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ORIGINAL ARTICLE

Saroglitazar Deactivates the Hepatic LPS/TLR4 Signaling Pathway and Ameliorates Adipocyte Dysfunction in Rats with High-Fat Emulsion/LPS Model-Induced Non-alcoholic Steatohepatitis

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Abstract— The most epidemic liver disorder non-alcoholic steatohepatitis (NASH) is characterized by hepatic steatosis and inflammation with hepatocellular damage. Recently, it is predictable to be the extensive cause for liver transplantation. The absence of an approved therapeutic agent for NASH is the reason for investigating saroglitazar (SAR) which showed promising effects as a dual PPAR-α/γ agonist in recent studies on NASH. Here, we aimed to investigate the effect of SAR on NASH induced in rats by the administration of high-fat emulsion (HFE) and small doses of lipopolysaccharides (LPS) for 5 weeks. Rats were divided into three groups: negative control group (saline and standard rodent chow), model group

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Abbreviations: *NASH*, Non-alcoholic steatohepatitis; *LPS*, Lipopolysaccharide; *HFE*, High-fat emulsion; *SAR*, Saroglitazar; *ALT*, Alanine aminotransaminase; *AST*, Aspartate aminotransaminase; *LDL*, Low-density lipoprotein; *NAFLD*, Non-alcoholic fatty liver disease; *HOMA*, Homeostasis model of assessment, *TLR4*, Toll-like receptor 4; *TNF-α*, Tumor necrosis factor-α and; *TGF-β1*,Transforming growth factor-β1; *NAFLD*, Non-alcoholic fatty liver disease; *HCC*, Hepatocellular carcinoma; *PPARs*, Peroxisome proliferator-activator receptors, *CMC*, Carboxymethyl cellulose; *FFAs*, Free fatty acids; i.p, Intraperitoneal; *H&E,* Hematoxylin and eosin; *LH*; Lobular hepatitis; *HB*, Hepatocellular ballooning; *PH*, Portal hepatitis

(HFE(10 ml/kg/day, oral gavage) + LPS(0.5 mg/kg/week, i.p)), and SAR-treated group (HFE(10 ml/kg/day, oral gavage) + LPS(0.5 mg/kg/week, i.p.) + SAR(4 mg/kg/day, oral gavage) starting at week 3.Treatment with SAR successfully ameliorated the damaging effects of HFE with LPS, by counteracting body weight gain and biochemically by normalization of liver function parameters activity, glucose, insulin, homeostasis model of assessment (HOMA-IR) score, lipid profile levels, and histopathological examination. Significant changes in adipokine levels were perceived, resulting in a significant decline in serum leptin and tumor necrosis factor-α (TNF-α) level concurrent with adiponectin normalization. The positive effects observed for SAR on NASH are due to the downregulation of the LPS/TLR4 pathway, as indicated by the suppression of hepatic Toll-like receptor 4 (TLR4), NF-κB, TNF-α, and transforming growth factor-β1 (TGF-β1) expression. In conclusion, this work verified that SAR ameliorates NASH through deactivation of the hepatic LPS/TLR4 pathway and inhibition of adipocyte dysfunction.

KEY WORDS: NASH; lipopolysaccharide; saroglitazar; PPAR-α/γ agonist; toll-like receptor 4; adipocyte.

INTRODUCTION

The prevalence of non-alcoholic fatty liver disease(NAFLD) is increasing rapidly worldwide, and it is now the most epidemic liver disorder from which 1 billion people suffer around the world located mainly in the Middle East and South America with an estimated prevalence rate of ≥ 30% [72, 67]. This worrying fact and NAFLD widespread risk factors as inactive lifestyle, overnutrition, hepatitis C, different pharmacological agents, and genetic predispositions lead to put NAFLD in one of the main concerns for global health [67].NAFLD comprises a wide spectrum of chronic liver diseases, ranging from simple steatosis to non-alcoholic steatohepatitis (NASH) with its serious complications which include liver fibrosis, liver cirrhosis, and hepatocellular carcinoma (HCC) [63]. The enlarged uptake of free fatty acids (FFA) from high-fat diet accumulate as triglycerides in hepatocytes developing liver steatosis the first marked stage of NAFLD [56]which in turn progress to NASH that is characterized by steatosis, liver inflammation, hepatocellular ballooning, and progressive liver fibrosis [2].

To date, there is an urgent need for approved pharmacological therapies for NASH; therefore, recent studies work on emerging new effective therapeutic agents targeting different molecular mechanisms underlying NASH and associated metabolic disorders [45, 25]. Peroxisome proliferator-activated receptors (PPARs) are among the potential targets which exert a promising pivotal regulator role in modulation of NASH as reported in the preclinical trials [25]. Unfortunately, neither PPAR-α agonist nor PPAR-γ agonist could be the approved one due to the weak potency of fibrates, PPAR-α agonist, and the side effects of Thiazolidinediones, PPAR-γ agonists, like fluid retention, weight gain, bone fractures, most liable cardiovascular, and bladder cancer risk which inhibit their clinical use [59]. Hence; there is a need for agents that could avoid both PPAR-α and PPAR-γ limitations but in the same time join the anti-inflammatory and the lipid-lowering properties of PPAR-α agonists with the adipocyte differentiation and the insulin-sensitizing effects of PPAR-γ agonists [59, 45] through their dual activation which will exert a synergistic effect in NASH treatment [62, 15].

The new chemical entity saroglitazar (SAR, Lipaglyn®) was the first glitazar approved for diabetic dyslipidemia; it acts as a dual PPAR-α/γ agonist [3]; furthermore, it overcomes the conventional side effects of fibrates and pioglitazone [29]. However, clinical trials on SAR are still going on for its effectiveness and safety in the treatment of NASH [25, 45].

