Original Paper

A Novel Role of SIRT1/ FGF-21 in Taurine Protection Against Cafeteria Diet-Induced Steatohepatitis in Rats

Azza H. Abd Elwahab^a Basma K. Ramadan^a Mona F. Schaalán^b
Amina M. Tolba^c

^aDepartment of Physiology, Faculty of Medicine for Girls (Cairo), Al-Azhar University, Cairo,

^bDepartment of Biochemistry Faculty of Pharmacy, Misr International University, Cairo, ^cDepartment of Anatomy, Faculty of Medicine for Girls (Cairo), Al-Azhar University, Cairo, Egypt

Key Words

Taurine • Cafeteria diet • Hepatokine • FGF-21 • SIRT1

Abstract

Background: Non-alcoholic fatty liver disease (NAFLD) is one of the alarmingly rising clinical problems in the 21st century with no effective drug treatment until now. Taurine is an essential amino acid in humans that proved efficacy as a non-pharmacological therapy in a plethora of diseases; however, its impact on NAFLD remains elusive. The aim of the current study is to evaluate the protective mechanism of taurine in experimental steatohepatitis induced by junk food given as cafeteria-diet (CAF-D) in male albino rats. **Methods:** Forty adult male albino rats of local strain between 8-10 weeks old, weighing 150 ± 20 g, were divided into four equal groups: Group I (control group), Group II (Taurine group), Group III (CAF-D for 12 weeks) and Group IV (CAF-D +Taurine). CAF-D was given in addition to the standard chow for 12 weeks, where each rat was given one piece of beef burger fried in 15 g of sunflower oil, one teaspoonful of mayonnaise, and one piece of petit pan bread, weighing 60g/ piece. In the serum, liver function tests; ALT, AST, ALP, GGT and the lipid profile; TG, TC, HDL-C added to reduced glutathione (GSH) were assessed colorimetrically, while fibroblast growth factor (FGF)-21, adiponectin & interleukin (IL)-6 via ELISA. The same technique was used for the assays of the hepatic levels of FGF-21, silent information regulator (SIRT1), malondialdehyde (MDA), IL-10, tumor necrosis factor-α (TNF-α) as well as the apoptotic markers; caspase-3 and B-cell lymphoma (Bcl-2). **Results:** The cafeteria-diet induced steatohepatitis was reflected by significantly increased body and liver weight gain, elevation of liver enzymes; ALT, AST, ALP and GGT added to the dyslipidemic panel, presented as increased TC, TG, LDL-C and decreased HDL-C levels. The steatosis-induced inflammatory milieu, marked by elevated serum levels of FGF-21, IL-6, hepatic TNF-α, as well as reduced IL-10 and adiponectin, was associated with steatosis- induced hepatic oxidative stress, reflected by increased hepatic MDA and decreased GSH levels, along with stimulated caspase-3 and decline in Bcl-2 hepatic levels. These pathological disturbances were significantly ameliorated by taurine supplementation and evidenced histopathologically. The cross talk between hepatic FGF-21 and SIRT1 and their association to the induced perturbations are novel findings in this study. Taurine's efficacy in

Dr. Mona F. Schaalán

Department of Biochemistry, Faculty of Pharmacy, Misr International University, Km 28, Cairo-Ismaïlia road, Cairo PO Box 1, Heliopolis, Cairo (Egypt)
Tel. 202/22400800; 20102011100, E-Mail Mona.Schaalan@mieu.egypt.edu.eg

restoration of hepatic structure and function is partially via the increase in SIRT1 and associated reduction of FGF-21. **Conclusion:** The findings of the current study prove the protective role of taurine in NAFLD via a novel role in the amelioration of FGF-21/ SIRT1 axis, which could be considered a new therapeutic target.

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Introduction

Obesity is a major health problem and its incidence is increasing at an alarming rate not only in adults, but also among children and adolescents. The rising prevalence of obesity is attributed to changes in dietary habits with increased consumption of palatable junk food [1]. A “cafeteria” diet is a self-selected high-fat diet, providing an excess of energy, which proved to induce obesity. Excessive dietary lipid intake hampers glucose utilization, hence eliciting insulin resistance and abnormal postprandial lipid biotransformation [2]. Obesity predisposes to a variety of liver pathologies known as nonalcoholic fatty liver disease (NAFLD). It is not a homogeneous disease entity; depending on the severity of the pathological process, but may present as an isolated fatty degeneration (simple hepatic steatosis), nonalcoholic steatohepatitis (NASH), or lead to liver cirrhosis and hepatocellular carcinoma (HCC) [3, 4].

The development of hepatosteatosis arises as a result of an imbalance between triglyceride deposition and removal. Epidemiologically, NAFLD is associated with type 2 diabetes, suggesting that hepatosteatosis and the development of insulin resistance are causally linked and play a central role in the metabolic syndrome [5]. Molecular mechanisms responsible for the accumulation of fat in the liver are not fully understood; however, certain cytokines derived from inflammation sites, particularly from extrahepatic adipose tissues, can trigger this process. More prominently, insulin resistance is emerging at a core phase for the immense metabolic dysregulations of NAFLD that instigate and exacerbate hepatic steatosis. Pathological features of NASH include simple hepatic steatosis and, more characteristically, liver cell damage and accompanying inflammation and/or fibrosis. Recently, a plethora of pathogenic mechanisms have been determined for the evolution from simple steatosis to NASH, such as lipotoxicity, oxidative stress-induced mitochondrial dysfunction, as well as endoplasmic reticulum stress [6]. The “two-hit” hypothesis has been proposed to explain NAFLD pathogenesis with steatosis representing the “first hit”, which increases the vulnerability of the liver to various “second hits” that in turn lead to inflammation, fibrosis and cellular death. Notably, oxidative stress is considered one such second hit, added to the inflammatory response, including the production of numerous proinflammatory molecules and adipokines; all together have key roles in the initiation and progression of the disease [7]. The current understanding of “the multiple parallel hypothesis” refers to NAFLD as a systemic, multifactorial disease involving multiple organs, such as adipose tissue, muscle and the intestine, as well as organelles such the endoplasmic reticulum and the mitochondria [8].

