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Pharmacological study of the possible ameliorative effect of derivative of isoflavones in a dietary rat model of established non-alcoholic steatohepatitis

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ABSTRACT

Insulin resistance induces nonalcoholic fatty liver disease and nonalcoholic steatohepatitis (NASH). We used a high-fat, high-calorie solid diet (HFD) to create a model of insulin resistance and NASH in nongenetically modified rats and to study the relationship between visceral adipose tissue and liver. Obesity and insulin resistance occurred in HFD rats, accompanied by a marked reduction in visceral adipose tissue adiponectin-mRNA and deterioration of lipid profile. These modifications lead to hepatic steatosis accompanied by oxidative stress phenomena, necroinflammation, and hepatocyteballooning .Oral administration of ipriflavone (IP), to HFD-treated animals restored adipose tissue adiponectin expression, highly effective in decreasing the levels of serum total cholesterol (TC), triglycerides (TG), low-density lipoprotein cholesterol (LDL-C), alanine aminotransferase (ALT) and aspartate aminotransferase (AST). It could decrease lipid accumulation in the hepatocytes and ameliorated the degree of liver injury. Thus, our model mimics the most common features of NASH in humans and proved that ipriflavone had significant therapeutic benefits and could be explored as a potential promising candidate for the prevention of NASH.

KEYWORDS: Nonalcoholic steatohepatitis; Insulin resistance; High fat diet; ipriflavone

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is commonly associated with the clinical features of the metabolic syndrome such as obesity, type II diabetes, and dislipidemia. The clinical importance of NAFLD is due to its high prevalence (25% of the general population) and its wide spectrum of histological damage ranging from simple steatosis, which is generally nonprogressive, to nonalcoholic steatohepatitis (NASH), which can lead to cirrhosis, hepatocellular carcinoma, and liver failure [1].

Insulin resistance is central to the pathogenesis of the metabolic syndrome, and recent data indicate that NAFLD should be considered the hepatic manifestation of the metabolic syndrome. It has been demonstrated that a series of molecular alterations in insulin signaling occurs in the setting of insulin resistance, finally resulting in triglyceride accumulation in the liver. Recently, many studies have shown that lipid peroxidation is the trigger factor responsible for the transition from simple fat accumulation to eventually more progressive steatohepatitis or NASH [2]. Oxidative stress (found to be the main mechanism for pathogenesis) is associated

with, and exacerbated by, a striking depletion of antioxidant enzyme activities. Antioxidant supplement is one of possible strategies to maintain the redox homeostasis by directly quenching excessive ROS, or protecting endogenous antioxidative enzyme activities against oxidative stress [3] (Figure I).

Ipriflavone (IP) (7-isopropoxy-3-phenyl-4H-1-benzopyran-4-one) falls into the large group of isoflavonoids. It is a non hormonal isoflavone derivative (derived from the soy isoflavone, daidzein) currently used as a bone-building agent [4]. It is an attractive adjunct or alternative to conventional hormone (estrogen) replacement therapy in the prevention and treatment of osteoporosis [5]. It has been fully proved that IP inhibited bone resorption, prevented bone loss, promote activity of the bone-building cells in vitro and *in vivo* in experimental models of osteoporosis and reduce the pain of osteoporotic fractures [4]. It was also investigated as a potential anti-anginal agent and was seen to influence the mitochrondrial energetics in a positive manner, with an oxygen sparing effect [6]. The role of ipriflavone in the control of the metabolic diseases was not studied. We thus took advantage of a high-fat, high-calorie solid diet to pursue the aims of our study 1) to create a model of insulin resistance and NASH in nongenetically modified animals, 2) to study the relationship between the visceral adipose tissue and the liver in this model, and 3) to evaluate the possible anti-steatogenic effects of ipriflavone.

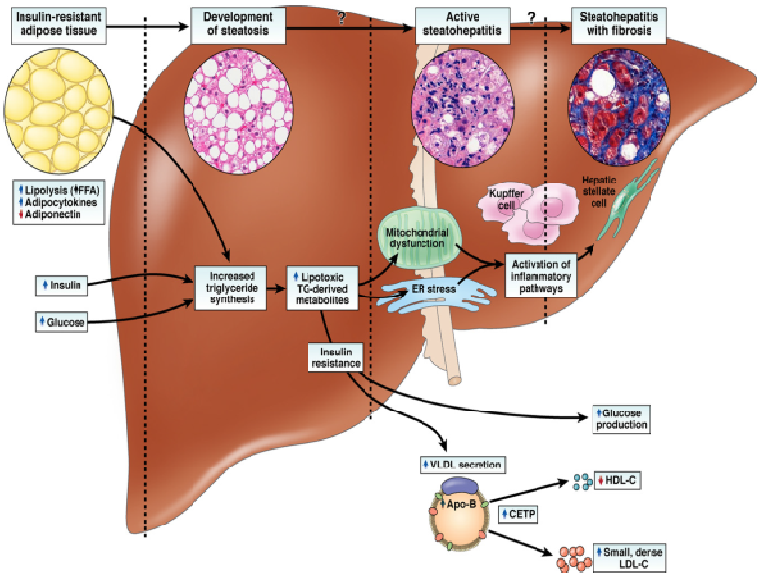


Fig. I: Schematic representation of the pathophysiology of NASH [7].

