed in liver sinusoidal endothelial and stellate cells, enhancing the hypoxia- induced angiogenesis. VEGF, the master manager of angiogenesis, is also implicated in fibrogenesis by activation of hepatic stellate cells (HSCs) ***(Lemoinne et al., 2016; Poisson et al., 2017 and Zadorozhna et al., 2020).*** Angiogenesis occurs under physiological conditions during normal wound healing and also in pathological

contexts, so that anti angio-genic molecules are used in the treatment of hepatocellular carcinoma (HCC) ***(Gordan et al., 2021).***

Training enhances lipid metabolism and decrease liver fat content leading to amelioration of hepatic steatosis (***Keating et al., 2015 and Houghton et al., 2017)***. Furthermore, exercise increases expression levels of anti-inflammatory cytokines in adipose tissue ***(Szostak and Laurant, 2011),*** improves mitochondrial performance and thus decreases ROS production and maladaptive immune response ***(Peeri and Amiri, 2015***). Moreover, exercise increases anti-inflammatory adipokine expression levels of adiponectin and inhibited proinflammatory adipokine expression levels of leptin and resistin ***(Fang and Tang, 2017).***

Exercise plays an important role in endothelial function through increasing laminar shear stress activation to decrease ROS activity and to preserve endothelial NO bioavailability (***Szostak and Laurant, 2011***). Moreover, it inhibited HFD-induced angiogenesis in skeletal muscle. Thus, regular exercise and dietary intervention have been recommended to alleviate NAFLD through weight reduction ***(Han et al., 2019).***

Although, it is still unclear whether angiogenesis represents a simple response to maintain homeostasis and repair hepatic injury ***(Elpek , 2015)*** and angiogenesis suppression aggravates hepatic fibrosis ***(Xi et al., 2017),*** or angiogenesis exerts a pathological role leading to liver injury and interference with angiogenesis might be a potential target to avoid fibrosis progression ***(Zhao et al., 2017***).

II. MATERIAL AND METHODS:

This study was carried out on healthy adult male albino rats (60 male weighing (180-200 g). They were housed in steel wire cages measured 90cm x 40cm x 30cm (6-8 cages) at the animal house in Medicine Faculty, Zagazig University under clean environment. They were fed standard chow, had free access to water, kept at comfortable temperature (20 to 24 ºC) and were maintained on a 12 h light/dark cycle. The rats were accommodated to animal house conditions before the study start ***(Gui et al., 2004).*** The experimental design was permitted according to the ethics guidelines of Zagazig University on laboratory animal, the national institute of health guide for the careand use of laboratory animal (NIH publication No, 80-23, revised 1978) was adopted and approved by Institutional Review Board (IRB) of Zagazig University (Approval number: 4562/22-4-2018). Standard chow (12.6kJ/g) which was consisted of 25.8 % protein, 62.8% carbohydrate and 11.4% fat was received to rats in control groups ***(Ahrén and Scheurink, 1998)***. While, high-fat (HF) chow was received to rats in high fat-fed groups, which was consisted of 18% protein, 24% carbohydrate and 58.0% fat a total (23.4kJ/g) ***(Cha et al., 2000)***, (Diets were get from agriculture faculty, Zagazig University). 60 male albino rats were divided randomly into two groups; group I: was divided equally to 3 sub group, group Ia (4 weeks control), rats were fed normal chow for 4 weeks. Group Ib, (4 weeks HFD), rats were fed HFD for 4 weeks for induction of steatosis. Group Ic, (4 weeks HFD + exercise), rats were fed HFD for 4 weeks for induction of steatosis and then expose to chronic moderate exercise for 12 weeks, The rats continuous on HFD till the finish of experiment. Group II: was divided equally to 3 sub group, group II a (12 weeks control), rats were fed normal chow for 12 weeks. Group II b (12 weeks HFD), rats were fed HFD for 12 weeks for induction of NASH. Group II c (12 weeks HFD+ exercise), ratswere fed HFD for 12 weeks for induction of NASH then expose to chronic moderate exercise for 12 weeks, tillthe end of experiment, the rats continuous on HFD.

Plan of swimming exercise

In exercising groups, the rats were at first qualified for 15 minutes/day and the period was gradually elevated 5 min/day till the rats were capable of performing exercise for one hour/day to keep away from water induced stress (Lu et al., 2016). Swimming rats were assigned to swim for one hour/day, 5 days/week for 12 successive weeks to achieve chronic swimming exercise (Luciano et al., 2002). Swimming was done between 9:30-10:30 am in a cylindrical container of 80 cm high, 45 cm diameter and 60 cm deep packed with tap water at 33–35 °C (Lapmanee et al., 2012). In order to achieve an exercise of moderate intensity a small amount of detergent was added to the water to prevent floating (Fleshner et al., 1998).When the rats are allowed to swim continuously without floating even without adding heaviness to the body or tail of rat, the exercise is well thought-out of moderate intensity (Musch et al., 1990). At the end of each exercise session; a warm environment was needed to keep dry animals.

