Original Article

Effect of Beta-adrenergic blockade in High-fat-induced nonalcoholic fatty liver disease

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Abstract

Objective: Previous studies have reported that Non-alcoholic fatty liver disease (NAFLD) is relevant to insulin resistance, and autonomic blockade improves insulin sensitivity. The current study aimed to evaluate the effect of propranolol as a beta-blocker on insulin resistance and biochemical parameters in the rat model of non-alcoholic fatty liver disease. Materials and Methods: Male Sprague-Dawley rats were divided into three groups (n= 8 in each group): normal control group (NC), high-fat diet group (HFD), and HFD and propranolol group (HFD+P). After six weeks, the rats were sacrificed and blood was collected for measurement of biochemical and ELISA analyses and also liver for hepatic biochemical indices. Liver tissue was collected for measurement of GLUT4 expression by immunoblot analysis. Results: After six weeks, the plasma level of triglyceride (TG), alanine aminotransferase (ALT), aspartate aminotransferase (AST), insulin resistance, TNF-α, hepatic content of malondialdehyde (MDA), and triglyceride (TG) increased in HFD group. Treatment by propranolol significantly attenuated these alterations. Conclusions: Administration of propranolol decreased insulin resistance, improved dyslipidemia, and increased expression of GLUT4 in the liver of rats.

Keywords: Non-alcoholic fatty liver disease, Propranolol, insulin resistance, GLUT4

Introduction

A high-fat diet (HFD) and excess energy intake adversely affect the health of humans and induce obesity, insulin resistance and hepatic damage [1,2]. Non-alcoholic fatty liver disease (NAFLD) is considered as one of the most common liver diseases. It is resulted by fat accumulation in the liver or steatosis followed by inflammation in the liver cells or non-alcoholic steatohepatitis (NASH), which could lead to fibrosis and finally cirrhosis [3]. NAFLD is mainly associated with insulin resistance. Insulin resistance increases glucose levels and causes adipocyte lipolysis that can induce hepatic steatosis and fatty liver [4]. Hepatic insulin resistance is mediated by partitioning of free fatty acids to the liver and by an imbalance of adipocytokines (decreased adiponectin and increased TNF-α) [5].

Glucose is a key fuel in mammals and it has to be transported from the circulation into target cells. The facilitative transporters (GLUT) utilize the diffusion gradient of glucose across plasma membranes [6]. Glucose transporter 4 (GLUT4) is encoded by the Slc2a4 gene and has a critical role in glycemic homeostasis. Binding of insulin to its receptor leads to translocation of GLUT4 to the plasma membrane and facilitates glucose uptake. Impaired GLUT4 expression has been related to insulin resistance [7, 8]. Expression of GLUT4 in the hyperglycemic liver is higher compared to the muscles [9] and expression of this transporter decreases in the liver of diabetic rats [10].

Propranolol (Prop) is a β-adrenergic receptor blocker. It has antioxidant and anti-inflammatory properties. Prop reduces intracellular Ca^{2+} and attenuates mitochondrial dysfunction. It is used traditionally for hypertension, myocardial infarction and cirrhosis [11]. Autonomic blockade improves

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insulin sensitivity [12]. Prop decreases hyperglycemia and increases GLUT4 expression in rats [7]. In the present study, we aimed to evaluate the effect of propranolol as a beta-blocker on high-fat diet-induced non-alcoholic fatty liver disease in Sprague–Dawley rats. For this study, biochemical parameters in plasma and liver were determined and histological analysis and hematoxylin and eosin (H&E) staining were performed. Following detection of GLUT4 protein expression was carried out by western blotting in liver tissue.