MATERIAL AND METHODS

2.1. Animals:

Healthy adult female albino rats weighing 100 ± 10 g were used in the present study. They were obtained from the animal house of the Research Institute of Ophthalmology (El-Giza, Egypt). Animals were kept under suitable laboratory conditions for one week for adaptation and to exclude any intercurrent infection. They were maintained in stainless steel cages at normal temperature of 27 ± 5ºC as well as under good ventilation. Animals had free access to water *ad libitum* and to two dietary regimens.

2.2. Dietary formula:

Composition of the rat diets (g/kg diet) are shown in table 1. Diet ingredients as casein, corn starch, cholesterol and sucrose were purchased from Oxford laboratories, Mumbai, India; DL- Methionine, bile salts, cellulose, and calcium carbonate were procured from S.D. Fine-Chem Ltd., Mumbai, India; potassium citrate, sodium chloride and calcium phosphate were obtained from Pharmaceutical Chemicals Co. Egypt; and the other components were obtained from commercial sources. Diets were prepared in the Department of Nutrition, Faculty of Veterinary Medicine, Beni-Suef University, Beni-Suef, Egypt, at intervals according to requirements and stored at 4°C till use (Table 1).

2.3. Tested drug:

Ipriflavone (IP) was purchased from Mfd. By Neutraceutical Corp., USA, suspended in 0.5% carboxy methyl cellulose (CMC) and it was administered in a dose of 50 mg/kg in intact animals. All the chemicals and reagents used were of analytical grade.

Table 1: Composition of the rat diets (g/Kg diet).

Ingredients	Control Diet (C)	High Fat Diet (HFD)
Casein	200	200
DL- Methionine	3	3
Corn Starch	612	—
Corn Oil	50	—
Beef tallow	—	400
Cholesterol	—	10
Bile salts	—	2
Sucrose	—	250
Cellulose	50	50
Vitamin and mineral mix	30	30
Calcium phosphate, dibasic	18	18
Calcium carbonate	16	16
Potassium citrate	16	16
Sodium chloride	5	5

2.4. Experimental design:

The animals were randomly distributed into 4 groups of each 12 animals: control (C) fed on control diet, control rats fed on control diet and supplemented with ipriflavone (IP), obese (Ob) fed on HFD, and obese rats treated with ipriflavone (Ob+IP). The rats were administered the diets and water ad libitum fed for ten weeks after which they were euthanized. After an overnight fast (throughout the 12-hour light cycle), blood glucose levels were measured in tail blood. At the end of the experiment, animals were sacrificed after an overnight fast; relevant tissues were removed and snap frozen in liquid nitrogen for subsequent analysis. A part of the median or left lobes of the liver was homogenized with 4 volumes of isotonic ice-cooled normal saline using a homogenizer to prepare 25% homogenates.

2.5. Serum markers:

Glucose concentration was determined according to the method of Siest *et al*. [8] using reagent kits provided by Bio-Mérieux Chemical Company (France). Insulin concentrations were measured in previously frozen and thawed serum samples by means of an enzyme-linked immunosorbent assay (ELISA) kit (Ultrasensitive Rat Insulin ELISA from Mercodia) according to the method of Olsson and Carlsson [9]. Both alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were assayed according to the method of Reitman and Frankel [10] using reagent kits purchased from Sclavo Company (Italy). Serum triglycerides concentration was determined according to the method of Kaplan [11] using reagent kit obtained from Spinreact Company (Spain). Serum cholesterol, high density lipoprotein cholesterol (HDL) and low density lipoprotein cholesterol (LDL) were estimated according to the method of Allain *et al*. [12] using the reagent kit purchased from Spinreact Company (Spain).

2.6. Real-time reverse transcriptase polymerase chain reaction:

Total RNA was isolated from adipose tissue samples using TRIzol (Gibco BRL, Life Technologies, Roskilde, Denmark); RNA was quantified by measuring absorbance at 260 and 280 nm, with a ratio of 1.8 or greater using a NanoDrop 8000 spectrophotometer (Thermo Fisher Scientific Inc, MA, USA). The integrity of the RNA was checked by visual inspection of the 2 ribosomal RNAs, 18S and 28S, on an agarose gel. Complementary DNA was synthesized with the Verso cDNA kit AB 1453 (Thermo Fisher Scientific Inc) using random hexamers. Real time polymerase chain reaction (PCR) for target genes were done with messenger RNA (mRNA) levels of 18S as an internal control, and this expression did not change during intervention.