Collection of blood

Samples of blood were get at the end of the experimental time after overnight fasting, blood samples obtained from animals that practiced exercise through 48h after the finish of the last training session to diminish the acute effects of exercise ***(Teixeira-Lemos et al, 2011)*** (between 9:00-11:00 a.m.), blood samples were obtained from sinus orbitus vein of each rat after ether inhalation ***(Yang et al.,2006).*** Blood samples for serum separation were collected in tubes which were (dry clean and screw capped). Blood samples were allowed to clot for 30 minutes then sera were separated by centrifugation at 3000 RPM (run per minute) for 15 minutes. Automatic pipetteswere used to separate the clean, clear sera. Also, dry sterile samples tubes were used to receive it. The separated sera were stored deeply frozen at -20˚C. Repeated freezing and thawing were avoided ***(Nishizawa et al., 2002).***

Biochemical parameters measurements

Serum **NOX level** was determined colorimetrically by Griess method ***(Miranda et al., 2001).*** **Hepatic NOX** was assessed by using acommercially available kit (no. 780001, Cayman Chemical, Ann Arbor, MI) that measures total nitrite and nitrate, stable derivatives of NO ***(Sakemi et al 1998*****). Serum glucose level was determined**, using glucose enzymatic (GOD-PAP)-liquizyme Kits (Biotechnology, Egypt), according to **Tietz, (1995), Seruminsulin level was estimated**, using rat insulin enzyme-linked immunosorbent assay kit, (Product Number:RAB0904, Sigma-Aldrich Chemie GmbH, U.S.A).according to **Temple**, ***(1992),*** **Calculation of homeostasis model assessment of insulin resistance (HOMA-IR),** via this equation; [insulin (µU/mL) x glucose (mg/dl) /405] (**Matthews et al., 1985 and Sun et al., 2007**), **Estimation of serum total cholesterol (TC) level** via using rat cholesterol enzyme-linked immunosorbent assay kit, (Catalog Number: 2011-11-0198, Shanghai Sunred biological technology, China). According to **Flegg, (1973)** and **Allain et al. (1974)**, **Serum triglycerides (TGs) level was estimated** by using rat triglycerides enzyme-linked immunosorbent assay kit (Catalog Number: 201111-0250, Shanghai Sunred biological technology, China**)**. According to **Nagele et al. (1984)**, **Estimation of Serum high density lipoproteins (HDL) level** by using rat HDL-cholesterol enzyme-linked immunosorbent assay kit, (Catalog Number: 2011-11-0255, Shanghai Sunred biological technology**,** China). According to ***Warnick et al. (1985)***, **Serum C-reactive protein (CRP) estimation** via using rat Immuno-enzymometric assay kits, (Monobind Inc Lake Forest, Ca 92630, USA), according to **Ridker et al. (1998).**

Tissue sampling

Laparotomy was conducted after the animals were sacrificed by cervical dislocation under mild ether anesthesia. Livers were quickly excised and cleaned totally, 200 mg of liver was harvested from the rats and precisely weighed. Subsequently, according to the tissue mass, saline was added: Saline volume=1:9(w/v). Following homogenization at 4˚C by a DY89- electric homogenate (Ningbo Scientz Biotechnology Co., Ltd., Ningbo, China), the homogenates was centrifuged at 1,100xg for 15 min at room temperature. **Hepatic malondialdehyde (MDA) level estimation** by using Sandwich-ELISA technique, (Sunred Bio Shanghai 201-11-0637, CHINA). According to ***Satoh, (1978)***, **Hepatic super oxide dismutase (SOD) Level Estimation** by using Sandwich- ELISA technique according to Thermofisher, USA, coiorimetric assay, Ca EI ASODC. According to ***Nishikimi et al.(1972),*** **Hepatic reactive oxygen species (ROS) level estimation** by using Sandwich-ELISA technique, Catalog NO. MBS164653, CHINA) According to ***Comar et al. (2013).***

Histopathological examination of liver and scoring

Through 48-60 hours, the remaining parts of rat livers were fixed in 10% buffered formalin solution. Then, tissue samples were processed through ethyl alcohol and xylene series, and embedded in paraffin blocks. Specimens of liver were sectioned (5µm thick), then stained by hematoxylin and eosin ***(Altunkaynak, 2005).*** Via using Light microscope with camera attachment, an expert pathologist evaluated and scored the stained samples in a blind fashion. The Pathological Committee of the NASH Clinical Research Network proposed the histopathologic scoring of NAFLD followed the NAFLD Activity Score (NAS) ***(Kleiner et al., 2005).*** The steatosis as (0 <5% of, 1: 5%- 33% , 2 : >33% -66%, and 3: > 66% .The degree of hydropic degeneration as follows: 0 for no hydropic degeneration, 1 for < 25%, 2 for between 25% and 50%, and 3 for > 50%.Portal tract inflammation, graded as none, mild, moderate, and severe (0–3),.0: no portal inflammation, 1 (sprinkling of inflammatory cells in, 1/3 of portal tracts), 2 (increased inflammatory cells in 1/3–2/3 of portal tracts),3: (dense packing of inflammatory cells in 0.2/3 of portal tracts**).**