Fibroblast growth factor-21 (FGF-21) is a metabolic hormone with multiple metabolic functions. It belongs to the FGF superfamily that is involved in cell proliferation and differentiation, neural development, angiogenesis, and metabolism. Physiologically, FGF-21 is reported to be expressed from tissues involved in metabolism such as the liver, adipose tissues, skeletal muscle, and pancreas [9]. Secreted FGF-21 could regulate whole-body metabolism via its impact on the function of several tissues including brain and adipose tissues in an endocrine manner [10]. In white adipocytes, FGF-21 stimulates glucose uptake in an insulin-independent manner, modulates lipolysis and enhances mitochondrial oxidative capacity, and potentiates PPAR γ activity [11]. There is also good evidence that FGF-21 is involved in the thermogenic functions of brown adipocytes [12, 13]. Notably, starvation is reported to increase hepatic expression of FGF-21, which then acts centrally to increase hepatic gluconeogenesis and fatty acid oxidation [14]. However, in obesity, fatty liver and high fat diet-induced metabolic pathologies, FGF-21 is expected to show different metabolic actions.

Sirtuins type 1 (SIRT1), an important histone deacetylase, is a potential molecule and main determinant of whole body homeostasis in mammals by regulating many transcriptional regulators in metabolic tissues such as liver, skeletal muscle and adipose tissues [15]. SIRT1 and its activators play a key role in lipid and glucose homeostasis and insulin sensitivity via regulating mitochondrial biogenesis and β -oxidation, and improving anti-inflammatory activities. Previous studies indicated that the activation of SIRT1 in the liver protects against high-fat diet (HFD)-induced metabolic damage [15, 16], and that the regulation of hepatic lipid metabolism is postulated by SIRT1 deacetylation of sterol regulatory element-binding protein (SREBP)-1c, thus decreasing its expression [17, 18]. Despite the high prevalence of NAFLD, an approved standardized treatment is still lacking, contributing to a serious challenge to health care systems. Diet and lifestyle modification remain the major non-pharmacologic therapy of NAFLD for the purpose of weight loss [19]. Insulin receptor sensitizing agents and antioxidants have been tested for the treatment of NAFLD, though their clinical efficacy and safety remain to be established [20]. Thus, it is necessary to explore novel therapeutics with high efficacy and fewer side effects. Amino acids have been recognized as important signaling mediators in different cellular functions. Taurine is a sulfur-including amino acid that is present in mammalian tissues in minute levels. It is endogenously synthesized from methionine and cysteine metabolism and is also provided in diets comprising fish and shellfish. Taurine is involved in various physiological functions, including bile salt conjugation, calcium homeostasis, osmoregulation, membrane stabilization, added to its anti-oxidant and immunomodulatory effects [21]. Global epidemiological studies revealed that people who consume a lot of sea food, containing taurine in abundance, have a lower risk of developing metabolic diseases such as obesity, diabetes, dyslipidemia, and hypertension [22]. To this end, the present study aims to examine the potential protective effects of taurine supplementation in CAF-D induced steatohepatitis in adult male albino rats, moreover, to determine the involved mechanisms.

Material and Methods

The composition of commercial rat chow diet (balanced diet) included 23.5% protein, 48.8% carbohydrate, 5% lipid, 12% water, 5% ash, 5% cellulose, and a 0.7% mixture of vitamins and minerals. The diet was designed at the National Research Institute for Nutrition, Giza, Egypt, according to the National Nutrition Database for Standard Reference. The diet was purchased from EL Nasr Pharmaceutical Chem. Co., New Maadi, Cairo, Egypt. The cafeteria diet (CAF) is also known as "Junk food" diet model: "JF" diet is a well-established model to study obesity; it is a highly palatable hyperlipidemic diet that mimics Western diet, inducing voluntary hyperphagia and rapid weight gain in rodents [2, 23]. CAF diet was composed of beef burger, bread and mayonnaise (purchased from local commercial sources). Each piece of beef burger weighed 150g before cooking and looses between 15 and 20g after cooking, resulting in an average new weight of 130–135g/piece. Each 100g of uncooked beef burger was composed of 18% protein, 17.7% total fat, 6.12% total saturated fatty acid, 45% carbohydrates, 12.3% water, and 0.8% vitamin mixture, according to the nutritive values recorded on the package. As regards mayonnaise, each teaspoonful gives 110 calories and is composed of 12g fat, 2g saturated fat, 10g cholesterol, and 75mg sodium, according to the nutritive values recorded on the package [24].

Taurine (2-amino ethane sulfonic acid) was supplied by GALL PHARMA, Austria Pharmaceutical formed as taurine 500 mg GPH capsules.

Experimental animals and design

Forty adult male albino rats (8-10 weeks old, weighing 130-150 g) were purchased from the Nile Pharmaceuticals Company (Cairo, Egypt). They were housed in laboratory standard cages (2 rats/25X30X30 cm cage), under specific pathogen-free conditions in facilities maintained at controlled room temperature with a natural light/dark cycle. All animals had free access to chow diet and water ad libitum and were acclimated for one week prior to initiation of the experiment in the laboratory of Physiology, Faculty of Medicine, Al-Azhar University. All procedures were approved by the Animal Care Committee and the rules and principles of laboratory animal care were followed, as per the specific institutional guidelines.

The rats were divided into 4 equal groups (10 rats/group). Group I (Control): rats were maintained on the standard diet for 12 weeks and served as control group. Groups II (Control/Tau): rats treated orally with taurine, freshly prepared by dissolving 500 mg/kg/b.w/day in distilled water [25], administered by gastric gavage tube for 12 weeks. Groups III (CAF-D): rats fed a cafeteria diet in addition to the standard chow for 12 weeks. Each rat was given one piece of beef burger fried in 15 g of sunflower oil, one teaspoonful of mayonnaise, and one piece of petit pan bread, weighing 60g/ piece [26]. Group IV (CAF-D/Tau): rats were given taurine at a daily dose of 500mg/kg/day dissolved in water by means of a gastric tube in addition to the 'junk food' as the same as group III for 12 weeks. The animal diet was introduced daily at 10 a.m. and the left over was not removed to ensure complete consumption by all rats.

