Basic Study

Resveratrol and fenofibrate ameliorate fructose-induced nonalcoholic steatohepatitis by modulation of genes expression

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Abstract

AIM: To evaluate the effect of resveratrol, alone and in combination with fenofibrate, on fructose-induced metabolic genes abnormalities in rats.

METHODS: Giving a fructose-enriched diet (FED) to rats for 12 wk was used as a model for inducing hepatic dyslipidemia and insulin resistance. Adult male albino rats (150-200 g) were divided into a control group and a FED group which was subdivided into 4 groups, a control FED, fenofibrate (FENO) (100 mg/kg), resveratrol (RES) (70 mg/kg) and combined treatment (FENO + RES) (half the doses). All treatments were given orally from the 9th week till the end of experimental period. Body weight, oral glucose tolerance test (OGTT), liver index, glucose, insulin, insulin resistance (HOMA), serum and liver triglycerides (TGs), oxidative stress (liver MDA, GSH and SOD),

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serum AST, ALT, AST/ALT ratio and tumor necrosis factor-α (TNF-α) were measured. Additionally, hepatic gene expression of suppressor of cytokine signaling-3 (SOCS-3), sterol regulatory element binding protein-1c (SREBP-1c), fatty acid synthase (FAS), malonyl CoA decarboxylase (MCD), transforming growth factor-β1 (TGF-β1) and adipose tissue genes expression of leptin and adiponectin were investigated. Liver sections were taken for histopathological examination and steatosis area were determined.

RESULTS: Rats fed FED showed damaged liver, impairment of glucose tolerance, insulin resistance, oxidative stress and dyslipidemia. As for gene expression, there was a change in favor of dyslipidemia and nonalcoholic steatohepatitis (NASH) development. All treatment regimens showed some benefit in reversing the described deviations. Fructose caused deterioration in hepatic gene expression of SOCS-3, SREBP-1c, FAS, MDA and TGF-β1 and in adipose tissue gene expression of leptin and adiponectin. Fructose showed also an increase in body weight, insulin resistance (OGTT, HOMA), serum and liver TGs, hepatic MDA, serum AST, AST/ALT ratio and TNF-α compared to control. All treatments improved SOCS-3, FAS, MCD, TGF-β1 and leptin genes expression while only RES and FENO + RES groups showed an improvement in SREBP-1c expression. Adiponectin gene expression was improved only by RES. A decrease in body weight, HOMA, liver TGs, AST/ALT ratio and TNF-α were observed in all treatment groups. Liver index was increased in FENO and FENO + RES groups. Serum TGs was improved only by FENO treatment. Liver MDA was improved by RES and FENO + RES treatments. FENO + RES group showed an increase in liver GSH content.

CONCLUSION: When resveratrol was given with half the dose of fenofibrate it improved NASH-related fructose-induced disturbances in gene expression similar to a full dose of fenofibrate.

Key words: Fructose; Nonalcoholic steatohepatitis; Supressor of cytokine signaling-3; Sterol regulatory element binding protein-1c; Fatty acid synthase; Malonyl CoA decarboxylase; Leptin; Adiponectin; Transforming growth factor-β; Tumor necrosis factor-α

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INTRODUCTION
Nonalcoholic fatty liver disease (NAFLD) and nonalcoholic steatohepatitis (NASH) are now the number one cause of liver disease in Western countries, in the Middle East, Far East, Africa, the Caribbean and Latin America[1]. The most reproducible risk factors for NAFLD/NASH are central obesity, insulin resistance, fasting hyperglycemia and hypertriglyceridemia[2].

Induction of NASH seems to be a multi-factorial process. Thus, suppressor of cytokine signaling (SOCS) proteins appears to play a key role in the induction of insulin resistance in obese rodents[3]. Furthermore, studies in obese mice proved that SOCS-3 overexpression was linked to decreased tyrosine phosphorylation of insulin receptor (IR) and insulin receptor substrate (IRS) proteins. Besides, increased expression of SOCS-3 was supplementary to enhanced expression of sterol-regulatory element-binding protein (SREBP)-1c, a transcriptional activator of all lipogenic enzymes[4]. These changes would eventually end up by an increased rate of fatty acid synthesis and would lead to classic fatty liver and increased lipogenesis[5].

Moreover, adipose tissue plays a key role in energy homeostasis, since its metabolic products, adipokines (leptin and adiponectin) exert local, peripheral and central effects. Leptin is thought to participate in NASH development while adiponectin is considered as an anti-inflammatory adipokine[6].

Recent studies ascertained that obesity is a systemic, low-grade inflammation[7] and that enhanced expression of tumor necrosis factor-α (TNF-α) is one of the earliest events after liver injury and that it represents a major trigger of the cytokine response[8]. Currently, the role of oxidative stress in disease progression from steatosis to steatohepatitis and potentially cirrhosis cannot be ignored[9].

On the other hand, it is well known that fructose is a highly lipogenic sugar molecule, which triggers the accumulation of TGs and FFAs into the hepatic tissues as well as in circulating blood, and leads to insulin resistance[10]. Fructose feeding has therefore been historically utilized as a model for studying various aspects of hepatic dyslipidemia and insulin resistance.