Sequences of the used primer (Forward-5′- GCTCAGGATGCTACTGTTGCAA-3′, Reverse-5′- AACGTCATCTTCGGCATGACT -3′)

The PCR reactions were performed in duplicate using KAPA SYBR FAST qPCR Kit (Kapa Biosystems, Inc, Woburn, MA, USA) in an ICycler from Bio-Rad Laboratories (Hercules, CA, USA) using the following protocol: 1 step at 95°C for 10 minutes and then 95°C for 3 seconds, 57°C for 20 seconds, and 72°C for 1 second. The increase in fluorescence was measured in real time during the extension step. The threshold cycle was calculated, and the relative gene expression was calculated [13] (PerkinElmer Cetus, Norwalk, CT, USA).

2.7. Oxidative stress measurement:

A part of the median or left lobes of the liver was homogenized with 4 volumes of isotonic ice-cooled normal saline using a homogenizer to prepare 25% homogenates. The homogenates was used to estimate hepatic thiobarbituric acid reactive substances (TBARS) content, estimated as malondialdehyde (MDA) after centrifugation at 1000 xg for 15 minutes. It was determined according to the method described by Uchiyama and Mihara [14].

2.7.1. Estimation of reduced glutathione:

Another portion of the homogenate was deproteinized with 12% ice-cooled trichloroacetic acid, then the clear supernatant was used to determine hepatic reduced glutathione (GSH) content according to the method described by Sedlak and Lindsay [15].

2.7.2. Antioxidant enzyme activity:

The supernatant so obtained was further centrifuged at 105000 xg for 15 minutes at 4^0C using cooling ultra-centrifuge (Sorvall combiplus T-880, Du Pont, USA), which was used to assay superoxide dismutase (SOD) activity according to the pyrogallol autoxidation method of Marklund and Marklund [16].

2.8. Histopathological examination:

Portions of the liver of individual rats in normal and treated groups were fixed in 10% neutralized formalin and embedded in paraffin. Liver sections were stained with hematoxylin and eosin (H & E) for light microscopy according to the method of Bancroft and Steven [17] by the National Institute, Cairo University, Egypt.

2.9. Statistical analysis:

Tukey-Kramer method for post-hoc analysis was used to compare the data between different groups. The data expressed as mean ± SE. Statistical significance interval is considered as $p<0.05$ for all data. All results were analyzed using Statistical Package for Social Science (SPSS) version 22 software [18].

Results:

3.1. Effect of IP on serum parameters in adult obese rats:

Feeding HFD resulted in marked increase in serum glucose, insulin, HOMA-IR, ALT and AST, as compared to the control group value. Similarly, serum TG, cholesterol, LDL and vLDL concentrations of the obese group were significantly elevated. Treatment with IP at a dose of 50 mg/kg induced a potential alleviation of the serum levels of all of these parameters (Tables 1, 2).

On the other hand, serum HDL of the obese group was decreased with highly significant degree when compared to the control one. While, the obese rats treated with IP showed a profound and progressed elevation of the serum HDL level (Table 2).

3.2. Changes in oxidative stress biomarkers:

HFD produced a detectable decline of the adiponectin expression in the adipose tissue in addition to significant elevation in hepatic level of MDA, along with marked GSH depletion and SOD reduction. Treatment with IP significantly up-regulated adiponectin expression and produced a marked reduction of the hepatic MDA level as well as marked elevation of GSH and SOD activities (Table 3).

3.3. Effect of IP on HFD-induced changes in hepatic morphology:

Histological examination of livers from-obese rats revealed degenerative changes, micro- and macro-vesicular steatosis and focal hepatic necrosis associated with inflammatory cells infiltration. Most hepatocytes were replaced by fat cells with signet ring appearance, vacuolated cytoplasm with flat peripheral nuclei. IP suppressed most of histopathological changes resulting in reappearance of the normal architecture of hepatic lobule but there is still few fatty change of sporadic hepatocytes and slightly congested central vein (Figs. A-E).

Table 1: Effect of treatment of IP on serum glucose, insulin, homeostatic model assessment of insulin resistance (HOMA-IR), ALT and AST in obese rats

Treatment	Glucose (mg/dl)	Insulin (µIU/L)	HOMA-IR	ALT (U/L)	AST (U/L)
Control (C)	76.80 ± 2.458^{a}	15.02 ± 1.221^{a}	2.85 ± 0.021^{b}	33.50 ± 0.224^{a}	46.17 ± 0.477^{a}
Ipriflavone (IP)	74.23 ± 3.283^{a}	14.63 ± 1.106^{a}	2.68 ± 0.011^{a}	34.17 ± 0.401^{a}	46.77 ± 0.430^{a}
Obese (Ob)	245.43 ± 1.756^{c}	28.12 ± 0.164^{b}	4.84 ± 0.007^{d}	93.95 ± 0.839^{c}	209.13 ± 0.352^{c}
Obese + IP	103.23 ± 3.304^{b}	13.33 ± 1.171^{a}	3.4 ± 0.068^{c}	50.65 ± 0.679^{b}	96.95 ± 0.561^{b}
P-value	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$

Values represent means of 6 rats ± SE of the mean.
Values with the same superscript letter are similar (non-significant, $P > 0.05$) whereas others aren't (significant, $P < 0.05$). $P < 0.001$ is significant effect at $\alpha = 0.001$.