Several pathways have been implicated recently in NASH onset and progression [21]. One of these is the TLR4 signaling pathway, which results in the production of the TNF-α proinflammatory cytokine. Activation of the TLR4 signaling pathway in the liver by its main ligand, LPS, is actively involved in alcoholic and non-alcoholic liver disease pathogenesis by mediating innate and adaptive inflammatory responses [20].On the other hand, recent studies have focused on the adipokines secreted upon adipocyte dysfunction associated with obesity and endotoxemia [22, 49] (including leptin, adiponectin, and TNF-α) as the key mediators in the pathogenesis of NASH, based on the influence of these mediators on insulin resistance, steatosis, and the inflammatory response [48, 68]. Accordingly, the present study investigates new insights into the mode of action of SAR to alleviate NASH

which have not been investigated on SAR before through inhibiting LPS/TLR4 signaling pathway activation and recovering adipocyte dysfunction.

Recently, investigators have begun to use combined chemical and dietary models to induce NASH and to study possible interventions, owing to the scarcity of animal models that are fully analogous to human steatohepatitis pathogenesis [13, 33].

Thus, the current study assessed SAR efficacy in the treatment of NASH using the high-fat emulsion (HFE) animal model [74] combined with small doses of lipopolysaccharides (LPS) which accelerates hepatic lipid accumulation and damage [9].

Finally, estimating serum biochemical parameters, serum adipokines, histopathological examination of liver tissue, and immunohistochemical expression of TNF-α, TGF-β1, and NF-κB in hepatic tissues successfully verified the aim of the study to evaluate the efficacy of SAR in targeting TLR4 signaling pathway and adipocyte dysfunction to treat NASH.

MATERIAL AND METHODS

Ethics Statement

Experimental design and animal handling procedures were approved by the Faculty of Pharmacy, Cairo University Research Ethics Committee, Cairo, Egypt: PT number (1742) and complied with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication no. 85–23, revised 1996). Every effort was made to minimize the number and suffering of animals used in this study.

Animals

Adult female Wistar rats (140–160 g) were obtained from the animal house colony of the National Research Centre (NRC, Egypt), and acclimatized for 1 week before the experiments started. The rats were housed in stainless steel cages (three rats per cage) and kept at a controlled temperature of 24 ± 1 °C with a 12–12 h light-dark cycle (light cycle, 07:00–19:00). The rats were supplied with commercially available standard chow diet and water *ad libitum*.

Chemicals and Antibodies

Lipopolysaccharides (*Escherichia coli*, serotype O111: B4) were purchased from Sigma-Aldrich, Germany, and SAR from Cadila Healthcare Limited, Ahmadabad, India. All other chemicals were of analytical grade. Polyclonal antibodies specific to TNF-α (catalog number: sc-130,220, 1:50 dilution; Santa Cruz Biotechnology, USA), NF-κB (catalog number: sc-109, 1:200 dilution, Santa Cruz Biotechnology, USA), and TGF-β1 (catalog number: PA1-29020, 1:200 dilution, Thermo Fisher Scientific Inc., USA).

Experimental Design

Animals were divided randomly into three experimental groups ($n = 6$). All rats were supplied with standard rodent chow and free access to drinking water. Rats in group 1 (the control group) received saline (10 ml/kg/daily, oral gavage) while rats in group 2 (the HFE/LPS model group) and group 3 (the SAR-treated group), steatohepatitis was induced by the administration of HFE (10 ml/kg/day, oral gavage) [74] and LPS (0.5 mg/kg/week, i.p) [19]. Besides, they received free access saccharose solution (18%) until the end of the experiment that continued to 5 weeks. Notably, the high-fat diet is designed in an emulsified form administered *via* gavage in order to counteract the natural inadequate consumption of the high-fat diet and control daily diet intake using a non-invasive feeding method [74]. Moreover, rats in group 3 were administered with SAR (4 mg/kg/day, oral gavage) [27] and suspended in 0.5% carboxymethyl cellulose (CMC) and HFE (10 ml/kg/day, oral gavage) /LPS (0.5 mg/kg/week, i.p)) that started at the 3rd week until the end of the experimental period (5 weeks).

Depending on the pilot study, the duration and optimum effective dose of LPS and SAR was modified.

Body weight was determined at 1-week intervals during the experimental period (5 weeks).

Blood Sampling and Serum Preparation

At the end of the experiment, the rats were fasted for 18 h to minimize feeding-induced variations in lipid patterns, and blood samples were drawn from the retro-orbital sinus under light anesthesia. The blood samples were allowed to clot at a temperature of 25 °C, and the serum was separated by centrifugation of the blood at 1409×g for 15 min using a centrifuge (Hettich Universal 32A, Germany). Each sample was divided into several aliquots, one for each of the biochemical parameters to be estimated in order to assess the effect of SAR on the biochemical changes induced by NASH, and stored at − 20 °C until analysis was performed.

Tissue Sampling

Animals were euthanized by decapitation. The liver and epididymal fat were excised carefully and rapidly. The removed livers were washed with cold normal saline and dried on filter paper. The liver lobes were homogenized in ice-cold saline using a homogenizer (Heidolph Diax 900, Germany) to prepare a 20% homogenate. The prepared homogenate was divided into several aliquots that were stored at − 20 °C until later assayed for the estimation of the chosen biochemical parameters. The remaining part of the large hepatic lobe was fixed with 10% formaldehyde for histopathological examination.

Histopathological Examination

Liver tissues from all groups were fixed in 10% buffered formalin. The tissues were dehydrated, embedded in paraffin wax, cut into sections of 4-μm thickness, stained with hematoxylin and eosin (H&E), and conventional histopathological examination was carried out under light microscopy by a pathologist who was blinded to the therapeutic strategy. Images were acquired with a Leica ICC50 HD digital camera attached to a Leica motorized light microscope system.

Lesion scoring was evaluated in ten random microscopic fields by an evaluator blinded to the therapeutic strategy to minimize bias and variability. A semi-quantitative lesion scoring approach for the assessment of non-alcoholic steatohepatitis was used, following the method of Kleiner et al. and Mitchell et al. [32, 41], with some modifications. Table 1 illustrates the scoring approach for the assessment of non-alcoholic steatohepatitis.