As for the management of NASH, treatments other than lifestyle modification by diet and exercise have not been fully established[11]. Nonetheless, the use of antihyperlipidemic agents stems from the association of dyslipidemia with NAFLD[12]. Additionally, several antioxidants were found to be effective in NAFLD treatment[13]. One of these is...
resveratrol, a polyphenol with known antioxidant and anti-inflammatory effects. Although the antioxidant effects of resveratrol have been proposed to contribute to its beneficial effects, the underlying molecular mechanism is not completely understood. Resveratrol treatment in mice fed a high calorie diet consistently improved various health parameters including glucose homeostasis and survival\(^{[13]}\), and has therefore been suggested to act as a calorie restriction mimic. Moreover, resveratrol decreased NAFLD severity in rats\(^{[14]}\).

In the present study, we investigated the effect of resveratrol treatment on fructose-induced NASH in rats and explored the potential molecular mechanisms through which the protective effects of resveratrol may work. We also explored the possible modulatory effects of fenofibrate and resveratrol given separately and in combination (half doses) on fructose-induced NASH. Besides, the expressions of a number of genes known to be critically involved in lipid metabolism were targeted.

### MATERIALS AND METHODS

**Animals and diet**

Adult male albino rats, weighing 150-200 g, were used in the present study. They were purchased from the animal house of the National Cancer Institute (Cairo, Egypt). The animal protocol was designed to minimize pain or discomfort to the animals. Animals were housed under controlled environmental conditions: constant temperature (25°C ± 2°C), humidity (60% ± 10%), and a 12/12-h light/dark cycle. Standard chow diet and water were allowed ad libitum two weeks prior to experimentation. All animals except the normal group were given 10% fructose in drinking water for 12 wk in order to induce NASH\(^{[15]}\). All experiments on laboratory animals were performed in accordance with the protocol approved by Faculty of Pharmacy, Cairo University Research Ethics Committee, Cairo, Egypt. PT number (660).

**Treatments**

The tested agents and doses used in the present study were: fenofibrate (100 mg/kg; po) (Eva Pharm)\(^{[16]}\) and resveratrol (70 mg/kg po) (Finest Nutrition, Walgreen)\(^{[17]}\). The drugs were suspended in 0.5% carboxymethyl cellulose (CMC).

**Experimental design**

After a period of adaptation, animals were randomly assigned into a normal control group and a fructose enriched diet group (FED). The FED group was distributed into 4 groups, a control FED group and 3 treated groups, each composed of 8-12 rats. The normal control group was fed a normal chow diet consisting of 66% carbohydrates, 22% protein, 6% fats, 3% fiber and 3% minerals and vitamins mixture and purchased from Alfa Media Trade (Giza, Egypt). The high fructose fed groups received the same diet plus fructose (10%) in drinking water for a period of 12 wk. Treatments were carried out by each drug separately and in combination of half the dose of each, for 4 wk from the 9\(^{th}\) week until the end of the 12 wk experimental period. Group I: normal control, II: control FED, III: fenofibrate 100 mg/kg (FENO), IV: resveratrol 70 mg/kg (RES), V: fenofibrate combined with resveratrol 50 mg/kg + 35 mg/kg (FENO + RES).

Body weight was determined during the experimental period (12 wk) at two weeks intervals.

**Blood sampling and serum preparation**

At the end of the treatment period, blood samples were taken from retro-orbital sinus of rats under ether anesthesia, after being fasted for 18 h, to minimize feeding-induced variations in lipid pattern. Blood samples were allowed to clot at room temperature then serum was separated by centrifugation of blood at 3000 rpm for 15 min using a centrifuge (Hettich universal 32A, Germany). Each sample was divided into several aliquots, one for each estimated biochemical parameter, and stored at -20°C until analysis was performed.

**Tissue sampling**

Animals were then sacrificed by cervical dislocation. Livers and epididymal fats were carefully and rapidly excised. The removed livers were washed with cold normal saline and dried on filter papers then weighed for the determination of liver index. Liver index percent was determined (= liver weight / body weight × 100).

Samples of the liver, taken 5 mm away from the edge of the largest hepatic lobe were frozen at -80°C for the determination of hepatic genes expressions. The other lobes of the liver were homogenized in ice-cold saline, using a homogenizer (Heidolph Diax 900, Germany), to prepare 20% homogenate. The prepared homogenate was divided into several aliquots that were stored at -20°C until assayed later for estimation of the chosen biochemical parameters. The remaining part of the large hepatic lobe was fixed with 10% formaldehyde for histopathological examination.

Besides, the epididymal fat located above the epididymis was dissected and frozen at -80°C for estimation of adipose tissue gene expression.

The dead bodies were frozen till incineration.

**Extraction of liver lipids**

Liver lipids were extracted according to the method of Bligh and Dyer\(^{[18]}\) for the determination of liver triglycerides.

**Histopathological examination**

The liver fixed with 10% formaldehyde was dehydrated and embedded in paraffin wax, cut into sections of 7-10 μm thickness, stained with hematoxylin and eosin (HE) and then examined under light microscope.
Hepatic and adipose tissue genes expression: Hepatic genes expressions of SOCS-3, SREBP-1c, fatty acid synthase (FAS), malonyl CoA decarboxylase (MCD), TGF-β1, and adipose tissue genes expressions of leptin and adiponectin were measured using real-time polymerase chain reaction (RT-PCR).

