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Telmisartan and/or chlorogenic acid attenuates fructose-induced non-alcoholic fatty liver disease in rats: Implications of cross-talk between angiotensin, the sphingosine kinase/sphingoine-1-phosphate pathway, and TLR4 receptors

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A B S T R A C T

Renin-angiotensin-aldosterone system (RAS) has been implicated in non-alcoholic fatty liver disease (NAFLD); the most common cause of chronic liver diseases. There is accumulating evidence that altered TLR4 and Sphingosine kinase 1(SphK1)/sphingosine1phosphate (S1P) signaling pathways are key players in the pathogenesis of NAFLD. Cross talk of the sphingosine signaling pathway, toll-4 (TLR4) receptors, and angiotensin II was reported in various tissues. Therefore, the aim of this study was to define the contribution of these two pathways to the hepatoprotective effects of telmisartan and/or chlorogenic acid (CGA) in NAFLD. CGA is a strong antioxidant that was previously reported to inhibit angiotensin converting enzyme. Male Wistar rats were treated with either high-fructose, with or without telmisartan, CGA, telmisartan + CGA for 8 weeks. Untreated NAFL rats showed characteristics of NAFLD, as evidenced by significant increase in the body weight, insulin resistance, and serum hepatotoxicity markers (Alanine and Aspartate transaminases) and lipids as compared to the negative control group, in addition to characteristic histopathological alterations. Treatment with either telmisartan and/or CGA improved aforementioned parameters, in addition to upregulation of antioxidant enzymes (Superoxide dismutase and Glutathione peroxidase). Effect of inhibiting RAS on both sphingosine pathway and TLR4 was evident by the suppressing effect of telmisartan and/or CGA on high fructose-induced upregulation of hepatic SPK1 and S1P, in addition to concomitant up-regulation of Sphingosine-1-Phosphate receptor (S1PR)3 protein level and increased expression of S1PR1 and TLR4. As TLR4 and SPK/S1P signaling pathways play important roles in the progression of liver inflammation, the effect on sphingosine pathway and TLR4 was associated with decreased concentrations of inflammatory markers, enzyme kB kinase (IKK), nuclear factor-kB and tumor necrosis factor-α as compared to untreated NAFL group. In conclusion, the present data strongly suggests the cross-talk between angiotensin, the Sphingosine SPK/S1P Axis and TLR4 Receptors, and their role in the pathogenesis of fructose-induced NAFLD, and the protection afforded by drugs inhibiting RAS.

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is by far the most prevalent form of liver dysfunction encountered worldwide, affecting roughly 20–30% of the general population. The association of excessive dietary fructose with the development of NAFLD has attracted much attention. A high flux of fructose to the liver disturbs normal hepatic carbohydrate metabolism leading to an enhanced rate of *de novo* triglycerides synthesis [1].

NAFLD ranges from simple steatosis to non-alcoholic steatohepatitis (NASH), which may progress to cirrhosis and hepatocellular carcinoma. Although the exact mechanism of NAFLD pathology is unknown, several hypotheses suggest that liver damage is multifactorial. According to the "multiple hit" hypothesis, fat accumulation in the liver is the 'first hit'. Subsequently, the liver becomes vulnerable to other hits; involving a combination of lipid peroxidation, oxidative stress, and inflammation. Macrophages and other immune cells are recruited to the liver and secrete reactive oxygen species (ROS), and pro-inflammatory cytokines

such as "nuclear factor-kappaB" (NF-κB), tumor necrosis factor α (TNF-α), interleukin (IL)-1, and IL-6 [2,3]. Kanuri et al. reported that TNF-α plays a key role in the onset of fructose-induced liver damage [4].

Toll-like receptors (TLRs) are sensors of microbial and endogenous danger signals that are expressed and activated in innate immune cells and in liver parenchymal cells and they contribute to the progression of NAFLD. TLR4 signaling pathway plays an important role in the progression of liver inflammation and fibrosis, by triggering the expression of pro-inflammatory cytokines through activation of IκB kinase (IKK), which activates the transcription factor (NF-κB) which is normally kept inactive in the cytosol complexed with the inhibitory protein IκBα [5].

Sphingosine 1-phosphate (S1P); a potent bioactive lipid mediator; is synthesized from sphingosine by sphingosine kinase 1 (SphK1) or SphK2. S1P binds to five high-affinity G protein-coupled receptors; S1PR1, S1PR2, S1PR3, S1PR4, S1PR5 with subsequent generation of downstream signals that play essential roles in cell proliferation, differentiation, angiogenesis, and inflammation. Impairment of SphK1/S1P signaling pathway may be a risk factor for impaired insulin signaling [6]. It was found that SphK1 and S1P are necessary for phosphorylation of IkBα and thus NF-κB activation. There is evidence of an interplay between the above-mentioned signaling pathways; SphK1/S1P and TLR4 leading to a cooperative up-regulation of the inflammatory cytokine production in various tissues [7].

Recent data suggest that angiotensin II (AT-II) plays an important role in the pathogenesis of NAFLD [8]. Moreover, it was found that AT-II cross-talks with both TLR4 and sphingosine pathway. A cross-talk between sphingosine pathway and angiotensin was reported by Wilson et al, 2015 [9], who found that activation of SphK1 is essential for the activity of AT-II in blood vessels. Additionally, Ohkura et al. 2017 [10] reported that AT-II upregulates S1PR1 in cardiac fibroblasts. Another cross-talk with TLR4 contributes to the development of liver fibrosis in NASH [11].

Telmisartan (TEL); an AT-II receptor blocker; showed hepatoprotective effects in NAFLD, and decreased hepatic fibrosis via antioxidant, and anti-inflammatory activity related to its ability to prevent the activation of NF-κB signaling pathway. A Previous study demonstrated that TEL reduced liver oxidative stress and fibrogenesis in patients with NAFLD and chronic hepatitis C [12]. Chlorogenic acid (CGA) is one of the most abundant polyphenols in nature, with the highest concentration in coffee and tea. The antioxidant, anti-inflammatory, anti-oxidant, hypocholesterolemic, and anti-apoptotic effects of CGA were reported in several animal models [13]. CGA was also found to affect AT-II via angiotensin converting enzyme (ACE) inhibitory action [14,15]. Nowadays, much attention is being directed towards natural products as adjuncts or alternative to conventional therapy, aiming to increase efficacy with probably less side effects.

Given that TLRs and S1P are key players in inflammation, and that angiotensin II interact with both, the present study was designed to study the effect of AT-II receptor blocker; TEL; and CGA; a natural product with reported ACE inhibitory action; on SphK1/S1P and TLR4 signaling pathways and its contribution to their hepatoprotective effects in NAFLD.

2. Materials and methods

2.1. Materials

2.1.1. Drugs and chemicals

TEL, fructose and CGA were purchased from Sigma-Aldrich® (St. Louis, MO, USA). Standard diagnostic kits of Total Cholesterol (TC) (1–250 mg/dl), Triglycerides (TG) (3.125–200 mg dl^{-1}), glucose (0.7–300 mg/dl), alanine aminotransferase (ALT) (7–55 U L^{-1}) and aspartate aminotransferase (AST) (8–48 U L^{-1}) were purchased from Sigma-Aldrich® (St. Louis, MO, USA). Kits for glutathione peroxidase (GPx), and superoxide dismutase (SOD) were purchased from Abcam® (Cambridge, MA, USA). Serum glucose level was assayed by a standard

diagnostic kit purchased from Quimica Clinica Aplicada (Amposta®, Catalonia, Spain) (0.7–300 mg/dl). Enzyme-linked immunosorbent assay (ELISA) kits for insulin (HOMA-IR: 1.7–2) and TNF-α (0.8–110 ng m^{-1}) were purchased from Sigma-Aldrich® (St. Louis, MO, USA). ELISA kits for SphK1 activity (31.2–2000 pg ml^{-1}), IKK (0.25–8 ng ml^{-1}.), S1P (62.5 nmol/L − 2000 nmol L^{-1}), and TLR4 (0.156–10 ng ml^{-1}) were purchased from MyBioSource®, Inc. (San Diego, CA, USA). Primary and secondary antibodies were obtained from Abcam® (Cambridge, MA, USA), Sigma-Aldrich® (St. Louis, MO, USA), and Norvocasta® (Newcastle upon Tyne, UK).

