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Interrelation of liver vascularity to non-alcoholic fatty liver through a comparative study of the vasodilator effect of carvedilol or nicorandil in rats

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ABSTRACT

Aim: An experimental study of the effect of two vasodilators, carvedilol (B blocker with alpha-antagonist) and nicorandil (NO donor) on nonalcoholic fatty liver (NAFLD) induced by hypercholesterolemia and fatty diet in rats through studying the possible anti-inflammatory and antioxidant mechanisms.
Main methods: The rats were divided into 4 groups (6 rats each): The first (negative control group). The second, third and fourth groups were fed with cholesterol and fat- enriched diet for one month that stopped and continued on the standard diet for another month without treatment in the second group but treated with carvedilol and nicorandil in the third and fourth group respectively.
Key findings: They revealed that both improved NAFLD especially nicorandil treated proved by the reduction of liver enzymes (AST, ALT), the fatty infiltration determined histologically and biochemically (decrease liver triglycerides). This may be due to either being antioxidants (reduced malondialdehyde and elevated reduced glutathione) or anti-inflammatory (decreased of TNF-α) together with the reduction of insulin resistance and adiponectin elevation or gene expression (increased liver NF-κB and decreased eNOS expression) and finally maybe by their obvious effect on improvements of lipid parameters.
Significance: Carvedilol and nicorandil improved NAFLD through the interrelationship between inflammatory cytokines, antioxidants and insulin resistance.

1. Introduction

Hypercholesterolemia is a common risk factor in the initial stages of hepatic damage, accelerating the onset and worsening of non-alcoholic fatty liver disease (NAFLD) [1]. The stages of NAFLD can be presented from simple steatohepatitis (NASH) to cirrhosis and hepatic carcinoma [2] regardless of the body-mass index [3].

Changes in liver blood flow and vascular function have been demonstrated in the disease of fatty liver, as a result of the abnormal accumulation of triacylglycerol with the hepatic cytoplasm [4]. Reduction of sinusoidal perfusion in hepatic cytoplasm was associated with the gravity of fat accumulation in parenchymal cells in steatosis induced by diet where the acuity of steatosis greatly affected microcirculation [5]. Studies had clarified that steatosis led to an increase in the resistance to the portal blood flow intrahepatically [6]. So, it was found that a high-cholesterol diet that significantly reduced tissue oxygenation and hepatic microcirculation could develop steatosis [7].

Insulin attaches to its receptors at the peripheral endothelial cells [8] which in turn activates the phosphorylation of the substrate of insulin-receptor initiating a phosphorylation of a series of downstream substrates, including the pathway PI3K/Akt [9]. PI3K/Akt pathway activates eNOS which in turn increase nitric oxide (NO) production leading to vasodilation [10]. In IR, this pathway is malfunctioned in the metabolic process without affection of other pathways of signaling of insulin, including the Ras/MAPK pathway (important in the control of cell proliferation). So, an imbalance between insulin functions done by the PI3K pathways and MAPK [11] occurs leading to decreased activation of eNOS with decreased production of NO. Decreased NO leads to endothelial dysfunction producing an environment of a pro-fibrogenic, pro-inflammatory and a pro-thrombotic, that impairs regeneration after injury of liver; that accelerates the transition from steatosis to steatohepatitis and cirrhosis [12].In addition, the pro-inflammatory

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cytokines secreted from adipose tissue such as TNF-a which is an important mediator in the NAFLD as it regulates diverse functions as metabolic, immune and inflammatory processes including IR cytokines [13]. The presence of NO can be affected by the oxidative stress so; antioxidants could maintain microcirculation in conditions of compromised liver perfusion [14].

Carvedilol (a non-selective beta blocker with potent competitive alpha1 blocking effect, is a potent anti-oxidant. In physicochemical, biochemical and cellular assays, carvedilol and several of its metabolites inhibit lipid peroxidation, scavenge oxygen free radicals, inhibit the formation of reactive oxygen radicals and prevent the depletion of endogenous antioxidants, such as vitamin E and glutathione [15]. Nicorandil (a balanced vasodilator that acts as both NO donor and arterial K (+) ATP channel opener, by their effects in different parameters involved in this pathogenesis [16].

The present study evaluated whether the two vasodilator drugs have a potential efficiency in the treatment of fatty liver through different possible mechanisms.

2. Materials and methods

2.1. Animals

About 24 healthy adult male albino rats (Sprague Dawley) weighing 200–220 g were used in this study. Rats were bred in the animal house of the ophthalmic institute and divided into 4 groups. Each rat was housed in a metal cage measuring 42 cm L x 26 cm W x 22 cm H at ordinary room temperature, natural daily light-dark cycle, humidity and fed with the standard diet which was composed of protein 21%, fat 2.7% and fibers 2.6% in the control group while cholesterol-enriched diet 1% was added in the other groups and drank clean water. The rats were left for an initial adaptation period for at least one week before subjecting them to the experimental manipulation(s). All the experiments were performed between 09.00 a.m. and 03.00 p.m. and were conducted according to the regulations of the committee of bioethics for animal experiments of Kasr al-Ainy.