RNA isolation and RT-PCR
Total RNA was isolated from the liver samples stored for determination of hepatic genes expressions using Thermo Scientific GeneJET RNA Purification Kit following the manufacturer’s protocol. Total RNA was purified using the supplied purification column, after which it was reverse transcribed using Thermo Scientific RevertAid First Strand cDNA Synthesis Kit. RT-PCR analysis was performed using the SYBR Green procedure and Stratagene Mx3000P instrument. Expression of mRNA values was normalized relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal standard.

The following primer pairs were employed (Primer-Blast program was used to assist in the design of primers) (Table 1).

Estimation of biochemical parameters

Blood glucose and oral glucose tolerance test: Two days before the end of the treatment period, rats were fasted for 18 h, with free access to water, to minimize feeding-induced variations in glucose pattern. Glucose level was determined in a blood sample obtained from the tail vein. The blood glucose and oral glucose tolerance test (OGTT) was carried out using glucometer test strips (Fine test, Korea). Rats were given oral glucose (20% solution in a dose 2 g/kg of body weight), and droplets of blood from the tail vein were withdrawn at 0 (prior to glucose administration), 15, 30, 60 and 120 min after glucose load, to evaluate the resulting blood glucose concentrations.

Serum insulin and Insulin resistance: Insulin was determined using DRG® Insulin (Rat) ELISA (EIA-2048) version 8.0, 2013. Insulin resistance was calculated using homeostasis model of assessment (HOMA) = blood glucose (mmol/L) × serum insulin (pmol/L)/155 [19].

Serum and liver TGs: Serum and liver TGs were determined according to the method of Bucolo and David [20].

Oxidative stress parameters: Liver homogenate was used for determination of thiobarbituric acid reactive substances (TBARS), measured as MDA [21], reduced glutathione (GSH) [22] and superoxide dismutase (SOD) [23].

Liver function tests: Serum alanine transaminase (ALT) and aspartate aminotransferase (AST) were determined according to the method of Reitman and Frankel [24].

Serum TNF-α: TNF-α was determined using Quantikine® ELISA Rat TNF-α Immunoassay kit. c2012 R&D Systems, Inc.

Statistical analysis
Data are expressed as means ± SE. Comparisons between means were carried out using one way analysis of variance test followed by Tukey Kramer multiple comparison’s test. For all statistical tests, the level of significance was fixed at P < 0.05.

GraphPad Prism® software package, version 6 (GraphPad Software, Inc., United States) was used to carry out all statistical tests.

The statistical methods of this study were reviewed by Dr. Nelly Alieldin, professor of biostatistics and cancer epidemiology, National Cancer Institute, Egypt.

RESULTS

Body weight and liver index percent
As presented in Figure 1A, there was a gradual gain in

Table 1  Primer pairs sequences

| Primer name | Forward primer | Reverse primer |
|-------------|----------------|----------------|
| GAPDH | 5'-ACAAGATGGTGAGGTGGCTGA-3' | 5'-TTGAACTTGCCGTGAGCTGA-3' |
| SOCS-3 | 5'-CTGCCGCCGCCGCTCCTGGG-3' | 5'-ACGGCACTCCAGTGAGCTCA-3' |
| SREBP1c | 5'-GGAGCCATGGATTGCACATT-3' | 5'-GCTTCCAGAGAGGAGGCACGG-3' |
| FAS | 5'-AGAGCTCTTCAAGGAGCAGG-3' | 5'-AGGTTCACTCCCCAGAGGAAGT-3' |
| MCD | 5'-CGGCACCTTCCTCATAAAGC-3' | 5'-GGGTATAGGTGACAGGCTGGA-3' |
| Leptin | 5'-TCAAGGCTGGCTCATATACACAGG-3' | 5'-TGAAGCCCGGAGTGAATCT-3' |
| Adiponectin | 5'-GGCAAACTTGTCAGGAGGTGAAG-3' | 5'-GGGTCACTTGGACACCAAGAA-3' |
| TGF-β1 | 5'-TGAGTCGCTCTTTTGACCCAG-3' | 5'-ACTTCCACCCAGTCCCTTCG-3' |
in body weight in all groups although the extent was variable. Comparing body weights on week 12, fructose-fed rats reached a body weight of 330.0 g compared to 289.8 g in control rats, indicating a 10% more weight gain in the FED group. This rise in body weight was virtually normalized by all treatment regimens.

Figure 1B shows that there was no change in liver index in the FED group compared to control and that treatment with FENO alone or FENO + RES significantly increased liver index% nearly to the same extent i.e., 3.83% and 3.75% respectively.

**OGTT, blood glucose, insulin and HOMA**

Figure 2A depicts the impairment of glucose tolerance in the fructose-fed group as compared to control. Thus, in the control group ingestion of glucose caused a marked steady rise in blood sugar level that reached a peak of 126.7 mg/dL at 15 min; it was maintained at 30 min (130.0 mg/dL) and became nearly normal 2 h after glucose loading. In FED rats, blood glucose peaked at 15 min, there was another peak at 60 min, and was still 26% higher than normal after 2 h. Moreover, the peak blood glucose level in the FED group was higher (156.9 mg/dL) and delayed. Glucose tolerance was improved but not normalized by all treatments.