Estimation of body weight and liver weight. Animals were regularly weighed and at the end of experiment they were sacrificed and their livers were quickly removed and weighed.

Serum biochemical analysis. At the end of the experiment, after overnight fasting, rats were anesthetized in the morning, and blood samples were collected from retro-orbital venous plexus by capillary tubes under light pentobarbitone anesthesia. The blood was then centrifuged at 3000 rpm for 15 minutes for serum collection. Serum was separated in aliquots in Eppendorf tubes and stored frozen at -80°C until analysis.

Assessment of hepatic enzymes. Serum alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP) and gamma glutamyl transferase (GGT) were calorimetrically determined as described by Ochei and Kolhacker [27] using commercial kits obtained from` Diamond Diagnostic\$, Egypt.

Lipid profile. Serum total cholesterol (TC) and triglycerides (TG) were estimated as described by Sharma et al. [28]. Serum high density lipoprotein cholesterol (HDL-C) was measured according to the method described by Grillo et al. [29], while low density lipoprotein cholesterol (LDL-C) according to Friedewald et al. [30] as follows: $LDL-C = [TC] - [HDL-C] - [TG/5]$.

Glycemic Indices. Serum glucose level was assessed according to Tinder (1969) [31] using glucose enzymatic (GOD-PAP)-liquizyme Kits (Biotechnology, Egypt). Serum insulin was determined by a solid phase enzyme amplified sensitivity immunoassay according to Starr et al. [32] using KAP1251-INS-EASIA (Enzyme Amplified Sensitivity Immunoassay) Kits (BioSource Europe S.A., Belgium). Estimation the Homeostasis Model of Assessment of Insulin Resistance was performed according to Matthews et al. (1985), where $HOMA-IR = \text{fasting insulin } (\mu\text{U/ml}) \times \text{fasting blood glucose (mg/dl)} / 405$ [33].

Assessment of serum hepatokine and adipokine markers. Serum Fibroblast Growth Factor-21 (FGF-21), IL-6 and adiponectin concentration were measured by ELISA using a commercially available kits according to the manufacturer's instructions [34]. FGF-21 was purchased from MyBioSource, Inc., San Diego, California, USA and adiponectin from Assaypro, Saint Charles, Missouri , USA). Serum IL-6 level was measured by commercial ELISA kits (Ray Bio® Rat, Ray Biotech, Norcross, GA, USA).

Preparation of liver tissue homogenate

The livers of the sacrificed albino rats were excised and the liver samples were cut into small pieces (1 g or gm), minced and then homogenized in 1 ml lysis buffer [20 mM HEPES (pH 7.5), 150 mM NaCl, 1% NP-40, 0.1% SDS, 1 mM EDTA, and 1.0 mM DTT] added to protease inhibitors (2 µg each of aprotinin, leupeptin, pepstatin A, and 0.5 mM phenylmethylsulfonyl fluoride) then incubated on ice for 30 min, and centrifuged at 10,000 xg at 4°C for 20 min [35]. The resulting supernatant [cell lysate] was separated and stored at -70 °C until used for further analysis.

Assessment of liver oxidative stress profile. Malondialdehyde (MDA) level in the liver homogenate was assayed for products of lipid peroxidation by monitoring thiobarbituric acid reactive substance formation [36] using the colorimetric method. On the other hand, the method of GSH is based on the reduction of 5,5 dithiobis (2-nitrobenzoic acid) (DTNB) with reduced glutathione (GSH) to produce a yellow compound. The reduced chromogen is directly proportional to GSH concentration and its absorbance can be measured at 405 nm. Both oxidative stress indicators, MDA and GSH, were assayed using a commercial kit (Biodiagnostic, Egypt).

Assessment of inflammatory cytokines. Both interleukin-10 (IL-10) and tumor necrosis factor-alpha (TNF-α) in liver homogenate were detected by quantitative enzyme-linked immunosorbent assay (ELISA) using ELISA commercial kits (Quantikine, R&D Systems, Minneapolis, USA) according to the manufacturer's instructions and according to Smith (1994) for TNF-α [37], while IL-10 was according to Croft et al. (2012) [38].

Assessment of apoptosis in the liver. Bcl-2 protein content and Caspase-3 activity were measured in liver lysates by employing the quantitative sandwich enzyme immunoassay technique using ELISA kits supplied by Uscn Life Science Inc.(NYC,USA) and Quantikine (R&D Systems, Minneapolis, USA); respectively [39]. The procedure was performed according to instructions of manufacturer.

Hepatokine marker in liver homogenate. Hepatic Fibroblast Growth Factor-21 (FGF-21) and SIRT1 concentration was measured by ELISA using a commercially available kit (My Bio Source, Inc., California, USA) according to the manufacturer's instructions.

Histopathological Study

Samples of liver tissues from each animal were fixed in 10 % neutral buffered formalin for 24 h. Washing was done with tap water and then serial dilutions of ethyl alcohol were used for dehydration. Specimens were cleared in xylene and embedded in paraffin at 56°C in hot air oven for 24 h. Paraffin wax tissue blocks were prepared for sectioning at 4 µm thicknesses by sledge microtone. The obtained tissue sections were collected on glass slides, deparaffinized, and stained with hematoxylin and eosin stain and Masson trichromate stain. Prepared slides were examined under a light electric microscope by a pathologist unaware of the treatment protocol.

Statistical analysis

All results were expressed as means ± SD. Differences between groups were assessed by one-way analysis of variance (ANOVA), followed by a Bonferroni post hoc multiple comparison test. The association between the parameters was determined using the Pearson's correlation coefficient. A p value <0.05 was considered statistically significant. Statistical analysis was performed using the SPSS version 20.0 statistical software package (SPSS Inc., Chicago, IL).

Results

Since there was no difference between control group receiving taurine (Control/ Tau) and control group, only those for control are utilized in the current study for statistical comparison.