Immunohistochemical Expression of TNF-α, TGF-β1, and NF-κB in Hepatic Tissues

Four-μm-thick liver tissue sections were deparaffinized in xylene, rehydrated in graded alcohol, and incubated in 3% hydrogen peroxide to block endogenous peroxidase activity. Sections were pretreated in citrate buffer (pH 6) in a microwave oven. Subsequently, sections were incubated with polyclonal antibodies specific for TNF-α, TGF-β1, and NF-κB. Finally, demonstration of immunoreactivity was carried out with diaminobenzidine (DAB) (Sigma, USA). Evaluation of TNF-α, TGF-β1, and NF-κB immune reactive cells was performed semi-quantitatively according to the method of Ribeiro et al. [55]. Depending on the percentage of positive cells in the microscopic high-power field (HPF) (× 40), the samples were scored as 0 (no staining), 1 (positive staining in < 30% of cells per HPF), 2 (positive staining in 30–70% of cells per HPF), or 3 (positive staining in > 70% of cells per HPF). A total of ten random high-power fields were used to estimate the percentage of positive cells.

Estimation of Biochemical Parameters

1. Determination of Liver Function

Serum alanine aminotransaminase (ALT) and aspartate aminotransaminase (AST) levels were determined by colorimetric assay according to the method of Reitman and Frankl [54], using colorimetric kits (Biodiagnostic, Egypt).In brief, serum aliquots were mixed with 2,4-dinitrophenylhydrazine (1 mmol/L) and then incubated at 37 °C for 30 min. The absorbance was determined (wavelength = 505 nm) using a double beam spectrophotometer (Thermo Electron Corporation, England).

2. Determination of the Serum Lipid Profile

Serum total cholesterol, triglycerides, LDL, and HDL were determined using colorimetric kits (Biodiagnostic, Egypt) using a UV-visible spectrophotometer (UV-1601PC, Shimadzu, Japan) following the manufacturer's instructions.

3. Determination of Fasting Blood Glucose

After the last dose of drugs, rats fasted overnight. Fasting blood glucose was determined with an automatic blood glucose meter (Super Glucocard, ARKRAY, Japan) using blood samples from the tail tip.

Table 1. Non-alcoholic Steatohepatitis Scoring System

Steatosis grade		Lobular hepatitis (× 20 microscopic field)	Hepatocellular ballooning (× 10 microscopic field)	Portal hepatitis (× 200 microscopic field)
0	< 5%	No foci	No ballooned hepatocytes	No foci
1	5–33%	< 2 foci per × 20 field	Few ballooned hepatocytes	< 2 foci per ×200 field
2	34–66%	2–4 foci per × 20 field	Many ballooned hepatocytes	2–4 foci per × 200 field
3	> 66%	> 4 foci per × 20 field	ND (not determined)	> 4 foci per × 200 field

4. Determination of Serum Insulin and Insulin Resistance

Insulin levels were determined using a Rat Insulin (INS) ELISA kit (Cusabio Biotech, USA, catalog number CSB-E05070r) according to the manufacturer's instructions. Insulin resistance was calculated using the homeostasis model of assessment (HOMA) formula: blood glucose (mg/dL) × serum insulin (U/mL)/405 [40].

5. Determination of Serum Adipokines

Adiponectin, leptin, and TNF-α levels were determined using ELISA Rat Immunoassay kits (Cusabio Biotech, USA, catalog numbers CSB-E07271r, CSB-E07433r, and CSB-E11987r, respectively) from R&D Systems, Inc., according to the manufacturer's instructions.

6. Determination of Hepatic Toll-like receptor4 (TLR4) Concentration

Toll-like receptor 4 levels were determined in liver tissue using an ELISA Rat Immunoassay kit (Cusabio Biotech, USA, catalog number: CSB-E15822r) from R&D Systems, Inc., according to the manufacturer's instructions.

Statistical Analysis

The data are expressed as the mean ± standard deviation (SD). Comparisons between means were carried out using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons tests, except for the mean histopathological score, which was analyzed by the Kruskal-Wallis test followed by Dunn's *post hoc* multiple comparison tests. For all statistical tests, the level of significance was set at $P < 0.05$. The Graph Pad Prism® software package, version 6 (Graph Pad Software, Inc., USA) was used to carry out all statistical tests.

RESULTS

Effect of SAR on the Body Weight of HFE and LPS Treated Rats

There was a gradual increase in body weight in all groups (Fig. 1). In week 5, rats in the HFE/LPS model group had a mean body weight of 238.5 ± 1.64 g, compared to 191.2 ± 1.47 g in control rats, indicating a significant increment in the body weight of the model group by 25% compared to the control group. This increase in body weight in the model group was normalized by SAR treatment.

For comparison, data from rats fed with only the HFE/LPS diet and rats fed with standard chow (CTRL) are shown. The data are presented as the means ± SDs and are representatives of a single independent experiment; $n = 6$. Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison tests. a, significantly different from the control group. b, significantly different from HFE/LPS model group. Differences were considered statistically significant if $P < 0.05$. CTRL, control; LPS, lipopolysaccharide; HFE, high-fat emulsion.

Effect of SAR on the Adipocyte Dysfunction Induced by HFE and LPS Model

Serum TNF-α was augmented by 323% in the HFE/LPS model group than in the control group (Fig. 2A). In addition, feeding rats with HFE and administering an intraperitoneal injection of LPS resulted in an increase by 537% in the leptin level and a decline by 76.8% in the adiponectin serum level compared to the control group (Figs 2B and C).

Treatment with SAR noted a decline by 47.6% in serum TNF-α level compared to that in the model group (Fig. 2A). The decrease in adiponectin was successfully normalized by SAR treatment (Fig. 2B). Furthermore, the leptin level was significantly reduced, by 58.6%, in the SAR-treated group compared to that in the HFE/LPS model group (Fig. 2C).