Beside the alterations in OGTT, feeding rats with fructose caused an increase in blood glucose level to 93.00 mg/dL compared to control (76.25 mg/dL), an increase in serum insulin level to 0.753 µg/L compared to control (0.560 µg/L) leading to a 56% rise in HOMA index. Treatment did not affect blood sugar level but it caused a recovery of both insulin level and HOMA index. It was noticed that the improvement produced by a full dose of FENO was equivalent to that of half the dose given with RES. Figure 2B, C and D illustrate the changes in glucose, insulin and HOMA respectively.

**Serum and liver TGs**

The changes in serum and liver triglycerides are illustrated in Figure 3A and B respectively. Fructose fed rats showed a rise of 44.5% in serum TGs and 86.3% in hepatic triglycerides content as compared to the control group. FENO, being a well-documented antihyperlipidemic drug, was effective in preventing the accumulation of both serum and liver TGs. RES, on the other hand was much less effective in this respect.

**Hepatic MDA, GSH contents and SOD activity**

Figure 4 shows the changes in the redox balance in the liver. Fructose feeding significantly elevated liver MDA by 59% without affecting both liver GSH content and SOD activity. Being an antioxidant free radical scavenger, RES alone, or in combination FENO + RES prevented the upsurge of MDA. Moreover, the effect of the drug combination on GSH superseded that of either treatment alone.

**Serum AST, ALT, AST/ALT ratio and TNF-α**

As shown in Figure 5A-C, feeding fructose amplified the activity of AST without affecting ALT; there was a 2 fold increase in the AST/ALT ratio. Additionally, Serum TNF-α (Figure 5D) was augmented (1.6 fold) compared to control. All treatments opposed the injurious effect of FED and normalized both AST/ALT ratio and serum TNF-α level. Interestingly, RES enhanced the effect of half the dose of FENO in the above-mentioned parameters such that it was equivalent to the effect of a full dose.

**Hepatic expression of SOCS-3, SREBP-1c, FAS, MCD and TGF-β1**

The expressions of SOCS-3, SREBP-1c, FAS, MCD and TGF-β1 in the control group were 1.287, 0.177, 1.04, 1.21 and 0.0084 respectively. Fructose feeding displayed profound changes in these signaling proteins.
pathways. Thus, there was about 7-fold increase in SOCS-3 gene expression, 2-fold increase in SREBP-1c, 8-fold increase in FAS, whereas MCD genes expressions revealed a 4-fold decline compared to control group.

Treatment of the rats given FED with FENO, RES and FENO + RES trimmed down SOCS-3 gene expression to 5.958, 3.933 and 3.707 respectively compared to FED (9.500). Again in this context, the decrease in SOCS-3 gene expression by half the dose
of FENO when combined with RES was 38% more compared to FENO indicating that insulin sensitivity was better in the combination (Figure 6A).

Although FENO treatment did not modify SREBP-1c gene expression, RES alone or combined with FENO caused moderation by 68.7% and 56% respectively.

The hepatic expression of the FAS gene, responsible for de novo lipogenesis, was reduced by FENO, RES and FENO+RES treatments to 5.34, 5.14 and 3.78 respectively compared to FED (8.60).

Furthermore, MCD gene expression in liver was increased in FENO, RES and FENO + RES treatment groups by 2.5, 3.0 and 3.5-fold respectively compared to FED group. The improvement afforded by FENO + RES combination was significantly better (38.6%) compared to FENO alone (Figure 6B, C and D).

TGF-β gene expression was upregulated in fructose fed rats (1.4-fold) as compared to the control counterpart and was normalized by treatment with either FENO or RES, while FENO + RES group showed 86.6% suppression in TGF-β gene expression compared to the FED group. Again, RES enhanced the effect of FENO such that the amelioration offered by the combination of half doses exceeded that of the full FENO dose as shown in Figure 6E.

Adipose tissue genes expression of leptin and adiponectin

Figure 7 represents the alterations in the expression of leptin and adiponectin genes in adipose tissue. It is obvious that feeding rats with fructose produced an almost 5-fold augmentation in leptin gene expression and 2-fold suppression in adiponectin expression. The upregulation of leptin gene expression was successfully normalized by all treatments. Furthermore, adiponectin gene expression was only adjusted by RES treatment alone.

Histopathological examination and measurement of steatosis area

Representative photomicrographs of liver sections are described in Figure 8 and the steatosis area is represented in Figure 9. Feeding rats with 10% fructose in drinking water for 12 wk showed marked steatosis reaching 32.02 µm²/image. Histopathological examination revealed cellular infiltration, cytoplasmic vacuolations, increased Kupffer cells' number, pyknosis and apoptosis of hepatic nuclei. Treatment with FENO completely prevented the induction of steatosis and preserved normal liver histology with irregular blood sinusoids and some degree of cytoplasmic vacuolations in hepatic cells. Notably, RES and FENO + RES treatments caused a marked improvement in the general structure of the liver tissue and markedly decreased the average steatosis area to 46% and 89% respectively and prevented most of the histopathological abnormalities observed in the FED group. The only abnormality seen was macrovesicular steatosis together with hepatic cell vacuolations, both of which are less than observed in the FED group.