Table 2: Effect of IP treatment on serum lipid profile in obese rats

Treatment	Triglycerides (TG) (mg/dl)	Cholesterol (mg/dl)	HDL-Cholesterol (mg/dl)	LDL-Cholesterol (mg/dl)	vLDL.Cholesterol (mg/dl)
Control (C)	109.55 ± 2.979^{a}	186.22 ± 0.878^{a}	142.67 ± 1.300^{c}	21.65 ± 1.961^{a}	21.91 ± 0.596^{a}
Ipriflavone (IP)	115.55 ± 1.152^{a}	182.85 ± 1.364^{a}	147.03 ± 0.968^{c}	21.70 ± 1.061^{a}	23.11 ± 0.230^{a}
	378.23 ± 4.999^{c}	283.50 ± 4.583^{c}	66.67 ± 5.919^{a}	141.18 ± 7.596^{c}	75.65 ± 0.999^{c}

Obese (Ob)					
Obese + IP	174.18 ± 3.801[b]	204.54 ± 5.951[b]	121.10 ± 2.296[b]	48.60 ± 6.223[b]	34.84 ± 0.760[b]
P -value	*P* < 0.001	*P* < 0.001	*P* < 0.001	*P* < 0.001	*P* < 0.001

Values represent means of 6 rats ± SE of the mean.
Values with the same superscript letter are similar (non-significant, $P > 0.05$) whereas others aren't (significant, $P < 0.05$). $P < 0.001$ is significant effect at $\alpha = 0.001$.

Table 3: Effect of treatment of IP on adiponectin m-RNA expression in adipose tissue and liver MDA, GSH as well as SOD in obese rats

Treatment	Adiponectin	MDA (nmol/g tissue)	GSH (nmol/100 mg tissue)	SOD (U/g tissue)
Control (C)	2.89 ± 0.048[c]	47.99 ± 2.902[a]	35.34 ± 2.314[bc]	1.86 ± 0.178[b]
Ipriflavone (IP)	2.82 ± 0.058[c]	38.81 ± 3.178[a]	37.99 ± 2.285[c]	2.09 ± 0.202[b]
Obese (Ob)	0.97 ± 0.075[a]	120.93 ± 2.183[b]	13.49 ± 1.326[a]	0.63 ± 0.114[a]
Obese + IP	1.86 ± 0.031[b]	51.52 ± 4.598[a]	28.78 ± 2.110[b]	1.62 ± 0.172[b]
P-value	P < 0.001	P < 0.001	P < 0.001	P < 0.001

Values represent means of 6 rats ± SE of the mean.
Values with the same superscript letter are similar (non-significant, $P > 0.05$) whereas others aren't (significant, $P < 0.05$). $P < 0.001$ is significant effect at $\alpha = 0.001$.

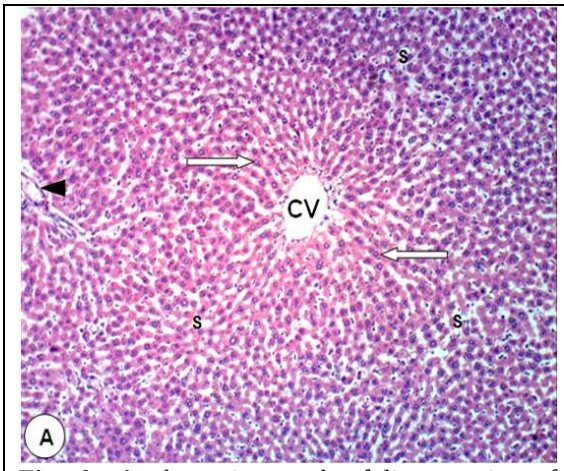


Fig. A: A photomicrograph of liver section of control rat showing cords of polyhedral hepatocytes arranged radially (arrows) around central vein (**CV**) and separated by blood sinusoids (**S**). Note the presence of portal tract (arrowhead) (H & E x 100).