For comparison, data from rats fed with only the HFE/LPS diet and rats fed with standard chow (CTRL) are shown. A) serum TNF-α level, B) serum Leptin level, and C) serum Adiponectin level. The data are presented as the means ± SDs and are representatives of a single independent experiment; $n = 6$. Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison tests. a, Significantly different from the control group. b, Significantly different from the HFE/LPS group. Differences were considered statistically significant if $P < 0.05$. TNF-α, tumor necrosis factor-α; CTRL, control; LPS, lipopolysaccharide; HFE, high-fat emulsion.

SAR Attenuated Steatohepatitis Through Downregulating the Hepatic LPS/TLR4 Signaling Pathway Activated by HFE and LPS Model

1. Hepatic TLR4

Feeding rats with HFE and administering small doses of LPS resulted in profound changes in the TLR4 signaling pathway in the liver. Specifically, there was an approximate

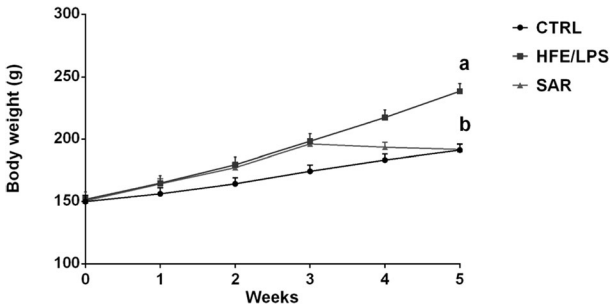

Fig. 1. Effect of SAR on the body weight of HFE and LPS treated rats.

increment by 484.5% in the hepatic TLR4 levels compared to that in the control group. This TLR4 activation was reduced by 58.5% in rats treated with SAR (Fig. 3A).

2. Immunohistochemical Reactivity of NF-κB, TNF-α, and TGF-β1 Observed in Hepatic Tissue

HFE/LPS administration resulted in a significant increase in NF-κB (Fig. 3B (b and d), TNF-α (Fig. 3C (b and d) and TGF-β1 levels (Fig. 3D (b and d) in hepatic tissues compared with the levels of these cytokines in the normal control group, which appeared normal, without any immunoreactivity (Fig 3Ba, Ca, and Da). In contrast, a suppression of NF-κB (Fig. 3B (c and d), TNF-α (Fig. 3C (c and d) and TGF-β1 (Fig. 3D (c and d) immunoreactivity in hepatic cells were demonstrated in the SAR group compared to the HFE/LPS model group.

For comparison, data from rats fed with only the HFE/LPS diet and rats fed with standard chow (CTRL) are shown. A) The concentration of Toll-like receptor 4 (TLR4) in liver tissue. B) Expression of translocated nuclear factor-κB (NF-κB) in rat liver tissues (immunohistochemical staining; magnification × 40). Control group (a),HFE/LPS model group (b),SAR-treated group (c),Quantification of NF-κB optical density (d).C) Expression of tumor necrosis factor-α (TNF-α) in rat liver tissues (immunohistochemical staining; magnification × 40). Control group (a), HFE/LPS model group (b), SAR-treated group (c), Quantification of TNF-α optical density (d).D) Expression of transforming growth factor-β1

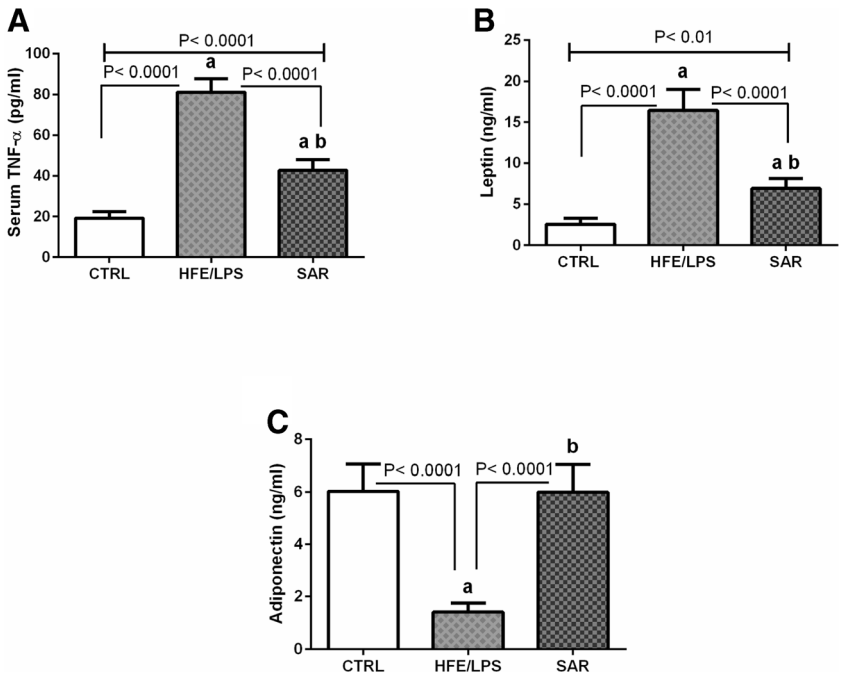

Fig. 2. Effect of SAR on the adipocyte dysfunction induced by HFE and LPS model.

(TGF-β1) in rat liver tissues (immunohistochemical staining; magnification × 40). Control group (a), HFE/LPS model group (b), SAR-treated group (c), Quantification of TGF-β1optical density (d). The data are presented as the means ± SDs and are representatives of a single independent experiment; $n = 6$. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. a, Significantly different from control group. b, Significantly different from HFE/LPS group. Differences were considered statistically significant if $P < 0.05$. CTRL, control; LPS, lipopolysaccharide; HFE, high-fat emulsion.

Effect of SAR on the Serum Biochemical Parameters of HFE and LPS Treated Rats

1. Liver Function Test

The extensive hepatic damage induced by the use of the HFE/LPS model to induce NASH was assessed by estimating both ALT and AST enzyme activity; in particular, there was an elevation with 3-fold in AST activity, whereas ALT activity increased with 4-fold (Table 2). Treatment with SAR reversed the injurious effect of HFE/LPS and normalized AST and ALT activity to levels comparable to those in the control group (Table 2).