2.1.2. Experimental animals

Adult male albino Wistar rats (110–120 g) were obtained from the Animal Care Center at College of Pharmacy, King Saud University, Riyadh, Saudi Arabia. The animals were housed (5/ cage) in standard polypropylene cages and given free access to standard chow and tap water. Rats were kept in a controlled environment at 25 ± 1 °C under a 12-h dark/light cycle and were acclimatized for one week prior to the experiments. All experiments were carried out in accordance with the recommendations of the Experimental Animals Ethics Committee Acts of King Saud University in accordance with the accepted international standards for the handling of experimental animals. Animal studies are reported in compliance with the ARRIVE guidelines.

2.2. Methods

2.2.1. Experimental design

Forty rats were randomly allocated into 5 groups (n = 8) according to weight range to ensure uniform distribution. Normal control rats were administered normal saline solution (0.9% NaCl; the drug vehicle) daily by oral gavage and maintained on regular chow and tap water for 8 weeks. Thirty-two rats were given 10% (wt vol^{-1}) fructose in drinking water (by dissolving 10 g fructose in 100 ml drinking water) along with 10% fructose diet (10 g fructose per 100 g regular chow) for 8 weeks to induce NAFLD (modified from [6,16]. Fresh drinking water was renewed every 2 days. These high fructose-fed (HFF) rats were randomly divided into the following 4 subgroups: **NAFL group**: rats did not receive any treatment. **TEL + NAFL group**: NAFL rats treated with TEL at a dose of 10 mg kg^{-1} daily [17,18]. **CGA + NAFL** group: NAFL rats treated with CGA at a dose of 40 mg kg^{-1} daily [19,20]. **TEL + CGA + NAFL** group: NAFL rats treated with a combination of TEL and CGA in the previously mentioned doses.

TEL and CGA were dissolved in saline, given once daily by oral gavage. Treatments were started from the first day of the experiment and continued for 8 weeks. Body weight (BW) of experimental animals were recorded weekly. All animals were sacrificed at the end of week 8.

2.2.2. Blood and liver sample collection

At the end of the 8-weeks period, animals were fasted overnight, anesthetized with ether, decapitated and blood samples were collected from the neck vessels. Blood samples were collected, allowed to coagulate, centrifuged at 3000 rpm at 4 °C for 30 min. The obtained sera were collected in 2-ml Eppendorf tubes, divided into aliquots and stored at −80 °C for biochemical analysis.

The liver was rapidly dissected out of each animal, washed with ice-cold phosphate-buffered saline. A part of liver tissue was excised and stored in 4% formaldehyde for histopathological and immunohistochemical studies. Another part was frozen in liquid nitrogen and stored at −80 °C for western blot analysis. The remaining excised liver tissues were homogenized in phosphate buffered saline using tissue homogenizer (Omni international Inc, Kennesaw, GA, USA). Homogenates were then centrifuged at 10,000 rpm at 4 °C for 15 min to remove cellular debris, and the supernatants were subsequently collected and stored at −80 °C until used.

2.2.3. Histopathological examination

Samples of the liver tissues were fixed with 4% phosphate-buffered formaldehyde (pH 7.4) for 24 h, dehydrated in ascending grades of alcohol and embedded in paraffin. Paraffin blocks were cut by microtone to prepare 5 μm-thick sections and were applied to glass slides and kept at room temperature until use. These sections were deparaffinized by incubation in a 60 °C-heated oven for one hour followed by immersion in xylene for 10 min, then rehydrated by descending concentrations of ethanol and stained with hematoxylin and eosin (H and E) for the detection of hepatocellular degeneration and fatty infiltration. Hematoxylin stains nuclei blue, while, eosin stains the cytoplasm and connective tissue in a variety of red or pink color [21]. Images were taken using light microscopy at 400x magnification for analysis.

2.2.4. Determination of serum parameters

2.2.4.1. Liver function tests, and lipid profile.
Serum activity of ALT and AST were assayed spectrophotometrically using kinetic kits. Serum levels of TG, TC and VLDL were determined according to the kit instructions. Tests were carried out as duplicates to minimize error.

2.2.4.2. Insulin resistance.
Serum glucose level was assayed with the standard diagnostic kit. Serum insulin was measured by ELISA kit according to the kit instructions. IR was calculated using the Homeostasis Model Assessment-Insulin Resistance (HOMA-IR);

HOMA-IR = [fasting glucose (mg ml^{-1}) × fasting insulin (μIUml^{-1})] ÷ 40.5.

2.2.5. Measurement of liver homogenate parameters

The supernatant obtained from liver homogenates were used for measuring the following parameters:

2.2.5.1. Hepatic oxidative stress markers.
GPx and SOD activity were measured using Kinetic kits according to the kit instructions.

2.2.5.2. ELISA for determination of SphK1 activity, S1P levels, TNF-α, IKK, and TLR4.
The supernatants of the liver homogenates were evaluated by the corresponding sandwich ELISA kits according to the manufacturer's instructions. Concentration was expressed as pg ml^{-1}. ELISA measurements were carried out as duplicates to ensure accuracy and reproducibility.

2.2.6. Western blot analysis

Western blot analysis was used to determine the protein levels of S1PR1 in liver homogenate. The samples containing 50 μg of protein were mixed with an equal volume of Laemmli loading buffer. Then, samples were denatured by incubation in thermomixer at 99 °C for 5 min. Denatured samples were separated using the vertical discontinuous sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli [22]. Samples were then loaded on SDS-polyacrylamide with 10–12% resolving gel and 5% stacking gel. Gels were run in a mini-gel apparatus (Bio-Rad®, Hercules, California, USA) in Tris/glycine/SDS running buffer (25 mM Tris, 192 mM glycine and 0.1% SDS, pH 8.6) at 150 V for approximately 90 min. Molecular weights of the different proteins were estimated using protein markers of known molecular weight (Bio-Rad Laboratories®, Hercules, CA, USA). The separated proteins were electrophoretically transferred to methanol-activated Polyvinylidene difluoride (PVDF) membranes (0.2 μm, Immun-Blot, Bio-Rad Laboratories®, Hercules, CA, USA). After electrotransfer, the membrane was washed twice in Tris-buffered saline and 0.1% Tween 20 (TBST) buffer. Subsequently, membranes were incubated overnight at 4 °C with a primary antibody: anti-S1PR1 rabbit polyclonal antibody (1:1000 dilution), and anti-GAPDH rabbit polyclonal antibody (1:1000 dilution) diluted in TBST. Blots were then incubated for one hour with HRP-conjugated anti-rabbit (1:20,000) secondary antibody diluted in TBST buffer. Finally, the protein bands were visualized by Image Quant LAS 4000 mini (GE Health Care®, UK) and

quantified by using ImageJ version 1.45 software. The S1PR1 protein level was normalized against the loading control (GAPDH) by dividing the value of the target protein by the value of the GAPDH. The relative value was normalized to the control whose value was fixed arbitrarily to one and assigned as a fold of induction.

