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RESEARCH ARTICLE

Rice bran oil ameliorates hepatic insulin resistance by improving insulin signaling in fructose fed-rats

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Abstract

Background Insulin resistance is an inadequate metabolic response of the peripheral tissue to circulating insulin. It plays an important pathophysiological role in type 2 diabetes mellitus. The purpose of the study was to investigate the molecular effects of rice bran oil (RBO) on the gene expression of insulin receptor (IR), insulin receptor substrate-1 (IRS-1), glucose transporters-4 and 5 (GLUT-4 and 5) in insulin-resistant rats induced by high fructose diet (HFD).

Methods Rats were divided into six groups (10 rats each) as follows: Groups 1 and 2: rats received a standard diet with corn oil or RBO (as the sole source of fat), respectively. Group 3: animals fed on HFD, which was furtherly divided into 2 sub-groups: rats fed HFD either for one (HFD1) or for 2 months (HFD2). Group 4, rats fed HFD containing RBO for 1 month (HFD1 + RBO), while rats in group 5 fed HFD for 30 days then RBO was added to the diet for another 30 days (HFD2 + RBO). Serum levels of glucose and insulin, as well as hepatic gene expression of insulin receptors and glucose transporters were determined. Livers were isolated for histopathological study.

Results HFD induced insulin resistance with a reduction in the hepatic level of insulin receptor and glucose transporters at both protein and molecular levels. Addition of RBO improved the insulin sensitivity and up-regulated the expression of the tested genes.

Conclusion HFD impaired the insulin sensitivity of the hepatocytes by down-regulating the insulin receptor genes. Addition of RBO alleviated all the hazardous effects.

Keywords Insulin resistance · Rice bran oil · Insulin receptors · Glucose transporters

Introduction

Insulin resistance is increasing at an alarming rate, becoming a major public and clinical problem worldwide. Insulin resistance is defined as an impaired ability of insulin to promote glucose uptake and exert its metabolic effects in the liver, skeletal muscle and adipose tissue [1, 2]. Experimental studies in animals documented that the general increase in fructose consumption is correlated with hyperglycemia, dyslipidemia and insulin resistance [3–5]. Fructose, a simple sugar found in honey, fruit, and high-fructose corn syrup, has a unique metabolism that results in oxidative stress and lipogenesis [6, 7]. Fructose intake has increased markedly due to the increased intake of beverages sweetened with sucrose (50% fructose) and high fructose corn syrup (55–90% fructose) [8].

Rice bran oil (RBO) is unique among edible oil as a result of its nutritional and functional properties such as γ-oryzanol, phytosterols, and tocopherols [9]. These bioactive compounds reduce oxidative stress which causes many diseases such as diabetes, cancers, and neurodegenerative diseases [10]. Several studies have demonstrated that RBO possesses hypoglycemic activity [5, 11] since chronic exposure to hyperglycemia may induce dysregulation of gene expression that converges on impaired insulin secretion and increased apoptosis [12].

Since insulin serves as the major physiological anabolic hormone, promoting the synthesis and storage of glucose, this study was designed to monitoring the metabolic effects

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of the RBO on the expression of two insulin signaling genes, insulin receptor (IR) and insulin receptor substrate-1 (IRS-1), glucose transporter 5 (GLUT5) and glucose transporter 4 (GLUT4) in insulin-resistant rat liver, as a central organ in carbohydrate metabolism.

Materials and methods

Animals

A total of 60 adult female Sprague Dawley rats weighing 140–220 g were used throughout this study. Animals were purchased from the breeding unit of the Egyptian Organization for Biological Products and Vaccines (Helwan, Egypt), and were housed in steel mesh cages (4/cage). Rats were maintained for a week acclimatization period on a commercial pellet diet. Food and water were provided ad libitum.

Preparation of diets

The standard, high fructose (60 g/100 g) diets and the diet containing 10% RBO were prepared as previously described [13, 14].

Study design

Rats were allocated into 5 groups. Normal Control group (NC): Rats fed standard diet. Rice Bran Oil group (RBO): Rats fed standard diet contains 10% RBO as the sole source of fat. High Fructose Diet group (HFD): this group was subdivided into 2 sub-groups: rats fed HFD for only 1 month (HFD1) and rats fed HFD for 2 months (HFD2) serving as reference groups for the corresponding treated groups. Rats fed HFD containing 10% RBO for 1 month (HFD1+ RBO). Rats in this group fed HFD for 30 days and then received HFD with 10% RBO for another 30 days (HFD2+ RBO). Animals were maintained in their designed groups for 4 weeks except group 5.