2. Serum Lipid Profile

Rats in the HFE/LPS model group showed a significant increase in total cholesterol (178.7%), triglyceride (295.6%), and LDL (326.6%) levels compared to those in the control group, while, there was a significant decline in the HDL level (65.5%) in the HFE/LPS group compared to that in the control group. The HFE/LPS-cause dyslipidemia was efficiently alleviated by SAR treatment resulting in serum lipid levels comparable to those in the control group (Table 2).

3. Blood Glucose, Insulin, and HOMA-IR

Feeding rats with HFE induced an increment in the fasting blood glucose level with 3- fold compared to that in the normal control group and an elevation in the serum

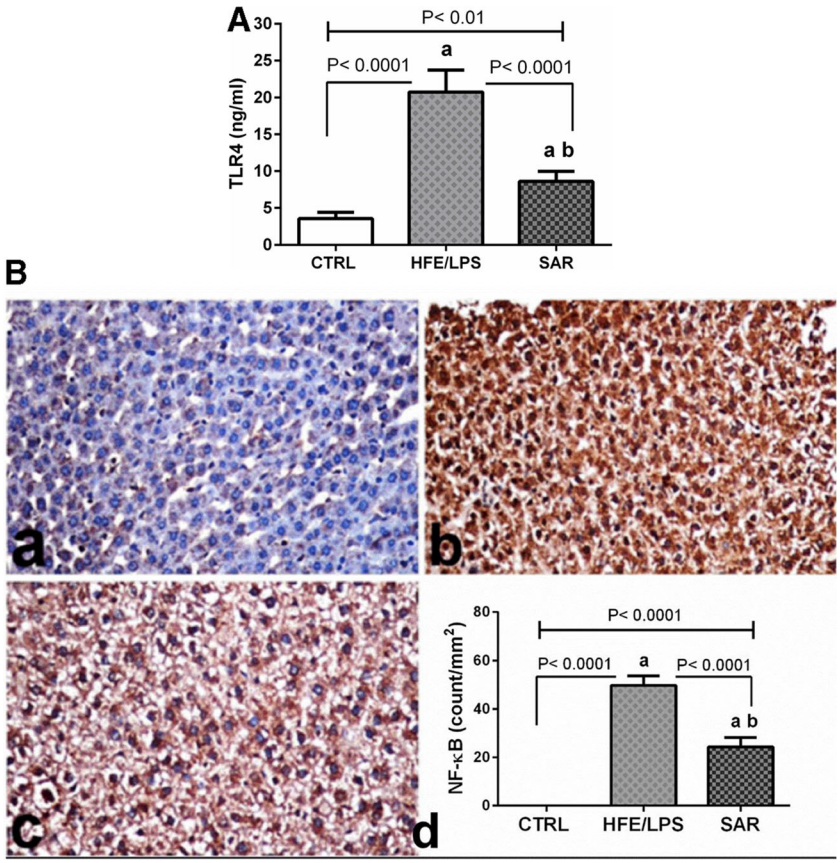

Fig. 3. SAR attenuated steatohepatitis through downregulating hepatic LPS/TLR4 signaling pathway activated by HFE and LPS model.

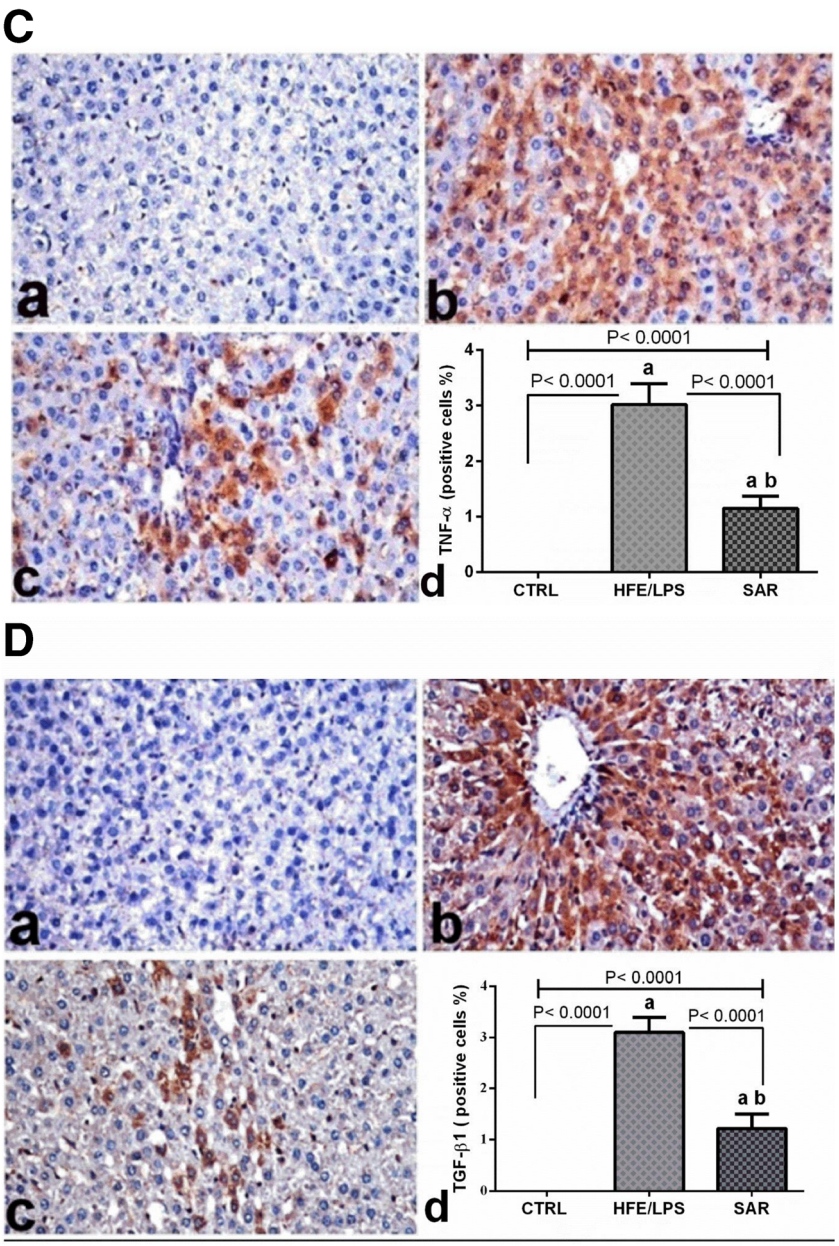

Fig. 3. (continued)

insulin level are estimated with 5- fold compared to that in the normal control group, leading to an escalation in the HOMA index with 18-fold.