Materials and Methods

Reagents and materials

Propranolol was obtained from Sigma–Aldrich, St. Louis, MO, USA. Cholesterol (Cholesterol, extra pure, Ph Eur) was purchased from Merck, Germany. Commercial kits used for the evaluation of glucose, triglyceride (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), alanine aminotransferase (ALT), and aspartate aminotransferase (AST) were purchased from Pars Azmun Company, Iran. Serum adiponectin measurement kit (Rat adiponectin, ADP ELISA kit) was purchased from ALPCO USA. Serum tumor necrosis factor α (TNFα) and insulin measurement kits (Rat TNF-α ELISA kit; Rat Insulin, INS ELISA kit) were purchased from CUSABIO Diagnostic, Japan.

Animals and treatment procedure

Male Sprague-Dawley rats (200 ± 20 g) were purchased from Razi Serum and Vaccine Institute Karaj, Iran. The experimental protocol was performed in accordance with the international guidelines established in the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, 1996) and further approved by the University's Internal Ethics committee. Animals were kept in plastic cages at a temperature of 22-24 °C under a 12-h light/dark cycle. They were allowed free access to standard rodent chow and water. Animals were kept at the laboratory for one week. After adaptation, they were weighed and divided into the following three groups (n= 8 in each group): normal control group (NC), high-fat diet group (HFD), and HFD and propranolol group (HFD+P).

Induction of non-alcoholic fatty liver disease using high-fat emulsion diet

NAFLD was induced by a high-fat emulsion diet (HFD) as previously described [13]. HFD was prepared based on 77% of its energy from fat, 14% from total milk powder and 9% from carbohydrate [13]. In this emulsion, fat was provided by corn oil (shown in Table 1). HFD was administered to the rats once a day via oral gavage for six weeks.

Treatment of non-alcoholic fatty liver disease

Propranolol (25 mg/kg) was administered for propranolol therapy. For the determination of propranolol dosage, previous studies were considered [14]. Rats in the drug-administered group were treated by oral gavage with a solution of water containing the propranolol at the indicated concentration, once a day for six weeks simultaneous with the induction of NAFLD. Rats in the non-drug-administered group received water without propranolol, according to the same time schedule.

Sample collection

At the end of the test and 14 hours after fasting, the animals were weighed and anesthetized by diethyl ether in inhalation way. Blood sampling was performed using a 5ml syringe from the heart ventricle. Blood samples were placed at room temperature and then serum was separated by centrifuge and kept at a temperature of -20 °C until analysis. The liver was immediately removed with caution, washed by normal saline and weighed. One part of the liver tissue was quickly isolated for Western blot analysis. It was submerged in liquid nitrogen and immediately stored at -80 °C until analysis. One part of the liver tissue was placed in 10% formalin buffer solution for histological evaluations. Other parts of the liver tissue were homogenized in a 50 mM phosphate buffer solution (pH = 7) and kept at -80 °C until the test time.

Serum biochemistry

Serum levels of LDL-C, HDL-C, TG, TC, glucose, AST, ALT, insulin, Adiponectin, and TNFα were determined by using standard animal diagnostic kits and Erba XL200 Auto Analyzer. The insulin resistance index was evaluated by the homeostasis model assessment (HOMA) formula [15].

HOMA-IR = fasting serum Insulin (μU/ml) × fasting plasma glucose (mmol/l) / 22.5

Hepatic biochemistry

The activity of superoxide dismutase (SOD) was evaluated by measuring the percentage inhibition of the pyrogallol auto-oxidation by SOD according to the method of Marklund & Marklundand [16]. Malondialdehyde (MDA) was estimated by the method proposed by Buege and Aust [17]. The hepatic concentration of TC and TG was estimated after chloroform-methanol extraction according to Floch's method [13].

Histopathological evaluation

Histological changes in liver tissues were detected by hematoxylin-eosin (H&E) staining as previously described [13]. Liver tissue was placed in 10% formalin solution for dehydration. After 48 h, paraffin was added to the tissue and staining was performed by H&E. The samples were cut into 5-μm sections according to routine protocols and examined by optical microscopy [13].