2.2. Drugs

Carvedilol (orally daily at 2 mg/kg for one month) Roche Pharmaceutical Chemicals Company, Cairo, Egypt) dissolved in distilled water immediately before use.

Nicorandil (orally daily at 3 mg/kg for one month) Merck Pharmaceutical Chemicals Company, Cairo, Egypt) dissolved in distilled water immediately before use.

2.3. Kits

1– Serum aspartate aminotransferase (AST)
2– Serum alanine aminotransferase (ALT)
3– Serum total cholesterol, triglycerides, High-density lipoproteins (HDL) were determined.
4– Fasting serum insulin level
5– Liver TNF-α content (ELISA)
6– Hepatic triglycerides
7– Malondialdehyde
8– Reduced glutathione contents
9– Measurement of adiponectin
10– Detection of gene expression of NF-κB and eNOS by the real-time-polymerase chain reaction (rtPCR)

2.4. Experimental design

The rats were divided into 4 groups (6 rats each) and subjected to the study for 2 months: The first and the second groups (negative and positive control group respectively). The second, third and fourth groups were fed with cholesterol and fat-enriched diet for one month that stopped and continued on the standard diet for one month without treatment in the second group but treated with carvedilol and nicorandil in the third and fourth group respectively.

2.5. Pharmacological estimation

2.5.1. Blood pressure measurement

Systolic blood pressure (SBP) of each rat was measured before and after the induction of hypercholesterolemia. SBP was determined using non-invasive blood pressure methodology in conscious rats [17]. It consists of using a tail-cuff sphygmomanometer (Panlab, USA) with a photoelectric sensor placed on the tail of the animal to prevent the blood flow. Pulsations disappear when the cuff is inflated. When the cuff is deflated, pulsations start to appear when the pressure in the cuff equals SBP. Exposure to high-fat diet tends to elevate SBP after the tests, so the difference between measurements before and after the tests was recorded [18] for each rat, SBP values were calculated as the mean of a minimum of three measurements. Then % change in blood pressure was calculated as follows: % change of blood pressure = (Systolic blood pressure after tests in mmHg - Systolic blood pressure before tests in mmHg)/Systolic blood pressure before tests in mmHg x 100.

2.6. Biochemical estimation

Blood samples were collected using citrate as anticoagulant from the tail rat. Samples were centrifuged at 3000 rpm for 10 min at +4 °C. The yellow plasma samples were collected with a pipette without disturbing the white buffy layer. The rats were sacrificed by cervical dislocation, the abdomen was dissected and the liver was extracted and divided into two parts for biochemical and pathological estimation:

2.6.1. Estimation of plasma cholesterol, triglycerides, LDL, HDL and glucose

They were measured in plasma, using commercially available kits according to the manufacturers' protocols (Biodiagnostic, Egypt).

2.6.2. Estimation of liver damage

ALT and ASTwere estimated in plasma, using commercially available kits according to the manufacturers' protocols (Biodiagnostic, Egypt).

2.6.3. Measurement of reduced glutathione (GSH)

It depends on the reduction of 5,5dithiobis (2-nitrobenzoic acid) (DTNB) with reduced glutathione (GSH) producing a yellow compound. The reduced chromogen is directly proportional to GSH concentration and its absorbance can be measured at 405 nm by using its kit [19].

2.6.4. Measurement of malondialdehyde (MDA)

One hundred milligrams of the liver tissue was homogenized in 1 mL PBS, pH 7.0 with the micropestle. 20% Trichloroacetic acid (TCA) was added to pancreatic homogenate precipitating the protein and then centrifuged. Thiobarbituric acid (TBA) solution was added to the supernatants after their collection, then was boiled for 10 min in a water bath, then the absorbance was measured. The concentration of MDA in supernatants of liver homogenate was calculated using the standard curve [20].

2.6.5. Measurement of insulin and adiponectin and TNF- α, by ELISA

Plasma insulin, adiponectin and liver **TNF- α** were determined by enzyme-linked immunosorbent assay kits, which were supplied by DRG, Union County, New Jersey, USA and Cloud-Clone CorP Company, Houston, Texas, USA, Biospes, China respectively.

Plasma insulin, adiponectin and liver **TNF- α** levels were quantified in duplicate using ELISA kit, according to the manufacturer's instructions. Standards at a series of concentrations were run in parallel with the samples, The samples were run from individual animals. The

Table 1
The primer sequence of the studied gene.

	Primer sequence
NF-kB	Forward primer: 5′- CATTGAGGTGTATTTCACGG −3 Reverse primer 5- GGCAAGTGGCCATTGTGTTC -3
e NOS	Forward primer: 5-TGA CCCTCACCGATACAACA3'- Reverse primer: 5- CGGGTGTCTGATCCATGC-3
Beta actin	Forward primer: 5′–GGTCGGTGTGAACGGATTTGG −3 Reverse primer:5′- ATGTAGGCCATGAGGTCCACC-3

readings were measured at 450 nm wavelengths.

2.6.6. Quantitative analysis of gene expression of NF-KB, eNOS by real-time PCR

Quantitative gene expression of **NF-kB** and **eNOS** was done by Real-time PCR. According to the manufacturer's protocol, total RNA was extracted from tissues by using SV Total RNA Isolation System supplied by Promega (Promega, Madison, WI, USA). RNA purity was determined spectrophotometrically at 260 nm.