Body weight of the animals in all groups was recorded weekly and body weight gain was calculated at the end of the feeding period. All animal experiments were carried out in accordance with the principles outlined in the Declaration of Helsinki (Adopted by the 18th WMA General Assembly, Helsinki, Finland, June 1964).

Blood collection and tissue sampling

Rats were anesthetized with Urethane (99%, Aldrich) at a dose of 1 g/kg body weight intraperitoneally, and then blood samples were taken from the retro-orbital venous plexus after overnight fasting. Blood was immediately centrifuged. Serum samples were aliquoted and stored at −20 °C until aminotransferases and insulin analyses, except for fasting glucose which was determined on the same day without delay.

The liver was quickly excised and rinsed from blood in phosphate buffer saline (PBS, pH 7.4), dried and weighed. The entire liver was divided into weighed portions, one portion was dropped into a test tube containing 30% (*w*/*v*) KOH for glycogen determination, another part was used for determination of insulin receptor (IR) and insulin receptor substrate-1 (IRS-1) and the third part of the fresh liver tissue was used for RNA extraction for PCR.

Biochemical assay

Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured using commercial assay kits (Diamond Diagnostics, Egypt). Fasting serum glucose level was assayed by the enzymatic colorimetric method [15], while serum insulin was assayed using the enzyme-linked immunoassay (Rat insulin ELISA kit, Glory science Co., USA) [16]. Homeostasis model assessment insulin resistance index (HOMA-IR) was calculated: HOMA- IR = [Fasting insulin (μIU/ml) × fasting glucose (mmol/L)] / 22.5 [17]. Hepatic glycogen was determined by the colorimetric method [18]. Immunoblotting was performed to examine the protein levels of hepatic insulin receptor (IR) and insulin receptor substrate-1 (IRS-1) [19].

Polymerase chain reaction (PCR)

Total hepatic RNA was extracted using TRIzol® Reagent (Invitrogen, Carlsbad, CA), treated with DNase I and then 1 μg of total RNA was used to synthesize cDNA using high-capacity cDNA reverse transcription kits (Promega, Madison, WI, USA) in accordance with the manufacturer's instructions. For real-time PCR, cDNA and primers were prepared with a SYBR Green PCR master mix (Applied Biosystems) according to the manufacturer's instructions. The primer sequences of IR, IRS-1, and glucose transporters 4 and 5 (GLUT-4 and GLUT-5) used for real-time PCR are shown in Table 1. All values were normalized to β-actin which was used as the control housekeeping gene.

Histological analysis

Liver sections (three independent rats from each group) were fixed in 10% neutral-buffered formalin then were paraffin embedded. The paraffin embedded sections were cut into 4-μm slices and stained with hematoxylin and eosin.

Statistical analysis

Data are expressed as means ± standard error of mean. Differences between the mean values were assessed with

Table 1 Primers sequences

Genes	Primer sequence	Product length (bp)	Accession No
IR	Forward: 5′CTTCTCGCGGAGTATGTCCC3′ Reverse: 5’CAGCACCGTTCCACAAACTG3′	703	NM_017071.2
IRS1	Forward: 5′CTGCATAATCGGGCAAAGGC3′ Reverse: 5′CATCGCTAGGAGAACCGGAC3′	916	NM_012969.1
GLUT4	Forward: 5′GATTCTGCTGCCCTTCTGTC3′ Reverse: 5′ATTGGACGCTCTCTCTCCAA3′	168	XM_006246596.3
GLUT5	Forward: 5′GTGTCTGTGACACTGGGAGG3′ Reverse: 5’GTGACATGGCTGGGTCAGAA3′	439	NM_031741.1
β-actin	Forward: 5′TCTGGCACCACACCTTCTACAATG3′ Reverse: 5′AGCACAGCCTGGATAGCAACG3′	166	NM_031144.3

bp Base pair

one way analysis of variance (ANOVA) and followed by post-hoc test (least significant difference analysis, LSD). A p value ≤0.05 was considered significant. The statistical analyses were applied using computer-based software (SPSS) version 16.

