Archives of Physiology and Biochemistry
The Journal of Metabolic Diseases

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/iarp20

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To cite this article: Mona M. Allam, Reham M. Ibrahim, Walaa Bayoumie El Gazzar & Mona A. Said (2021): Dipeptedyl peptidase-4 (DPP-4) inhibitor downregulates HMGB1/TLR4/NF-κB signaling pathway in a diabetic rat model of non-alcoholic fatty liver disease, Archives of Physiology and Biochemistry, DOI: 10.1080/13813455.2021.1975758

To link to this article: https://doi.org/10.1080/13813455.2021.1975758

Published online: 20 Sep 2021.

ARCHIVES OF PHYSIOLOGY AND BIOCHEMISTRY
https://doi.org/10.1080/13813455.2021.1975758

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ORIGINAL ARTICLE

Dipeptedyl peptidase-4 (DPP-4) inhibitor downregulates HMGB1/TLR4/NF-κB signaling pathway in a diabetic rat model of non-alcoholic fatty liver disease

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ABSTRACT

Context: Inflammatory and immune pathways play a crucial role in the pathophysiology of non-alcoholic fatty liver disease (NAFLD). Sitagliptin blocks the dipeptidyl peptidase-4 (DPP-4) enzyme, mechanisms that alter inflammatory pathways and the innate immune system, and by which Sitagliptin affects the pathogenesis of NAFLD weren't previously discussed.

Objective: This study aims to understand the interaction between Sitagliptin and innate immune response in order to meliorate NAFLD.

Methods: Thirty- two Wistar male albino rats were categorised into four groups. Rats have received a standard diet or a high-fat diet either with or without Sitagliptin. Serum HMGB1, protein and mRNA expressions of hepatic TLR4 and NF-κB, inflammatory cytokines, and histopathological changes were analysed.

Results: An ameliorative action of Sitagliptin in NAFLD was demonstrated via decreasing HMGB1-mediated TLR4/NF-κB signalling in order to suppress inflammation and reduce insulin resistance.

Conclusion: Sitagliptin may in fact prove to be a beneficial therapeutic intervention in NAFLD.

ARTICLE HISTORY
Received 22 June 2021
Accepted 27 August 2021
Published online 20 September 2021

KEYWORDS
Steatohepatitis; toll-like receptor 4; sitagliptin; high-mobility group box 1 protein; nuclear factor-κB

Introduction

Long-standing obesity has been known to be the main contributor to the development and pathogenesis of non-alcoholic fatty liver disease (NAFLD), which is by far the most prevalent type of liver disease worldwide, thus making it a global health concern (Mitra *et al*. 2020). NAFLD includes a wide spectrum of chronic liver disorders with serious complications affecting the liver including hepatic fibrosis and hepatic cirrhosis (Takeuchi *et al*. 2014). Non-alcoholic steatohepatitis (NASH) is considered an important turning point along the NALFD course. Several pathways have been implicated recently in NASH onset and progression including the immune and the inflammatory pathways (Han *et al*. 2016).

Damage-associated molecular patterns (DAMPs) as well as their "pattern recognition receptors", are important components of the inflammatory pathways mediated by the innate immune system which are demonstrated under different pathological states including NAFLD (Mihm 2018). A specific nuclear protein known as the High-mobility group box 1 protein (HMGB1), has the ability to translocate between the nucleus and the cytoplasm to be subsequently released into the extracellular matrix in response to cellular stress, damage, and death, which functions as a DAMP (Lu *et al*. 2014). Toll-like receptor 4 (TLR4), a pattern recognition receptor, is one of the receptors to which HMGB1 has the capability of binding.

Accumulating data has shown that TLR4 is involved with NAFLD pathogenesis. Upon activation, TLR4 collaborates with its downstream molecules in order to stimulate nuclear factor-κB (NF-κB) transcription factor, which enhances the development of pro-inflammatory cytokines which drive the inflammatory response (Miura *et al*. 2010). Thus, the depression of TLR4 ligands, stimulation of the TLR4/NF-κB signalling pathways, as well as the downstream inflammatory effectors may be considered an innovative method to ameliorate NAFLD.