Protein expression detected by Western blot

Western blot analyses were performed as previously described with some modifications [18, 19]. For western blotting, the tissue lysates were mixed with an equal volume

of 2X Laemmli sample buffer. Quantitation of proteins was measured using the Bradford assay. Lysates (15 µg) were then subjected to SDS-PAGE after a 5 min boiling and subsequently transferred to a 0.2 µm immune-Blot™ polyvinylidene difluoride (PVDF) membrane (Cat No: 162-017777; Bio-Rad Laboratories, CA, USA). The membranes were then blocked with 5% BSA (Cat No: A-7888; Sigma Aldrich, MO, USA) in 0.1% Tween 20 for 1h. Then, the membranes were incubated with Anti-Glucose transporter type 4 (GLUT-4) (Cat No: ab654, Abcam) and anti-beta actin-loading control antibodies (Cat No: ab8224; Abcam) for 1h at room temperature. Subsequently, membranes were washed thrice with TBST and incubated with HRP-conjugated secondary antibody. The membranes were then incubated with enhanced chemiluminescence (ECL) for 1–2 min. Protein expression was normalized to β-actin. Densitometry of protein bands was performed using the ImageJ Version 1.44 software (NIH, USA), such that, the percentage area under the curve of each band was divided by the percentage area under the curve of its corresponding actin band, and then calculated values were compared between groups as we described previously [20].

Statistical analysis

One-way analysis of variance (ANOVA) was used, and the results were expressed as the mean ± SEM (standard error of the mean) followed by Tukey's post-hoc test. The level of statistical significance was set at $p < 0.05$. Experiments were replicated at least two times.

Results

Evaluation of NAFLD rat model by feeding HFD

Induction of non-alcoholic fatty liver disease was established by feeding a high-fat emulsion diet and evaluated by H&E and transaminase activities. H&E staining displayed obvious fat droplets and macro-vesicular steatosis in liver sections of the HFD group. Liver histological changes were not observed in rats fed a normal diet. Liver transaminase activities (ALT and AST) were increased significantly in the HFD group compared with the NC group. Insulin resistance, hyperlipidemia, steatosis, transaminase activities and elevation of hepatic content of MDA in the animals showed the successful formation of a NAFLD animal model.

Bodyweight gain and liver weight alterations

Bodyweight gain, food intake, and liver weight alterations are shown in Table 2. After six weeks of oral administration of the high-fat emulsion diet, no significant differences were observed in the body weight gain in the NC group compared with the HFD group and HFD+P group (P>0/05). HFD and HFD+P groups showed a marked reduction in food intake compared with the NC group (P<0/001). The liver index significantly increased in the HFD group compared with the NC group (P<0/05) but no significant change was observed in the HFD+P group compared with the NC group (P>0/05).

Serum biochemistry alterations

Biochemical parameters' alterations are reported in Table 3. After six weeks, a high-fat emulsion diet induced a significant increase in serum insulin level and insulin resistance based on the HOMA index (P<0/05). In the HFD+P group, a significant decrease was observed in the levels of these factors compared with the HFD group (Fig.1A) (P<0/05). High-fat emulsion diet induced a significant increase in serum TG, LDL-C and a significant decrease in serum HDL-C in the HFD group compared with the NC group (P<0/05). Serum FFA level significantly was increased in the HFD group compared with the NC group (P<0/05) but no significant change in FFA level was observed in the HFD+P group compared with the NC group (P>0/05) (Fig.1B). High-fat emulsion diet significantly increased serum TNF-α level and decreased adiponectin levels in the HFD group compared with the NC group (P<0/05), but in the HFD+P group, no significant change was observed compared with the NC group (P>0/05). High-fat emulsion diet induced a significant increase in serum AST and ALT levels compared with the NC group (P<0/05), but these elevations were decreased by administration of propranolol (P<0/05) (Fig.1C).