Following RNA extraction, reverse transcription of the isolated RNA into cDNA was performed using Access RT-PCR System kit supplied by Promega (Promega, Madison, WI, USA) according to the manufacturer's instructions.

Using an Applied Biosystem with software version 3.1 (StepOneTM, USA), Real-time PCR amplification and analysis was done. The reaction contained SYBR Green Master Mix (Applied Biosystems).

Reverse transcription-polymerase chain reaction (RT-PCR) for b-actin (housekeeping gene) was performed to confirm the integrity of extracted RNA.

The sequences of the primers used for the real-time PCR are listed in Table 1.

2.7. Histological estimation

2.7.1. Specimens were taken and sectioning

After euthanasia of animals, specimens from liver were immediately dissected out, fixed in 10% formol saline for 24 h at room temperature, dehydrated in ascending grades of alcohol (70%, 95%, 100%), cleared in of xylene then embedded into paraffin wax (Department of Histology, Faculty of Medicine, Cairo University). Sections of 5 µm thickness were subjected to the following:

1. Hematoxylin & eosin (H.&E.) stain [21].
2. Periodic Acid-Schiff (PAS) reaction to demonstrate glycogen granules in hepatocytes [22].
3. Immunohistochemical staining for alpha-smooth muscle actin (α-SMA) to evaluate activated hepatic stellate cells (Myofibroblasts) [22] α-SMA Ab-1 (Clone 1A4) is a Mouse Monoclonal Antibody (Novocastra Lyphilized Corporation laboratories, UK, catalog number NCL-SMA).It was supplied as 200 µg/ml. then prepared in 10 nM phosphate buffer saline (PBS), pH 7.4, with 0.2% Bovine serum albumin (BSA) and 0.09% sodium azide. It is reactive to human, mouse, and rat. α-SMA immunopositivity staining appeared as cytoplasmic brown deposits.

2.7.2. Morphometric study

Using Leica Qwin 500 LTD (Cambridge UK) computer-assisted image analyzer,the area percent represented the areas of the positive reaction or staining, which were masked by a binary color to the area enclosed within a standard measuring frame (it was 7286.783 µm^2) on using magnification x400. The following parameters were measured:

1– Optical density of periodic acid –schiff reaction (PAS OD), to detect if there is depletion or preservation of glycogen in each group.
2– Area percent of α-SMA immunopositive cells.
3– 3-Sinusoidal diameter.

2.7.3. Statistical methods

Data were coded and entered using the statistical package SPSS version 20. Data were summarized using mean and standard error. Comparisons between groups were done using analysis of variance (ANOVA) with multiple comparisons post hoc test. P-values < 0.05 were considered as statistically significant.

3. Results

3.1. Effects of vasodilators on SBP in cholesterol-fed rats

Rat SBP significantly increased in the diseased group ($P < 0.01$) compared with the control group (Fig. 1). Both drugs significantly lower SBP compared with the diseased group ($P < 0.01$).

3.2. Effects of vasodilators on plasma liver enzymes, lipid profile and glucose in cholesterol-fed rats

Liver enzymes (ALT and AST) levels were significantly ($P < 0.01$) increased in the diseased group compared with the control group. ALT and AST levels decreased significantly ($P < 0.01$) with treatment with carvedilol and nicorandil compared to the diseased group (Table 2).

Likewise, statistically significant differences in total cholesterol (TC), triglycerides (TG), low-density lipoproteins (LDL) and high-

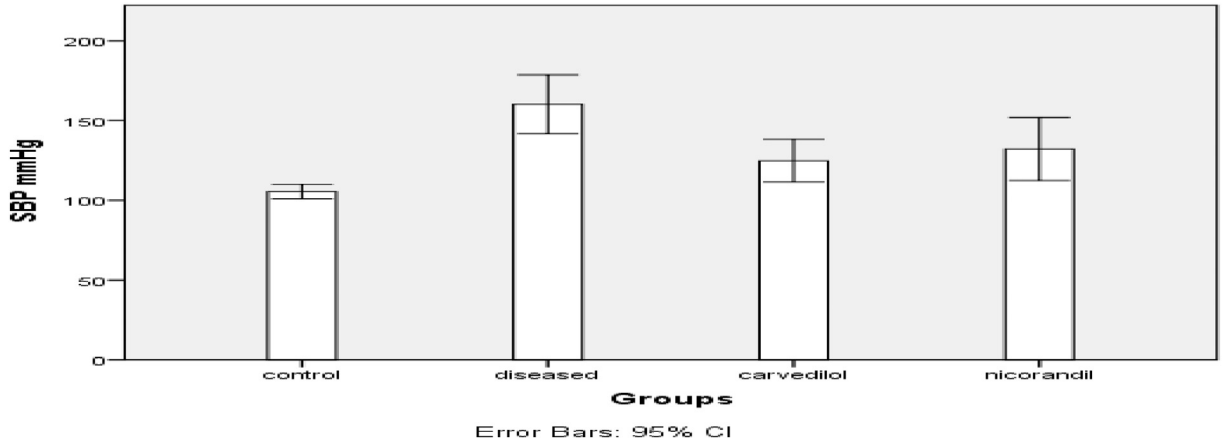

Fig. 1. Systolic blood pressure (SBP) mmHg in all groups. Data are presented as mean ± SE.
*, #: statistically significant compared with the control group and diseased group, respectively ($P < 0.05$).