DISCUSSION

Several studies emphasized that consumption of high fructose diets led to obesity, fatty liver, hypertri-
glyceridemia and insulin resistance\textsuperscript{[26,27]}. One of the contributing factors is the fast hepatic uptake of fructose from the portal circulation compared to glucose and its bypassing the phosphofructokinase step in glycolysis. In the liver, fructose increases de novo synthesis of fatty acids\textsuperscript{[28]} and increases inflammation\textsuperscript{[29]}, both contribute to increased hepatic lipogenesis.

In the present study fructose accelerated the accumulation of fat in the liver of rats, as evidenced by histological analysis and hepatic triglyceride content. In addition to hepatic fat accumulation, fructose induced hyperglycemia, hyperinsulinemia and insulin resistance. These results are in harmony with other previous studies\textsuperscript{[10,30]}.

One mechanism of fructose-induced insulin resistance is the defective transduction of insulin signaling\textsuperscript{[31]}. Decreased number of IR in liver and skeletal muscle could provide an alternative explanation\textsuperscript{[32]}.

Several studies found that fructose-induced insulin resistance is correlated with serum concentration of triglycerides\textsuperscript{[33,34]}. Although insulin resistance is, at least partly, responsible for hypertriglyceridemia the possibility that hypertriglyceridemia itself may contribute to insulin resistance should not be ruled out\textsuperscript{[33]}. As a consequence of increased circulatory TGs, the current study showed an accumulation of TGs in the liver. This is an important cause of NASH and may be the major risk factor for the observed glucose intolerance, hyperglycemia and insulin resistance in the FED rats. The primary source of hepatic TGs synthesis are the adipose tissue derived FFAs\textsuperscript{[35]}. Although an excessive availability of plasma fatty acids is an important determinant of steatosis, lipogenesis is also considered as an important contributing factor. In fact, prolonged consumption of fructose also increases de novo lipogenesis and this contributes to fat accumulation in the liver\textsuperscript{[26,36]}.

Another important factor which plays an important role in NASH development by high fructose diet is fructose-induced oxidative stress\textsuperscript{[30]}. This was demonstrated in this study by increased hepatic MDA content, reflecting enhanced lipid peroxidation. The fructose-induced oxidative stress could be attributed to accumulation of advanced glycation end-products\textsuperscript{[37,38]}.

In parallel with these abnormalities, the AST/ALT ratio was elevated. Most causes of liver cell injury are associated with a release of AST and ALT from damaged hepatocytes into the blood. The magnitude of AST and ALT elevations vary depending on the
cause of the hepatocellular injury[39,40].

The role of fructose in the development of hepatic oxidative stress and predisposition to necroinflammation and fibrogenesis has been emphasized[41]. Indeed we found a significant increase in the gene expression of hepatic fibrogenic marker TGF-β1 and this result was in agreement with that of Sivakumar and Anuradha[42]. Moreover, fructose-induced increase in leptin gene expression could be another cause of augmented TGF-β1 expression. This is because a positive correlation exists between the expression of TGF-β1 in the liver and insulin resistance, obesity and steatosis[43]. Clinically, TGF-β1 plasma concentration is elevated in NASH patients compared to patients with hepatic steatosis and healthy subjects, suggesting that this cytokine is involved in fibrogenesis in NASH[44].

The possible molecular mechanism responsible for all these detrimental effects of fructose may
be explained by the changes observed in hepatic SOCS-3, SREBP-1c, FAS and MCD genes expression in addition to adipose tissue leptin and adiponectin genes expression.

Regarding the SOCS-3 gene, its enhanced expression would result in decreased tyrosine phosphorylation of IRS proteins and consequently attenuation of insulin action. Another contributing role of SOCS-3 arises from its ability to increase fatty acid synthesis by up-regulation of SREBP-1c expression, presumably through suppression of STAT3 phosphorylation and persistent hyperinsulinemia\cite{41}.

As for the increased SREBP-1c gene expression, this leads to increased lipid synthesis\cite{42}. Beside SREBP-1c, FAS gene expression was increased. These results are in accordance with another study that reported the upregulation of FAS and SREBP-1c protein expression in high-fructose-fed rats\cite{43}. Increased FAS expression, which is responsible for fatty acid synthesis, together with decreased MCD expression, which is responsible for fatty acid oxidation, would collectively explain the increased fat accumulation in the liver currently noticed in the FED rats. Overproduction of fatty acids results in further insulin resistance, creating a vicious cycle. The overexpression of SOCS proteins may be a critical step in this vicious cycle\cite{44}.

Aside from hyperinsulinemia and insulin resistance, fructose feeding caused an increase in leptin gene expression in epididymal adipose tissue. This could possibly be mediated by elevation in serum TNF-\textalpha.. Previous studies pointed to a direct correlation between TNF-\textalpha and leptin levels in mice\cite{46}, hamsters\cite{47} and in diabetic patients\cite{48}. Likewise, leptin gene expression was increased in diet-induced obese mice, in mice with genetic obesity (db/db)\cite{49} and in obese patients\cite{50}.

Leptin plays an important role in limiting TGs accumulation in liver and skeletal muscle through direct activation of AMPK\cite{51} and consequently increases fatty acid \textbeta-oxidation. However, leptin resistance was found to be associated with reduction of leptin-mediated JAK-STAT signaling, and induction of SOCS-3\cite{52}. Therefore, the observed hypertriglyceridemia and hepatic steatosis induced by fructose in the present study would have resulted from a reduction in the hepatic catabolism of fatty acids driven by a state of leptin resistance.