Hepatic biochemistry alterations

As shown in Table 4, after six weeks, hepatic content of TC and TG were significantly increased in the liver of HFD group compared with the NC group (P<0/05). In the HFD+P group, a significant decrease was observed in the level of hepatic content of TG compared with the HFD group (P<0/01) (Fig.1. D). Hepatic SOD activity significantly decreased and hepatic content of MDA significantly increased in the HFD group compared with the NC group (P<0/01). In the HFD+P group, a significant increase was observed in the levels of SOD activity and a significant decrease was observed in the levels of MDA compared with the HFD group (Fig.1E) (P<0/05).

Histological analysis

As shown in Fig.3, after six weeks of high-fat emulsion diet feeding, liver sections from the high-fat emulsion model group displayed obvious fat droplets and macro-vesicular steatosis in liver sections of the HFD group that was confirmed by H&E staining. Liver histological changes were not observed in rats fed a normal diet (Fig. 3A). Administration of propranolol attenuated the hepatic steatosis and fat droplets in the liver (Fig. 3B and 3C).

Expression of GLUT4 protein in liver tissue

We then analyzed protein level changes of GLUT4 in the liver. The results showed that propranolol significantly increased protein levels of GLUT4 in the liver tissue of the HFD+P group compared with the HFD group (P>0/05) (Fig.2).

Discussion

A high-fat diet and excess energy intake often cause oxidative stress that may be associated with various liver injuries and NAFLD [1,13]. In this study, oral administration of high-fat emulsion diet to rats induced oxidative stress, lipid peroxidation, steatosis and increasing in liver transaminase activities (ALT and AST). These results showed the successful formation of a NAFLD animal model.

Previous reports suggest that dietary fat intake plays a major role in body weight gain and saturated fatty acids have more effects on obesity than unsaturated fatty acids [21]. Corn oil contains high values of unsaturated fatty acids [22]. The present study showed that a high-fat emulsion diet (with corn oil) did not increase body weight in the HFD group compared with the normal control group. The results of this study are in agreement with these reports.

Previous studies have indicated that sympathetic activation seems to influence the development of insulin resistance [23]. Autonomic blockade improves insulin sensitivity in obese subjects [12]. Stimulation of the β-adrenergic receptor inhibits both insulin-induced glucose uptake and autophosphorylation of the insulin receptor substrate. Phosphorylation of insulin receptors was inhibited by propranolol [24]. GLUT utilizes the diffusion gradient of glucose across plasma membranes. Propranolol increases GLUT4 expression and improves insulin sensitivity in rats [7]. In this study, a high-fat emulsion diet increased insulin resistance based on the HOMA index and serum insulin level. We found that propranolol (a non-selective β-blocker) significantly decreased insulin resistance and improved Hyperinsulinemia in the HFD+P group and it may play a role in the improvement of insulin resistance in NAFLD.

The plasma level of FFA is related to lipolysis and insulin resistance [25]. Patients with metabolic syndrome and NAFLD often have dyslipidemia [26]. Insulin resistance, due to ectopic lipid, increased hepatic de novo lipogenesis and hyperlipidemia [27]. Insulin resistance is associated with increased TG levels and LDL-C [28,29]. Propranolol increases GLUT4 expression and improves insulin sensitivity [7]. In our study, a high-fat emulsion diet increased the serum level of FFA, TC, TG, LDL-C, and decreased HDL-C, but the levels of these parameters were not decreased in treated groups compared with the HFD group. Our finding suggests that propranolol may reduce hyperlipidemia produced by insulin resistance.

The elevated serum AST and ALT are laboratory abnormalities in most patients with NAFLD. Adiponectin is decreased and TNF-α is increased in patients with NAFLD [3]. TNF-α is a key factor in insulin resistance and NAFLD. High TNF-α and low adiponectin levels are associated with insulin resistance [30]. Propranolol has antioxidant and anti-inflammatory properties [12] and improves insulin sensitivity [7]. In this study, a high-fat emulsion diet increased serum AST, ALT, TNF-α level and decreased adiponectin levels compared with the NC group, but in the treated group with propranolol, no significant change was observed compared with the NC group. Improvement of inflammation and insulin resistance by propranolol may play an important role in modifying the increase in liver transaminase activities (ALT and AST), adiponectin and decrease in TNF-α level.