Table 2
Mean of plasma liver enzymes and lipid profile.

	Control	Diseased group	Carvedilol group	Nicorandil group
ALT U/L	14.33 + 0.615	56.33 + 7.762*	28.67 + 1.856#	27.50 + 3.191#
AST U/L	18.50 + 0.563	51.83 + 2.725*	30.67 + 2.486*#	25.50 + 1.607#
TC mg/dl	129.50 + 2.377	196.17 + 4.362*	152.67 + 6.254*#	146.17 + 5.582#
TG mg/dl	73.67 + 0.715	125.17 + 6.819*	102.50 + 7.496*	102.50 + 4.137*
LDL mg/dl	83.68 + 3.207	187.2 + 4.399*	120.53 + 6.805*#	115.27 + 5.070*#
HDL mg/dl	60.55 + 1.248	34.00 + 1.802*	52.63 + 1.424#	51.40 + 3.730#
Glucose mg/dl	92.60 ± 4.32	191.61 ± 3.23*	136.98 ± 5.34*#	135.37 ± 6.28*#

Total cholesterol (TC), Triglycerides (TG), low density lipoproteins (LDL) and high-density lipoproteins (HDL). Data are presented as mean ± SE.
*, #: statistically significant compared to the control group and diseased group, respectively ($P < 0.05$).

density lipoproteins (HDL) levels were seen between the control and the diseased groups ($P < 0.01$). Treatment with carvedilol and nicorandil significantly ($P < 0.01$) lower TC and LDL levels and increase HDL levels compared with the diseased group (Table 2). Lastly, a modest insignificant ($P = 0.051$) decrease in the TG levels was observed with vasodilator treatment compared with the diseased group (Table 2).

Plasma glucose was significantly increased in the diseased group compared to the control group ($P < 0.01$). Even both vasodilators decrease significantly ($P < 0.01$) the plasma glucose level compared to the diseased group ($P < 0.01$), plasma glucose level did not return to the normal level and was significantly higher than control group ($P < 0.01$) (Table 2).

3.3. Effects of vasodilators on plasma insulin, adiponectin, and TNF-α in cholesterol-fed rats

Insulin and TNF-α levels were increased significantly while adiponectin levels were decreased in the diseased group compared with the control group ($P < 0.01$) (Table 3).

Insulin was significantly decreased in the nicorandil group ($P < 0.05$) but insignificantly ($P = 0.277$) in the carvedilol group compared with the diseased group. Both vasodilators significantly ($P < 0.01$) decreased TNF-α and increased adiponectin levels as compared with the diseased group.

3.4. Effects of vasodilators on TG content, e NOS,NF-kb relative expression and oxidative stress in the hepatic tissue in cholesterol-fed rats

e NOS relative expression ($P < 0.01$) was significantly decreased while NF-kb relative expression ($P < 0.05$) was significantly increased in addition to TG content and oxidative stress biomarkers in the rat liver of the diseased group ($P < 0.01$). The data in Table 4 demonstrated that administration of both drugs significantly decreased TG contents and ameliorated the effects of oxidative stress. Both vasodilators significantly increased e NOS relative expression ($P < 0.01$) and significantly lowered NF-kb relative expression ($P < 0.05$), and lowered the content of GSH, MDA ($P < 0.01$) compared with the diseased group (Table 4).

3.5. Histological results

3.5.1. Hematoxylin and eosin results

Histological examination of the rat liver sections from control group revealed normal liver parenchyma with a preserved architecture of hepatic lobules containing hepatocyte cords radiating from central veins (Photo 1a). Group II revealed marked disruption of hepatic architecture (Photo 1b). In group III, some hepatocytes displayed normal vesicular nuclei, while others showed pyknosis (Photo 1c). Group IV revealed picture closely near to that of the control group (Photo 1d).

3.5.2. PAS-stained liver sections

Strong PAS reaction was seen in the cytoplasm of the hepatocytes from the control group (Photo 2a). Group II displayed marked depletion of PAS-positive glycogen granules in most of the hepatocytes (Photo 2b). Group III showed diminished PAS reaction of some hepatocytes (Photo 2c). Group IV showed preservation of the glycogen contents in the cytoplasm of most of hepatocytes (Photo 2d).

3.5.3. Immunohistochemical results for α-SMA

Examination of liver sections from control group revealed weak immunoreactivity for α-SMA (Photo 3a). In group II, strong positive immunoreactions were seen (Photo 3b). Group III showed moderate positive immunoreactivity (Photo 3c). While group IV showed mild positive immunoreactivity (Photo 3d).