There was an increase in leptin gene expression together with a significant reduction in epididymal adipose tissue adiponectin gene expression. In turn this would lead to insulin resistance\cite{53} through accumulation of hepatic TGs and down-regulation of insulin signalling.

It is now known that adipose tissue has a pivotal role in obesity, insulin resistance, NASH and other metabolic abnormalities and that obesity is associated with insulin resistance\cite{54}, inflammation\cite{55}, macrophages accumulation\cite{56} and expression of TNF-\textalpha.. This explains the rise in TNF-\textalpha in rats given high fructose diet. The possible cause of serum TNF-\textalpha increase is the rise observed in leptin gene expression as this confirms the finding of a dramatic increase in serum TNF-\textalpha level after leptin injection\cite{57}.

Fenofibrate has been used for many years to treat dyslipidemias and has also recently been shown to have anti-inflammatory effects. In view of the lipid lowering properties of fenofibrate, we attempted to study its efficacy in controlling the metabolic derangements in rats fed fructose to induce NASH. Our study showed that fenofibrate, besides decreasing body weight, it restored liver histology to normal, prevented hepatic steatosis and maintained irregular blood sinusoids and this was reflected by improvement in liver function tests. These effects are in harmony with those reported by many investigators\cite{58,59}.

The histological improvement observed by fenofibrate was accompanied by restoration of insulin sensitivity and reduction in hepatic and serum TGs.

![Figure 7](image-url)
Abd El-Haleim EA et al. Resveratrol and fenofibrate effects on NASH
The current study offers possible molecular mechanisms responsible for fenofibrate beneficial effects. Genes expressions were remodeled either in the liver or adipose tissue where the drug caused a decrease in hepatic genes expressions of SOCS-3 and FAS and increased MCD without affecting that of SREBP-1c.

Enhanced liver MCD expression would result in decreased malonyl CoA and, subsequently, decreased free fatty acid and TGs deposition in liver. This confirms Lee et al. finding that, PPAR-α activates rat hepatic MCD transcription. In support, the highly elevated malonyl-CoA levels in the skeletal muscle and liver of pre-diabetic, high fat fed-rat were significantly reduced by fenofibrate.

An unexpected finding was the suppression of FAS expression by FENO although SREBP was not decreased to any significant extent. This might be by virtue of the activation of AMPK as reported by Chen et al. who showed that, the lipid lowering effects of fenofibrate may be exerted through a PPAR-α/AMPK dependent pathway leading to increased fatty acid β-oxidation. AMPK activation by fenofibrate would also suppress FAS expression.

Because fibrogenesis is an integral part of steatohepatitis, another devastating effect that was ameliorated by fenofibrate is the elevated expression of hepatic TGF-β1, a fibrogenic marker. The reduction in TGF-β1 expression was associated with attenuated obesity, reduced adipose leptin expression, increased serum TNF-α and hepatic SOCS-3 expression, factors that precipitate fibrosis. In adipose tissue, fenofibrate caused an improvement in leptin sensitivity as it decreased adipose tissue leptin gene expression. This was associated with decreased SOCS-3 gene expression. This adds support to the finding, SOCS-3 insufficiency enhances leptin sensitivity. Several studies showed
that, PPAR-α agonists decrease leptin levels in obesity induced animal models and in type 2 diabetic patients with hypertriglyceridemia\textsuperscript{[70]}. Adiponectin is implicated to be an anti-inflammatory adipokine and TNF-α antagonist. Accordingly, its expression is related to obesity, insulin resistance and TNF-α. However, in the current model, fenofibrate caused no change in adipose tissue adiponectin gene expression. This effect was in harmony with the study showing that, fenofibrate treatment in high fat fed rats failed to decrease adiponectin plasma level or gene expression in adipose tissue\textsuperscript{[71]}. Similarly, in obese women with type 2 diabetes, serum adiponectin concentration was not significantly affected by fenofibrate treatment\textsuperscript{[72]}. One of the explanations afforded is that PPAR-α activation, besides reducing adiposity of body, it increases adiponectin receptor-1 expression in adipose tissue and this could enhance adiponectin effects although no change can be detected in circulating adiponectin levels\textsuperscript{[73]}.

The effect of fenofibrate on oxidative stress is a matter of controversy, depending on the condition in which it is used. In the present study 4 wk treatment with fenofibrate did not alter the oxidative stress parameters in the liver. This was consistent with the results of El-Sheikh and Rifaa\textsuperscript{[74]} who reported that, PPAR-α could not ameliorate cyclophosphamide-induced oxidative stress. However, some studies have shown a detrimental effect of long term fenofibrate use\textsuperscript{[75]}. Others have shown a beneficial effect in streptozotocin-induced diabetic rats\textsuperscript{[76]}. Resveratrol is a natural dietary polyphenol which has been shown to combine antioxidant, anti-inflammatory and antihyperlipidemic effects\textsuperscript{[14,17]}. In the present study, when resveratrol was given to rats fed high fructose diet it prevented the gain in body weight and modulated the severity of liver damage seen microscopically and relieved the development of NASH. It ameliorated insulin resistance via reducing hepatic lipid deposition, prevented lipid peroxidation and ROS formation and inhibited inflammation by suppressing the production of TNF-α.