Superoxide dismutase (SOD) is an enzyme, which disables oxidizing agents such as active oxygen species [31]. Oxidative stresses also cause lipid peroxidation and MDA formation [32]. Reactive oxygen species (ROS) such as anion superoxide are free radicals created as a result of oxygen cellular metabolism. It can attack lipids and unsaturated fatty acids in the cell and cause lipid peroxidation [4]. The result of these reactions is the production of compounds with a carbonyl group such as MDA. It can migrate and cause harmful effects on proteins and DNA in the cell [33]. MDA is a toxic compound that is considered as a marker for lipid peroxidation [34]. Propranolol has antioxidant and anti-inflammatory properties [12]. In the HFD group of this study, hepatic content of MDA increased and SOD activity decreased, but in the treated group with propranolol, no significant change was observed. These findings suggest that Propranolol reduced oxidative stress and lipid peroxidation that were produced by HFD.

Dietary excess of fat contributes to intrahepatic fat accumulation [5]. The accumulation of cholesterol in the liver is one of the causes of hepatotoxicity [2]. Cholesterol in the liver causes inflammation and liver damage [35]. Previous studies have indicated that propranolol prevents lipid accumulation in the liver [36]. Propranolol can inhibit acute hepatic fat accumulation [37]. The results of this study indicated that Propranolol decreased intrahepatic cholesterol and triglyceride.

Previous reports have shown that expressions of GLUT4 in the liver reduce hyperglycemia and insulin resistance [38-41]. Propranolol increases the expression of GLUT4 in rats [7]. The present study showed that the expressions of GLUT4 in the liver significantly increased in the treated group with propranolol compared with the HFD group.

Conclusion

The results of this study suggest that propranolol reduces insulin resistance, oxidative stress, and the level of serum and hepatic lipid profile in male Sprague–Dawley rats fed with a high-fat emulsion diet. Thus, it seems that propranolol may be an effective drug for the improvement of insulin resistance, dyslipidemia and oxidative stress resulting from a high-fat diet and in the treatment of NAFLD.

Ethical Issues

The experimental protocol was performed in accordance with the international guidelines established in the Guide for

the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, 1996) and further approved by the University's Internal Ethics committee (approval code: IR.IAU.SRB.REC.1397.122). Concerning Ethical Clearance, the protocol was approved by the Animal Ethics Committee of the Science and Research Branch, Azad University, Tehran.

Conflict of interest statement

The authors declare that they have no conflict of interest.