SAR restored blood sugar levels, insulin levels, and the HOMA-IR (Table 2).

Histopathological Examination

The mean pathological score recorded in all groups is shown in Fig. 4d. There were no abnormal histopathological alterations in the livers of the normal control group (Fig. 4a). However, the livers of the HFE/LPS model group appeared with the typical hepatic lesions of NASH, since we observed the diffuse intracytoplasmic aggregation of small and large lipid droplets (Fig. 4b) associated with lobular hepatitis and hepatocellular degeneration. Hepatocellular ballooning, with greatly expanded hepatocytes and cytoplasmic reticulation, was one of the characteristic lesions of this group, in addition to megamitochondria, which appeared as an eosinophilic globular

Table 2. Effect of Saroglitazar on Serum Parameters in Rats fed a High-fat Emulsion and Administered with LPS

Parameter	Control group	HFE/LPS model group	SAR-treated group
AST(U/L)	28.2 ± 2.71	92.9 ± 2.04[a]	29.2 ± 1.69[b]
ALT(U/L)	14.4 ± 0.45	61.5 ± 2.38[a]	13.4 ± 0.57[b]
Cholesterol(mg/dL)	159 ± 4.18	443.1 ± 5.00[a]	155.3 ± 3.19[b]
TG(mg/dL)	50 ± 1.84	197.8 ± 2.34[a]	49.8 ± 1.18[b]
HDL(mg/dL)	69.9 ± 3.64	24.1 ± 3.32[a]	66.6 ± 7.17[b]
LDL(mg/dL)	73 ± 3.11	311.4 ± 3.65[a]	69.1 ± 2.18[b]
Fasting blood glucose(mg/dL)	66.2 ± 6.10	200.8 ± 1.73[a]	69.7 ± 5.04[b]
Fasting insulin(IU/mL)	2.1 ± 0.19	10.6 ± 0.97[a]	2.4 ± 0.29[b]
HOMA-IR	0.3 ± 0.04	5.3 ± 0.47[a]	0.4 ± 0.06[b]

The data are presented as the means ± SDs; $n = 6$. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests

[a] Significantly different from the control group

[b] Significantly different from the HFE/LPS model group. Differences were considered statistically significant if $P < 0.05$

ALT, alanine aminotransferase; *AST*, aspartate aminotransferase; *IR*, insulin resistance; *TG*, triglycerides; *HFE*, high-fat emulsion; *HOMA*, homeostasis model of assessment; *LPS*, lipopolysaccharide; *SAR*, saroglitazar

body in the hepatocellular cytoplasm. Notably, in the SAR group, hepatocytes appeared to be normal in size and very similar to those of the normal control group. Furthermore, there was a marked decrease in the inflammatory reaction (Fig. 4c).

DISCUSSION

The current research investigated the potential therapeutic effects of SAR on HFE/LPS model-induced NASH in rats through inhibiting hepatic TLR4 signaling pathway activation and improving adipocyte dysfunction.

The absence of a reliable integrated animal NASH model stands as an obstacle during the preclinical investigation of new therapeutic agents [36]. To overcome this challenge, we fed rats with a combination of HFE which is characterized by high fat, cholesterol, protein, and sucrose in an emulsified form and repeated small doses of LPS which accelerate the development and progression of NASH in only 5 weeks in comparison with previous models who induced NASH by using combined high-fat diet and intraperitoneal injection low-dose LPS rat model [30, 24]. Simply, this model produced the typical features of NASH in humans starting from the significant increase in body weight, dyslipidemia, and amplified liver function parameters until the adipocyte dysfunction which revealed in the significant elevation of serum TNF-α and leptin accompanied with pronounced adiponectin reduction level. In addition, there was an apparent activation of the hepatic LPS/TLR4 signaling pathway as suggested by the increase in TLR4 levels in hepatic tissue with a downstream increase in NF-κB and TNF-α and ended with early fibrotic steatohepatitis which was assessed by TGF-β1.

Recent preclinical studies have shown that SAR improves typical aspects in NASH pathogenesis [26, 6]. However, its effect on adipocyte differentiation and gut-derived lipopolysaccharide (LPS) endotoxemia activating TLR4 signaling pathway in the liver has not been investigated yet. Treatment with SAR during the last 2 weeks of HFE/LPS model administration resulted in a significant decrease in serum TNF-α and leptin levels. Consistent with this, SAR treatment deactivated the LPS/TLR4 signaling pathway in the liver of rats, as evidenced by the reduction inTLR4, TNF-α, and NF-κB levels, together with a decrease in TGF-β1 levels. Moreover, SAR treatment resulted in beneficial and significant differences in adiponectin levels, body weight gain, glucose homeostasis, and lipid profiles, as well as liver function parameters. These improvements are likely to be due to the pivotal regulatory role of PPARs in the modulation of NASH [25].

The current findings revealed a significant increase in body weight of the HFE-fed animals compared to those in the normal control group fed with a standard diet. This finding is in accordance with previous literature [74]. The increase in body weight stimulates the sensitivity of liver cells to the damaging effects of LPS [71, 17].