Effect of taurine on body and liver weight, serum lipid profile, liver function tests and glycemic indices

CAF-D induced a robust increase in body and liver weights (1.3, 2.25 fold; respectively); effects that were significantly ameliorated by taurine administration by 14% and 30.5%; respectively. Furthermore, the induced dyslipidemia was presented by a significant elevation of TG, TC, LDL-C (2.72-, 1.5-, 2.2-fold; respectively) and decrease in HDL-C by 37.7%. These effects were associated with elevation of liver function tests; ALT, AST, ALP and GGT (2.5, 2.3, 2, 2.1 fold; respectively) and perturbation of the glycemic indices; fasting glucose, insulin and HOMA-IR; by 1.3-, 1.6-, 2.4-fold; respectively. Notably, administration of taurine successfully ameliorated the aforementioned deleterious effects, as illustrated in Table1.

Table 1. Effect of taurine on physiological and serum laboratory indices in adult male albino rats with steatohepatitis. Values are presented as means ±SDM. (*) Value significantly different vs. control group or (#)from CAF-D group, at P<0.05

Groups/ Parameters	Control	Control/Tau	CAF-D	CAF-D +Tau
-Final body weight(g)	221.66± 4.7	219.0 ±4.1	295.16 ± 6.2 *	254.16 ± 6.2 **
- Liver weight (g)	4.05 ± 0.5	3.98 ± 0.3	9.0 ± 0.39 *	6.25 ±0.6 **
Serum Lipid Profile				
TG (mg/dl)	73.71 ± 5.3	73.61 ± 6.6	200.58 ± 13.5 *	135.76 ± 22**
TC (mg/dl)	90.32 ± 9	89.67 ± 7	131.79 ± 17.3*	107.98 ± 10.69 #
HDL-C(mg/dl)	46.94 ± 9.5	49.64 ± 6.5	29.21 ± 7.87*	43.26 ± 9.83#
LDL-C(mg/dl)	28.6 ± 6	27.61 ± 6.6	62.46 ± 16.86*	37.56 ± 11 *
Serum liver enzymes				
ALT (IU/L)	35.65 ± 7.11	35.68 ± 6.79	89.71 ±13.64*	43.92 ± 6.3*
AST(IU/L)	36.71 ± 5.87	36.1 ±4.77	83.75 ±8.56*	40.74 ± 6.54#
ALP (IU/L)	82.31 ± 2.27	81.5 ±4.36	165.57 ± 4.33*	101.55 ± 3.36**
GGT (IU/L)	19 ± 8.9	22.3± 3.8	61.3 ± 3.7 *	27.8 ± 3.8 **
Glycemic indices				
Fasting glucose(mg/dl)	80.68 ± 4.92	79.48 ±4.96	104.14 ± 4.81*	86.98 ±5.26#
Fasting insulin (µU/ml)	30.55 ± 4.8	28.84 ±3.9	50.34 ± 7.4*	36.88 ±5.54#
HOMA-IR	5.46 ± 0.32	4.99 ±0.78	12.97 ± 2.3*	7.91 ±1.51**
Serum Adiponectin (ng/ml)	59.58 ± 1.68	62.32 ± 4.97	30.8 ± 2.84*	52.09 ± 3.24**
Serum IL-6 (pg/ml)	2.2 ± 1.2	1.9 ± 1.1	8.4 ± 2.2*	4.3± 2.1**
Serum FGF-21 (pg/ml)	75.23 ± 2.7	73.6 ± 2.5	157.7 ± 6.4*	93.05 ± 3.63 **

Fig. 1. Effect of taurine on hepatic levels of SIRT1 (Fig.1A), FGF-21 (Fig.1B), TNF- α (Fig.1C), IL-10 (Fig. 1D), MDA (Fig. 1E), GSH (Fig. 1F), casapase-3 (Fig. 1G) and Bcl-2 (Fig. 1H) in different experimental groups. Data represent the means $\pm$ SDM, (*, #) compared with control, CAF-D, respectively, at $P < 0.05$, using one-way ANOVA followed by Bonferroni post hoc multiple comparison test.

Effect of taurine on CAF-D – induced NAFLD changes in adiponectin, IL-6, FGF-21 and SIRT1 levels

Rats fed CAF-D showed marked decrease in serum adiponectin (-48.3%) together with elevation of serum levels of IL-6 and FGF-21 (2 fold, 4 fold their control levels; respectively). Conversely, taurine administration concomitantly with CAF-D decreased both serum levels of FGF-21 and IL-6 (47%, 50%; respectively), while increased serum adiponectin by 1.7 fold; data illustrated in Table 1. Concerning the hepatic levels of SIRT1 (Fig.1A) and FGF-21 (Fig. 1B), they showed opposite patterns. While hepatic level of FGF-21, in the CAF-D group, showed a two-fold elevation of the control level, administration of taurine restored normal level via a 45.7% significant reduction. Conversely, hepatic SIRT1 was significantly decreased (50%) upon experimental induction of NAFLD model, and taurine significantly ameliorated this effect by 1.74 fold increase in its level.

Effect of taurine on CAF-D –induced hepatic inflammatory and oxidative stress markers

CAF-D for 12 weeks showed an evident disturbance in the hepatic pro-/ antiinflammatory equilibrium, by causing a prominent increase in levels of hepatic proinflammatory marker, TNF- α (2.9 fold; Fig. 1C) and decrease in anti-inflammatory cytokine, IL-10, by 53.8% (Fig.