3.6. Histo-immunological evaluation

3.6.1. Study of oxidative stress of hepatic tissue

Oxidative stress was evaluated by PAS OD and alpha-SMA. PAS OD is a marker for glycogen content in the liver that significantly decreased in the liver of the diseased group ($P < 0.01$), indicating severe stress. Alpha-SMA which is secreted from hepatic stellate cells in response to liver injury and stress was significantly increased ($P < 0.01$) in the rat liver of the diseased group compared to the control group (Table 5). So, administration of carvedilol and nicorandil significantly increase PSA OD and lower alpha-SMA area % compared with the diseased group.

3.6.2. Assessment of sinusoidal diameter

Sinusoids diameter was significantly decreased in the diseased group compared to the control group ($P < 0.01$). Both vasodilators increase significantly ($P < 0.01$) the diameter of sinusoids compared

Table 3
Plasma INSULIN, adiponectin and TNF-α levels.

	Control	Diseased group	Carvedilol group	Nicorandil group
Insulin ng/ml	2.16 + 0.053	3.49 + 0.213*	2.98 + 0.184*	2.72 + 0.177#
Adiponectin ng/ml	8.55 + 0.585	3.59 + 0.450*	6.62 + 0.578#	6.48 + 0.386#
TNF-alpha pg/ml	35.80 + 1.343	122.17 + 6.590*	71.93 + 5.913*	65.75 + 4.170*

Data are presented as mean ± SE.
*, #: statistically significant compared to the control group and diseased group, respectively ($P < 0.05$).

Table 4
TG content and biomarkers of oxidative stress in liver tissue.

	Control	Diseased group	Carvedilol group	Nicorandil group
NF-kb relative expression	1.00 + 0.0	6.32 + 0.824*	3.59 + 0.0.388*#	3.23 + 0.571#
e NOS relative expression	1.00 + 0.0	0.29 + 0.053*	0.70 + 0.044*#	0.73 + 0.042*#
GSH mmol/mg ptn	52.67 + 0.835	28.38 + 2.120*	39.97 + 1.765*#	44.08 + 3.201#
MDA mom/ng ptn	11.51 + 0.372	86.63 + 11.443*	43.08 + 5.301*#	33.13 + 2.446#
TG mg/g ptn	35.27 + 1.496	82.22 + 5.858*	50.43 + 2.575*#	47.67 + 2.037#

Data are presented as mean ± SE.
*, #: statistically significant compared to the control group and diseased group, respectively (P < 0. 0.05)

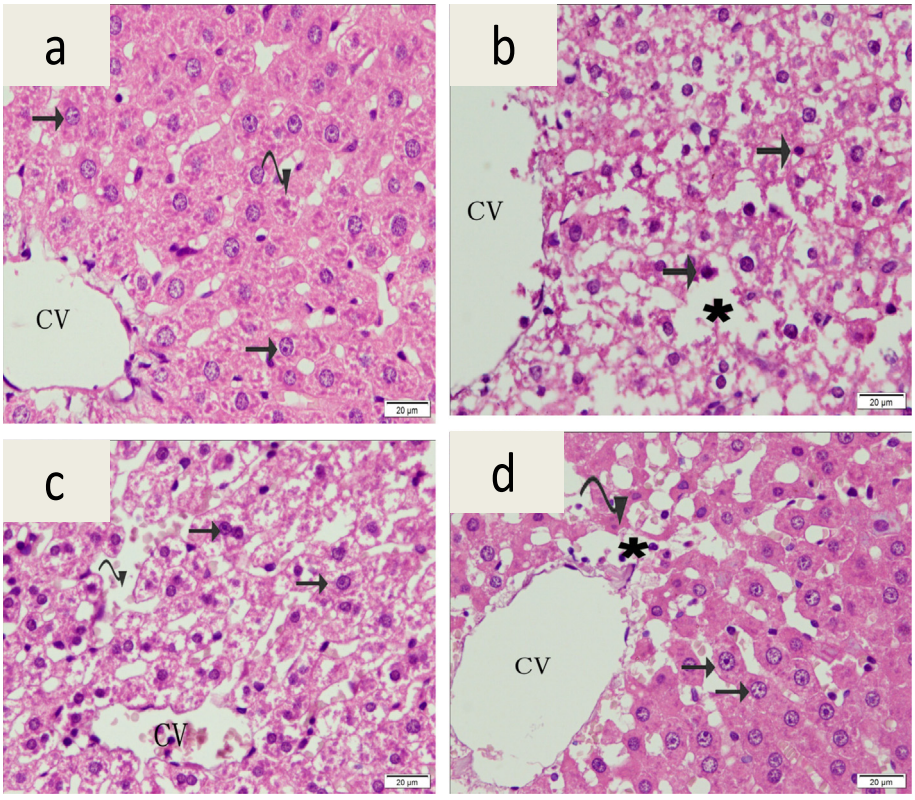

Photo 1. Photomicrographs of sections in the liver of rats from: (H&E X400).
(a): control group showing hepatic lobule containing hepatocytes arranged in plates radiating from the central vein (CV). The hepatocytes display acidophilic cytoplasm and vesicular nuclei with prominent nucleoli (arrows). The plates are separated by blood sinusoids (curved arrow),
(b): group II showing marked disrupted and vacuolated hepatocytes with dilated blood sinusoids. Some hepatocytes have pyknotic nuclei (arrows) and other devoid of nuclei with lysis (asterex),
(c): group III showing less vacuolations with preservation of the hepatocyte arrangement (arrows). Note the irregular dilated blood sinusoids (curved arrow),
(d): group IV showing hepatocytes with acidophilic cytoplasm and vesicular nuclei with prominent nucleoli (arrows). Few hepatocytes exhibit deeply acidophilic cytoplasm and pyknosis (curved arrow). Also, dilated sinusoids are seen (asterex),

to the diseased group (P < 0.01). The effect of nicorandil was significantly higher than carvedilol (P < 0.01) (Table 5).