Acknowledgment

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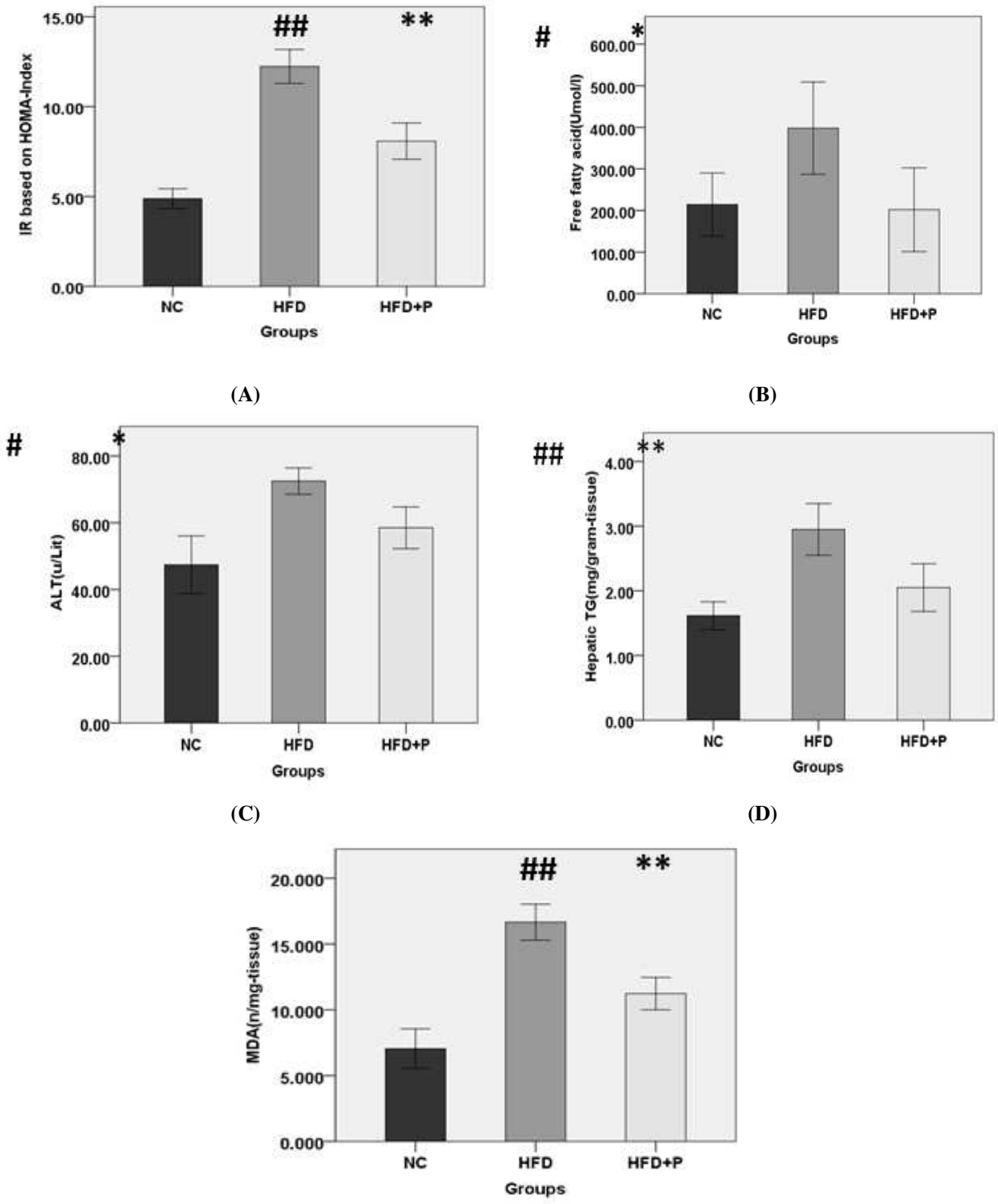


Figure 1. Effect of propranolol therapy on the concentration of (A) IR, insulin resistance based on HOMA index, (B) FFA, Serum-free fatty acid serum, ALT, (C) alanine aminotransferase level (D) hepatic content of TG, triglyceride and (E) MDA, malondialdehyde. Normal control group (NC), High fat diet group (HFD), HFD and propranolol group (HFD+P). Data are expressed as means ± SEM. Statistical analysis was performed using one-way ANOVA with Tukey's post-hoc test. * represents $p < 0.05$ compared with the HFD group. ** represents $p < 0.01$ compared with the HFD group. # represents $p < 0.05$ compared with the NC group. ## represents $p < 0.01$ compared with the NC group (n=8/group)