A specific protease which plays a role in the inhibition of incretin hormones like glucagon-like peptide 1 (GLP-1) is Dipeptidyl peptidase-4 (DPP-4) (Itou *et al*. 2013), inhibitors of which are used to treat hyperglycaemia (McIntosh *et al*. 2005) by increasing the levels of GLP-1 levels as well as deactivating the release of glucagon, which subsequently suppresses insulin secretion. Also, they were reported to ameliorate liver enzymes and hepatocyte ballooning in NASH patients with T2DM (Itou *et al*. 2013). Moreover, a clinical study showed that patients with NAFLD have higher serum DPP-4 activity, which correlated positively with the grade and degree of hepatosteatosis (Balaban *et al*. 2007). The DPP-4 inhibitor, Sitagliptin, is a widely available and used medication for the treatment of T2DM, which has been evaluated for its use in diabetic patients presenting with symptoms of NAFLD (Ideta *et al*. 2015).

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However, mechanisms of action of such a DPP-4 inhibitor in diabetic individuals with NALFD or animal models are poorly described, so further investigations are required to clarify its underlying mechanisms of action. In order to evaluate the effects of Sitagliptin on NAFLD progression, it was necessary to establish an animal model of NAFLD, which was done by providing the rats a high-fat diet (HFD). Furthermore, the involvement of the HMGB1/TLR4/NF-κB signalling pathway was investigated as a novel mechanism for this effect in such a model if found.

Methods

This study was conducted in the Physiology Department of the Faculty of Medicine, Benha University. The study protocol was approved by the Local Ethical Committee, Benha Faculty of Medicine.

Animals

Thirty-two adult Wistar male albino rats obtained from the Faculty of Agriculture, Moshtohor, Egypt, were included in the study. These rats were ages 6–8 weeks and weighing approximately 180 to 200 grams. Animals were categorised into 4 groups of 8, and each group of rats was placed in separate cages. The cages were maintained at a temperature of 25 °C with 12 h light and dark cycles, and all animals had access to food, water, and libitum. One week before conducting any experimentation, all rats were acclimatised to the laboratory setting. After finalisation of the study and sample collection, animals were euthanized and disposed of using an incinerator.

Study design

The animals were categorised into four groups of eight as follows;

Group I (control)**:** animals were given a standard diet for twenty weeks which consisted of 10, 20, and 70 kcal % of fats, protein, and carbohydrates respectively. Using a gavage, rats were given distilled water orally in the final 8 weeks of the study (Shen *et al*. 2018).

Group II (sitagliptin): This group received the same standard diet as the control group for a duration of twelve weeks then received 100 mg/kg/day of sitagliptin orally using a gavage + standard diet for an additional eight weeks (Shen *et al*. 2018).

Group III (HFD): rats received HFD for twenty weeks (45 kcal % fat, 20 kcal % protein, and 35 kcal % carbohydrate) (Shen *et al*. 2018).

Group IV (HFD+sitagliptin): rats initially received HFD for a duration of twelve weeks then received 100 mg/kg/day of sitagliptin orally using a gavage + HFD for an additional eight weeks (Shen *et al*. 2018).

The animals fasted overnight prior to the day of sample collection (after the twelve weeks of observation). Initially, rats underwent anaesthesia using urethane (1.5 g/kg; i.p.), subsequently, samples were collected using the following method:

- Initially, body weight was checked and recorded before animals were sacrificed by decapitation
- The epididymal fat, an indicator of visceral adipose tissue was removed and weighed. It has been used
- The collection of blood samples was via the abdominal aorta.
- After exposure to the abdominal cavity, the whole liver was excised from the animal and isolated. The liver was washed multiple times using 0.9% sterile ice-cooled saline to remove excess blood. Excess saline was absorbed using 2 filter papers pressed around the organ. The livers of each rat were weighed and the liver index was calculated (liver weight/body weight × 100%).