Results

Body weight gain

Non-significant changes in the body weight gain were observed in HFD-fed rats (HFD1), while the addition of RBO (HFD1 + RBO) producing significant reduction ($p < 0.02$), compared to the control group. However, feeding HFD for 8 weeks (HFD2) increased significantly ($p < 0.03$) the body weight gain, compared to HFD1 group (Table 2).

Serum ALT and AST levels

Serum ALT levels in HFD1 and HFD2 were significantly elevated ($p < 0.001$) as compared to the control. However, significant reductions ($p < 0.01$) were observed in both (HFD1 + RBO) and (HFD2 + RBO) groups, compared to HFD1 and HFD2 groups, respectively. With regard to the

Table 2 Summary of the effect of RBO body weight gain and serum levels of ALT, AST, glucose and insulin in addition to calculated HOMA-IR and hepatic glycogen concentration in all experimental groups

Groups	Body weight gain (%)	ALT (U/L)	AST (U/L)	Glucose (mg/dL)	Insulin (mU/L)	HOMA-IR	Glycogen (g/100 g liver)
NC							
Mean ± SE	7.40 ± 1.2	26.45 ± 1.10	13.47 ± 0.69	120 ± 3.95	7.46 ± 0.36	2.24 ± 0.11	0.82 ± 0.26
Range	(0.00–11.76)	(21.67–29.99)	(11.33–16.32)	(108–136)	(6.50–10.1)	(1.81–275)	(0.53–1.09)
RBO							
Mean ± SE	7.60 ± 1.32	32.61 ± 0.94	17.93 ± 1.05[a]	117.6 ± 4.37	8.85 ± 0.36	2.56 ± 0.13	0.81 ± 0.57
Range	(1.25–14.29)	(29.00–37.6)	(13.65–23.32)	(90–134)	(7.50–10.5)	(1.67–3.00)	(0.26–1.78)
HFD1							
Mean ± SE	6.57 ± 0.87	40.54 ± 3.09[ab]	8.42 ± 0.28[ab]	180.4 ± 10.13[ab]	14.64 ± 0.83[ab]	5.37 ± 0.56[ab]	2.51 ± 0.88[ab]
Range	(2.78–10)	(29.00–52.00)	(7.00–9.66)	(134–230)	(10.6–16.7)	(2.24–7.33)	(1.76–4.07)
HFD1 + RBO							
Mean ± SE	3.52 ± 1.06[ab]	32.14 ± 1.83[c]	9.70 ± 0.25[ab]	142.7 ± 2.48[abc]	11.79 ± 0.75[abc]	4.14 ± 0.26[abc]	1.47 ± 0.74[abc]
Range	(0.00–10)	(27.01–43.34)	(8.33–10.66)	(135–159)	(9.03–14.90)	(3.05–5.33)	(0.55–2.86)
HFD2							
Mean ± SE	10.34 ± 1.08[c]	35.95 ± 3.9[a]	12.12 ± 1.13[c]	134.2 ± 4.93[c]	21.54 ± 1.16[ac]	7.17 ± 0.55[abc]	1.37 ± 0.25[abc]
Range	(6.25–13.89)	(25.00–56.47)	(9.66–18.6)	(121–161)	(17.6–25.5)	(5.51–10.13)	(1.15–1.78)
HFD2 + RBO							
Mean ± SE	9.8 ± 1.35	26.86 ± 1.53[d]	11.89 ± 0.66[b]	125.5 ± 7.23	11.55 ± 0.73[abd]	3.53 ± 0.26[ad]	1.02 ± 0.36
Range	(3.13–16.67)	(22.00–36.92)	(9.66–15.36)	(99–148)	(8.90–15.7)	(2.64–5.58)	(0.62–1.43)

[a] Significance vs NC

[b] Significance vs RBO

[c] Significance vs HFD1

[d] Significance vs HFD2, the mean difference is significant at $p < .05$, each group contains 10 rats

serum level of AST, significant reduction ($P < 0.001$) was recorded in HFD1 and HFD1 + RBO, compared to control group. Serum AST showed significant elevation ($P < 0.001$) in HFD2 group, compared to HFD1 group (Table 2).