2.2.6.1. Quantitative immunohistochemical analysis of NFκB, S1PR1, and TLR-4.
Paraffin-embedded liver sections of 3 μm thickness were rehydrated first in xylene and then in graded ethanol solutions. Immunohistochemistry was performed by using streptavidin-biotinylated HRP method (Novalink Max Polymer detection system, Novocastra®, product NO. RE7280-K). The endogenous peroxidase activity was inhibited by 3% H_2O_2 in distilled water for 5 min, and then the sections were washed in Tris-buffered saline (Sigma-Aldrich®, ST. Loui, MO, USA) twice; 5 min for each. Non-specific binding of antibodies was blocked by incubation with a protein block for 5 min. Sections were subsequently incubated with one of the following primary antibodies; rabbit polyclonal anti-rat NF-κB (p65) (Cat.# ab16502), rabbit polyclonal antibody to rat EDG1 / S1P1R (Cat.# PRS480 from Sigma Aldrich®, ST. Louis, MO, USA) or mouse monoclonal antibody to rat TLR4 (Cat.# [76B357.1]; Abcam®, Cambridge, MA, USA) at a concentration of 1 μg·mL^{-1} and incubated overnight at 4 °C. Sections were washed in tris buffer for 3 times each for 3 min, then incubated with biotinylated anti-mouse IgG for NF-KB, anti-rabbit IgG for TLR4 and anti-rabbit IgG for S1PR1 (Novocastra®, Newcastle upon Tyne, UK) for 30 min. After washing the slides with TBS, the sections were incubated with the corresponding biotinylated secondary antibody for 10–15 min. After that, the horseradish peroxidase-conjugated streptavidin solution was added and incubated at room temperature for 10–15 min. Sections were then washed with TBS and incubated for 5–10 min in a solution of 0.02% diaminobenzidine containing 0.01% hydrogen peroxide. Counterstaining was performed using Mayer's hematoxylin, and the sections were mounted in DPx (a mixture of distyrene, plasticizer and xylene) to preserve the stain. For negative control sections, the same procedure was followed with the omission of incubation with the primary antibody. The samples were analyzed using a bright field light microscope (DMRBE, Leica®, Bensheim, Germany) equipped with a video camera (ProgRes, Kontron Instruments®, Watford, UK). Immunohistochemical quantification was performed by measuring the percentage of the immunopositive area using image analysis software (ImageJ®, 1.46a, NIH, Bethesda, Maryland, USA).

2.2.6.2. Statistical analysis.
The data and statistical analysis in this study comply with the recommendations on experimental design and analysis in pharmacology [23]. Data are expressed as the mean ± standard error of the mean (SEM). Differences between the groups were determined using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test using GraphPad Prism 7 software (GraphPad Software®, San Diego, CA, USA). Differences were considered statistically significant at P < 0.01.

3. Results

3.1. Effects of TEL and/or CGA on BW in high Fructose-Induced NAFL rats

High fructose feeding for 8 weeks caused an increase of more than 3-fold in BW of untreated NAFL rats versus 2-fold-increase in BW of normal control group compared to their mean baseline BW. On the other hand, there were no significant differences in BW among the control and treated groups, Fig. 1.

3.2. Effects of TEL and/or CGA on the histopathological changes in liver sections stained with H and E in high Fructose-Induced NAFL rats

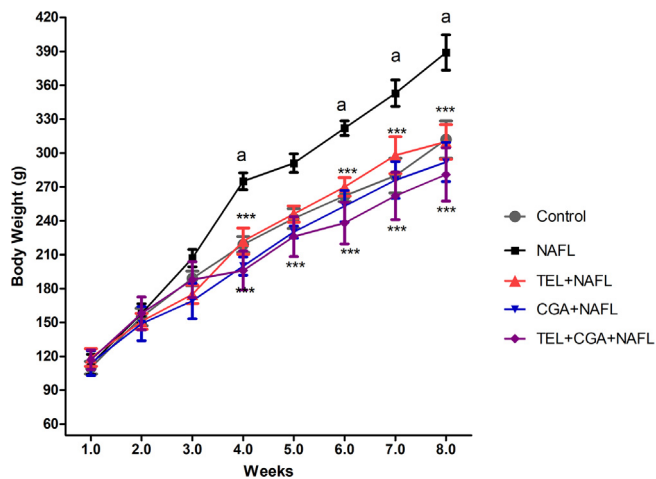H and E-stained hepatic sections from normal control rats show

Fig. 1. Effects of telmisartan and/or chlorogenic acid on body weight in high fructose-induced NAFL rats over 8 weeks. Control: normal control rats; **NAFL rats:** untreated HFF rats; **TEL + NAFL:** NAFL rats treated with TEL (10 mg/kg/day, p.o.); **CGA + NAFL:** NAFL rats treated with CGA (40 mg/kg/day, p.o.); **TEL + CGA + NAFL:** NAFL rats treated with TEL and CGA in the previously mentioned doses. Data are presented as the mean ± SDM. ***P < 0.01 vs. NAFL group, using Two-Way ANOVA (n = 8).

normal hepatic architecture in which the hepatocytes and blood sinusoids appear normal, Fig. 2-A. On the other hand, livers of untreated NAFL rats show marked fatty changes and loss of normal hepatic architecture with excessive fatty degeneration of hepatocytes cytoplasm that appears vacuolated and most of the hepatocyte nuclei are pyknotic, Fig. 2-B. Livers of TEL-treated rats show marked attenuation of the

cytoplasmic fatty degeneration and nuclear pyknosis, Fig. 2-C. Similarly, livers of CGA-treated rats show moderate improvement of the hepatocellular degeneration, Fig. 2-D. Livers of NAFL rats treated with TEL and CGA show apparently normal hepatocytes in which the cytoplasmic and nuclear degenerations disappeared, Fig. 2-E.

3.3. Biochemical studies

3.3.1. Effects of TEL and/or CGA on serum liver aminotransferases (ALT and AST) in high Fructose-Induced NAFL rats

High levels of liver enzymes, in general, signify some form of liver injury. Data collected from this study revealed that the activity of serum ALT and AST was significantly increased in untreated NAFL rats compared to the normal control group. Treatment of HFF rats with TEL or CGA or their combination ameliorated the liver injury and significantly decreased the serum levels of AST and ALT to nearly control values, Table 1.