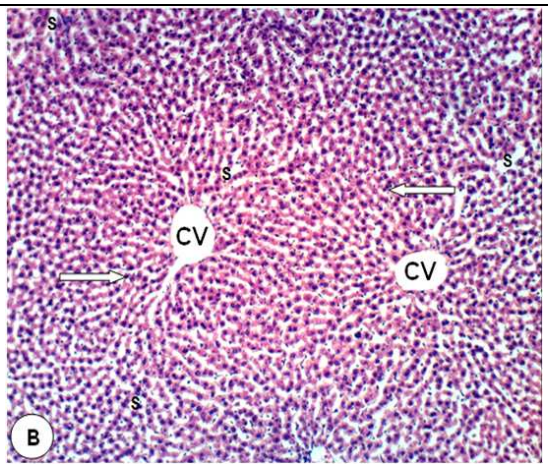
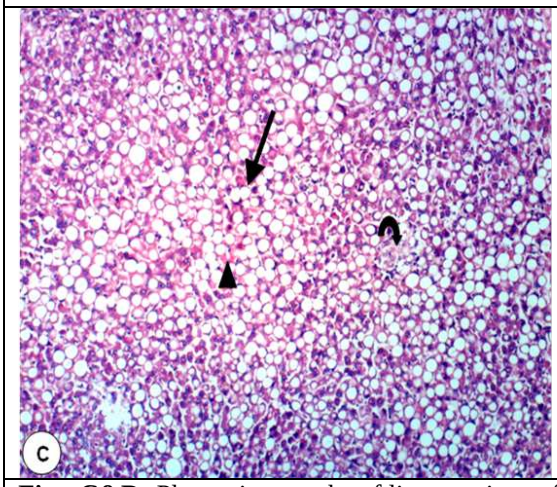
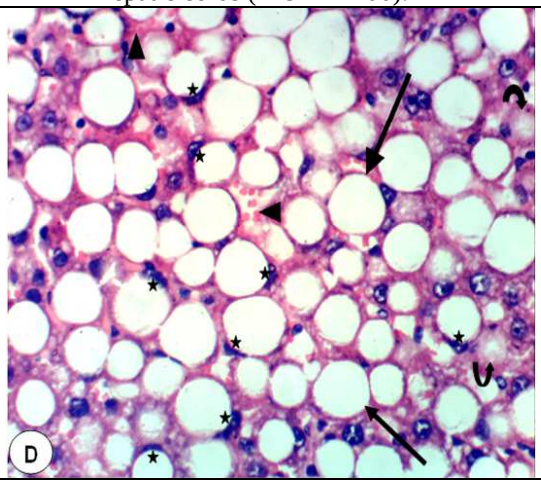


Fig. B: A photomicrograph of liver section of control rat treated with IP showing normal architecture of the classic hepatic lobule with normal central vein (**CV**). Polyhedral hepatocytes (arrows) are arranged in radiating cords and blood sinusoids (**S**) are enclosed between the hepatic cords (H & E x 100).





Figs. C&D: Photomicrographs of liver sections of obese rat showing **(C):** heavy fatty infilteration with signet ring appearance (arrow). There is focal hepatic necrosis (curved arrow) and extravasation of blood (Hemorrhage) (arrowhead) (H & E x 100). **(D):** micro- and macro-vesicular steatosis where most hepatocytes are replaced by fat cells with signet ring

appearance (arrows), vacuolated cytoplasm (curved arrows) and flat peripheral nuclei (stars). There is extravasation of blood (Hemorrhage) (arrowheads) (H & E x 400).

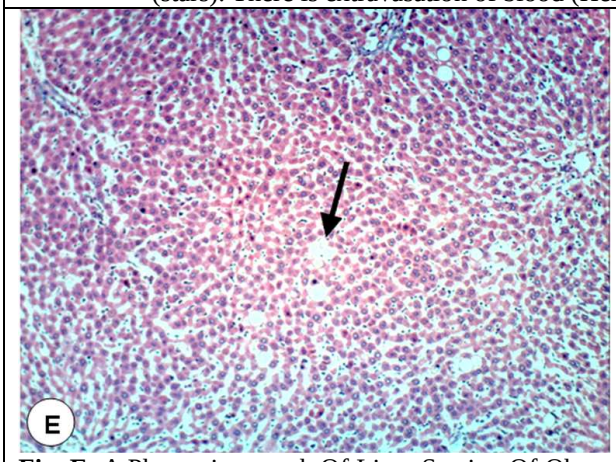


Fig. E: A Photomicrograph Of Liver Section Of Obese Rat Treated With Ip Showing Reappearance Of The Normal Architecture Of Hepatic Lobule But There Is Still Few Fatty Change Of Sporadic Hepatocytes (Arrow) (50 Mg/Kg) (H & E X 100).

Discussion:

Progress in the understanding of the pathophysiology of NASH has been hampered by the lack of experimental models adequately reproducing features of the human disease. Most of the data gained so far have been obtained using rodent models based either on genetic leptin defects, which are resistant to the development of fibrosis during chronic liver injury, or on the MCD diet. In the MCD model, steatosis, necroinflammation, and little fibrosis develops in zone 3 as a result of a nutritional deficiency to which rodents are selectively sensitive but that is uncommon in patients with NASH, because animals lose weight and are cachectic [19]. Recently a high-fat, high-calorie liquid diet was able to reproduce some features of NASH in rats. However, the liquid diet represents a time-consuming method that was used for only 3 weeks and did not induce body or liver weight gain. Moreover no information on adipose tissue or insulin resistance was provided in the study [19]. Thus, we used a developed model based on a commercially available high-fat, high-calorie solid diet. Using such a model, we have been able to demonstrate in the present study that visceral obesity in animals without genetic modifications is associated with insulin resistance, deterioration of lipid profile that could stimulate hepatic oxidative stress which finally lead to development of NASH.