Histological analysis

Histopathological observations of H&E staining of livers were performed as supporting evidence in biochemical analysis. Figure 1a showed the normal morphological characteristics of the hepatic cells, whereas the hepatic cells of HFD-fed rats (for 1 and 2 months) showed cytoplasmic vacuolation and focal hepatic necrosis associated with mononuclear cells infiltration as illustrated in Fig. 1 (b and c, respectively). Slight cytoplasmic vacuolation of hepatocytes of HFD1 + RBO group, in addition to activation of Kupffer cells of HFD1 + RBO and HFD2 + RBO groups were observed in Fig. 1 (d and e, respectively).

Glucose, insulin and HOMA-IR

Current results showed a state of moderate insulin resistance in the fructose-fed rats, as demonstrated by hyperinsulinemia and the increase of HOMA-IR value in HFD1 and HFD2 groups. Beside, hyperglycemia was observed in rats fed HFD for 4 weeks, while rats fed HFD diet for 8 weeks revealed significant reduction in serum glucose ($p < 0.01$), compared to those fed HFD for 4 weeks (Table 2).

In spite of the improvement in the serum insulin level in (HFD1 + RBO) and (HFD2 + RBO) groups ($p < 0.01$ and $p < 0.001$, respectively), compared to their respective control groups (HFD1 and HFD2), but insulin level still highly elevated than the control group. Addition of RBO to the HFD diet (HFD1 + RBO) improves serum glucose ($p < 0.001$), as compared to HFD1. Moreover, the HFD contains RBO (HFD1 + RBO and HFD2 + RBO) reduced HOMA-IR significantly ($p < 0.02$ and $p < 0.001$, respectively) as compared to their respective control group.