Diet and chemicals

Both the standard and the high-fat diet were prepared as pellets by the faculty of Agriculture, Moshtohor, Egypt. Sitagliptin was purchased by Merck Sharp & Dohme (Italy) a subsidiary of Merck & Co., Inc. (MSD Company) the powder was dissolved in distilled water and given to rats by means of oral gavage in a total volume of 1 ml for each rat.

Biochemical analysis

Samples of blood were centrifuged at 3000 × *g*, 4 °C for 10 min. Samples were left to stand for a total duration of 30 min at room temperature. Sera were collected and stored at −20 °C till further assessment of the following parameters based on the guidelines of the manufacturer;

- Measurement of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activity levels by Colorimetric Activity Assay kits (Biodiagnostic, Cairo, Egypt).
- Measurement of serum glucose using the Colorimetric Assay kits (Spinreact, Spain).
- Rat insulin enzyme-linked immunosorbent assay (ELISA) kits (RayBiotech, USA) were used in order to estimate the serum insulin.
- Serum total cholesterol (TC) levels were measured using Rat total Cholesterol colorimetric assay ELISA kits (Cell Biolabs, San Diego, USA).
- Serum triglycerides (TG) levels were measured using rat triglycerides ELISA colorimetric assay kit (MyBioSource, San Diego, CA, USA).
- Serum high-mobility group box 1 protein (HMGB1), was measured using rat HMGB1 ELISA assay kits (Bioassay Technology Laboratory, Shanghai, China).
- The liver was divided into 5 parts, first, part was weighed and homogenized and used to measure the pro-inflammatory cytokines; tumour necrosis factor-alpha (TNFα) and interleukin 1β (IL-1β) levels using rat ELISA colorimetric assay kit (RayBiotech, Inc., USA), the second part was

weighed and homogenised and used to measure the triglycerides (TG) contents were measured using triglycerides ELISA colorimetric assay kit (MyBioSource, San Diego, CA, USA).

- Using the homeostasis model assessment insulin resistance ($HOMA_{IR}$) index, the insulin resistance was calculated (fasting insulin (U/l) × fasting glucose (mg/dl)/405) (Matthews *et al*. 1985).
- DPP-4 enzymatic activity was measured by the conversion of glycin-prolin-p-nitroanilide (Sigma, USA) to p-nitroanilide. Production of p-nitroanilide was measured by the absorbance at 405 nm. The results are expressed as nmol/min/ml (Baumeier *et al*. 2017).

Quantitative real-time PCR analysis of TLR4 and NF-κB gene expression

Twenty-five milligrams of liver (third part) tissue (that was previously stored in an RNA later solution – (RNA stabilising reagent) (Qiagen Inc., Valencia, CA)) was taken from each of the liver biopsies and homogenised using the Mixer Mill MM400 (Retsch, Germany). From the liver homogenates, RNA was extracted using the RNeasy Mini Kit (Qiagen, Germany) based on manufacturer guidelines.

Using a NanoDrop One spectrophotometer (Thermo Fisher Scientific, USA) at the absorbance of 260 and 280 nm, the purity and concentration of the RNA were evaluated. The A260/A280 ratio of pure RNA is 1.8–2.1. Using the Maxima First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA) and a T100 Thermal Cycler (BioRad, USA), 1 μg of RNA was reverse transcribed.

Maxima SYBR Green/ROX qPCR Master Mix kit (Thermo Fisher, USA) was required to perform the quantitative Real-Time PCR via the Step One Plus Real-Time PCR system (Life Technologies, USA). Primers sequences for TLR4, NF-κB and GAPDH (housekeeping gene) are shown in Table 1. The relative expression of target genes was obtained using the $2^{-\Delta\Delta CT}$ formula as described by (Livak and Schmittgen 2001) using GAPDH as a housekeeping gene.