Fig. 2. (A-G) Microscopic examination of hepatic tissues of the assessed groups shows the following results, (A) A photomicrograph from a liver of control rat showing normal hepatocyte with euchromic nuclei with no fibrosis around the central vein (blue arrow) and portal tracts (black arrow). X 200 H & E. (B) A photomicrograph from a liver of control rat showing normal distribution of the collagen fibers in the central vein (blue arrow) and the portal tracts (black arrows) X 100 masson-trichrome. (C) A photomicrograph from a liver of a rat in CAF-D group showing thick wall of hepatic artery, fatty degeneration (blue arrow), with inflammatory cell infiltration (black arrow) as lymphocytes and mononuclear cells with focal areas of degeneration, congestion and necrosis. (x 200 H & E). (D) A photomicrograph from a liver of a rat in CAF-D group showing excessive deposition of collagen fibers in the central vein and portal tracts X 100 masson trichrome. (E) A photomicrograph from a liver of taurine group (Control/Tau) showing the nearly normal architecture of hepatic lobule with no fibrosis around the portal tracts (x 200 H & E). (F) Liver cell of taurine pretreated CAF-D group showing congested vessels, hepatocytes with pyknotic hepatic tissue with moderate distribution of the nuclei (blue arrow) and some fat globules (black arrow) and cellular infiltrate. (x 100 H & E). (G) Liver of taurine pretreated CAF-D group showing some hepatocytes with pyknotic nuclei and moderate distribution of the collagen fibers in the central vein (blue arrow) and the portal tracts (black arrows) (X100 masson trichrome). (H) Schematic illustration of mechanisms of CAF-D induced NAFLD and the hypothesized ameliorative effect of taurine. Habitual consumption of CAF-D stimulates hepatic SIRT1, which in turn promotes expression, production and release of hepatic FGF-21. Induction of hepatic and circulating levels of FGF-21 stimulates lipogenesis, oxidative stress and inflammation. Activation of SIRT1-FGF-21 cascade may represent a liver-adipose tissue axis that contributes to hepatic steatosis and apoptosis leading to NAFLD. Taurine proved to inhibit SIRT1-FGF-21 axis and prevent the deleterious sequelae.

1D). These effects were associated with an increase in hepatic MDA level (2.8 times; Fig. 1E) and decrease in hepatic antioxidant marker, GSH (3.5%; Fig. 1F). On the other hand, animals given taurine showed a decline in TNF- α and MDA levels and increase in IL-10 and GSH levels.

Effect of taurine on CAF-D -induced NAFLD on hepatic apoptotic markers

CAF-D- induced NAFLD in rats produced significant apoptosis in hepatic tissue as manifested by increased caspase-3 (3.5 fold, Fig. 1G) and decreased Bcl-2 (50%; Fig. 1H), compared to their respective controls. On the other hand, taurine administration concomitantly with CAF-D decreased caspase-3 activity by 51%, as well increased Bcl-2 by 1.4 folds.

Histopathological effect of taurine on CAF-D - induced NAFLD changes

Microscopic examination of hepatic tissues of CAF-D supplemented group showed fatty degeneration with inflammatory cell infiltration of lymphocytes and mononuclear cells with focal areas of degeneration, congestion and necrosis. (Fig. 2C), and marked collagen fiber deposition around the central vein and portal tracts (Fig. 2D) compared to normal patterns (Fig. 2A and 2B). Taurine supplementation showed nearly normal hepatic architecture (Fig.

2E). Taurine supplementation for twelve weeks with CAF-D-intake resulted in ameliorative process of hepatic cells morphologies in the form of marked reduction of the amount of fat globules with decreased cellular infiltration (Fig. 2F). Also, the liver cells showed some hepatocytes with pyknotic nuclei and moderate distribution of the collagen fibers in the central vein and the portal tracts (Fig. 2G).

Correlation analysis

The correlation analysis, illustrated in Table 2, revealed significant association between serum vs. hepatic FGF-21 and the following markers; serum adiponectin ($r = -0.97, -0.95$; respectively), hepatic levels of TNF- α ($r = 0.97, 0.97$; respectively), IL-10 ($r = -0.97, -0.96$), GSH ($r = -0.97, -0.95$); MDA ($r = 0.92, 0.91$), Bcl2 ($r = -0.92, -0.88$) and caspase-3 ($r = 0.98, 0.99$), all respective to serum vs hepatic FGF-21, at $p < 0.05$. The correlation between hepatic SIRT1 and assessed parameters revealed negative association with HOMA-IR ($r = -0.86$), hepatic levels of FGF-21 ($r = -0.89$), TNF- α ($r = -0.87$), MDA ($r = -0.88$) and caspase-3 ($r = -0.88$); whereas positive correlation with serum adiponectin ($r = 0.88$) and hepatic levels of IL-10 ($r = 0.87$), GSH ($r = 0.87$); and Bcl2 ($r = 0.83$), all at $p < 0.05$.

Table 2. Correlational analysis between serum and hepatic FGF-21, h-SIRT1 and the assessed parameters

	s-FGF-21	h-FGF-21	h-SIRT1
Serum levels			
TG	0.92	0.89	-0.83
LDL-C	0.81	0.89	-0.69
AST	0.97	0.68	-0.88
ALT	0.93	0.93	-0.79
HOMA-IR	-0.90	0.90	-0.86
Adiponectin	-0.97	-0.95	0.88
IL-6	0.95	0.94	-0.86
Hepatic levels			
h-FGF-21	0.99	-----	-0.89
h-SIRT1	-0.90	-0.89	-----
TNF- α	0.97	0.97	-0.87
IL-10	-0.97	-0.96	0.87
MDA	0.92	0.91	-0.88
GSH	-0.97	-0.95	0.87
Bcl-2	-0.92	-0.88	0.83
Caspase-3	0.98	0.99	-0.88