3.3.1.1. Effects of TEL and/or CGA on serum TG and TC in high Fructose-Induced NAFL rats. High-fructose feeding for 8 weeks induced dyslipidemia in rats as indicated by the significant increase in serum TG and TC concentrations of untreated NAFL rats compared to control normal rats. On the other hand, treated groups showed a marked decrease in serum TG and TC compared to untreated NAFL group. It was found that co-treatment with TEL and CGA elicited a significant lipid-lowering effect on serum TG and TC compared to monotherapy. Moreover, CGA caused a significant decrease in serum TC concentration compared to TEL. However, no significant differences were detected between TEL- and CGA-treated groups with respect to their effect on serum TG concentration, Table 1.
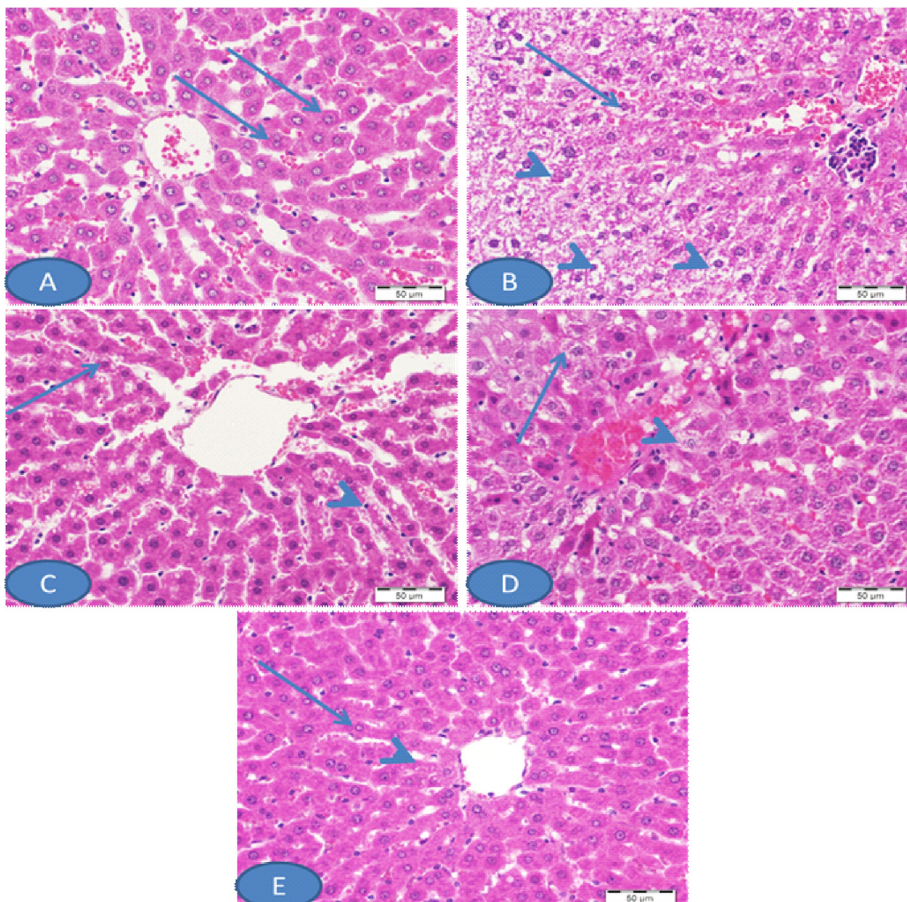

Fig. 2. Photomicrographs of H&E-stained liver sections. Scale bars = 50 μm. (A) A liver section from a normal control rat showing normal hepatocytes cytoplasm and nuclei (arrows). (B) A liver section from untreated NAFL rat shows fatty changes and large patches of hepatocytes with almost vacuolated cytoplasm (arrow) and pyknotic nuclei (arrowheads). (C) A liver section from a TEL-treated NAFL rat showing normal hepatocytes except for scanty scattered fatty infiltration (arrow) and few pyknotic nuclei (arrowhead). (D) A liver section from a CGA-treated NAFL rat shows few groups of degenerated hepatocytes cytoplasm (arrow) and nuclei (arrowhead). (E) A liver section from a NAFL rat treated with a combination of TEL and CGA shows almost absence of cytoplasmic fatty degeneration (arrow) and nuclear pyknosis (arrowhead).

Table 1
Effects of Telmisartan and/ or Chlorogenic acid on Serum ALT, AST, TC and TG Levels, as well as Serum Glucose and IR in High Fructose-Induced NAFL Rats.

Group	ALT U/L	AST U/L	TC (mg/dl)	TG (mg/dl)	Glucose (mg/dl)	HOMA-IR
Normal control	39.3 ± 1.69	53.2 ± 2.53	178 ± 3.23	172 ± 3.43	91.8 ± 1.64	0.948 ± 0.033
Untreated NAFL rats	88.2 ± 2.49[a]	110 ± 3.88[a]	598 ± 6.75[a]	350 ± 7[a]	149 ± 3.72[a]	5.57 ± 0.295[a]
TEL + NAFL	42.3 ± 1.72[***]	67.8 ± 1.32[a,***]	350 ± 3.99[a,***]	304 ± 5.42[a,***]	71.7 ± 1.28[a,***,$]	0.77 ± 0.05[a,***]
CGA + NAFL	48.2 ± 1.72[a,***]	72.4 ± 3.3[a,***]	307 ± 3.75[a,***,#]	284 ± 8.29[a,***]	105 ± 2.69[a,***]	1.3 ± 0.105[a,***]
TEL + CGA + NAFL	39.3 ± 0.89[***,$]	64.2 ± 2.24[a,***]	263 ± 7[a,***,#,$]	215 ± 4.2[a,***,#,$]	79.8 ± 2.1[a,***,$]	0.73 ± 0.05[a,***]

Data were shown as means ± SEM (n = 8).
[a] P < 0.01 compared with control group.
[***] P < 0.01 compared with NAFL group.
[#] P < 0.01 compared with TEL group.
[$] P < 0.01 compared with CGA group.

3.3.1.2. Effects of TEL and/or CGA on serum glucose level and insulin resistance in high Fructose-Induced NAFL rats. As shown in Table 1, serum glucose level and insulin resistance were significantly increased in NAFL rats when compared with that of the normal control group. However, treatment with TEL or CGA or their combination caused significant decreases in serum glucose levels, and serum HOMA-IR when compared to NAFL group and were maintained comparable to control.

3.3.2. Effects of TEL and/or CGA on oxidative stress markers; GPx and SOD in liver homogenates in high Fructose-Induced NAFL rats

The activity of the antioxidant enzymes; GPx and SOD were significantly decreased in the liver homogenate of NAFL rats when compared with their control peers. On the other hand, administration of TEL or CGA or their combination alleviated the oxidative stress induced by high-fructose feeding as evidenced by significant increases in the activity of GPx, and SOD as compared to NAFL group to levels comparable to control group, Fig. 3.

3.3.3. Effects of TEL and/or CGA on IKK, and TNF-α concentrations in liver homogenates in high Fructose-Induced NAFL rats

Fig. 4 shows a significant elevation in hepatic levels of IKK, and TNF-α in untreated NAFL rats compared to normal control rats. Administration of TEL or CGA or their combination markedly decreased IKK, and TNF-α levels compared with NAFL rats. Rats which received the combination therapy had lower IKK, and TNF-α concentrations than TEL or CGA treated groups. Also, TEL-treated rats showed significantly lower TNFα concentration than CGA-treated group.

3.3.3.1. Effects of TEL and/or CGA on TLR4 concentration in liver homogenates in high Fructose-Induced NAFL rats. By the end of the 8-week experiment, hepatic TLR4 concentrations of untreated NAFL rats were significantly high compared to the normal control group. Treatment with either TEL or CGA or their combination significantly decreased TLR4 concentrations compared to untreated NAFL rats. There was a significantly lower hepatic TLR4 concentration in TEL-treated group than CGA-treated group, Fig. 4C.

3.3.3.2. Effects of TEL and/or CGA on S1P concentration in liver homogenates in high Fructose-Induced NAFL rats. As shown in Fig. 5A, hepatic S1P levels were significantly increased in untreated NAFL rats compared with the normal control group. Treated groups showed highly significant decreases in S1P levels compared with the untreated NAFL rats. Furthermore, a significant decrease in S1P level was found in the combination vs. the monotherapy groups.

3.3.3.3. Effects of TEL and/or CGA on SphK1 activity in liver homogenates in high Fructose-Induced NAFL rats. The results of the present study clearly demonstrated that untreated NAFL rats showed a significant increase in SphK1 activity compared with normal control. Treatment with TEL and/ or CGA elicited significant decreases in SphK1 and S1P activity compared to the untreated NAFL rats. Both monotherapy and combination therapy maintained SphK1 activity comparable to that of the normal control group, Fig. 5B.