The administration of SAR significantly restored the normal body weight of the animals. The ability of SAR to inhibit any increase in body weight and counteract obesity is attributed to its activity as a dual PPAR-α/γ agonist [15]. SAR activates PPAR-γ, and thus regulates adipocyte differentiation and lipid storage, and it also activates PPAR-α, which is involved in the activation of fatty acid utilization [52].

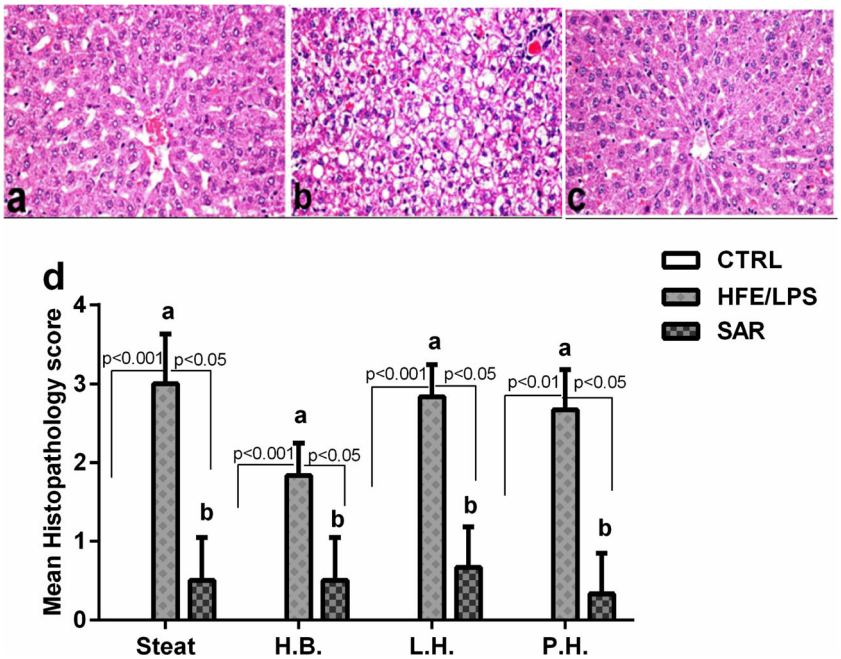

Fig. 4. Histopathological changes in the hepatic tissue of rats by using the light microscopy. The photomicrographs display the following groups: (a) control group, (b) HFE/LPS model group, (c) SAR-treated group, (d) the mean pathological score recorded in all groups (hematoxylin-eosin (H&E) stain, magnification × 40). The data are presented as the means ± SDs and are representatives of a single independent experiment; $n = 6$. Statistical analysis was performed using the Kruskal-Wallis test followed by Dunn's *post hoc* test. A, significantly different from the control group. B, significantly different from the HFE/LPS group. Differences were considered statistically significant if $P < 0.05$.CTRL, control; HB, hepatocellular ballooning; HFE, high-fat emulsion diet; LH, lobular hepatitis; LPS, lipopolysaccharide; PH, portal hepatitis; SAR, saroglitazar.

Lau et al.[33] demonstrated that upon feeding rats with high-fat diet, dietary free fatty acids(FFA) increase; this, in turn, initiates adipose tissue lipolysis. The released FFAs accumulate in liver cells as triglycerides, developing hepatic steatosis and hepatocellular damage [33, 67]. Also, Zu et al. [75] concluded that bacterial endotoxin stimulates adipocyte lipolysis. Consistent with this, our histopathological observations showed a significant increase in the mean pathological score for steatosis and hepatocellular ballooning. These observations are in accordance with those of Lieber et al. [34] who emphasized that in contrast to a lipid-rich chow diet, a high-fat liquid diet initiates intrahepatic lipid accumulation and promotes hepatic steatosis similar to that seen in humans.

The histopathological examination of the SAR group showed a reduction in hepatic triglyceride accumulation, as evidenced by a significant decrease in the mean pathological score of steatosis and hepatocellular ballooning. This improved antisteatotic effect of SAR is likely due to the double action of this drug on both PPAR-α and PPAR-γ [3], which results in increased β-oxidation of fatty acids and reduced fatty acid influx to the liver [15, 44, 45].

Marked hypoadiponectinemia noticed in the HFE/LPS model group played an important role in NAFLD progression, owing to the antilipogenic and anti-inflammatory effects of adiponectin [70]. Reduced adiponectin activity, together with the excessive over-secretion of TNF-α in the serum noted in the current HFE/LPS model group, promotes hepatocyte steatosis through the induction of insulin resistance [38, 18]. The latter events provoke signals that activate NF-κB within hepatocytes, thus increasing the generation of various inflammatory mediators, signifying the emergence of NASH and accelerating the progression of hepatic fibrosis [1, 61].

The administration of SAR restored the normal adiponectin serum level in rats, owing to the influence of both PPAR-α and PPAR-γ on upregulation of the expression of adiponectin and adiponectin receptors [15]. Elevated adiponectin not only prevented the progression of simple steatosis to NASH through the enhancement of insulin sensitivity and the attenuation of adipogenesis [37] but also attenuated hepatic inflammation and stellate cell proliferation [64, 39]. Along these lines, El-Haleim et al. [15] observed that adiponectin elevation occurred upon the addition of pioglitazone (a PPAR-γ agonist) to fenofibrate (a PPAR-α agonist), although fenofibrate alone did not affect hypoadiponectinemia.

The elevated serum leptin level observed in obese rats reflects the incidence of hepatic leptin resistance, in which leptin failed to stimulate hepatic lipid turnover [12, 65]. This observation supports the present results, in which leptin was significantly higher in the HFE/LPS model group than in the control group. Stojsavljević et al. and Tsochatzis et al. [61, 66] reported that leptin affects all stages of NASH development, contributing to insulin resistance and steatosis, and, through its proinflammatory role, promotes liver fibrosis and inflammation, as it induces the expression of procollagen-I, TGF-β1, smooth muscle actin, and TNF-α [23]. This cytokine imbalance between TNF-α and leptin elevation and adiponectin reduction has been investigated in the HFE/LPS model and found to promote the full development of NASH [5, 69].