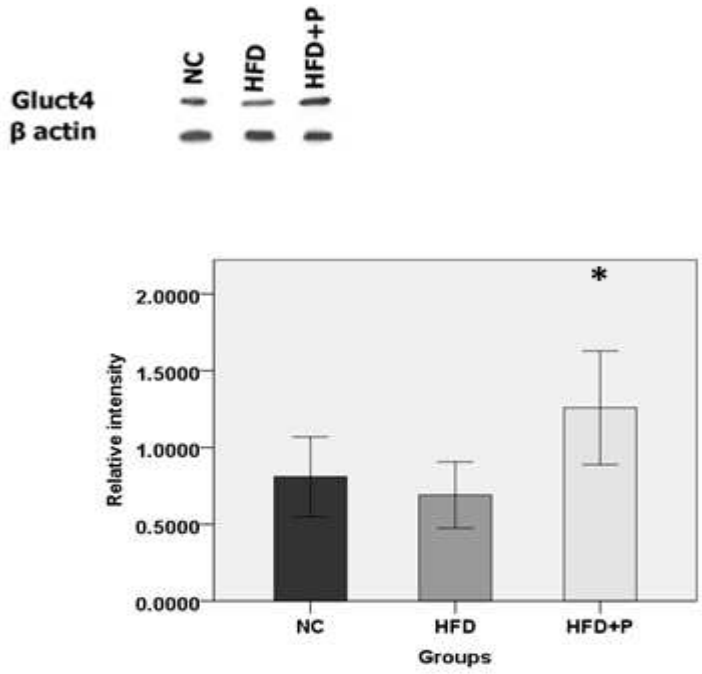
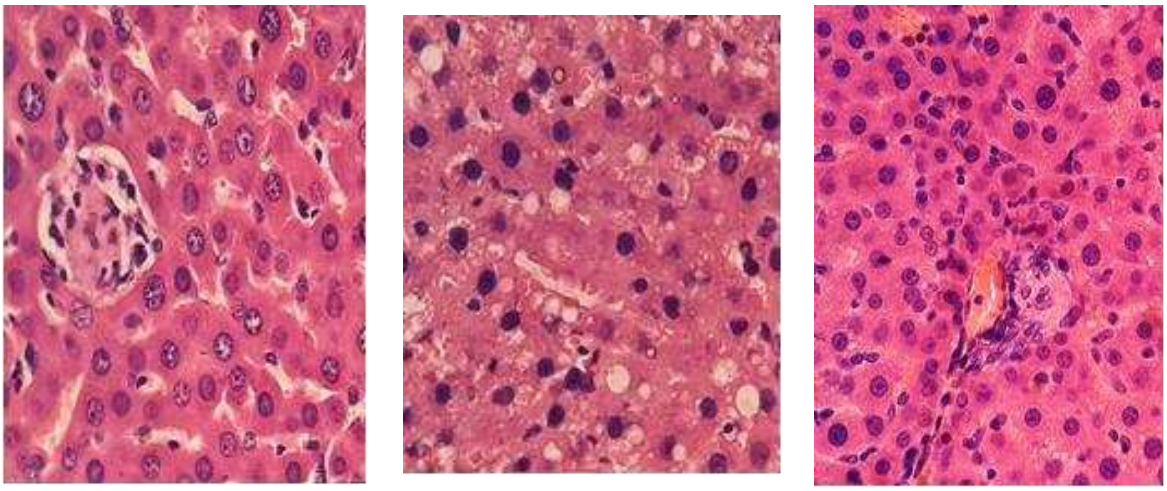


Figure 2. Western blots for GLUT4. Representative western blot images (A), and relative expression of GLUT4 in the liver. The percentage area under the curve of the protein band was divided by the percentage area under the curve of the corresponding β-actin band, and the normalized data were statistically compared between groups. Normal control group (NC), High fat diet group (HFD), HFD and propranolol group (HFD+P).
* represents $p < 0.05$ compared with the HF group. ** represents $p < 0.01$ compared with the HF group. # represents $p < 0.05$ compared with the NC group. ## represents $p < 0.01$ compared with the NC group.



(A) **(B)** **(C)**

Figure 3. Effects of propranolol therapy on histological alterations. The sections were stained for hematoxylin and eosin. (A) Normal control group (NC), (B) High fat diet group (HFD), (C) HFD and propranolol group (HFD+P).

Table 1: The composition and caloric content of the high-fat emulsion diet ingested via gavage in rat model of NAFLD [13].