3.4. Molecular studies

3.4.1. Effects of TEL and/or CGA on the hepatic protein level of S1PR3 in high Fructose-Induced NAFL rats using western blot analysis

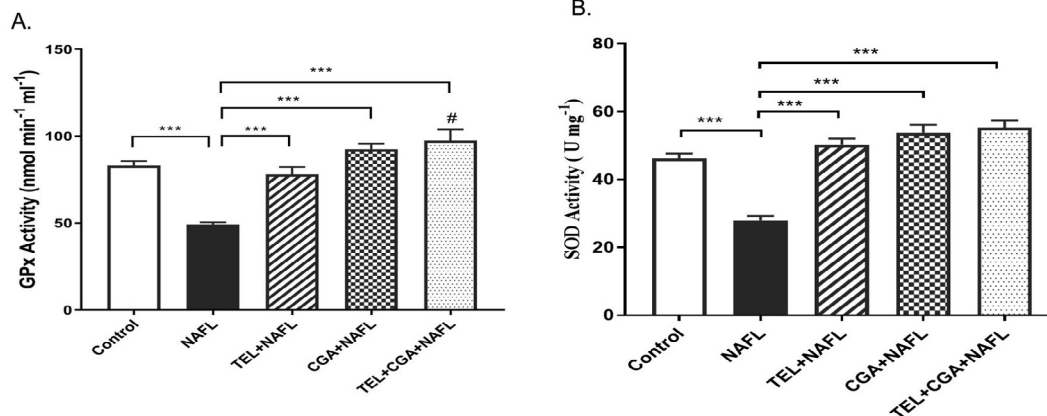Fig. 6 shows the western blot analysis of S1PR3 in the livers of the

Fig. 3. Effects of telmisartan and/or chlorogenic acid on oxidative stress markers; GPx (A) and SOD (B) in liver homogenates in high fructose-induced NAFL rats, after 8 weeks of treatment. **Control:** normal control rats; **NAFL rats:** untreated HFF rats; **TEL + NAFL:** NAFL rats treated with TEL (10 mg/kg/day, p.o.); **CGA + NAFL:** NAFL rats treated with CGA (40 mg/kg/day, p.o.); **TEL + CGA + NAFL:** NAFL rats treated with TEL and CGA in the previously mentioned doses. Data are presented as the mean ± SEM. ***P < 0.01 vs. NAFL group, #P < 0.01 vs. TEL group, using one way ANOVA followed by post hoc Tukey Kramer test (n = 8).
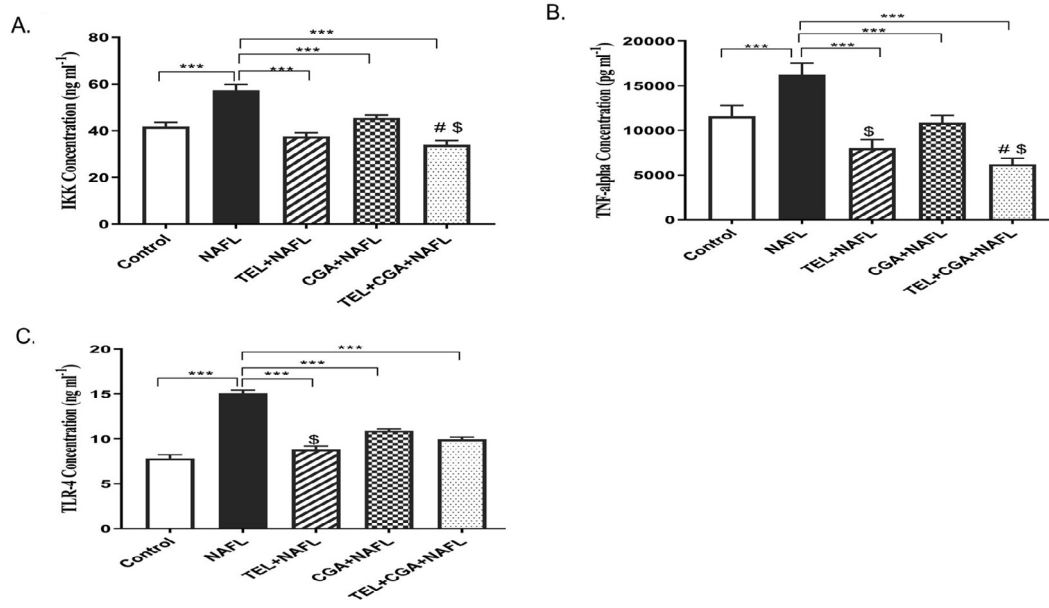
Fig. 4. Effects of telmisartan and/or chlorogenic acid on IKK (A), TNF-α (B), and TLR4 concentration (C) in liver homogenates in high fructose-induced NAFL rats, after 8 weeks of treatment. **Control:** normal control rats; **NAFL rats:** untreated HFF rats; **TEL + NAFL:** NAFL rats treated with TEL (10 mg/kg/day, p.o.); **CGA + NAFL:** NAFL rats treated with CGA (40 mg/kg/day, p.o.); **TEL + CGA + NAFL:** NAFL rats treated with TEL and CGA in the previously mentioned doses. Data are presented as the mean ± SEM. ***P < 0.01 vs. NAFL group, [#]P < 0.01 vs. TEL group, [$]P < 0.01 vs. CGA group using one way ANOVA followed by post hoc Tukey Kramer test (n = 8).

control and experimental groups. A representative immunoblot of protein levels is shown in Fig. 5-C, and the quantitative results of the blots are shown in Fig. 5-D.

After 8 weeks of fructose feeding, the liver protein level of S1PR3 was significantly higher in NAFLD group than in the control group. Treatment with either TEL or CGA or their combination significantly

decreased S1PR3 protein levels in comparison with untreated NAFL rats. Furthermore, a significant decrease in S1PR3 protein level was detected in TEL-treated group compared to CGA-treated group. Co-treatment with TEL and CGA produced the highest attenuation in the S1PR3 protein level compared to either drug alone and maintained S1PR3 protein level comparable to the normal control group, Fig. 5-D.
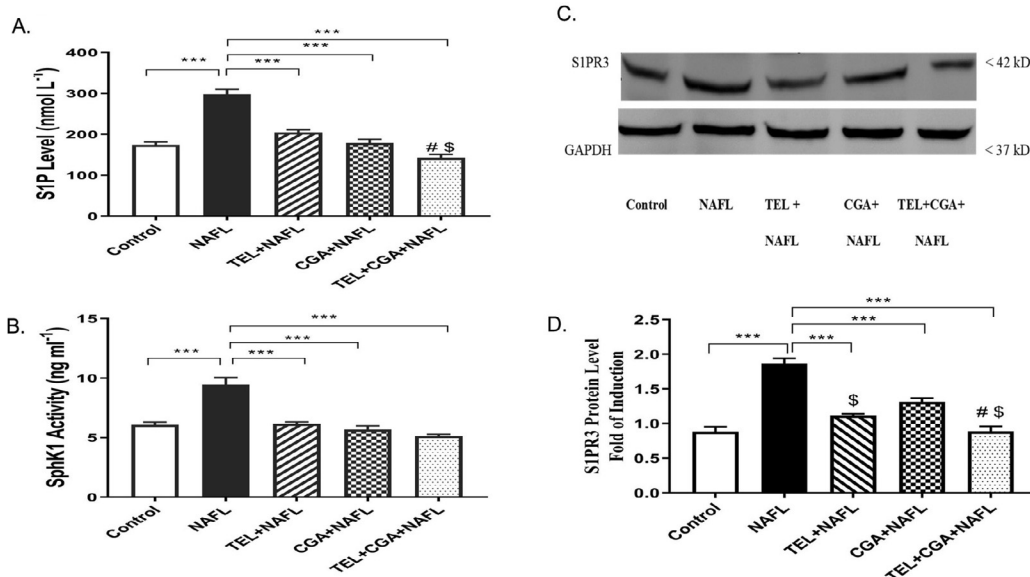