The present data indicated a hyperinsulinemia, followed by a concomitant rise in glucose concentration as compared to the control ones. These results run parallel with the results of Silva *et al*. [20] and Ferrari *et al*. [21]. The hyperglycemia observed in the obese dams could be due to a state of insulin resistance, confirmed by the elevation of the homeostatic model assessment of insulin resistance (HOMA-IR). The observed insulin resistance may be a result of feeding HFD which increase serum FFAs level. HFD-feeding play a key role in promoting the loss of insulin sensitivity, where it causes a decline in insulin receptors and insulin receptor substrate phosphorylation, which diminishes the cascade of insulin induced reactions and leads to muscle, adipose tissue and hepatic insulin resistance [2,7] that stimulates gluconeogenesis and resulting in excess glucose supply which by timing has worsened to peripheral IR [22]. Noteworthy, glucose primarily enters the cells via membrane protein-facilitated diffusion (glucose transporters; GLUT-1 to GLUT-5). GLUT-4 is the main protein that promotes glucose transport into skeletal muscle cells and adipocytes. When insulin receptors are not properly phosphorylated, the signal stimulating GLUT-4 transport activity is not created, and glucose capture by cells is consequently reduced while stimulation of insulin production remains continuous, resulting in IR [23].

Administration of IP induced a marked reduction in both serum insulin and glucose levels as compared to the obese group. These observations correspond with Nosrati *et al*. [24], where the tested materials significantly improved both the hyperglycemia and insulin resistance Mechanisms that augment its hypoglycemic action may be through stimulation of glucose uptake by peripheral tissues, increasing the expression of hepatocytes insulin receptor and improves cellular glucose consumption [22], reducing the intestinal glucose absorption or through its ability to scavenge the free radicals. Mukherjee *et al*. [25] previously found that isoflavones play important roles in preventing the progression of hyperglycemia, partly by increasing hepatic glycolysis and glycogen concentration and/or by lowering hepatic gluconeogenesis. Another theory centers around increased GLUT4 translocation in adipocytes [22] and/or decreased PPARγ expression in the pancreas, which decreased the synthesis of GLUT-2 mRNA, thus decreasing glucose transport into the pancreas and minimizing insulin release. Low insulin release might by itself minimizing peripheral IR and help in maintaining normal glycemia.

Marked alterations in lipid metabolism have been reported in the serum of the obese group. Where, the present results elucidated highly significant elevations of triglycerides (TGs), total cholesterol, low density lipoprotein cholesterol (LDL-cholesterol), vLDL-cholesterol and a concomitant reduction in high-density cholesterol (HDL-cholesterol).

In agreement with these results, Ying *et al*. [1] and Andersen *et al*. [26] reported remarkable impaired lipid profile of rats fed HFD. It is well known that when hyperglycemia took place this is accompanied with the increase in cholesterol, LDL, TGs and fall of HDL that may be contributed to the development of steatosis [22]. So, the observed hypertriglyceridemia in the gestational obese dams may be due to increased dietary TGs absorption from the small intestine following the intake of HFD, decreased TGs uptake in peripheral tissues as a result of dysfunction of insulin-dependent lipoprotein lipase (LPL), a key enzyme in triglycerides hydrolysis, or through increased hepatic production of triglycerides enriched very low density lipoprotein (vLDL-TGs) [27].

Additionally, gestational obese rats' hypercholesterolemia may be attributed to increase the input into system by the accelerated intestinal cholesterol synthesis [28], secondary to the elevated activity of the intestinal hydroxymethylglutaryl COA (HMG-COA) reductase; the key enzyme responsible for cholesterol synthesis or an increment of the rate of intestinal cholesterol absorption following exogenous consumption of diet rich in fat [29] depression of the output due to decreased synthesis of bile salts that follows depressed hepatic phenol-2-monoxygenase activity, the key enzyme responsible for the catabolism of cholesterol to bile acids and/or by decreased number of LDL receptors (LDLR) with consequent delayed clearance of cholesterol rich in LDL particles [28].

In humans, vLDL secretion rate increases linearly with increasing intrahepatic TGs accumulation but hepatic vLDL-TGs export is inadequate to normalize hepatic TGs content in NAFLD [7]. Fat accumulation in the NAFLD is the direct result of increased delivery of FFAs into the portal vein for conversion to TGs within the liver [30]. So, the increased up take of FFAs by the liver exceeds its capacity to metabolize them by secretion into the blood as vLDL [31]. Increased hepatic vLDL secretion lowers HDL-cholesterol levels and leads to small, dense, LDL-cholesterol molecules, the typical triad of NAFLD and other IR states [7]. As a consequence, liver steatosis develops, generally considered as a "benign" prerequisite of NASH [31].

Moreover, LDL-cholesterol elevation in the serum of the obese rats demonstrated in the present study could be due to a diminished number of LDL receptors or reduced LDL binding to its receptor or may be due to increased m- RNA levels for the LDL receptor in the small intestine of the obese dams [32].

In view of the present results, it was found that; treatment with IP evoked a profound improvement of the altered serum lipid variables. The present data coincides well with the work of Jungbauer and Medjakovic [33]. The tested materials significantly ameliorate the reduction in serum HDL-cholesterol level, exhibited a noticeable decrease in TGs, cholesterol, LDL-cholesterol levels and hasten removal of cholesterol from peripheral tissue to liver for catabolism and excretion.