Discussion

There is consensus that the pathogenesis of NAFLD is as a multi-hit process that includes lipotoxicity, oxidative challenge and endoplasmic reticulum (ER) stress [40]. In the current study, taurine administration inhibited the NAFLD multi-hit process, based on lipid profile optimization, improvement of glycemic indices, increased adiponectin level, mitigation of hepatic oxidative stress markers (through reduction of MDA), inflammation (IL-6&TNF- α), apoptosis (caspase-3), and enhanced hepatic GSH, IL-10 and Bcl-2 levels. The most important implication of the present study is the identification of novel cross-talk between nutrient sensor SIRT1 and FGF-21, in the current model of steatohepatitis, where SIRT1/ FGF-21 axis could represent a novel hepatocyte-derived endocrine signaling to potentially combat hepatic steatosis and obesity in humans. Several studies reported a positive association between SIRT1 and FGF-21 in starvation, feeding and obesity-related disorders, with the latter being the downstream regulator of the former. Interestingly, the assessment of hepatic levels of SIRT1 protein content, in the current CAF-D-induced NAFLD, revealed a marked decrease in its expression, with concomitant elevation in FGF-21; perturbations that were successfully normalized by taurine supplementation. The NAFLD-induced reduction of SIRT1 expression was previously evidenced in experimental and clinical studies. Deng et al. [41] reported a reduction of SIRT1 expression in rats with NAFLD induced by high-fat diets. In addition, Mariani et al. [42] observed that SIRT1 expression was significantly decreased in visceral adipose tissue of morbidly obese patients with severe steatosis, compared to patients with slight or moderate steatosis. Remarkably, the molecular mechanisms of the action of taurine in correction of NAFLD was reported in the study of Banks et al. [43], who explained that taurine increases the expression of SIRT1 in the liver which further stimulates the antioxidant capacity, by SOD induction and lowering the proinflammatory signals, TNF- α and interleukin 6 (IL-6). Moreover, taurine was reported to induce overexpression of SIRT1 in mice which provided protection against HFD induced hepatic steatosis through upregulation of fatty acid oxidation and downregulation of lipogenesis [15].

FGF-21, a hepatokine, has recently been shown to possess beneficial effects on lipid metabolism and hepatic steatosis [9]. In the present study, both serum and hepatic FGF-21

levels showed marked increase in CAF-D supplemented group when compared to normal control rats. Notably, Hondares et al. [12] reported that exogenous administration of FGF-21 increased browning of white adipose tissue, energy expenditure and glucose disposal. Additionally, FGF-21 regulates energy homeostasis in adipocytes through activation of AMPK and SIRT1, resulting in enhanced mitochondrial oxidative function. In alignment with our findings, several studies demonstrated that serum FGF-21 concentrations were significantly increased and associated with hepatic fat content especially in subjects with moderate hepatic steatosis [13, 44] and could, therefore, be used as useful circulating biomarker for predicting progression in NAFLD patients. This elevation of FGF-21 levels was consistent with the data of Wree et al. [45] who reported concomitant reduction of adiponectin. Moreover, they revealed that reduced adiponectin levels may establish proinflammatory changes, thus increasing vulnerability to lipotoxicity, exacerbating hepatocytes injury, factors that promote progression from simple steatosis to NASH and even advanced hepatic fibrosis. This is further confirmed by the current negative association between FGF-21 and adiponectin. Another explanation was reported by Yu et al. [46] that elevated serum FGF-21 is likely due to time-dependent expression of *FGF-21* mRNA in human hepatocytes, which is more related to unsaturated fatty acids but is opposite to the glucometabolic pattern of insulin and glucose.

Several clinical studies explained that the increased level of FGF-21, in patients with a cluster of obesity-related disorders including nonalcoholic fatty liver disease, is considered a compensatory, adaptive response to the induced proinflammatory milieu [47]. This is further evidenced by the significant positive correlation between FGF-21 and TNF- α in the current study. Taurine supplementation, in the current study, induced significant decrease in both serum and hepatic FGF-21. We speculate that the improvement in steatosis mediated by taurine is the result of the reduced FGF-21 levels. Furthermore, Terashima et al. [48] reported that taurine supplementation ameliorated oxidative stress and fibrosis which caused correction of the pathogenesis of NAFLD. Collectively, these results indicate that the secretion of FGF-21, in the context of hepatic damage, may serve as an endogenous, autoregulatory, hepatoprotective signaling pathway against pro-oxidative damage.

The novelty of the current study lies in the new relation between FGF-21 and SIRT1 in this experimental model of NAFLD and the mechanism of taurine in correcting this pathogenesis. A plethora of studies addressed the controversial relationship between FGF-21 and SIRT1, yet there no consensus about their causal relationship. A previous study of Li et al. reported that SIRT1 regulates FGF-21 expression in hepatocytes *in vitro* and *in vivo*; however, the relative contribution of FGF-21 to SIRT1's effects on overall hepatic metabolism in different hepatic disease states has not been investigated [49]. They characterized FGF-21 as a critical downstream regulator of SIRT1 that protects against hepatic steatosis, enhances expression of brown fat-like genes in white adipose tissue, and increases whole-body energy expenditure. This further supports the perception that SIRT1 induces gene and protein expression of FGF-21 in hepatocytes. The negative association between FGF-21 and SIRT1, added to CAF-D induced reduction in hepatic SIRT1 and concomitant elevation in hepatic and circulating FGF-21 levels are new findings in the current study. Similar findings, but in a heart failure model, were explained by Planavila et al. [50] who stated that SIRT1 activity can be modulated by the action of specific metabolic activators and mechanisms other than changes in its expression level. Thus, the up-regulation of FGF-21 in failing hearts may be mediated by post-transcriptional changes in SIRT1, although SIRT1 expression was induced in failing human hearts.

While most of the studies of NAFLD focus on SIRT1 and SIRT-3, the mitochondrial SIRT-4 deserves more consideration. Tarantino et al. [51] showed low circulating levels of SIRT4 in obese patients with NAFLD concomitant with its reduced mitochondrial expression to increase the fat oxidative capacity together with the hepatic and muscular mitochondrial functions. Interestingly, SIRT4 controls the metabolism of free fatty acids (FFA) reducing their high circulating levels but increasing ROS production as well.

At the metabolic levels, CAF-D for 12 weeks verified the reported pathogenic characteristics of NAFLD, including obesity, lipotoxicity, oxidative stress and apoptosis. The induced increase in mean final body and liver weights [24] was accompanied by overt dyslipidemia; both causing elevation of liver enzymes. Yang et al. [52] attributed this increase in the aforementioned weights first to the hyperphagia and consequently, high energy intake induced by adipocyte-derived leptin hormone secretion due to high fat diet intake. The second cause is the consumption of HFD contents of saturated fats, which are considered to be one of main contributors to overweight and obesity, because fat is digested into monoglycerides and fatty acids by lipase and the absorbed fat is hence accumulated in adipose tissue through excessive adipocyte differentiation.