4. Discussion

NAFLD is a multi-disorder which represents the hepatic part of insulin resistance and metabolic syndrome [23], ranging from steatosis to the end-stage liver cirrhosis, which is classified by clinical pathology of the liver [24].

To prove the occurrence of NAFLD, we measured the liver enzymes (AST, ALT) and systolic blood pressure which were significantly elevated in the diseased group and were decreased significantly in both treated groups. This was correlated to a study which indicated that most patients suffering from NAFLD are without obvious symptoms and the disease is usually suspected by raised AST, ALT levels in addition to other clinical and biochemical features, or discovered accidentally during abdominal ultrasonography. The mechanisms of NAFLD are still incompletely understood pathologically; however, it had strong correlations with insulin resistance, hyperlipidemia, oxidative stress and inflammation [25]. Also, innate immune system activation and release of proinflammatory cytokines from portal tract inflammatory cells might be the cause for the occurrence of hypertension in NAFLD patients [26], who were registered to have arterial stiffness among different populations [27].

NAFLD is characterized by excessive hepatic triglycerides and free fatty acids accumulation [28] which was obvious in our study by their elevation in the diseased group in addition to elevation of the lipid parameters determined by increased levels of serum cholesterol(TC), triglycerides (TG) and low-density lipoprotein (LDL) and decreased high-density lipoprotein (HDL). This is in accordance to a study which stated that NAFLD patients exhibit atherogenic dyslipidemia that is characterized by an increased plasma concentration of TG, reduced concentration of HDL cholesterol, and LDL particles that are smaller and denser than normal [29].

In this study, treatment with carvedilol and nicorandil significantly lower TC and LDL levels and increase HDL levels compared with the diseased group in addition to a modest insignificant decrease in TG levels was observed with treatment compared to the diseased group. This was in accordance to a study that proved nicorandil pre-treatment showed a significant decrease in TC, TG, LDL, very low-density lipoproteins-cholestero1(VLDL-C) and atherogenic index (Al) against high-fat diet model, suggesting that nicorandil possesses hypolipidemic activity which could be related to the release of nitric oxide property and inhibition of oxidative stress [30]. For carvedilol, a study proved that it is, in contrast to the conventional β-blockers, lacks the negative side effects on lipid profile [31]. So, we try to prove that the vasodilator

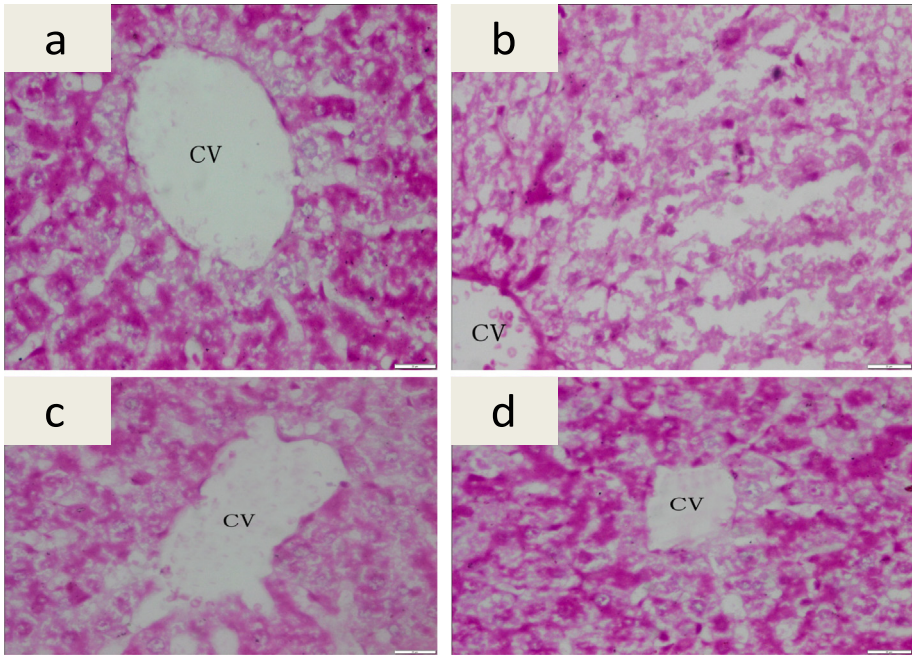

Photo 2. Photomicrographs of sections in the liver of rats from: (PAS x400)
(a): control group showing strong PAS + ve reaction,
(b): group II showing marked diminished PAS + ve reaction,
(c): **group III** showing localized diminished PAS + ve reaction,
(d): **group IV** showing PAS + ve reaction near to control.