Padhi *et al*. [34] detected that isoflavones, a class of molecules called flavonoids that belong to a large family of polyphenols, could modulate serum lipids. So, the hypolipidemic effect of IP that observed in the study herein could be returned to its free radical scavenging property [35] that effectively prevented lipid peroxidation, which paralleled the inhibition of membrane damage and prevented the release of FFAs from the adipose tissue that followed by increase in TGs clearance in the periphery by stimulating the LPL enzyme. Furthermore, IP appeared to provoke some protective effects against LDL oxidative modification, enhance the fractional catabolic rate of LDL and consequently lower plasma LDL level. Therefore, the decrease of LDL-cholesterol and the increase of HDL-cholesterol concentration in serum of obese rats treated with IP suggest that IP could affect cholesterol distribution in lipoproteins.

The present study indicated that HFD-fed rats caused extensive damage to the liver confirmed by the histopathological observations and reflected in the marked elevation of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) enzymes activities, which are the circulating markers of hepatocyte injury.

These findings are in accordance with Hamad *et al*. [36] and Lo *et al.* [37]. Elevated ALT levels correlated strongly with NAFLD. As it correlated with serum free fatty acids and hepatic TNF-α supporting that inflammatory-mediated dyslipidemia contributed to liver damage [3]. Significantly elevated activity of ALT in serum often suggests its leakage from damaged hepatic cells and reflects hepatocyte damage [38]. High level of serum AST indicates liver damage such as due to viral hepatitis and may also be elevated in diseases affecting other organs. So, serum ALT is more specific indicator of liver inflammation than AST and can be considered as a better screening and surrogate marker for detecting hepatic damage since ALT is a cytosolic enzyme found in very high concentration in the hepatocytes, while AST present in the cytoplasm as well as in the mitochondria and is rapidly inactivated [39].

The present data revealed a significant decrease in the activities of both ALT and AST after treatment with IP. These observations go parallel with that of Oliveira *et al*. [23].This positive effect on biochemical variables (ALT and AST) might account for the improvement of the liver histology and less fatty infiltration in hepatocytes of obese dams treated with IP. This might be ascribed to the improved liver function with return of

gluconeogenesis toward its normal level. In addition to IP anti-inflammatory [26] effect that could inhibit the production of the pro-inflammatory cytokines and its antioxidant-sparing action [1] which in turn, it could scavenge free radicals and inhibit peroxidation of lipids thereby preserve the structural integrity of hepatocellular membrane that prevents the leakage of liver markers into the circulation.

Obesity and insulin resistance occurred in HFD rats, accompanied by a marked decrease in adiponectin mRNA expression in the visceral adipose tissue. Adiponectin (30-kd hormone) is a collagen-like plasma protein, which is specifically synthesized in adipose tissue. It plays an important role in the regulation of whole-body insulin sensitivity, glucose and lipid metabolism in insulin-sensitive tissues in both humans and animals. Reduced plasma adiponectin levels, and the levels of adiponectin and AdipoR1/R2 expression have been shown in humans diagnosed with obesity as well as patients with NASH, that developed as a result of feeding HFD [40] thereby, reducing adiponectin sensitivity and leading to insulin resistance, which in turn aggravates hyperinsulinemia, creating a "vicious cycle" [41].

Although the three-dimensional structure of adiponectin closely resembles that of TNF-α, these two proteins have completely opposite effects, and both *in vivo* and *in vitro* experiments demonstrate that adiponectin and TNF-α antagonize each other's action in their target tissues [19]. Thus, it is reasonable to speculate that the association of visceral obesity and hepatic steatosis is needed for the progression from NAFLD to NASH, and this data confirms that our model reproduces most of the pathogenetic mechanisms leading to liver injury associated with the metabolic syndrome.

It is evident that IP markedly reverted back adiponectin expression most likely through impairment of adipose tissue inflammation. All of these findings are in line with the favorable effects of Chlorella, an antioxidant unicellular green alga, for improvements in diabetes predisposed people [42].

In addition to IP anti-inflammatory [35] effect that could inhibit the production of the pro-inflammatory cytokines, the protective effect of IP administration in our model, beyond its ability to up-regulate adiponectin expression in adipose tissue that has an antagonistic effect against TNF-α and beneficial effects on lipid metabolism by increasing fatty acids β-oxidation, has also direct anti-inflammatory effects [43]. Furthermore, adiponectin has insulin sensitizing effect which is attributed primarly to its activation of AMPK that leads to expression of gluconeogenic enzymes that suppress the hepatic glucose production in addition to enhancing fatty acids oxidation and energy dissipation, which in turn decreases lipid accumulation in liver and skeletal muscle causing an increase in insulin sensitivity [40].