Western blot analysis

The expression of TLR4 and NF-κB proteins in the 4th part of liver tissue was determined using the Western blot procedure. The procedure was as follows;

1. In order to evaluate the protein concentrate (each cell lysate) using the Bradford Assay, five milligrams of liver tissue were homogenised in a RIPA buffer.
2. Using an SDS loading buffer, 25 μg of protein (equal quantities) were boiled, then cooled on ice, and finally loaded in SDS-polyacrylamide gel.
3. After separation by the Cleaver electrophoresis unit (Cleaver, UK), using a Semi-dry Electro-blotter (Biorad, USA), the material was transferred on polyvinylidene fluoride (PVDF) membranes (BioRad).
4. The PVDF membrane was blocked using the Tris-buffered saline-Tween-20 (TBS-T) plus dry milk (5% non-fat). The membrane was left to incubate overnight with the primary antibodies for TLR-4 (Invitrogen, USA), NF-κB (Abcam, United Kingdom) and β-actin (Sigma-Aldrich, USA) at indicated dilution.
5. With the use of TBS-t, the blots were washed a total of three times. Subsequently, the membrane was incubated again with horseradish peroxidase-linked secondary antibodies (Dako, Denmark), and then washed again, also using TBS-T.
6. Chemiluminescent Western ECL substrate (Perkin Elmer, Waltham, MA) was added to the blot and using a Chemi Doc imager (Biorad, Hercules, CA, USA), the signals were captured to be finally analysed by the Bio-Rad Image Lab software with β actin normalisation.

Histopathological examination

For histopathological examination of the liver (5th part), the specimen needed to be preserved using a 10% buffered formalin solution (pH 7.8). Thin sections (4 μm) were cut from the preserved sample and stained using haematoxylin-eosin (HE). The steatosis degrees of individual liver samples were examined and scored, according to the percentage of hepatocytes containing lipid droplets (Uno *et al*. 2008) by a pathologist in a blinded manner.

Immunohistochemistry staining

Immunohistochemical staining for HMGB1 and CD68 expression in liver tissue was performed using a technique that was described previously (Bai *et al*. 2016). A rabbit anti-HMGB1 monoclonal antibody (1:200; Abcam, UK) was used as the primary antibody for detection of HMGB1, Anti-CD68 antibody (1:200; Abcam, UK) was used as the primary antibody for detection of CD68. A peroxidase-linked secondary antibody and diaminobenzidine (DAB) (Sungene Biotech Co., Ltd., Tianjin, China) were used to detect specific immunostaining.

Light microscopy examination was conducted in the Department of Pathology, Faculty of Medicine, Benha University.

Statistical analysis

All the data are presented as mean ± standard deviation (SD) ranges, numbers, and ratios. Evaluation of differences between groups was performed using one-way ANOVA with *post hoc* test (LSD) between groups using the Software, Statistical Package for Social Science, (SPSS Inc. Released 2009-PASW Statistics for Windows Version 19 Chicago: SPSS Inc.) for Windows statistical package. A value $p<.05$ was considered statistically significant.

Results

Sitagliptin suppressed HFD-induced body weight (BW) gain and increased visceral adiposity

Any changes in body weight (BW) of the rats (all groups) during the study (beginning and end of study) were

measured and recorded. A significant increase in BW was found among the group of rats which received an HFD for 20 weeks in comparison to the control. The group which received Sitagliptin (8 weeks) in HFD + Sitagliptin demonstrated a significant reduction of the mean BW.

In comparison to the control, the groups of rats which received an HFD demonstrated an eminent rise in visceral adiposity as determined by their liver index and weight of epididymal fat. Sitagliptin for 8 weeks in HFD + Sitagliptin group had a significant decrease in terms of epidydimal fat weight and liver index (Table 2).

Sitagliptin improved insulin resistance, serum lipid profile, hepatic triglycerides content, induced liver injury, and increased levels of serum HMGB1 and DPP-4 activity in HFD-fed rats

Regarding insulin resistance, the rats which were given an HFD for 20 weeks had a significant rise in fasting blood glucose, serum insulin levels, and $HOMA_{IR}$ compared to the control group. However, administration of Sitagliptin for 8 weeks in the HFD + Sitagliptin group resulted in a significant reduction of all these parameters compared to the HFD group (Table 3).