Taurine with CAF-D supplementation for twelve weeks induced significant reduction in body and liver weights, ameliorated the dyslipidemia and the liver function tests. The reduction in body and liver weights was explained by Murakami et al. [21] who clarified that taurine conjugation with bile acid and the neutralization of hypochlorous acid produced by phagocytic cells in the body, generate taurine-conjugated bile acids and taurine chloramines (Tau-Cl), respectively. These products are also active and exhibit a variety of physiological activities, including anti-inflammatory and antioxidative effects, modulation of glucose, and lipid metabolism and reducing the body and liver weights. Moreover, the antiobesity effect of taurine was also explained by Tsuboyama-Kasaoka et al. [53], who clarified that dietary taurine prevents the high-fat diet-induced obesity with increased resting energy expenditure through its effect in decreasing the white fat and increasing the brown fat weight. Pina-Zentella et al. [54] reported that taurine enhances the rate of lipolysis in isolated adipocytes by stimulating protein kinase A (PKA) activity. The aforementioned events explain the logic sequelae of dyslipidemia in the current study, evidenced by marked elevation in the total cholesterol, triglycerides, LDL-C and reduction of serum HDL-C. NAFLD is reported to closely associate with risk factors such as dyslipidemia and increased hepatic contents [55, 56]. In this study, CAF-D caused significant elevation of serum ALT, AST, ALP and GGT indicating hepatic dysfunction, cellular leakage and loss of functional integrity of membrane hepatocytes with more specificity of ALT enzyme in liver injury assessment [57]. In addition, elevated serum levels of ALP and bilirubin give a clue of hepatobiliary injury, especially interruption of the flow of bile from the liver, i.e. cholestasis [58]. Taurine supplementation, in the present work, showed improvement of the induced dyslipidemia which was explained by the reported physiologic hypocholesterolemic and antiatherogenic effects in experimental animals [59]. Notably, Fukuda et al. [60] reported that the lowering effect of dietary taurine of serum cholesterol was partly attributable to a reduced secretion rate of cholesteryl ester by the liver, and that reductions in hepatic secretion rate and hepatic accumulation of cholesteryl ester were inversely related to increased ketone body production, an index of fatty acid oxidation. This pattern of changes suggested that dietary taurine influences the metabolic fate of fatty acids, either they undergo esterification or oxidation. Moreover, Lam et al. [61] stated that taurine increases the expression level of cholesterol 7 α -hydroxylase (CYP7A1) mRNA in HepG2 cells, which decreases the cholesterol pool in liver causing some secondary changes, as enhancing the hepatic LDL- receptor activity and its binding of LDL-C. Other mechanisms have been reported such as the inhibition of very low-density lipoprotein (VLDL) secretion from the liver; the suppression of hepatic acyl-CoA: cholesterol acyltransferase (ACAT) activity, and the inhibition of the absorption of bile acid from the intestinal tract [62]. Plasma high-density lipoprotein (HDL) is considered as antioxidant protein and involved in antiatherogenic activities. Plasma HDL-C can reduce the level of nonesterified fatty acid hydroperoxides and participates in the antioxidant property of HDL-C [63]. The efficacy of taurine to cause amelioration in the lipid and metabolic profiles was reflected by a further correction in the ALT and AST levels, as corroborated by our histopathologic findings of liver tissues.

Insulin resistance (IR) is the key factor in the pathogenesis of NAFLD and is deeply entangled with the progression of fatty liver to advanced stages of fibrosis and cirrhosis [64]. During the course of NAFLD, in our rats, we found that fasting blood glucose and insulin

levels were significantly higher in NAFLD, compared to control rats, associated with a higher degree of insulin resistance index (HOMA-IR). Moreover, Milnar et al. [64] and Stanhope & Havel [65] referred this IR to that visceral adiposity, induced by HFD, that causes direct delivery of portal blood flow from visceral fat to the liver releasing FFAs. Increased amounts of FFAs is reported to directly affect insulin signaling, diminish glucose uptake in muscle, and induce gluconeogenesis in the liver. Another explanation was reported by El Mesallamy et al. [66] that HFD intake activates serine kinases coupled with inhibition of tyrosine phosphorylation of the insulin receptor which could result in IR. Changes in redox balance can activate certain stress induced serine kinases which can, in turn, decrease the extent of tyrosine phosphorylation, and is consistent with the attenuation of insulin action. All these changes were successfully ameliorated by taurine supplementation.

Adiponectin is a protective adipokine that antagonizes hepatic lipid accumulation in addition to insulin-sensitizing, anti-inflammatory and antifibrotic effects. Decreased adiponectin levels play a pivotal role in the pathogenesis of NAFLD [67]. This information supports our results that CAF-D induced significant reduction of serum adiponectin, which was corrected by taurine supplementation [68, 69]. Kim et al. [70] stated that taurine significantly increased serum adiponectin expression by inhibiting STAT-3 signaling in IL-1 β -stimulated adipocytes, independent of MAPK signaling, suggesting that taurine plays a significant role in modulating the expression of adipokines under inflammatory conditions. Concomitant with reduced adiponectin in the current study, CAF-D induced an elevation of TNF- α with significant reduction of IL-10. Intriguingly, taurine supplementation effectively ameliorated TNF- α and IL-10 levels, compared with CAF-D group. As the oxidative stress is tightly linked to mediators of inflammation, both playing a vital role in the pathogenesis of NAFLD, the proinflammatory cytokines, TNF- α and IL-6, were secreted from various cells/tissues including adipose tissue. Various clinical studies have suggested that drugs that reduce the levels of inflammatory markers [71], and elevate serum adiponectin level may provide a better treatment strategy for NAFLD [72]. On the other hand, IL-10 is an important anti-inflammatory cytokine that is known to be an important factor in maintaining homeostasis of overall immune responses [73]. Su et al. [74] observed that administration of taurine in cardiac patients induces a potent anti-inflammatory response in the form of decrease in serum IL-6 and an increase in serum IL-10 with significant improvement in arrhythmias. Another study of Yang et al. highlighted the ability of taurine to reduce epidural fibrosis in rat models after laminectomy via downregulating early growth response protein 1 (EGR1), an enhancer of fibrosis, both *in vivo* and *in vitro* [75].