The present investigation revealed that feeding female rats with HFD produced an elevation of hepatic enzymes which was coupled with a pronounced increase in hepatic oxidative stress and a marked suppression of the antioxidant defense system in the liver. Hepatic malonaldialdehyde (MDA) formation was markedly elevated and reduced glutathione (GSH) content was enormously depleted. Moreover the activity of hepatic antioxidant superoxide dismutase (SOD) was also depressed in the obese rats. The mechanisms leading to hepatic steatosis and subsequently NASH are often characterized by a "two-hit" hypothesis. The "first-hit," triggered by obesity, especially visceral fat accumulation, and insulin resistance [41], which were observed in our model and results in excess hepatic lipid accumulation causing hepatic steatosis and injury. Steatotic livers are vulnerable to "second-hits," mediated by inflammation and oxidative stress, resulting in lipid peroxidation, exacerbated hepatic injury, inflammatory infiltration, and NASH [39].

It is reported that, hyperglycemia and hypertriglyceridemia evoke the hepatic lipid peroxidation due to β-oxidation overload that is characterized by high thiobarbituric acid reactive species (TBARS) level [22] furthermore, when insulin resistance occurs, a higher than normal level of insulin leads to further lipid accumulation and lipid peroxidase stimulation [24]. Also, increased intrahepatic levels of fatty acids provide the appropriate environment for lipid peroxidation particularly if the system is under the condition of oxidative stress. This observation might account for disease progression from steatosis to steatohepatitis and cirrhosis due to enhanced levels of lipid peroxidation and cytokines induction [26].

Moreover enhanced levels of lipid peroxidation induced by HFD are accompanied by a decrease in the activities of enzymatic antioxidant (SOD) and non-enzymatic antioxidant (GSH). HFD-feeding resulted in decreased intracellular levels of NADPH, which is required for regeneration of GSH from its oxidized form (GSSG) [44]. Also, Hamad *et al*. [36] proved that hepatocyte and plasma glutathione (reduced form, GSH) decreased and glutathione disulfide (oxidized form, GSSG) increased in NAFLD patients.

The decrease in the activity of SOD in the liver of obese rats may be due to the increased lipid peroxidation or inactivation of the enzymes by cross-linking with MDA. Therefore, a reduction in the activity of SOD can result in a number of deleterious effects due to the accumulation of superoxide and H_2O_2 radicals, which could further stimulate lipid peroxidation [45]. Based on these predescribed studies, it is worth noting that the enhanced lipid peroxidation and the deterioration of the antioxidant defense system may play a significant role in the pathogenesis of NASH and the deleterious histological effects of HFD on liver reported in this study.

Treatment with IP (isoflavone), exhibited a profound amelioration of the hepatic dysfunction along with lipid peroxidation and restored the levels of GSH and SOD in obese dams. The aforementioned co-ordinate effects should contribute to the effective antioxidant potential of isoflavones [34]. The overall antioxidant

potential of isoflavones depends on the number and arrangement of the hydroxyl groups and the extent of structure conjugation. They can donate hydrogen atom from their hydroxyl groups and stabilize the phenoxy radical formed by delocalization of the unpaired electron within the aromatic structure [46]. Furthermore, mounting evidence has shown that IP could enhance GSH production may be via providing more substrate for reactive intermediates that promote detoxification mechanisms. This might a plausible explanation for the restoration of SOD.

In the current study, the gross and histological examination of liver sections of dams fed HFD revealed degenerative changes involve hepatocytes and cells lining the blood sinusoids. Sections showed pronounced hepatic steatosis and focal hepatic necrosis associated with abundant mononuclear inflammatory cells infiltration. Most hepatocytes were replaced by fat cells with signet ring appearance in addition to extravasation of blood. Hepatocytes also showed vacuolated cytoplasm with flat peripheral nuclei. Such changes lead to loss of the normal architecture of the classic hepatic lobule. Treatment with IP resulted in reappearance of the normal architecture of hepatic lobule and elicited a striking decrease in fat accumulation with no inflammatory infilterate as compared with the corresponding obese ones. It effectively improved hepatocytes degeneration, suppressed hemorrhage and but, there was still slightly congested central vein.

The possible hepatoprotective action might be due to the inhibition of hepatic inflammation, therefore, attenuated the production of inflammatory mediators in addition to the free radical scavenging property as well as membrane stabilizing effect of IP [26]. This subsequently improves the integrity of the hepatic architecture and accelerated regeneration. Moreover, physiological properties of this isoflavone are attributed to the ability of isoflavones to inhibit cell proliferation, promote differentiation and are modulators of tyrosine kinases [47].

Conclusion:

Taken together, we have demonstrated that ipriflavone up-regulates adiponectin expression, modifying the altered lipid profile, improved liver function and morphology, attenuating oxidative stress and restoring antioxidants content in a high-fat diet–treated rodent model. This is probably due to the antioxidant, free radical scavenging, anti-inflammatory and membrane stabilizing activities of ipriflavone. All of these effects could have worked in harmony to produce the general protective effect we have demonstrated.

Our study supports the importance of taking this well-tolerated and nontoxic compound, ipriflavone, into human randomized clinical trials to study the potential in the prevention and treatment of NASH.

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Conflict of interest:

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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