HFD fed rats for 20 weeks showed significantly higher levels of total serum cholesterol and triglycerides (Table 3), as well as a significant rise in hepatic triglycerides content. The Sitagliptin for 8 weeks in HFD + Sitagliptin group showed a significant reduction of total serum cholesterol, triglycerides, as well as hepatic triglycerides content.

Concentrations of serum AST and ALT were evaluated in order to determine whether Sitagliptin treatment for 8 weeks in HFD + Sitagliptin group could attenuate HFD associated hepatic damage. In comparison to the rats which received the standard diet, the rats which received an HFD had significantly elevated concentrations of serum AST and ALT. The administration of Sitagliptin significantly lowered the serum levels of AST and ALT (Sitagliptin group) (Table 3).

Higher levels of serum HMGB1 were demonstrated in the rats that were given the HFD (Table 4). Nevertheless, Sitagliptin treatment for 8 weeks in HFD + Sitagliptin group lowered serum HMGB1 levels in HFD-fed rats significantly compared to HFD group.

Serum DPP-4 activity showed higher levels in rats that received HFD, however, Sitagliptin treatment for 8 weeks in HFD + Sitagliptin group lowered serum DPP-4 activity levels in HFD-fed rats significantly compared to the HFD group (Table 4).

Sitagliptin reduced hepatic pro-inflammatory cytokines; tumour necrosis factor-α (TNF-α) and interlukin-1β (IL-1β) levels in HFD-fed rats

Hepatic TNF-α and IL-1β were much greater in the HFD fed group than in the control Table 4). Sitagliptin administration for 8 weeks in HFD + Sitagliptin group significantly reduced hepatic TNF-α and IL-1β levels induced by HFD.

Table 1. Sequences of real-time PCR primers.

Primer name	Sequence	Accession number
TLR4	Forward 5′-CCG CTC TGG CAT CAT CTT CA-3′ Reverse 5′-CCC ACT CGA GGT AGG TGT TTC TG-3′	NM_021297.3
NF-κB	Forward 5′-TAC CCT CAG AGG CCA GAA GA-3′ Reverse 5′-TCC TCT CTG TTT CGG TTG CT-3′	NM_008689.2
GAPDH	Forward 5′-AGC TGA ACG GGA AGC TCA CT-3′ Reverse 5′-CAT TGA GAG CAA TGC CAG CC-3′	NM_001289726.

Table 2. Bodyweight, epididymal fat weight and liver index in different experimental groups.

	Control	Sitagliptin	HFD	HFD + Sitagliptin
Initial bodyweight (g)	190.38 ± 6.28	190.05 ± 5.91	191.82 ± 5.14	190.89 ± 5.28
Final body weight (g)	315.98 ± 9.81	318.15 ± 4.49	436.42 ± 3.80*	375.97 ± 10.86*#
Epididymal fat weight (g)	10.64 ± 0.82	10.12 ± 0.81	15.59 ± 0.56*	12.30 ± 0.63*#
Liver index (%)	2.84 ± 0.13	2.82 ± 0.20	4.15 ± 0.09*	3.05 ± 0.24*#

HFD: high-fat diet. Data are expressed as mean ± standard deviation ($n = 8$). $p < .05$ is significantly tested by using one-way analysis of variance (ANOVA) and *post hoc* multiple comparisons (LSD). *$p < .05$ vs. control group; #$p < .05$ vs. HFD group.

Table 3. Non-alcoholic fatty liver disease (NAFLD) related metabolic parameters of different experimental groups.