drugs could be effective in treatment of NAFLD and to determine by which mechanism this could be done. For this, Insulin and tumor necrosis factor alpha (TNF-α) levels were measured which were increased significantly while adiponectin levels were decreased in the diseased group compared to the control group. Insulin was significantly decreased in the nicorandil but insignificantly in the carvedilol group compared with the diseased group. Both vasodilators significantly decrease TNF-α and increase adiponectin levels compared to the diseased group. This was in accordance to a study which stated that TNF-α appears to play the main role in the occurrence of hepatic steatosis being a proinflammatory cytokine that leads to oxidative stress, inflammation, and apoptosis or necrosis of liver cells [32]. In addition, adiponectin is a

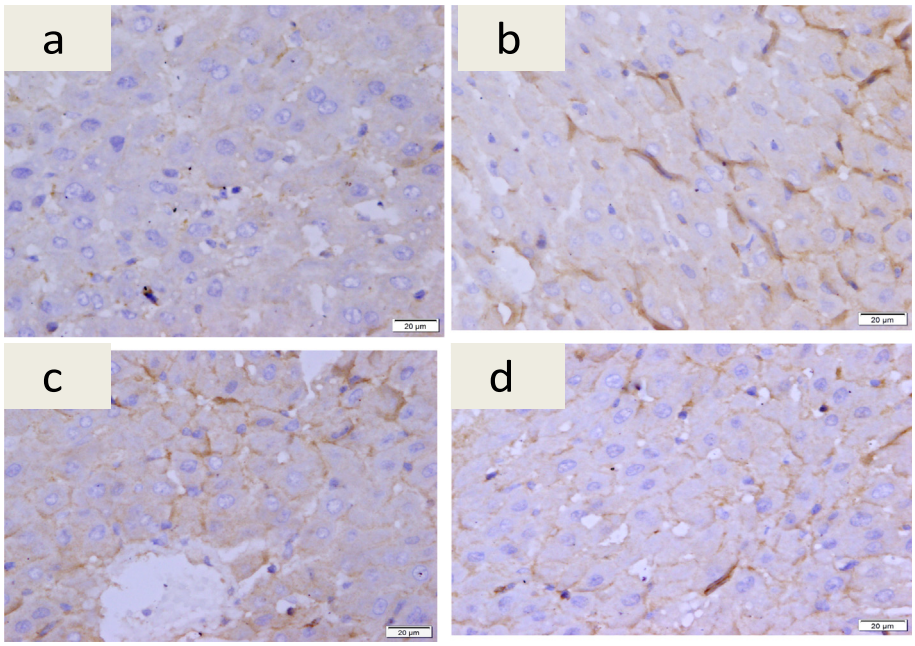

Photo 3. Photomicrographs of sections in the liver of rats from: (Immunohistochemical stain for α- SMA x400).
(a): control group showing weak immunoreactivity,
(b): group II showing strong immunoreactivity,
(c): **group III** showing moderate immunoreactivity,
(d): **group IV** showing mild immunoreactivity.

Table 5
PSA OD and alpha-SMA area % in liver tissue.

	Control	Diseased group	Carvedilol group	Nicorandil group
PSA OD	0.59 + 0.007	0.30 + 0.009*	0.44 + 0.014*#	0.57 + 0.0.009#
alpha- SMA area %	10.39 + 0.348	24.02 + 0.702*	17.36 + 0.321*#	12.49 + 1.003#
Sinusoids diameter μm	0.408 ± 0.010	0.332 ± 0.010*	0.600 ± 0.007*#	0.538 ± 0.0135*#$

Data are presented as mean ± SE.
*, #, $: statistically significant compared to the control group, diseased, carvedilol group, respectively ($P < 0.05$).

potent TNF-αneutralizer via suppression of its synthesis also it induces anti-inflammatory cytokines as IL-10 or IL-1 receptor antagonist leading to a balance between pro-inflammatory and anti-inflammatory cytokines [33].

Insulin resistance led to activation of eNOS with decreased production of NO initiating inflammation [12]. This was evidenced in this study by significant reduction of eNOS relative expression in the diseased group and its significant elevation in both treated groups, especially nicorandil group.

Nuclear factor-κB (NF-κB) is a nuclear transcription factor activated by different factors as cytokines (TNF-α, TNF-β, IL-1), growth factors (as insulin), stress response (oxygenation)leading to the expression of inflammatory cytokines together with activator protein-1 (AP-1) [34], making the inflammation worse [35] by initiating transcription of many genes as IL-1, IL-6, IL-8, TNF-α after activation. These gene products regulated by NF-κB are involved in liver inflammation, fibrosis, regeneration, and apoptosis, together with insulin resistance in the liver [36]. Both IR and inflammatory cytokines are involved in NAFLD pathogenesis. There were also experiments confirmed that NAFLD rat liver tissue NF-κB expression was significantly enhanced than the normal group [37]. Therefore, the NF-κB signaling pathway is probably involved in the pathological process of NAFLD, this was supported in our study which showed a significant increase of NF-κB relative expression in the diseased group and its significant reduction in both treated groups especially nicorandil.