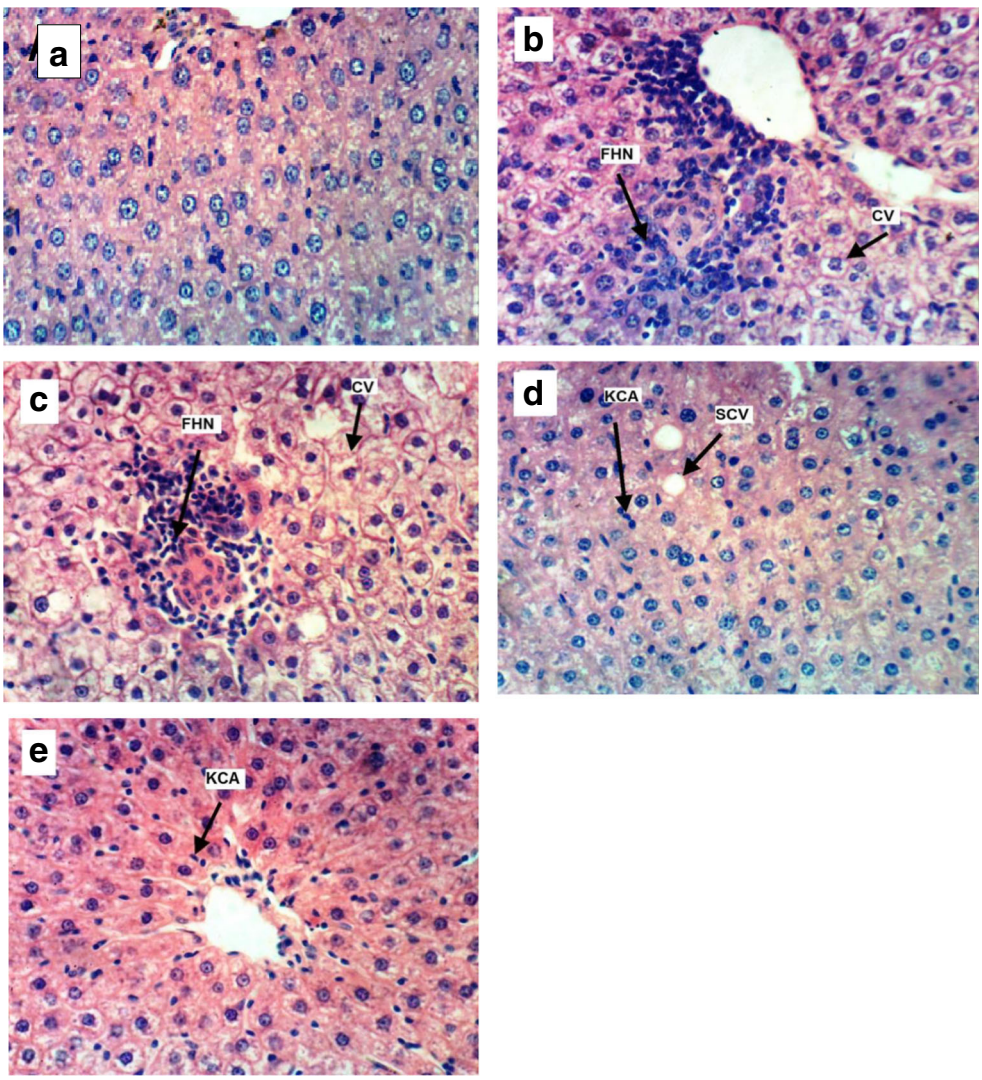

Fig. 1 Histopathological examination by light microscope of liver cells from control (**a**), HFD fed groups for one (**b**) and 2 (**c**) months and RBO groups [HFD1 + RBO (**d**) and HFD2 + RBO (**e**)] (X400- H & E). CV: Cytoplasmic vacullation, FHN: Focal hepatic necrosis, KCA: Kupffer cells activation, SCV: Slight cytoplasmic vacillation

Hepatic glycogen concentration

Rats fed on HFD for 4 and 8 weeks showed a significant elevation in hepatic glycogen concentration especially in HFD1 group. However, rats fed on the diets containing RBO revealed reduced levels (Table 2).

Hepatic IR and IRS-1 by western blot

Hepatic insulin receptor (IR) concentrations were significantly reduced ($p < 0.001$) in rats fed high fructose (HFD1 and HFD2), compared to NC group (Fig. 2). HFD1 group revealed a highly significant reduction ($p < 0.001$), compared to RBO group, while rats fed HFD for 2 months revealed significant elevation in hepatic IR concentration ($p < 0.025$) as compared to rats fed HFD for only 1 month. Although HFD1 + RBO and HFD2 + RBO groups revealed a highly significant increase in hepatic IR concentrations ($p < 0.001$) as compared to their corresponding controls (HFD1and HFD2, respectively), HFD1 + RBO group was significantly reduced ($p < 0.017$), compared to NC rats.

As regards to hepatic insulin receptor substrate-1 (IRS-1) concentration, a significant reduction was observed in HFD1 and HFD2 ($p < 0.001$) groups, compared to NC group (Fig. 3). Although HFD1 + RBO and HFD2 + RBO groups revealed a highly significant increase in hepatic IRS-1 concentrations ($p < 0.001$) as compared to their corresponding control (HFD1and HFD2, respectively) groups, but they did not reach the normal control value.

Real time-PCR

Significant down-regulation in hepatic IR, IRS-1 and glucose transporter-4 (GLUT-4) genes expression ($p < 0.001$) were observed in HFD1 and HFD2 groups, compared to NC group. Addition of RBO to these diets for 1 month either from the first day of regimen (HFD1 + RBO) or after 1 month of feeding (HFD2 + RBO) improved these results significantly ($p < 0.001$), compared to their respective controls as observed in Figs. 4, 5 and 6.

The mRNA level of hepatic GLUT-5 was significantly reduced ($p < 0.001$) in rats fed HFD either for 1 or 2 months, compared to NC group as illustrated in Fig. 7. Moreover, rats fed HFD for 2 months (HFD2) revealed a more pronounced reduction ($p < 0.001$), compared to HFD1. Addition of RBO to the HFD improved these reductions significantly in HFD1 + RBO and HFD2 + RBO ($p < 0.001$), compared to their respective control groups.