Fig. 5. Effects of telmisartan and/or chlorogenic acid on S1P activity (A) and SphK1 activity (B) in liver homogenates in high fructose-induced NAFL rats after 8 weeks of treatment. Representative immunoblot of the effects of telmisartan and/or chlorogenic acid on the protein level of hepatic S1PR3 (C) in high fructose-induced NAFL rats after 8 weeks of treatment. Sample proteins were resolved by SDS-PAGE and immunoblotted with EDG 3 antibody "upper panel" and after stripping with GAPDH antibody as loading control "lower panel". The densities of immunoblots were quantified using analysis software. The sizes of the molecular mass markers expressed in kDa are indicated at the right of the panel. (D) Quantitative results of the immunoblots. S1PR3 protein levels are expressed as the ratio of S1PR3/GAPDH. Five repeats of immunoblotting were used to calculate the quantitative results. The relative quantities were normalized to the control and expressed as a fold of induction. **Control:** normal control rats; **NAFL rats:** untreated HFF rats; **TEL + NAFL:** NAFL rats treated with TEL (10 mg/kg/day, p.o.); **CGA + NAFL:** NAFL rats treated with CGA (40 mg/kg/day, p.o.); **TEL + CGA + NAFL:** NAFL rats treated with TEL and CGA in the previously mentioned doses. Data are presented as the mean ± SEM. ***P < 0.01 vs. NAFL group, [$]P < 0.05 vs. CGA group, using one way ANOVA followed by post hoc Tukey Kramer test (n = 8 for A–C).
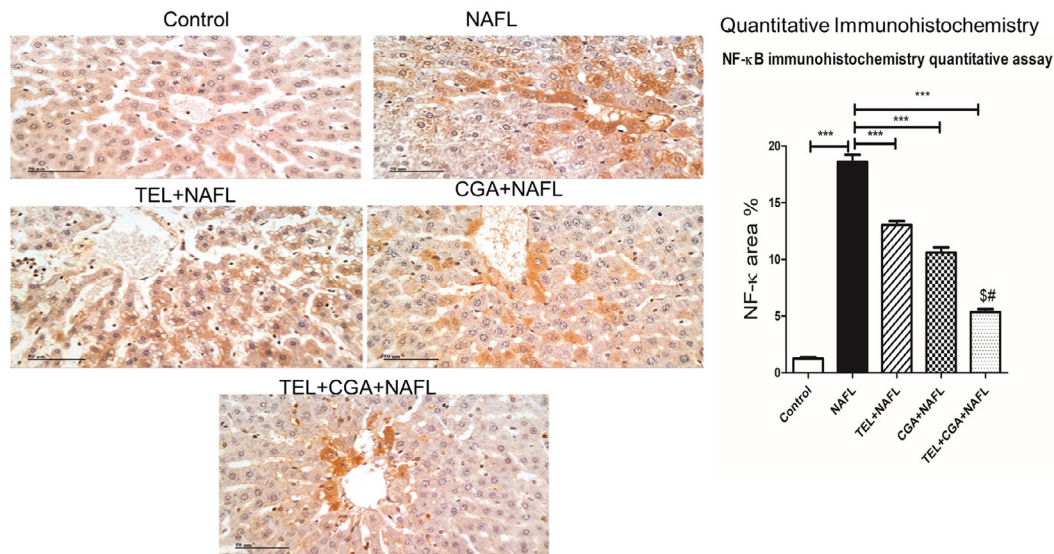
Fig. 6. Photomicrographs of anti-NFκB immunostained liver sections, liver section from a normal control rat showing the absence of immunopositivity in hepatocytes cytoplasm and nuclei. A liver section from NAFL rat showing a large number of hepatocytes with strong immuno-positive cytoplasm. A liver section from a TEL-treated NAFL rat showing few hepatocytes with cytoplasmic immunoreaction, few with pyknotic nuclei. A liver section from a CGA-treated rat shows many cells with a positive cytoplasmic immune reaction. A liver section from a NAFL rat treated with the combination of TEL and CGA shows few hepatocytes with a very weak immune reaction in the cytoplasm. Quantitative image analysis for immunohistochemical staining of NF-kB expressed as the percentage of stained area averaged across 6 different fields for each rat of at least five rats. Each column represents the mean ± SE.

3.4.1.1. Immunohistochemical studies

3.4.1.1.1. *Effects of TEL and/or CGA on the hepatic expression of NFκB in high Fructose-Induced NAFL rats.* Anti-NFκB immunostained liver sections from normal control rats show almost absence of staining in the cytoplasm and nuclei of hepatocytes. While livers of untreated NAFL rats show multiple large patches of hepatocytes with strongly stained cytoplasm and nuclei. Livers of TEL-treated rats show a marked decrease in the immunostained areas. On the other hand, liver sections from CGA-treated group show a moderate decrease in the strength of immunopositivity (still there are many immunopositive patches). Treatment with TEL and CGA markedly decreased the intensity and number of immunopositive hepatocytes compared to other treated groups, Fig. 6.

3.4.1.1.2. *Effects of TEL and/or CGA on the hepatic expression of TLR4 in high Fructose-Induced NAFL rats.* Anti-TLR4-immunostaining of liver sections from normal control rats shows very weak immunostaining of the cytoplasm and nuclei of few hepatocytes. While untreated NAFL rats show multiple strong immunopositivity of the cytoplasm and nuclei of the fatty degenerated hepatocytes. A liver section from a TEL-treated rat shows a moderate decrease in the immunopositive stained hepatocytes, few nuclei are positively stained. Livers of rats treated with CGA show a decrease in the strength of immunopositivity but less than TEL-treated group. Livers of TEL + CGA treated rats show a marked decrease in the intensity and number of immunopositive hepatocytes (i.e. immunostaining was restricted to the cell membrane), Fig. 7. Results were comparable to those of ELISA test, Fig. 4-C.

3.4.1.1.3. *Effects of TEL and/or CGA on the hepatic expression of S1PR1 in high Fructose-Induced NAFL rats.* The expression of S1PR1 was detected in liver tissue by immunohistochemical staining using anti-EDG1 antibody. Liver sections from normal control group show a normal weak immune reaction in the hepatocytes. On the other hand, fructose feeding caused enhanced expression of S1PR1 as detected by the increased intensity and distribution of the immunoreaction in the liver of untreated NAFL rats. TEL-treated rats showed a marked decrease in the expression of S1PR, as revealed by the marked depletion of the immunoreaction which was restricted to few hepatocytes. CGA-treated rats showed a moderate decrease in the

intensity and distribution of the immunoreaction. Treatment with TEL and CGA resulted in a marked reduction in the immune reaction in most of the hepatocytes, Fig. 8.

4. Discussion

NAFLD has become the most common manifestation of chronic liver diseases in developed countries and has frequently been associated with obesity, hyperlipidemia, and type 2 Diabetes. NAFLD may progress to liver cirrhosis and hepatocellular carcinoma which potentially requires liver transplantation [24]. However, treatment of NAFLD remains a challenge; up to date, no effective lines of treatment have been approved. Several mediators and pathways are involved in NAFLD pathogenesis, including angiotensin II, sphingolipids, and TLR4-mediated NF-kB activation. Cross talk between these biological mediators has been observed in various tissues. However, their contribution to NAFLD development, and the effect on inhibition of Angiotensin II on sphingolipids, and TLR4 was not previously elucidated.

In the present study, the high fructose feeding model; a classical rat model of NAFLD was used [25]. Although many experimental models of NAFLD are available, high fructose model represents many of the pathophysiological features of human NAFLD, and has proven reproducibility in many studies [26]. It is well known that high fructose feeding is associated with accumulation of TG in hepatocytes, abnormal hepatic metabolism, inflammation and impaired insulin signaling [6].

In the present study, HFF rats showed significant increases in BW, serum ALT and AST activity, serum TC & TG, blood glucose and IR compared to normal control rats, Fig. 1, Table 1. These findings, in addition to histopathological changes, Fig. 2, confirmed the induction of NAFLD in rats and are in agreement with previous studies [6,27].