The first observation with resveratrol was a desirable decrease in body weight. It has been reported that resveratrol supplementation decreases body weight\textsuperscript{[77]} and it has an anti-obesity potential\textsuperscript{[78]}. The decrease in body weight could also be attributed to the enhanced insulin sensitivity and decreased accumulation of body-fat mediated in part by adipokines changes as resveratrol reduced leptin and increased adiponectin mRNA levels. This is in harmony with a number of previous studies showing the importance of leptin adiponectin balance\textsuperscript{[79,80]}. In contrast, other studies denied beneficial effects of resveratrol on body weight and obesity\textsuperscript{[13,81]}. The discrepancy could be due to the duration of study, dosage of resveratrol, and age of animals.

In the histopathological examination, the only abnormality seen was macrovesicular steatosis together with hepatic cell vacuolations, both of which are less than that observed in the FED group. Several investigators have demonstrated a similar hepatoprotective effect of resveratrol against steatosis in mice\textsuperscript{[81]} or rats\textsuperscript{[82]} fed high fat diet.

The histological improvement of resveratrol was extended to involve an improvement in insulin sensitivity. This lends credit to previous studies showing improved insulin sensitivity in NAFLD rats\textsuperscript{[83]} fed high fat diet and in obese humans\textsuperscript{[84]}. The enhanced insulin sensitivity was reflected herein by decreased hepatic TGs deposition. This was in accordance with other investigators who demonstrated a reduction in hepatic fat deposition in experimental NAFLD models\textsuperscript{[14,83]}.

Another important property of resveratrol is having anti-inflammatory activity. This was shown in the present study by preventing the rise in serum TNF-α. Anti-inflammatory activity was also indicated by inhibition of inflammation in mice fed high fat diet\textsuperscript{[85]} in NAFLD rats\textsuperscript{[84]} and decreasing IL-6 and TNF-α mRNA and reduced the Kupffer cells number induced in the injured liver of mice after bile duct ligation\textsuperscript{[85]}. In obese humans, resveratrol decreased expression levels of genes of inflammatory pathways, and plasma levels of several inflammatory markers\textsuperscript{[84]}.

Being an effective antioxidant, resveratrol could also inhibit the progression of steatosis or steatohepatitis. In the fructose fed rats it decreased hepatic MDA content. Resveratrol has been shown to prevent free radicals and inflammatory cytokines-induced hepatic damage by scavenging ROS and decreasing lipid peroxidation\textsuperscript{[86]}. Resveratrol administration reduces oxidative stress in obese rats\textsuperscript{[87,88]} and in hyperlipidemic rats\textsuperscript{[17]}. Other studies supposed that, the intake of resveratrol containing preparations and polyphenols from muscadine grapes suppress or prevent oxidative stress induced by high fat high carbohydrate meal\textsuperscript{[89]}. The observed resveratrol-induced improvement in NASH related parameters and histopathology was associated with a significant hepatoprotective effect revealed by the reduction in AST/ALT ratio. Other study showed that, resveratrol significantly reduced both ALT, AST serum levels in cholestatic liver injury\textsuperscript{[85]}.

On the molecular basis, resveratrol decreased hepatic SOCS-3 gene expression, therefore preventing insulin and leptin resistance. Similar findings were reported clinically in normal, healthy subjects in the postprandial state where resveratrol suppressed oxidative and inflammatory stress responses to in high fat high carbohydrate meal\textsuperscript{[89]}. Additional findings reported in this study were the down-regulation of the expressions of the lipogenic genes; SREBP-1c and FAS. This was also reported in an earlier study\textsuperscript{[90]}. Resveratrol may improve fructose-induced metabolic abnormalities by activating AMPK pathway\textsuperscript{[83]}. Because resveratrol activates AMPK, one would expect that, AMPK activation in the liver shuts down anabolic processes like cholesterol and TG biosynthesis by reducing the activities of SREBP-1c and
FAS. AMPK activation also promotes catabolic processes. This would lead to a reduction in lipid synthesis and higher fatty acid oxidation rates and finally, prevention of liver steatosis. Furthermore, resveratrol positively activates SIRT-1 through activation of AMPK[91]. In turn, SIRT-1 activation gives protection against insulin resistance[92] and results in SREBP-1c inhibition.

The expression of hepatic MCD gene was upregulated by resveratrol leading to suppression of lipogenesis and enhanced β-oxidation.

As for adipose tissue, resveratrol attenuated the expression of leptin gene, indicating an enhancement of leptin sensitivity and at the same time it increased adiponectin expression. Both of these actions would, in turn, lead to activation of AMPK[93,94]. The reduction in adipose leptin gene expression was associated with decreased hepatic SOCS-3 expression with consequent enhancement of leptin sensitivity[89].

The reduction observed in oxidative stress, serum TNF-α and leptin expression in addition to stimulation of adiponectin expression was reflected in reduced TGF-β1 expression in liver by resveratrol treatment. As an anti-inflammatory, resveratrol decreased TGF-β1 mRNA expression. It has been reported that resveratrol decreases NF-κB which controls transcription of TGF-β1 induced by CCA[94].