CAF-D supplementation for 12 weeks induced significant oxidative stress in the form of elevated MDA and reduced GSH [76, 77]. Nobili et al. [78] reported that oxidative stress is one of the main causes of NAFLD, which is further exacerbated with steatohepatitis and represented with elevated malondialdehyde (MDA) and reduced glutathione (GSH), the biomarkers of oxidative stress. Taurine is known to improve cellular antioxidant defense system, stabilize biomembranes and reduce *in vivo* lipid peroxidation (LPO), thus preventing apoptosis and necrotic cell death [79]. Parvez et al. [80] attributed the anti-oxidant property of taurine to the inhibitory effect of mitochondrial superoxide generation, thus suggesting that the anti-oxidant properties of taurine are mediated by mitochondrial pathways. The molecular mechanisms involved in this process have been evaluated in *in vitro* studies of Jong et al. [81] by depleting mitochondrial taurine levels. The depletion of taurine in mitochondria causes a decline in the biosynthesis of mitochondrial-encoded proteins ND5 and ND6, which leads to the impairment of complex I and III activities and results in the excessive production of superoxide. Therefore, taurine functions as an indirect anti-oxidant which may help to prevent NAFLD.

The performed experimental NAFLD induced activation of caspase-3 which indicates an increase in the apoptotic activity in the liver tissue. Bcl-2 is a prosurvival multidomain protein that regulates apoptosis by preventing the release of proapoptogenic factors from the mitochondria (e.g. cytochrome c) and subsequent caspase activation [82]. In our study, the anti-apoptotic protein Bcl-2 level was significantly reduced with CAF-D intake; this dysregulation

of the fine-tuned apoptotic pathway is considered one of the mechanisms of CAF-D induced injury of the liver which might also explain the elevated liver enzymes. Moreover, Tarantino et al. reinforced our results, in his clinical study, that showed significantly lower serum Bcl-2 concentrations in nonalcoholic steatohepatitis (NASH) subjects, compared to fatty liver patients. Insulin resistance and GGT activity were also considerably higher in NASH subjects, concomitant with significantly more numerous apoptotic hepatocytes [83]. Several *in vitro* studies demonstrated that ER stress is the mediator of saturated FFA-induced apoptosis. As intracellular FFA are trafficked to and esterified within the endoplasmic reticulum (ER), it is not surprising, that the inundation of the liver with FFA (termed lipoapoptosis) disturbs ER function resulting in an ER stress response through depleting ER-calcium stores in liver cells [84]. JNK activation can be linked to dysregulation of this core cell death machinery, promoting cell death, as it induces mitochondrial dysfunction and apoptosis via BCL-2-associated Xprotein (Bax). Apoptotic signaling is activated when Bax migrates to the mitochondrial surface, promoting cyt-c and apoptosis inducing factor (AIF) release from the mitochondria to the cytosol, cleaving procaspase-9 to caspase-9, and activating caspase-3 to promote cell death [85]. Additionally, Guha et al. [86] demonstrated that apoptotic stimuli such as GSH depletion promote the translocation of mitochondrial Bax, which is a pro-apoptotic Bcl-2 family of proteins that decreases the mitochondrial membrane potential (MMP) and promotes the generation of ROS.

The protective role of taurine is highlighted in the adjustment of caspase-3 activities and Bcl-2 level towards the control values in order to properly control the proliferation and differentiation of liver cells. This anti-apoptotic effect of taurine is in consistence with Watanabe et al. [87] who documented an anti-apoptotic effect of oyster extract that is rich in taurine in NASH-model mice, and Gentile et al. [88] who found that taurine significantly mitigated palmitate-mediated caspase-3 activity, cell death, ER stress, and oxidative stress in H4IIE liver cells and primary hepatocytes.

Conclusion

Taurine administration inhibited the NAFLD multi-hit process, based on reduced lipotoxicity of CAF-D by lipid profile optimization, improvement of glycemic indices, increased adiponectin level, mitigating hepatic markers of oxidative stress through reduction of MDA, inflammatory marker (TNF- α) and apoptosis (caspase-3) as well as enhanced hepatic GSH, IL-10 and Bcl-2 levels, compared with CAF-D group. Intriguingly, the negative association between hepatic FGF-21 and SIRT1 in the NAFLD model is a novel finding in this study. This SIRT1/FGF-21 axis represents a novel hepatocyte-derived endocrine signaling to potentially combat hepatic steatosis and obesity in humans. Taurine's efficacy in restoration of hepatic structure [which was evidenced histologically] and function is partially due to its reduction of FGF-21 via increase in SIRT1 hepatic level. These findings indicate that taurine has antioxidant and anti-apoptotic effects on NAFLD model cells and may thus exert protective effects against NAFLD. Additionally, taurine has a number of advantages as a potential protection for NAFLD in that it is inexpensive, nontoxic, and can be administered orally. Our study supports the rationale for targeting FGF-21/ SIRT1 axis as a novel complementary approach to treat hepatic steatosis.

Limitation of the study

As FGF-21 has potential pleiotropic actions in hepatocytes and adipocytes, the authors acknowledge the inability to assess the levels of SIRT1 and FGF-21 in the adipocytes and relate them to the assessed hepatic levels. Moreover, the autocrine/paracrine actions of FGF-21 could be assessed in white adipose tissue and in brown adipocytes as well to delineate its thermogenic activity. Considering the reported strong involvement of the mitochondria in the pathogenesis of NAFLD, the authors would like to acknowledge the inability to isolate the mitochondrial hepatocytes to determine SIRT-4 expression, which is another important sirtuin in the pathogenesis of NAFLD.

Disclosure Statement

The authors declare having no funding sources for this research and no conflict of interest.

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