Several studies have demonstrated that oxidative stress is a major player triggering the progression of steatosis to steatohepatitis, as ROS play a significant role in the induction of inflammation [28]. High fructose feeding caused a significant decrease in the activity of hepatic GPx and SOD which are considered the primary defense machinery against ROS and oxidative stress, and increased levels of inflammatory markers; IKK, NFκB and TNFα, Fig. 3,4 and 6. Markedly, IKK plays a central role in the signal-dependent activation of NFκB [29].
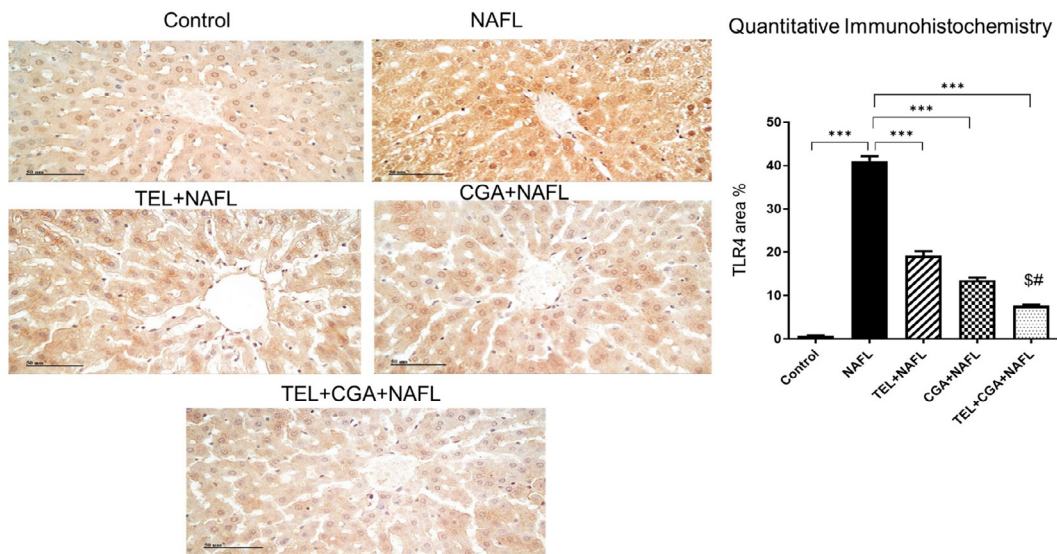
Fig. 7. Photomicrographs of anti-TLR4 immunostained liver sections. A liver section from a normal control rat showing the absence of immunopositivity in the cytoplasm and nuclei of most of the hepatocytes, only very few scattered cells show immunopositivity. A liver section from untreated NAFL rat showing fatty changes and a large number of degenerated hepatocytes with strong immunopositive reaction. A liver section from a TEL-treated NAFL rat showing a decrease in the number of hepatocytes with immunopositive reaction. A liver section from a NAFL rat treated with CGA shows few cells with positive cytoplasmic immunoreaction. A liver section from a NAFL rat treated with a combination of TEL and CGA shows very few hepatocytes with a weak immunopositive reaction in the cytoplasm. Quantitative image analysis for immunohistochemical staining of TLR4 expressed as the percentage of stained area averaged across 6 different fields for each rat of at least five rats. Each column represents the mean $\pm$ SE.
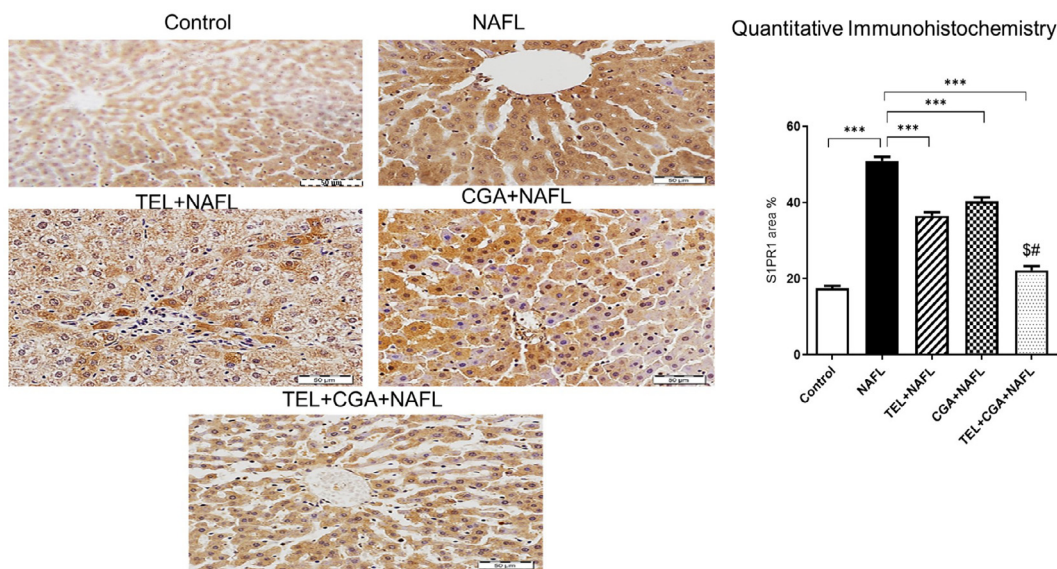

Fig. 8. Liver sections immunostained with EDG1 primary antibody from Norvocastra®. A normal control liver showing a weak immune reaction in the hepatocytes surrounding a central vein. A liver section from untreated NAFL rat shows fatty changes and scattered hepatocytes with a strong immune reaction. A liver section from a TEL-treated NAFL rat shows a decrease in the immunostaining of center-lobular hepatocytes. A liver section from a NAFL rat treated with CGA showing decreased immunoreaction and some center-lobular positive cells. A liver section from a NAFL rat treated with TEL and CGA and showing a marked diminution of the immunoreaction in most of the hepatocytes. Quantitative image analysis for immunohistochemical staining of S1PR1 expressed as the percentage of stained area averaged across 6 different fields for each rat of at least five rats. Each column represents the mean $\pm$ SE. Scale Bar = 50μm.

Overexpression of NFκB modulates the level of the inflammatory mediator "TNFα"; a key mediator involved in the development of IR, steatosis, and NASH [30]. Our results are in accordance with those of previous studies [31,32].

Potential pathways that may play a key role in the development of NAFLD are RAS, SphK1/S1P and TLR4 signaling pathways [6,33]. However, the role of these pathways, and their mutual interaction in fructose-induced NAFLD and their modulation by treatment modalities have not been fully investigated. So, the present research examined the divergent role of these signaling pathways in a rat model of NAFLD.

Considerable attention has been paid to explore the diverse biological effects of ceramides; the main precursors in the biosynthesis of various sphingolipids. Ceramides are deacetylated to form sphingosine, which is phosphorylated to S1P via SphK1. S1P binds with S1PR1 and S1PR3 which are widely expressed in various types of cells [34]. SphK1/S1P signaling pathway plays an important role in the regulation of cell proliferation & survival, and also in insulin action and lipid partitioning [35]. It is likely that disruption of SphK1/S1P signaling pathway could contribute to the metabolic derangement in NAFLD and hence modulation of this pathway may be of great therapeutic value. L

Cowart [36] revealed that inhibition of sphingolipid synthesis enhanced insulin sensitivity and resolved hepatic steatosis in obese rodents. In addition, Osawa et al., [37] reported that impairment of this signaling pathway was implicated in the pathological development of obesity and diabetes.