Immunohistochemical methods

BY using a specific mouse monoclonal antibody (conjugated streptavidin (Sigma), VEGF recognition was done. To prepare immune histochemical positive control, the primary antibody was omitted followed by incubation

with the secondary antibody only to detect any non specific binding. Then stained with diaminobenzene (DAB) as the chromogen and counterstained with hematoxylin (Fehrenbach et al.,1999). All stained slides were examined by the light microscope (LEICA ICC50 W) and analyzed in the Image Analysis Unit of the Anatomy and Embryology Department, Zagazig University.

For INOS, sections deparaffinized and rehydrated then were per meabilized with 0.1% Triton X-100 in PBS for 20 min. Nonspecific Antibody absorption was reduced by incubating the section in 2% pre immunized goat serum in PBS for 20 min. Endogenous biotin- or avidin-binding sites were blocked by sequential incubation for15 min with avidin and biotin. Then we put anti-iNOS polyclonal antibody (1:500 in PBS) to the Sections and they were incubated overnight. Then the sections were washed with PBS and left for incubation at room temperature for two hours(Cuzzocrea et al.,2003)

Statistical analysis: Via using SPSS program (version 18 for windows) (SPSS Inc. Chicago, IL, USA).

III. RESULTS:

HFD successfully induced hepatic steatosis and non- alcoholic steatohepatitis in groups (I b and II b), respectively which was accompanied by a significant progressive elevation in the serum levels of glucose, insulin, TC, TGs levels and calculated HOMA-IR ($P<0.05$) in comparison to control groups (I a &II a respectively) [table1]. However, there was a significant decrease in serum level of (NOX & HDL) and hepatic NOX in HFD groups (Ib, IIb) against control group (I a, IIa respectively) ($P<0.05$) [table1].There was also a significant progressive increase in serum CRP and tissue level of oxidative markers (MDA & ROS) in steatohepatitis group (II b) in comparison to control group (II a) ($P<0.05$) [table1] .On the other hand there was a significant progressive decrease in tissue level of anti oxidative marker SOD in same group(II b) versus group (II a) ($P<0.05$) [table1].

In the exercised groups (I c &II c), calculated HOMA-IR, serum levels of glucose, insulin levels and TC levels decreased significant in comparison to HFD groups (I b &II b) ($P<0.05$) [table1] . In groups (Ic, IIc) Serum levels of (NOX& HDL) and total hepatic NOX were increased significant in comparison to HFD groups (I b, IIb respectively) ($P<0.05$) [table1]. There was also a significant progressive decrease in serum CRP and tissue level of oxidative markers (MDA & ROS) in exercised group (II c) in comparison to group (II b) ($P<0.05$) [table1]. On the other hand there was a significant progressive increase in tissue level of anti oxidative marker SOD in same group in comparison to group (II b) ($P<0.05$) [table1]. There was significant statistical difference in pathologic scoring was found in HFD groups (4 and 12 weeks) versus control (4 and 12 weeks) respectively and versus each other ($P<0.001$) [table 2]. Histopathologic features and scoring decreased significantly in exercise groups (Ic and IIc) versus HFD (4 and 12 weeks) groups in that order ($P<0.001$) [table 2].

Table 1: Measured parameters in all studied groups (mean ± SD)

Parameters	4weeks control (Ia)	4 weeks HFD (Ib)	4 weeks HFD + Exercise (Ic)	12 weeks control (IIa)	12 weeks HFD (IIb)	12 weeks HFD + exercise (IIc)
Serum glucose(mg/dl)	77.14±12.36	112.2±15.76 [a]	83.6±10.53 [b]	79.7±12.25 [b]	130±13.80 [a,b,c,d]	105.9±10.4 [a,c,d,e]
Serum insulin(μIU\ml)	17.8±2.44	22.5±1.84 [a]	18.9±2.28 [b]	19.5±1.95 [b]	26.8±3.4 [a,b,c,d]	23.3±1.65 [a,c,d,e]
HOMA-IR	3.43±0.94	6.2±0.67 [a]	4.11±0.896 [b]	3.76±0.59 [b]	8.24±1.8 [a,b,c,d]	6,04±0.977 [a,c,d,e]
Serum TC (mg/dl)	89.6±7.49	191.30±14.4 [a]	97±8.6[b]	95.4±7.75[b]	241.8±25.9[a,b,c,d]	141.57±7.46[a,b,c,d,e]
Serum TG (mg/dl)	65.86±13.06	90.86±6.87[a]	86.86±5.27 [a]	70.86±12.99[b,c]	131.0±7.39[a,b,c,d]	124.43±7.99[a,b,c,d]
Serum HDL (mg/dl)	40.1±5.85	30.9±3.34[a]	39.9±5.64[b]	38.1±5.24[b]	21.3±5[a,b,c,d]	30.28±3.8[a,c,d,e]
Serum CRP(mg/l)	0.083±0.01	0.1±0.02	0.09±0.01	0.11±0.02	0.33±0.03[a,b,c,d]	0.17±0.03[a,b,c,d,e]
Serum NOX umol/l	11.7±1.8	8.56±0.78[a]	11.28±1.05 [b]	12.3±1.44[b]	5.47±1.55[a,b,c,d]	9.5±1.2[a,c,d,e]