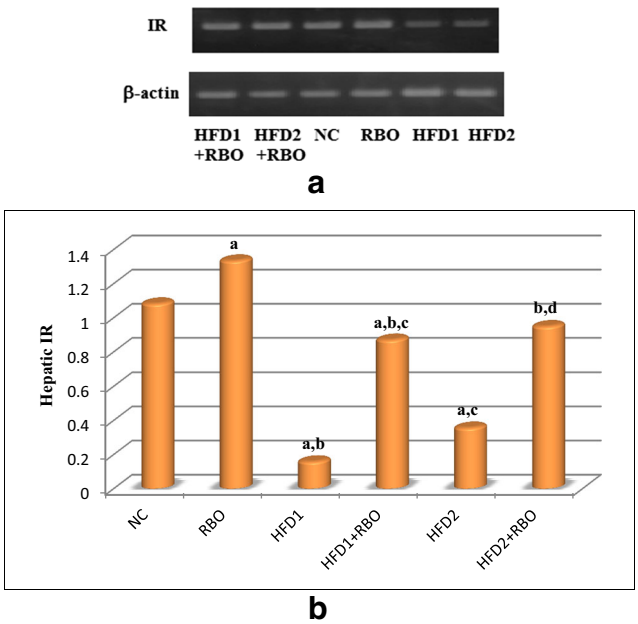

Fig. 2 Effect of RBO on hepatic insulin receptor. Effects on protein expression of the hepatic IR were determined by western blotting (**a**). The effects of RBO were analyzed using ANOVA followed by least significant difference analysis (LSD) for multiple comparisons (**b**). The mean difference is significant at $p < 0.05$. Each group contained 10 rats. a: significance vs NC, b: significance vs RBO, c: significance vs HFD1, d: significance vs HFD2

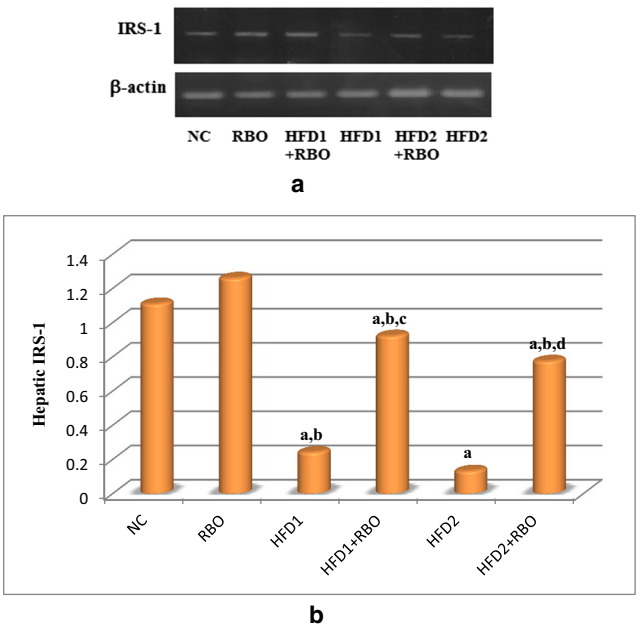

Fig. 3 Effect of RBO on hepatic IRS-1. Effects on protein expression of the hepatic IRS-1 were determined by western blotting (**a**). ANOVA was performed to analyze the effects of RBO followed by least significant difference analysis (LSD) for multiple comparisons (**b**). The mean difference is significant at $p < 0.05$. Each group contained 10 rats. a: significance vs NC, b: significance vs RBO, c: significance vs HFD1, d: significance vs HFD2

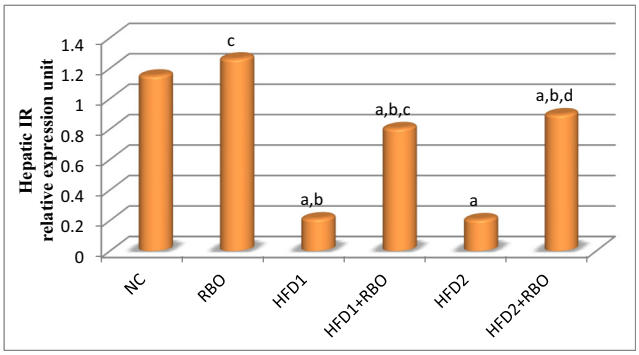

Fig. 4 Hepatic IR gene expression in the high fructose fed rats with or without RBO. Expression of the hepatic IR was determined by real time quantitative polymerase chain reaction and results were normalized by β-actin. The effects of RBO were analyzed using ANOVA followed by LSD for multiple comparisons. The mean difference is significant at $p < 0.05$. Each group contained 10 rats. a: significance vs NC, b: significance vs RBO, c: significance vs HFD1, d: significance vs HFD2