Component	High-fat emulsion
Corn oil (g)	400
Saccharose (g)	150
Total milk powder (g)	80
Cholesterol (g)	100
Sodium deoxycholate (g)	10
Tween 80 (g)	36.4
Propylene glycol (g)	31.1
Vitamin mixture (g)	2.5
Cooking salt (g)	10
Mineral mixture (g)	1.5
Distilled water (ml)	300
Total energy	(4342 kcal/l)

Table 2: Effects of Propranolol on body weight gain, food intake, and liver weight

Group	NC	HFD	HFD+P
Parameters			
Food intake(g)	18.80±.11	14.56±.03##	14.66±.04##
Body weight gain(g)	45.42±16.39	57.50±12.95	55.83±11.82
Liver Index	2.64±0.16	3.06±0.40#	2.75±0.10

Normal control group (NC), High fat diet group (HFD), HFD and Propranolol group (HFD+P). Data are expressed as means ± SEM. Statistical analysis was performed using one-way ANOVA with Tukey's post-hoc test.
* represents $p < 0.05$ compared with the HFD group. ** represents $p < 0.01$ compared with the HFD group. # represents $p < 0.05$ compared with the NC group. ## represents $p < 0.01$ compared with the NC group (n=8/group).

Table 3: Effects of Propranolol on serum biochemistry parameters

Group	NC	HFD	HFD+P
TC (mg/dl)	75.57±18.94	60.50±5.63	69.83±6.91
TG (mg/dl)	46.57±7.32	59.75±6.11##	54.16±6.55
HDL-C(mg/dl)	40.42±3.86	28.37±2.61##	32.83±4.44#
LDL-C(mg/dl)	33.85±2.11	64.25±3.77##	54.50±2.88**
FFA(Umol/l)	214±81	397±132#	201±95*
AST(U/L)	147±22	249±23#	169±30*
ALT(U/L)	47±9	72±4#	58±5*
Adiponectin(pg/dl)	2.07±0.37	0.66±0.11##	2.11±0.79**
TNFα(Pg/ml)	174±38	229±41##	182±29**

Glucose (mg/dl)	96.85±12.23	151.12±14.37##	130.83±10.22*
Insulin (µIU/ml)	21.40±1.75	33.03±3.32##	25.73±2.81**
HOMA Index	4.87±0.59	12.23±1.12##	8.07±0.96**

TG, triglyceride; TC, total cholesterol; HDL-C, high-density lipoprotein-cholesterol; LDL-C, low-density lipoprotein-cholesterol; FFA, Free fatty acid; AST, aspartate aminotransferase; ALT, alanine aminotransferase; Adiponectin, Glucose, Insulin level, and HOMA Index. Normal control group (NC), High fat diet group (HFD), HFD and Propranolol group (HFD+P). Data are expressed as means ± SEM. Statistical analysis was performed using one-way ANOVA with Tukey's post-hoc test.
* represents $p < 0.05$ compared with the HFD group. ** represents $p < 0.01$ compared with the HFD group. # represents $p < 0.05$ compared with the NC group. ## represents $p < 0.01$ compared to the NC group (n=8/group).

Table 4: Effects of Propranolol on hepatic biochemistry parameters

Group	NC	HFD	HFD+P
Parameters			
TC (mg/g-tissue)	1.98±.33	3.63±.36##	2.88±.42*
TG (mg/g-tissue	1.61±0.23	2.9±0.47##	2.05±0.35**
SOD(u/mg-tissue)	333±19	237±29##	277±25*
MDA(n mol/mg-tissue)	7.04±1.62	16.66±1.63##	11.23±1.17**

TG, triglyceride; TC, total cholesterol; SOD, superoxide dismutase; MDA, malondialdehyde. Normal control group (NC), High fat diet group (HFD), HFD and Propranolol group (HFD+P). Data are expressed as means ± SEM. Statistical analysis was performed using one-way ANOVA with Tukey's post-hoc test.
* represents $p < 0.05$ compared with the HFD group. ** represents $p < 0.01$ compared with the HFD group. # represents $p < 0.05$ compared with the NC group. ## represents $p < 0.01$ compared with the NC group (n=8/group).