Hepatic TNOX µmol/µg protein	4.97±0.33	2.20±0.32[a]	4.55±0.30[b]	4.7±0.038[b]	0.89±0.01[a,b,c,d]	2.60±1.1[a,c,d,e]
Hepatic SOD(µg/g tissue)	11.48±1.06	10.59±0.97	10.54±0.68	10.57±0.9	6.38±0.94[a,b,c,d]	8.11±0.71[a,b,c,d,e]
Hepatic MDA(µg/g tissue)	37.77±4.36	39.49±3.37	38.75±3.45	38.7±4.02	66.47±4.86[a,b,c,d]	50.6±3.2[a,b,c,d,e]
Hepatic ROS(nmol/mg)	1.22±0.08	1.28±0.06	1.26±0.06	1.25±0.076	2.97±0.195[a,b,c,d]	1.77±0.14[a,b,,c,d,e]

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

Table2: Scoring of Histopathologic changes (Steatosis, hydropic degeneration of hepatocytes and portal tract inflammatory cells) in rat livers of all studied groups:

Parameter	4weeks control (Ia)	4 weeks HFD (Ib)	4 weeks HFD + Exercise (Ic)	12 weeks control (IIa)	12 weeks HFD (IIb)	12 weeks HFD + exercise (IIc
	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 4weeks control, b: significant versus 4weeks HFD, c: Significant versus 4 weeks HFD + exercise, d: Significant versus 12 weeks control, e:significant versus 12 weeks. HFD

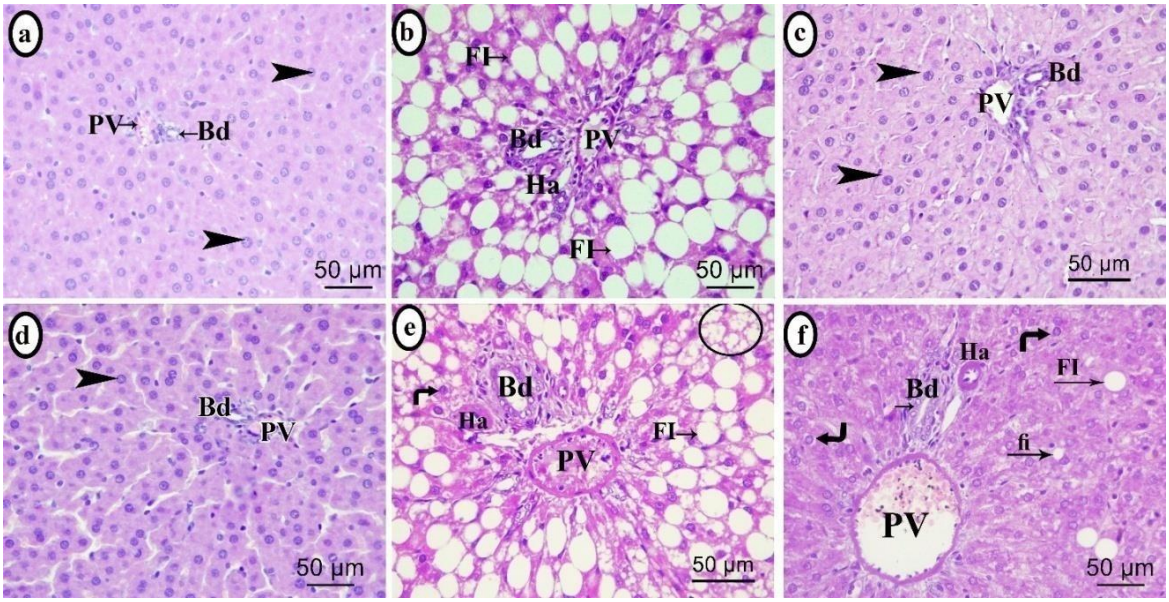

Figure 1 : photomicrographs of hematoxyin and eosin staining of rat liver tissue in the different groups a,b,c (4 weeks duration), d,e,f (12 weeks) ; a) control group, shows normal architecture of liver tissue, normal sizes of portal vein (pv) and bile duct (Bd) with normal hepatocyte (arrow heads) b)fatty liver group, shows large congested portal vein (PV),thickened walls of bile duct (Bd) &hepatic artery(Ha),large amount of fatty infiltrations(FI),and disturbed architecture of liver sinusoids c) exercise group, shows no fatty infiltration detected, hepatocyte returned to normal (arrow heads) with normal appearance of liver sinusoids ,d) control group , shows normal architecture of liver tissue e) fatty liver group, shows marked fatty infiltration (FI),vacuolated-ballooned hepatocytes surrounded by cytoplasm with centrally placed pyknotic nuclei (curved arrows),areas of apoptosis (encircled area), large congested portal vein (PV) ,thickened walls of bile