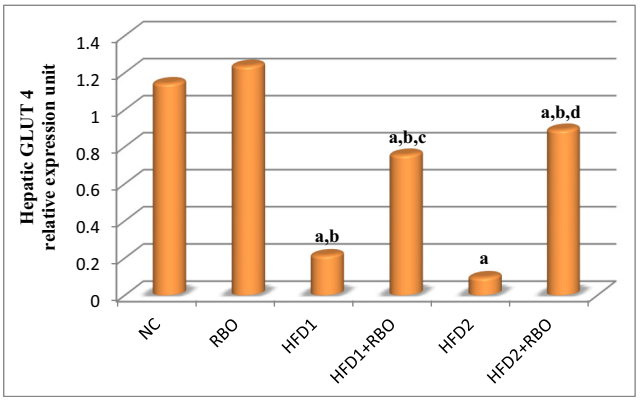

Fig. 6 Hepatic glucose transporter 4 gene expression in the different experimental groups. The expression was determined by real time quantitative polymerase chain reaction and results were normalized by β-actin. Data are presented as mean and were analyzed using ANOVA followed by LSD for multiple comparisons. The mean difference is significant at $p < 0.05$. Each group contained 10 rats. a: significance vs NC, b: significance vs RBO, c: significance vs HFD1, d: significance vs HFD2

Discussion

Insulin resistance syndrome is a cluster of related variables that included resistance to insulin-induced glucose uptake and hyperinsulinemia [20, 21]. Insulin resistance occurs at multiple levels in cells, from the cell surface to the nucleus, including insulin receptor desensitization and suppression of IRS protein and functionality, all of which can result from inhibition of IRS1 and IRS2 [22].

Previous studies confirmed that the high-fructose diet induces insulin resistance and oxidative stress in rat tissues [3, 5, 23, 24]. Rats fed high-fructose diet showed elevated levels of serum ALT and AST, the specific markers of hepatocellular injury [25, 26]. Previous studies agree with the current results only for serum ALT in HFD1 and HFD2 [25–27]. These elevations were significantly reduced in groups fed HFD containing RBO, compared to their respective control rats.

Based on the further histopathological examination, HFD groups revealed pathological changes in the liver architecture, as indicated by cytoplasmic vacuolation, hepatocyte necrosis, and mononuclear cells infiltration. These results confirm the induction of liver dysfunction. However, RBO administration reduced these pathological changes, showing near-normal appearance, which proved the protective and therapeutic effects of RBO.

Insulin resistance induced by a high fructose diet in rats is well documented [3, 5, 23, 28] and has been established in the present study. The degree of insulin resistance was higher in

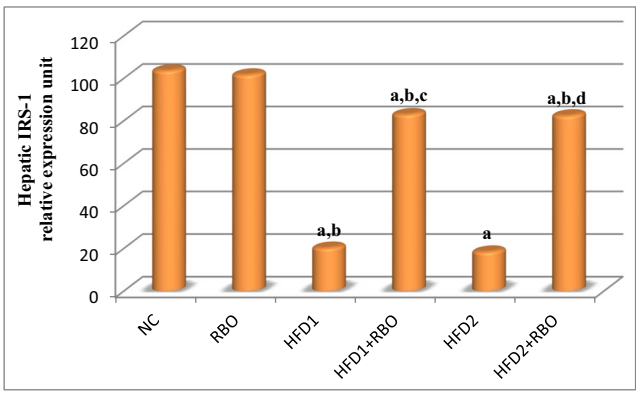

Fig. 5 Hepatic IRS-1 gene expression in the different experimental groups. The expression was determined by real time quantitative polymerase chain reaction and results were normalized by β-actin. Data are presented as mean and were analyzed using ANOVA followed by LSD for multiple comparisons. The mean difference is significant at $p < 0.05$. Each group contained 10 rats. a: significance vs NC, b: significance vs RBO, c: significance vs HFD1, d: significance vs HFD2

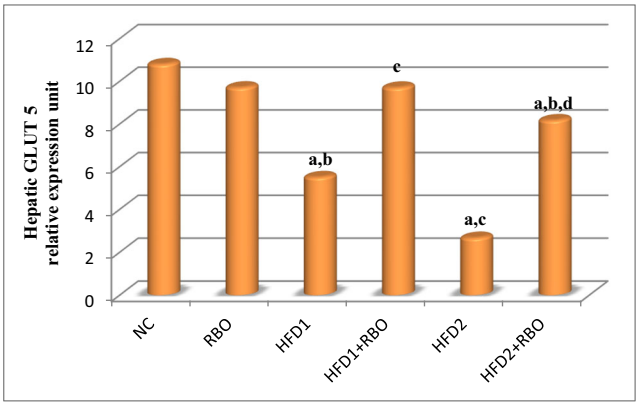

Fig. 7 Hepatic GLUT5 gene expression in the different experimental groups. The expression was determined by real time quantitative polymerase chain reaction and results were normalized by β-actin. The effects of RBO were analyzed using ANOVA followed by LSD for multiple comparisons. The mean difference is significant at $p < 0.05$. Each group contained 10 rats. a: significance vs NC, b: significance vs RBO, c: significance vs HFD1, d: significance vs HFD2

HFD1 and HFD2 groups as indicated by the significant elevation of serum insulin levels and HOMA-IR. The development of hyperglycemia in HFD1 group may be due to the formation of glucose from fructose by gluconeogenesis and impaired utilization of glucose by tissues, due to insulin resistance [29].