The data established by Houstis et al., [38] stated that elevation in ROS levels usually precede the IR occurrence and might be related to its cause, this was matched by our results where MDA was significantly elevated while GSH was significantly decreased in the diseased group compared to the control group, but these oxidative parameters were significantly improved in both treated groups.

Those previous results matched the study which reported that treatment with pioglitazone or nicorandil either alone or in combination successfully improved the bad effect of high fat diet(HFD) through a significant reduction in liver triglycerides, MDA content and TNF-α as well as a significant elevation in liver GSH content. In comparison with the control group; liver expression of NF-κB was significantly elevated while liver eNOS expression and nitric oxide content were significantly decreased in HFD group [39].

Also our results were in accordance to a study which stated that carvedilol can reduce the oxidative stress, inflammatory response and fibrosis in ethanol-induced liver injury in a rat model through the downregulation of Kupffer cells and hepatic stellate cells (HSCs) signaling with reduction in steatosis, fibrosis, necrosis and degeneration of the hepatic cord, this was evidenced by reduced levels of AST, ALT, TG, MPO, MDA, and proinflammatory cytokines (IL-1β and TNF-α,), and increased levels of the anti-inflammatory cytokine IL-10 and GSH in addition to increasing expression of suppressor of cytokine signaling 1(SOCS1), superoxide dismutase 1(SOD-1), and glutathione peroxidase1(GPx-1) and decreasing expression of IL-1β and NF-κB [40].

In this study, sections from HFD group (group II) showed liver injury characterized by disorganization, degeneration in addition to necrosis of the hepatocytes, where the necrotic cells exhibited deeply acidophilic cytoplasm. Fatty infiltration with marked vacuolation of the hepatocytes surrounding the central veins was also seen. These results are consistent with other researchers like Ganz et al., [41] who reported histological changes in the liver sections from rats exposed to HFD in the form of distorted hepatic architecture and apoptotic hepatocytes. Furthermore, there was a marked reduction of glycogen hepatocytes in this group. This was evident by a significant decrease in the mean area % of PAS-positive reaction of this group (group II) compared to the control (group I). This is further supported by a study done by Park, et al. [42] who described a pronounced glycogen depletion in the PAS-stained sections from the liver of rats exposed to HFD. They reported that the accumulation of lipids in the liver disturbs hepatocytes function, impairing hepatic glucose metabolism which finally leads to hyperglycemia. Increase the activity of the hepatic stellate cells (HSCs) in group II was proved by the significant increase in the mean area % of α-SMA immunoreactivity when compared to the control. HSCs were established as a contributor to hepatic oxidative stress and liver fibrosis [43]. This is further supported with observations done by Munsterman et al. [44] who reported the direct proportion of HSCs activity and fatty liver tissue.

In the present study, liver sections from rats in group III showed a decrease in the histological alterations caused by HFD. There was a significant increase in the optical density of PAS reaction and significance decrease in the mean area % of α-SMA immunoreactivity when compared to the HFD group. In agreement with these findings, Mosbah et al. [45] who detected that carvedilol improved the steatotic livers caused by prolonged exposure to cold ischemia, reduced hepatic injury and improved hepatic functionality. Moreover, Hamdy and El-Demerdash, [46] reported that carvedilol significantly reduced markers of liver fibrosis including collagen accumulation and the expression of α-SMA in HSCs.

On the other hand, group IV showed preserved a histological picture of liver sections closely to the control group. This is further emphasized by a significant increase in the optical density of PAS reaction as compared to the groups II & III indicating the ability of nicorandil to preserve the glycogen content of hepatocytes exposed to HFD. Similarly, there was a significant decrease in the mean area % of α-SMA immunoreactivity when compared to group II indicating the protective effect of the nicorandil against HFD induced liver injury. These findings are in line with the previous study that reported the role of nicorandil in the protection of hepatic injury induced by HFD by its antioxidant and anti-inflammatory [39]. Moreover, Mohamed et al., [47], reported a protective effect of nicorandil against liver fibrosis induced by bile duct ligation in rats as proved by a significant decrease in α-SMA expression in HSCs. This may be explained by the study done by [48] Zhu et al., who reported that nicorandil could restore redox homeostasis and protect cells from the ROS with further reduction in oxidative damage and inflammatory responses.

5. Conclusion

In this study, we suggest that vasodilatation itself has the major role in ameliorating the NAFLD whatever the mechanism which was beneficial in the results detected in both groups especially that treated by carvedilol, also we prove the relation between vasodilatation and such improvements.

6. Research involving human participants and/or animals

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed according to animal ethics of Helsinki.

Conflict of interest

The authors state no conflict of interest in carrying out, reporting or publishing this work.

Authors contribution

Dr. Ghada Farouk Soliman got the idea and designed the protocol, shared in the practical work and in writing the paper, Professor Dr. Laila Ahmed Rashed, Dr. Walaa Ibrahim and Dr. Heba Morsi performed the biochemical part of the study; Dr., Hanan Abdallah performed the histopathological part in addition to writing their parts in the manuscript, Dr. Nermeen Bastaway and Omnia Mohamed did the statistics, shared in the practical work and writing the paper.

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