duct(Bd)&hepatic artery(Ha) f)exercise group, shows large congested portal vein (PV),vacuolated hepatocyte (curved arrows),large fatty infiltration(FI),small fatty infiltration(fi).

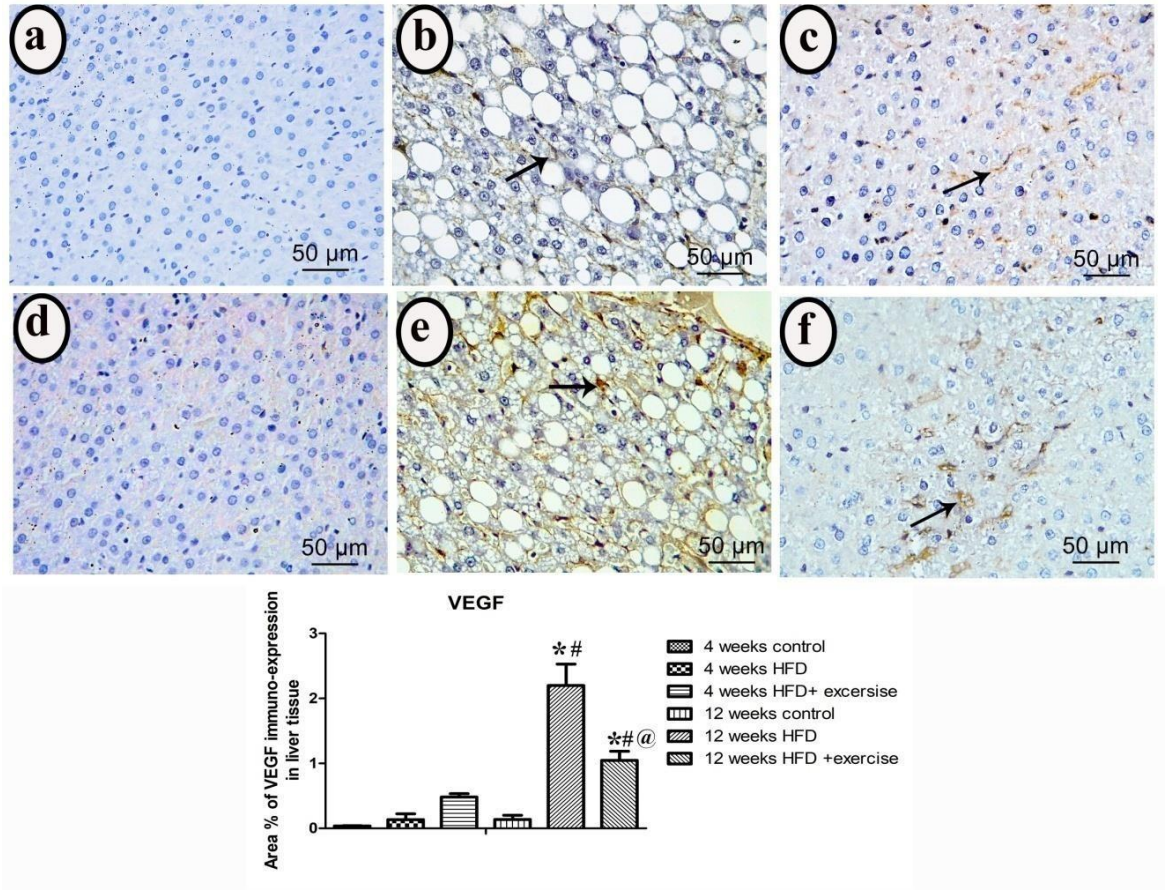

Figure 2: Photomicrographs of VEGF immune histochemical staining of rat liver tissue in the different groups a,b,c (4 weeks duration), d,e,f (12 weeks) characterized by brown coloration a) Control group shows normal weak reaction to VEGF . b) Fatty liver group reveals moderate reaction with many VEGF positive cells (arrow). c) Exercise group reveals weak reaction with a minimal number of VEGF positive cells (arrow), d) control group shows normal weak reaction to VEGF. e) Fatty liver group reveals strong reaction with an abundant amount of VEGF positive cells (arrow). f) Exercise group reveals mild reaction with a minimal number of VEGF positive cells (arrow). Scale bar, 50 µmx400 Immunohistochemical staining of VEGF. ***** Significant dissimilarity in comparison to 4 weeks HFD group, $P < 0.05$. **#** Significant dissimilarity in comparison to 12 weeks control group, $P< 0.05$. **@**Significant dissimilarity in comparison to 12 weeks HFDgroup, $P < 0.05$.