Several studies have shown that a reduction in leptin aids the amelioration of steatohepatitis, an effect ascribed to the strong correlation of leptin with body mass index, TNF-α level, and insulin resistance [76, 58]. Moreover, investigators observed that activating PAR-α has an inhibitory effect on adipocyte hypertrophy, which decreased leptin levels in obesity-induced animal models [60, 28]. This finding is in concordance with the results of this study as the SAR-treated group showed a significant decrease in leptin levels compared to the HFE/LPS model group.

In Kupffer cells, the LPS/TLR4 signaling pathway is considered the main pathway for the progression of NAFLD to NASH [42]. Once TLR4 binds with LPS, NF-κB downstream signaling is activated, triggering the production of inflammatory cytokines and chemokines such as TNF-α, the key proinflammatory cytokine in NASH pathogenesis [31, 35, 7]. The TLR4 signaling pathway not only activates Kupffer cells but also sensitizes hepatic stellate cell activation and hepatic fibrosis mediated by the profibrogenic signaling molecule TGF-β1 [14]. In the present study, the injection of small doses of LPS triggered significant activation of TLR4 in liver tissue with consequent upregulation of NF-κB and TNF-α expression compared to that in the normal control group, which reveals that the HFE/LPS model successfully targeted the main inflammatory pathway induced in NASH. Moreover, the upregulation of TGF-β1 expression and the elevation of leptin levels in the HFE/LPS model group provide proof of the incidence of early fibrotic steatohepatitis.

Previous studies showed that the activation of both PPAR-γ and PPAR-α attenuated LPS-mediated inflammatory pathways by interfering with the gene expression and activity of TLR4/ NF-κB signaling pathway with a consequent reduction in the activity of IL-6, TNF-α, and other inflammatory mediators, as proved in both *in vitro* and *in vivo* studies [11, 73, 57, 50]. This finding is corroborated by the present findings, which revealed a significant downregulation in TLR4, NF-κB, and TNF-α expression in the SAR-treated group compared to the HFE/LPS model group. Furthermore, hepatic TGF-β1 levels were downregulated by SAR treatment probably due to the ability of SAR to enhance adiponectin serum levels, which in turn downregulates connective tissue growth factors that stimulate hepatic fibrosis by activating TGF-β1 [15].On the other hand, the activation of PPAR-α by SAR has a key role in the suppression of fibrotic markers and reduces the number of stellate cells [51]. Chronic inflammation triggered by excessive TNF-α expression inhibits insulin sensitivity through direct interference with the insulin signaling pathway, thus inhibiting insulin activity in the liver [10].

Insulin resistance has a key role in the development of NASH; it increases the lipolysis of peripheral adipose tissue and fat afflux to the liver, the intrahepatic triglycerides accumulation, and the inhibition of fatty acid oxidation, with an accumulation of triglycerides resulting in hepatic steatosis [4, 46]. The significant increase in fasting insulin and glucose levels in addition to the HOMA-IR score of the current HFE/LPS model compared to that of the control group supports the finding of Lin et al. [35], who emphasized the strong association between insulin resistance and the pathogenesis of NASH in response to TNF-α through proinflammatory pathways. SAR normalized the induced hyperglycemia, hyperinsulinemia, and enhanced insulin sensitivity, as indicated by the normal HOMA values in the SAR-treated group. The therapeutic effects exerted by SAR are ascribed to its activity as a dual PPAR-α/γ agonist. One interpretation of the obtained results is based on studies by Musso et al. and Sun et al. [45, 62] who showed that activating PPAR-γ enhance insulin signaling and reduce gluconeogenesis.

The results obtained in the HFE/LPS model group showed a marked elevation in ALT and AST due to the extensive hepatic damage induced by LPS, which resulted in the loss of functional integrity of the hepatic membrane and the leakage of cellular enzymes [53, 43]. The administration of SAR in the present study enhanced liver function, as evidenced by the normalization of the ALT and AST levels, which is in agreement with recent studies reporting hepatocyte integrity in the investigation of SAR [47, 45].

In the current study, serum levels of TG, LDL, and total cholesterol in the HFE/LPS model group remained significantly elevated, whereas those of HDL markedly decreased, compared to these levels in the normal control group. These findings in agreement with that of Zou et al. [74] who confirmed that obesity, hyperglycemia,

hypertriglyceridemia, and low levels of HDL-cholesterol indicate the incidence of the actual human metabolic syndrome associated with steatohepatitis.

In this study, SAR treatment resulted in marked improvement of serum lipid profile parameters, and this is likely to be due to its activity as a dual PPAR-α/γ agonist [3]. These effects are generated by enhancing hepatic fatty acid uptake with consequent β-oxidation. In addition, SAR upregulates the expression of adiponectin, and genes involved in glucose and lipid metabolism, reduces TG-rich lipoprotein (TRL) biosynthesis, and increases HDL production and reverse cholesterol transport [8, 16]. These effects explain the normalization of the elevated TG, LDL, and total cholesterol levels by SAR in the present study.

CONCLUSION

The current findings suggest that the HFE/LPS model of NASH is a realistic experimental model: it induced obesity and LPS/TLR4 signaling pathway activation in rats, which resulted in a surge of damaging effects such as adipocyte dysfunction, insulin resistance, intrahepatic lipid accumulation, and fibrotic steatohepatitis. Treatment with SAR successfully reversed all these deleterious effects in the rats. The role of SAR as a dual PPAR-α/γ agonist likely explains the inhibition of LPS/TLR4 signaling pathway activation and the amelioration of all the consequent effects.

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COMPLIANCE WITH ETHICAL STANDARDS

Ethics Statement. Experimental design and animal handling procedures were approved by the Faculty of Pharmacy, Cairo University Research Ethics Committee, Cairo, Egypt: PT number (1742) and complied with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication no. 85–23, revised 1996). Every effort was made to minimize the number and suffering of animals used in this study.

Conflict of Interest. The authors declare that they have no conflicts of interest.

PUBLISHER'S NOTE

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