	Control	Sitagliptin	HFD	HFD + Sitagliptin
Glucose (mg/dl)	77.12 ± 2.54	76.91 ± 2.94	265.05 ± 5.52*	81.13 ± 2.11*#
Insulin (μIU/ml)	23.04 ± 1.74	22.10 ± 2.04	42.00 ± 2.56*	26.26 ± 1.55*#
$HOMA_{IR}$	4.38 ± 0.41	4.19 ± 0.36	27.49 ± 1.97*	5.26 ± 0.35#
TC (mg/dl)	81.50 ± 2.80	80.45 ± 2.23	279.81 ± 3.23*	198.92 ± 3.52*#
TG (mg/dl)	77.23 ± 2.89	76.72 ± 2.80	150.28 ± 2.13*	103.67 ± 2.11*#
ALT (U/L)	29.56 ± 1.34	30.23 ± 2.10	141.41 ± 4.88*	96.58 ± 3.05*#
AST (U/L)	25.78 ± 1.83	27.54 ± 1.32	165.86 ± 6.77*	84.77 ± 2.76*#
Hepatic TG (mg/gm protein)	9.79 ± 1.32	10.52 ± 1.43	19.51 ± 1.77*	12.56 ± 1.41*#

HFD: high-fat diet; $HOMA_{IR}$: homeostasis model assessment of insulin resistance; ALT: alanine aminotransferase; AST: aspartate aminotransferase; TC: total cholesterol; TG: triglycerides HMGB1; High-mobility group box 1 protein. Data are expressed as mean ± standard deviation ($n = 8$). $p < .05$ is significantly tested by using one-way analysis of variance (ANOVA) and *post hoc* multiple comparisons (LSD). *$p < .05$ vs. control group; #$p < .05$ vs. HFD group.

Table 4. Serum HMGB1, serum DPP-4 activity and proinflammatory cytokines in liver tissue in different experimental groups.

	Control	Sitagliptin	HFD	HFD + Sitagliptin
HMGB1 (ng/ml)	6.11 ± 0.82	5.96 ± 1.18	14.10 ± 1.70*	9.27 ± 1.18*#
DPP-4 activity (nmol/min/ml)	44.88 ± 1.91	43.73 ± 2.50	94.54 ± 3.45*	63.73 ± 2.69*#
Hepatic TNF-α (Pg/mg protein)	43.72 ± 2.20	44.71 ± 2.49	62.86 ± 4.60*	48.58 ± 1.22*#
Hepatic IL-1β (Pg/mg protein)	68.24 ± 2.87	69.56 ± 2.56	103.17 ± 4.20*	73.71 ± 2.23*#

HFD: high-fat diet; TG: triglycerides; TNF-α Tumour necrosis factor-alpha; IL-1β: Interleukin 1beta. Data are expressed as mean-±standard deviation ($n = 8$). $p < .05$ is significantly tested by using one-way analysis of variance (ANOVA) and *post hoc* multiple comparisons (LSD). *$p < .05$ vs. control group; #$p < .05$ vs. HFD group.