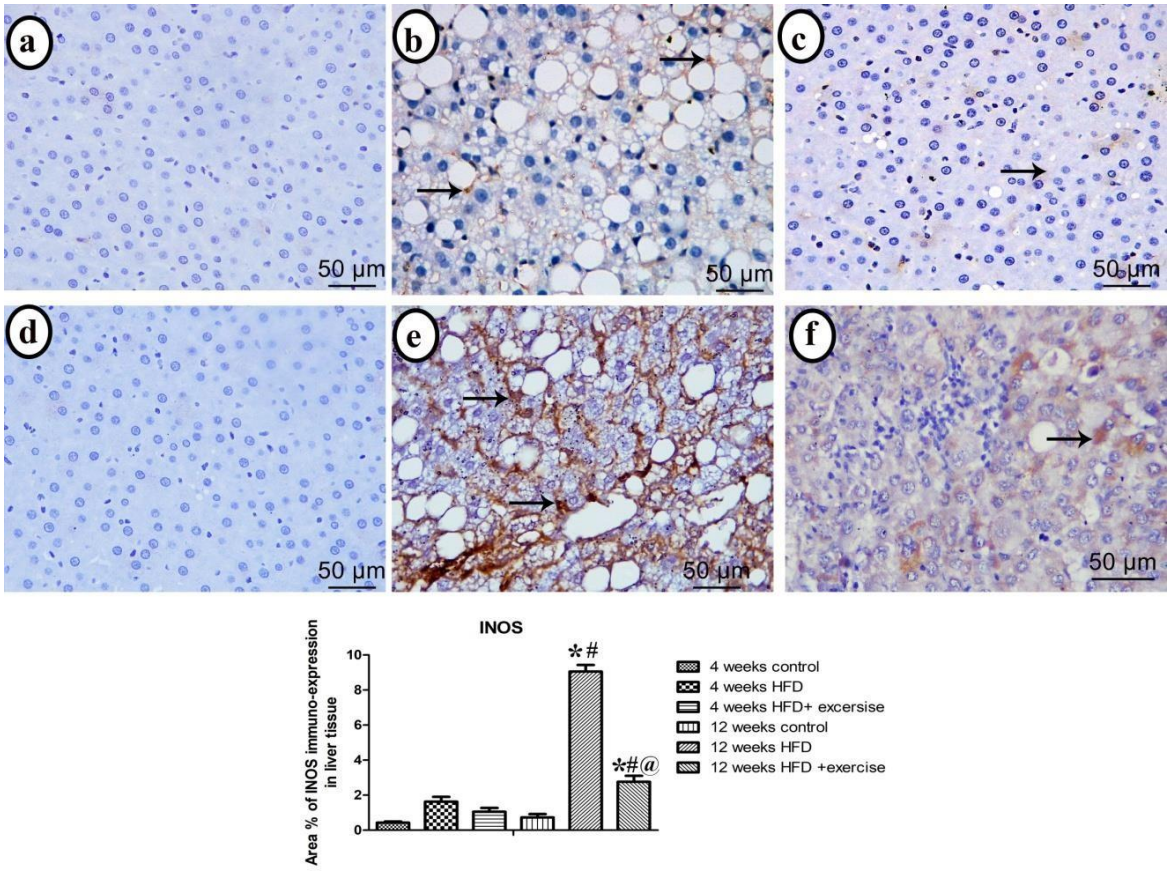

Figure3. Photomicrographs of iNOS immune histochemical staining of rat liver tissue in the different groups a,b,c (4 weeks duration), d,e,f (12 weeks) characterized by brown coloration a) Control group shows normal weak reaction to iNOS). b) Fatty liver group reveals moderate reaction with many of iNOS positive cells (arrows). c) Exercise group reveals weak reaction with low number of iNOS positive cells (arrow). d) control group shows normal weak reaction to iNOS. e) Fatty liver group reveals strong reaction with an abundant amount of iNOS positive cells (arrows).f) Exercise group reveals mild reaction with a minimal number of iNOS positive cells (arrow). Scale bar, 50 µmx400 Immunohistochemical staining of iNOS.***** Significant dissimilarity in comparison to 4 weeks HFD group, P < 0.05. # Significant dissimilarity in comparison to 12 weeks control group, P< 0.05. **@**Significant dissimilarity in comparison to 12 weeks HFDgroup, P < 0.05.