There is a interplay between obesity, leptin, adiponectin, TNF-α and TGF-β1.

Fenofibrate is an important lipid-regulating drug and could ameliorate NASH as shown in the present study. However, its use for long-term and in high-dose could induce the possibility of liver function damage[95,96]. In order to regulate blood lipid effectively, protect against NASH and avoid the occurrence of drug-induced liver injury, we investigated the effect of combining RES and half the dose of FENO. This will provide a definite experimental basis for the clinical use of this combination. The combination of half dose fenofibrate with half dose resveratrol showed a protection that was equivalent to the effect of the full FENO dose. Thus, there was a sort of additive or synergistic effect. The histopathological abnormalities observed in the FED group were prevented; the only abnormality seen was macrovesicular steatosis together with hepatic cell vacuolations, both of which are less than that observed in the FED group. This was accompanied by a correction of insulin resistance and a reduction in the deposition of TGs in the liver. Both effects could be, at least, additive.

One side effect of fenofibrate reported in the present investigation was increased lipid peroxidation. However, the combination improved the oxidative stress state as evidenced by a significant decrease in MDA as compared to fenofibrate alone and the decrease was even comparable to the full dose of resveratrol. In addition the combination produced a synergistic effect on liver GSH level.

Moreover, the effect of combination on serum TNF-α and liver function tests could be additive. The decrease in hepatic SOCS-3 gene expression by fenofibrate was substantiated when combined with resveratrol. There was a significant reduction in SREBP-1c expression as compared to fenofibrate alone possibly due to enhanced adiponectin expression. FAS gene expression in liver dropped off and this could be an additive effect. Besides, the combination exerted a favorable synergistic effect on MCD expression.

The reduction in adipose leptin gene expression, which was additive, was associated with a reduction in hepatic SOCS-3 expression. Fenofibrate has no effect on the reduced adiponectin expression caused by fructose, but it increased adiponectin receptors number, while resveratrol enhanced adiponectin expression. So, combining the two drugs produced a favorable effect on insulin sensitivity and TGs accumulation.

Furthermore, the combination exerted a beneficial synergistic effect on the fibrogenic marker, TGF-β1 possibly due to decreased leptin gene expression caused by both treatments in addition to enhanced adiponectin expression driven by resveratrol treatment.

However, the anti-hypertriglyceridemic effect of fenofibrate, in the combination, was attenuated, possibly due to lowering the dose of fenofibrate to one half.

In conclusion, the current work may justify the use of lower doses of fenofibrate in combination with resveratrol to protect the liver from fructose induced hepatic steatosis and damage. The synergistic effect may be due to antioxidant, anti-inflammatory and anti-hyperlipidemic effect of resveratrol. As an addition therapy, resveratrol can augment the beneficial outcome of a lower dose of fenofibrate and reduces its toxic or side effects.

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COMMENTS

Background

Obesity, insulin resistance, hypertriglyceridemia and oxidative stress all contribute to the development of steatosis and its progression to nonalcoholic steatohepatitis (NASH). Fenofibrate that corrects dyslipidemia and resveratrol, an antioxidant, are possible candidates for NASH therapy. This study aimed to investigate the potential molecular mechanisms through which these two
agents may work and the benefit from their combined use in lower doses.

**Research frontiers**

There is still no approved drug for the treatment of NASH. However potential targets for NASH therapy are insulin resistance, inflammatory signaling and fibrosis, both of the latter two effects are promoted by oxidative stress. PPARs agonists as fenofibrate inhibits the development of steatosis, improves insulin resistance, and reduces hepatic inflammation, while resveratrol gives favorable results through both its antioxidant effect and non-antioxidant properties on genes expressions, downregulation of the hepatic inflammatory pathways, and antifibrogenesis effects. Novel therapies with insulin-sensitizing effects but also anti-inflammatory and antifibrotic actions are under research and development.

**Innovations and breakthroughs**

Several studies have tested either fenofibrate or resveratrol individually in NASH, however neither of them alone can be an ideal therapy for NASH as fenofibrate, although it prevented steatosis and improved insulin resistance, it poses the risk of developing liver function damage which is both dose and time-dependent while resveratrol, although it blunts many of the underlying disturbances characteristic of NASH, its use individually is insufficient. Combining both, in half doses has given better results than the full dose of fenofibrate alone in terms of hepatoprotection and correction of the underlying genes expressions responsible for insulin resistance as SOCS3, lipogenesis as SREBP-1C and fibrogenesis as TGF-β1 that have been altered in NASH.

**Applications**

The combination, in the reduced doses, provides a promising experimental evidence for better hepatoprotection, and amelioration of the underlying insulin resistance, hepatic inflammation, and fibrogenesis in fructose-induced NASH and would thus establish the basis for further clinical investigation.

**Terminology**

"Non-alcoholic fatty liver disease" is the accumulation of fat in liver due to causes other than excessive alcohol use (also called steatosis). NASH "non-alcoholic steatohepatitis" is the accumulation of fat in liver that is associated with inflammation and often accompanied by fibrosis. Fenofibrate is an antihyperlipidemic drug. Resveratrol is an antioxidant polyphenol extracted from grape.

**Peer-review**

The paper shows that the use of lower doses of fenofibrate in combination with resveratrol to prevent the liver from fructose induced hepatic steatosis and damage.

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