Addition of RBO restored insulin sensitivity and reduced HOMA-IR, compared to normal control and fructose-fed rats (HFD1and HFD2). Diminishes in insulin level along with the reduction in glucose and HOMA-IR suggest that RBO acts as a hypoglycemic agent through improving insulin action rather than insulin secretion. These results agree with those of Abd elbast et al. [11] and Abd El-Wahab et al. [5], who recorded that addition of RBO to high fructose diet-fed rats, improves insulin resistance. The appreciable amount of oleic acid and tocotrienols in RBO may be the causes of glucose reduction and insulin sensitivity in rats fed HFD containing RBO [30, 31].

Because of the absence of glucose in our fructose diet, so the substrate for glycogen synthesis in the fructose-fed groups likely came from dietary fructose through the gluconeogenic pathway due to the induction of fructose-1,6-bisphosphatase [32].

As shown previously, rats fed HFD for 30 days had elevated levels of hepatic glucose-6-phosphatase which catalyzes the terminal reaction of both glycogenolysis and gluconeogenesis [33]. Moreover, phosphoenolpyruvate carboxykinase is another regulatory enzyme in gluconeogenesis and its activity is greater in animals fed high fructose diets [34]. Together with the current results, these findings suggest that the reduction in IRS-1/PI3-kinase association, due to impaired insulin signaling in the liver of rats fed HFD, can reduce the effects of insulin on glucose-6-phosphatase and phosphoenolpyruvate carboxykinase, and consequently increases the hepatic glycogen as observed in HFD1 and HFD2 groups. In addition, feeding fructose for 8 weeks (HFD2) increased the hepatic glucose release which promotes hyperinsulinemia and insulin insensitivity. AS a result of insulin sensitivity improvement due to the addition of RBO, the hepatic glycogen content was reduced significantly as compared to HFD groups.

The difference in initial metabolism of fructose from glucose not only acutely affects carbohydrate metabolism but also induces metabolic adaptation including changes in gene expression [32]. In the current study, HFD significantly suppressed IR and IRS-1 at both gene and protein expression levels in rat hepatocytes. Moreover, high-fructose levels down-regulates both GLUT4 and GLUT5 genes expression in the liver. These results agree with previous studies which reported impaired insulin action and a decrease in GLUT4 expression in insulin resistance [27, 35]. The current results established that exposure to high concentrations of fructose induces insulin resistance like conditions, including inhibition of the Akt/PI3K pathway. In this signaling pathway, Akt lies downstream of PI3K, facilitates glucose uptake in the hepatic tissue.

The fundamental action of insulin is the regulation of glucose uptake in the liver via GLUT4, which is the most important downstream site of the insulin receptor because it sits at the rate-limiting step in the insulin transduction signal pathway [36]. GLUT4 acts as the major transporter after being translocated from the cytoplasm to the plasma membrane [37]. GLUT5 has an exclusive specificity for fructose and its expression is dramatically stimulated by the introduction of dietary fructose [38–40].

Surprisingly, RBO markedly increased these insulin receptors, exhibiting a protective effect against high-fructose evoked down-regulation of the insulin signaling pathway. In addition, RBO significantly increased the expressions of GLUT4 and 5 in rat liver. Thus, the upregulation of GLUT4 may be one of the mechanisms involved in the effects of RBO in increasing insulin sensitivity and improving insulin resistance.

Conclusions

Results indicated that insulin pathway was impaired by high fructose, and subsequently hepatic glucose utilization was repressed through suppressing PI3K/Akt down signaling and GLUT4 expression. Notably, the supplementation of RBO alleviated this insulin signaling blockade by improving the function of IR and IRS-1 by promoting PI3K/Akt phosphorylation and activating GLUT4 expression.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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