IV. DISCUSSION

Our results revealed a significant progressive decline in serum and hepatic TNOX levels in HFD groups against control groups. The decrease in NOX was also significant in NASH group (IIb) versus SS group (Ib). There were conflicting findings in several previous studies examined the relationships between endothelial function and fatty liver. Many studies were in the line with our results, which reported a significant reduction in e NOS and so on decreased in level of NO ***(Pasarín et al., 2012; Xie, 2012; Sheldon et al., 2014 and Sheldon et al., 2019).*** Also, ***Gonzalez-Paredes et al. (2016)*** reported a reduction in e NOS activation and liver nitric oxide content in mice and rats fed a high-fat diet for 4 weeks and these changes were observed in the absence of inflammation and fibrosis, suggesting that endothelial dysfunction is an early feature associated with steatosis in NAFLD. In addition, ***Lee et al. (2019)*** showed that mice treated with relaxin-2 after methionine choline deficient (MCD) diet-induced NASH increased hepatic eNOS activation. Increasing hepatic eNOS and NO plays a therapeutic role to advanced liver disease progression by attenuating hepatic inflammation ***(Cunningham et al., 2020).*** The possible explanation of this disturbance in endothelial function in NAFLD groups is insulin resistance and dyslipidemia,as we found a significant progressive increase in metabolic and lipid profile parameters (glucose, insulin,HOMA-IR, TC and TG) and a significant decrease in HDL level in both HFD groups when compared with each other and with their control groups. These results were previously described by many studies which reported that,HFD successfully induced hepatic steatosis and steatohepatitis (***Marra et al., 2018 and Younossi et al., 2018***). ***Pasarín et al. (2017)*** reported that mice fed a high-fat diet had insulin resistance in their LSECs which lead to impairment of insulin-dependent vasodilatation. Also, nitric oxide consuming enzyme (NOX1) highly expressed in their LSECs which triggers endothelial dysfunction through increased nitro oxidative stress. ***Lei et al. (2021)*** stated that disturbance of LSEC appears early in the course of NAFLD because fat accumulation and lipo toxicityreduces sinusoidal perfusion which enhances tissue damage. In addition, expression of iNOS increased

significant in 12 weeks HFD group but not changed in 4 weeks HFD group. This result came in line with ***Tache et al. 2014*** who reported enhancing the expression of iNOS in hepatocytes, bile ducts and endothelial cells in chronic viral hepatitis patients. Also, ***Wang et al. (2018)*** stated that iNOS expression was increased in macrophages of lung due to inflammatory and oxidative markers.

According to VEGF expression in NAFLD, Our study showed that significant increase in VEGF expression in 12 weeks HFD group but no significant change in its expression in 4 weeks HFD group. There were manyconflicting studies about process of angiogenesis in fatty liver even in humans or animals, likewise to our result, in animal models of NASH, hepatic expression of VEGF was increased in multiple studies ***(Coulon et al., 2013; Fang et al., 2017 and Rezk et al., 2019).*** Several studies reported increasing Pathologic angiogenesis with NASH, but not in individuals with simple steatosis or normal livers (***Coulon et al., 2011; Coulon et al., 2012; Tamaki et al., 2013 and Lefere et al., 2018)***. In addition, Coulon and colleagues stated that improving liver vasculature and decreases liver inflammatory gene expression in a mouse model of NASH was treated by anti- VEGFR2 antibody. In another research, ***Van Steenkiste et al. (2011)*** stated that circulating level of placental growth factor (PlGF), which belongs to the VEGF family, increased in the patients with cirrhosis, which showed its relationship with the stage of fibrosis. Also, ***Belghaisi-Naseri et al. (2018)*** reported increasing in plasma VEGF in NAFLD patient.

In contrast to our findings, no significant difference was denoted in serum VEGF levels in the patients with NAFLD compared to the controls ***(Yilmaz et al., 2011)***. In another study, ***Papageorgioua et al. (2017)*** reported lower VEGF levels in the patients with NASH compared to healthy controls and those with simple fatty liver.

The possible explanation of up-regulation of VEGF, iNOS and Progression of simple steatosis to steatohepatitis is might be the consequence of inflammation and oxidative stress accompany IR. As we found a significant increase in serum CRP and hepatic oxidative stress markers (increased MDA, ROS, and decreased SOD) in 12 weeks HFD group. In NAFLD an obvious oxidative stress and inflammatory response is found because the consumptionof high caloric diets rich in fats and refined sugars result in deregulation of metabolism and oxidative stress (***Friedman et al., 2018). Zadorozhna et al., (2020)*** showed that**,** this chronic inflammation and capillaries LSECscause up-regulation of vascular endothelial growth factor (VEGF) and its receptors which are expressed in liver sinusoidal endothelial and stellate cells.

There is difference among the views on the position of angiogenesis in either declaration or exasperation of chronic liver disease (CLDs) and NASH. ***Elpek. (2015)*** revealed that in CLDs, angiogenesis might contribute to the progression of disease. Also, ***Xi et al. (2017)*** suggested that suppression of angiogenesis could deteriorate fibrogenesis. Also, ***Rezk et al. (2019)*** showed that Angiogenesis occurs before fibrogenesis and promotes HSC activation and fibrosis development, so suppression of angiogenesis might be a potential target to counteract the progression of CLDs.