Our results revealed an increase in the activity of hepatic SphK1 and S1P levels in untreated NAFL rats with concomitant up-regulation of S1PR3 protein and increased expression of S1PR1, Figs. 5 and 8. Theoretically, activation of S1PR1 and S1PR3 causes phosphorylation of IKK which in turn activates the signaling of NFκB and release of TNFα which in-turn stimulates the production of ceramides. The interaction between TNFα and ceramides; besides causing IR may also increase the mitochondrial generation of ROS, thus promoting apoptosis and recruitment of inflammatory cells to the liver, leading to worsening of hepatic inflammation and steatosis [38].

TLR4; the most studied receptor among TLRs; is expressed by all parenchymal and non-parenchymal cell types. More recently, evidence has emerged that TLR4 activation plays a key role in NAFLD. Ferreira et al., [33] demonstrated that TLR4 deficiency enhanced fatty acid oxidation in the liver of mice and prevented TG accumulation. Kiziltas, 2014 showed that TLR4 gene mutation in humans may have a preventive role against the development of NAFLD [39]. In the present study, high fructose feeding induced a marked increase in TLR4 level and expression in the liver of NAFL rats, Figs. 4 and 7.

Evidence of the interplay between the two signaling pathways; SphK1/S1P and TLR4 has been previously reported. S1PRs and TLR4 signaling lead to a cooperative up-regulation of the inflammatory cytokine production in human gingival epithelial cells [7] and interstitial cells of human aortic valve [40]. In addition, the immunoprecipitation studies showed an increased S1P/TLR4 interaction and enhanced allergic inflammatory response in mice [41]. An important new finding in the present study is that SphK1/S1P and TLR4 signaling pathways are implicated in the pathogenesis of fructose-induced NAFLD in rats.

The RAS is believed to play a significant role in the development of NAFLD. RAS was found to directly favors steatosis, inflammation and fibrogenesis via enhancing activation of hepatic stellate cells. Therapy with RAS inhibitors such as ACE-inhibitors or angiotensin receptor blockers has been associated with improvement of liver damage [42]. In the present study, we studied the effect of AR blocker; TEL; and a natural antioxidant with reported ACE inhibitor activity; CGA; alone and in combination in NAFLD and their modulatory effects on Sphingosine pathway, and TLR4 mediated NF-κB activation. TEL has been reported to possess marked antifibrotic and hepatoprotective effects in different animal models [43,44] and in humans [45]. Similarly, CGA; found in coffee mostly and a lot of plant compounds; has important biological effects related to its antioxidant and anti-inflammatory activities. The hepatoprotective effects of CGA have been documented in several animal models [19,46].

Daily administration of TEL for 8 weeks in NAFL rats caused marked improvement in measured parameters affected by fructose feeding, emphasizing that TEL represents a promising therapeutic tool for the prevention and treatment of NAFLD, Figs. 1–3 and Table 1. TEL significantly lowered IKK, TNFα levels and down-regulated NFκB expression in the liver of NAFL rats Figs. 4 and 6. Our results are consistent with previous studies that reported the anti-inflammatory activity of TEL in various animals models [47,48] and humans [49]. The anti-inflammatory activity of TEL may be explained in part by blocking RAS which plays an important role in the induction, maintenance of inflammation and oxidative stress [50,51].

Recently, Ohkura et al., [10] suggested a connection between AT-II and the activation of sphingosine pathway; AT-II upregulated S1PR1 expression in the heart and caused activation of TGFβ and induction of cardiac fibrosis. Similar effects of AT-II on sphingosine pathway may exist in the liver. In this study, the block of AT-II receptors with TEL markedly decreased SphK1 activity, S1P, and NFκB levels and simultaneously down-regulated S1PR3 protein level and S1PR1

expression in the liver of NAFL rats, thus TEL inhibited sphingosine pathway activation, Figs. 5,6, and 8. Therefore, a possible mechanism of the hepatoprotective effects of TEL against fructose-induced NAFLD is through suppressing hepatic SphK1/S1P signaling pathway-induced NF-κB activation.

Shirari et al., [11] reported that AT-II induces an inflammatory process by TLR4 activation via the AT1 receptor. Similar results were demonstrated by Ji et al., [52] who reported that treatment with a neutralizing anti-TLR4 antibody, decreased the inflammatory processes induced by AT-II in rats. In the present study, TEL treatment of NAFL rats significantly decreased the hepatic expression of TLR4 and NFκB; indicating that TEL may inhibit liver inflammation and steatosis through the inhibition of TLR4-dependent NFκB activation which resulted in down-regulation of the transcription of inflammatory molecules, Figs. 4, 6 and 7.

Regarding CGA, the present study demonstrated its ability to combat the oxidative stress that was induced by fructose feeding as it could significantly increase the activity of hepatic GPx and SOD, Fig. 3. This finding is in agreement with other previous studies [53,54]. Additionally, treatment of NAFL rats with CGA caused a significant decrease in the hepatic concentration of TNFα and IKK with down-regulation of NFκB expression as compared to untreated NAFL rats, Fig. 4. These finding could be attributed to the anti-inflammatory effects of CGA [55], its ability to up-regulate PPARγ expression and to inhibit ROS-induced NFκB activation, and inhibition of RAS through ACE inhibition activity [56].

In the present study, CGA caused a marked decrease in SphK1 activity, S1P level with a concomitant decrease in S1PR1, and S1PR3 protein level in liver tissue, Figs. 5 and 8. This may give the clue that the hepatoprotective effects exerted by CGA are likely due to inhibition of SphK1/S1P-dependent NFκB signaling pathway. Our results are supported by A Belkaid, J-C Currie, J Desgagnés and B Annabi [57] who reported that CGA inhibited the cell migration induced by S1P in brain tumors.

Concomitantly, our results showed the reduction in hepatic TLR4 level and expression by CGA treatment; this effect plays a role in suppressing the inflammatory response and steatosis most likely through blocking the high fructose-induced TLR4/NFκB signaling pathway, Figs. 4, 6 and 7. This finding is consistent with previous studies with different animal models [58,59].

As expected, treatment of NAFL rats by a combination of TEL and CGA resulted in a significant better substantial improvement in the biochemical and metabolic derangements induced by high fructose feeding compared to treatment with either drug alone for some markers such as serum lipids and the hepatic concentrations of antioxidant enzymes, IKK and TNFα. Surprisingly, the liver histopathological examination of TEL + CGA-treated NAFL rats showed no fatty changes, inflammation or vacuolization. Therefore, CGA may be of great therapeutic value for the prevention and treatment of NAFLD as an adjuvant to TEL or other treatment modalities due to its anti-inflammatory, antioxidant and anti-steatosis effects.

In conclusion, the present study reveals the implication of SphK1/S1P and TLR4-signaling pathways in the pathogenesis of fructose-induced NAFLD in rats, and their modulation by antioxidants, and drugs affecting RAS. Obviously, the hepatoprotective effects of TEL or CGA rely; at least in part; on their antioxidant and anti-inflammatory effects which is likely mediated through the modulation of RAS, SphK1/S1P and TLR4-dependent NFκB activation and subsequently inhibition of TNF-α release. Further studies are needed to better understand the molecular mechanisms involved in the development and progression of NAFLD.

5. Bullet unit summary

1- What is already known:
 o Telmisartan and Chlorogenic acid are promising drugs for NAFLD,

approved in many models
- o Sphingosine pathway and TLR-4 receptors play a role in NAFLD pathogenesis
2- What is new:
- o Telmisartan and Chlorogenic Acid effects on S1P/SPK, and TLR-4 contribute to their efficacy in NAFLD
- o Telmisartan and Chlorogenic acid administered together provided greater liver protection compared to individual drugs
3- Clinical Implication
- o This work emphasis efficacy of Telmisartan and Chlorogenic Acid in NAFLD that may be helpful clinically
- o Combination of both drugs may be more effective clinically than each, may direct toward the clinical study

Conflict of interest

To the best of our knowledge, no conflict of interest, financial or others, exists. Authors are fully aware of this submission.

Acknowledgements

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