Serum VEGF may not be a good indicator due to the platelet-mediated secretion of VEGF in the clotting process and that what leaded to this discrepancies in previous findings. Therefore, it can be concrete that angiogenesis plays a pivotal role in the progression of NAFLD.

After rats were subjected to chronic moderate exercise training in groups (Ic, IIc) (4 and 12 week HFD + exercise respectively), there was a significant increase in serum and hepatic level of NOX was noticed in association with an improvement of metabolic and lipid profile parameters including a decrease in (glucose, insulin, calculated HOMA and TC) and an increase in HDL levels. Attenuation in hepatic cell injury was also found in these groups detected by the significant decrease in pathologic features and histopathologic score of steatosis, hydropic degeneration and portal tract inflammation. There are conflicting findings in several previous studies examined the relationships between aerobic exercise and NO concentrations. Our result came in agreement with many studies ***(Bender et al., 2011 and Mikus et al., 2012). Rector et al. (2011) and Sheldon et al. (2014)*** reported the prevention of NAFLD development with chronic running exercise, indicating an important role for exercise in activation of eNOS in liver. On the other side ***Shekarchizadeh et al. (2012)*** reported that resistance training have no effect on the plasma level of effective factors on angiogenesis including NO. An improvement in endothelial cell function in exercise groups could be attributed to the decrease in hepatic cellular stress mediated by the beneficial effect of exercise in remission of NAFLD. Insulin resistance and its linked metabolic syndrome, a consistent feature of NAFLD, were considered the motivating force in endothelial function disturbance in fatty liver ***(Pasarín et al., 2017 and Tilg et al., 2017***). So, improving insulin resistance by exercise as was found in our

study is along with the proposed ways by which physical exercise improve NAFL ***(Haus et al., 2013 and Oh et al., 2015).*** Also, exercise training enhances lipid metabolism and decrease liver fat content leading to amelioration of hepatic steatosis and improvement of endothelial function ***(Keating et al., 2015, Houghton et al., 2017 and Hammoutene and Rautou, 2019).***

According to expression of VEGF and iNOS, there was significant decrease in their expression in group II c (12 weeks HFD + exercise).***Fang and Tang. (2017)*** agreed with us, they proved that swimming exercise significantly decreased HFD-induced VEGF expression levels in the gastrocnemius skeletal muscle samples. In contrast, proliferation and VEGFR expression were significantly increased in normal mouse hearts following an endurance training protocol ***(Bellafiore et al., 2019).*** Moreover ***Javad et al (2017)*** reported that aerobic training for 8 weeks leaded to increase VEGF in sedentary women**.** For instance, ***Fulghum and Hill, (2018)*** proved that endurance exercises such as running or swimming performed for prolonged periods are a significant physiological stimulus leading to the formation of new capillary in normal myocardium**.** ***Di Raimondo et al. (2017)*** proved that circulating factors which were released by the exercising skeletal muscle might have a role in supporting angiogenesis of healthy hearts.

On the contrary, many studies revealed that systemic levels of VEGF were frequently unaltered in response to exercise ***(Rojas et al., 2010; Ranjbar et al., 2011; Shekarchizadeh et al., 2012; Nourshahi et al., 2013 and Landers-Ramos et al., 2014).***

As mentioned above, there were several studies examined the relationships between aerobic exercise of different intensity and angiogenesis in different tissue (skeletal muscle & heart). Our study is the first study to show the effect of chronic moderate aerobic exercise on VEGF expression in liver tissue of NAFL rat model. Our explanation about decreasing VEGF and iNOS expression after exercise was due to the beneficial effect of exercise on metabolism of liver and that proved by the improvement in inflammatory, oxidative markers and histopathology of liver in exercised groups. Our results demonstrated a reduction in serum level of CRP in 12 weeks HFD + exercise group and that result came in agreement with ***van der Windt et al. (2018)***. On the other hand, ***Houghton et al. (2017)*** reported that 12 weeks of exercise in patients with NASH did not affect circulating markers of inflammation. A significant decrease in oxidative stress parameters (MDA and ROS) and an increase in SOD were also found in this study indicating attenuated inflammatory response and oxidative stress in 12weeks HFD + exercise group by the effect of exercise. Multiple clinical trials matched with our result which had shown that exercise reduces ROS formation ***(Oh et al., 2013; Oh et al., 2014 and Oh et al., 2015)***. In addition, training was reported to increase antioxidant enzymes including SOD1and SOD2, catalase and glutathione peroxidase in the liver with reduction in oxidative damage ***(Lima et al., 2015)***. Further studies will be necessary: (i) for a better understanding of the precise cellular and molecular mechanisms by which exercise affects on angiogenesis in NAFLD development and progression. (ii) to elucidate Whether the decrease in VEGF expression in the exercised groups was a consequence or a cause of this improvement, needs further investigations to detect expression of different types of VEGF receptors.

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