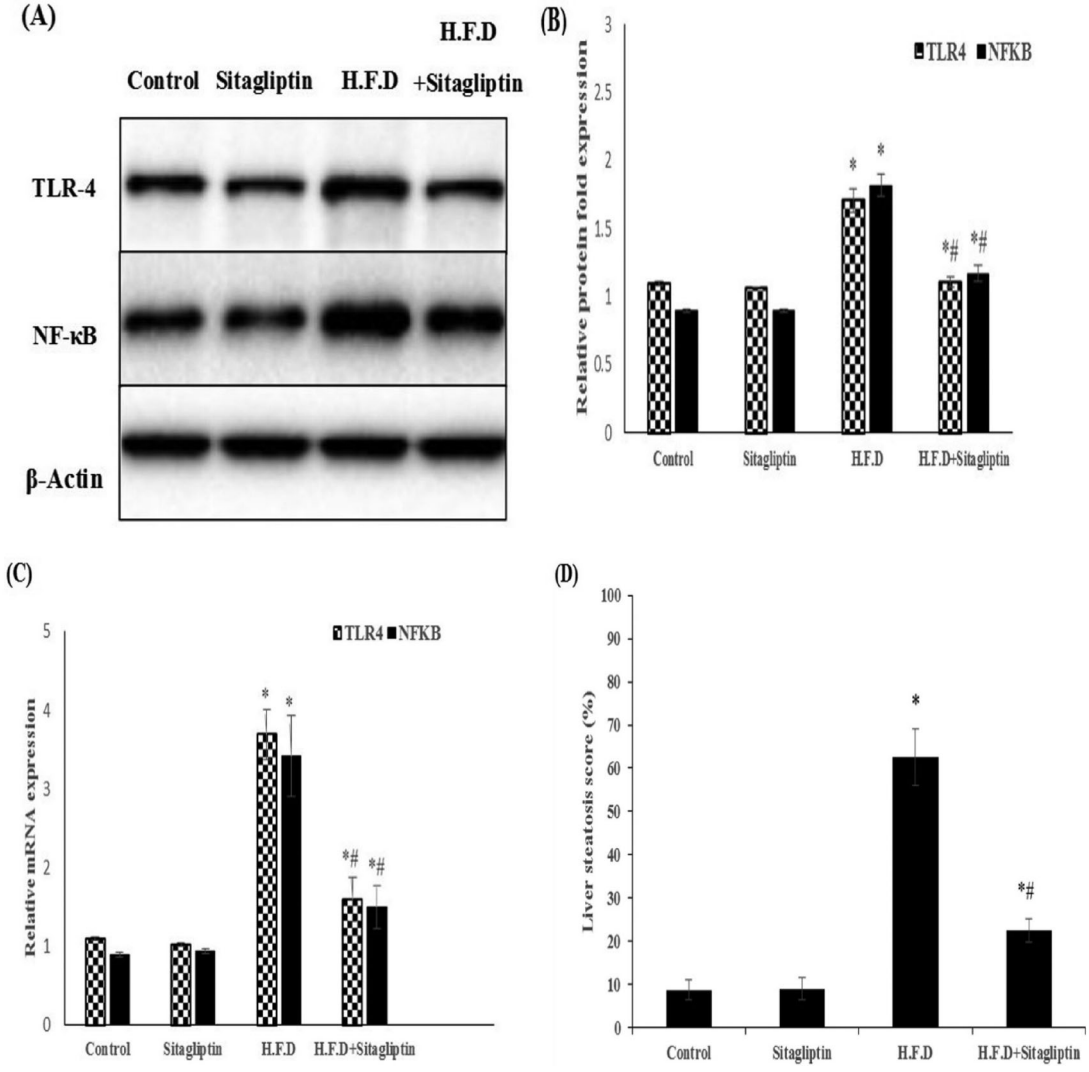

Figure 1. Administering sitagliptin downregulated hepatic TLR4 and NF-κB expression in the HFD-fed group. (A) Western blotting was used to analyse TLR4 and NF-κB protein expression, (B) TLR4/actin and NF-κB/actin ratios are demonstrated. (C) Relative mRNA expression levels of TLR4 and NF-κB. (D) Scores of hepatic steatosis of rat livers. The scores were determined, according to the percentage of hepatocytes containing lipid droplets. Data are expressed as mean ± SD of each group ($n = 8$). The results are expressed as mean ± SD ($n = 8$). *$p < .05$ versus control group; #$p < .01$ versus HFD group.

Sitagliptin ameliorated hepatic TLR4 and NF-κB expressions in HFD-fed rats

Real-time-PCR and Western blotting analysis indicated that hepatic TLR4 and NF-κB mRNA and protein level expressions respectively in rats fed HFD for 20 weeks were significantly up-regulated compared with those in the control group who received normal diet (Figure 1(A–C)). Additionally, expressions were significantly reduced in Sitagliptin treated group for 8 weeks in HFD + Sitagliptin group compared with the HFD group.

Effects of sitagliptin on histopathological changes of hepatic tissues in rats

Both the control and the Sitagliptin group demonstrated normal hepatic cellular architecture and no signs of inflammation/cellular infiltrate under light microscopy (normal lobule with radially arranged hepatocytes and a central vein (Figure 2(A,B)). The tissue samples from the HFD rats demonstrated signs of inflammation (cellular infiltrate) as well as ballooning and bubble-like hepatocyte steatosis (Figure 2(C)). Administration of Sitagliptin for 8 weeks in HFD + Sitagliptin

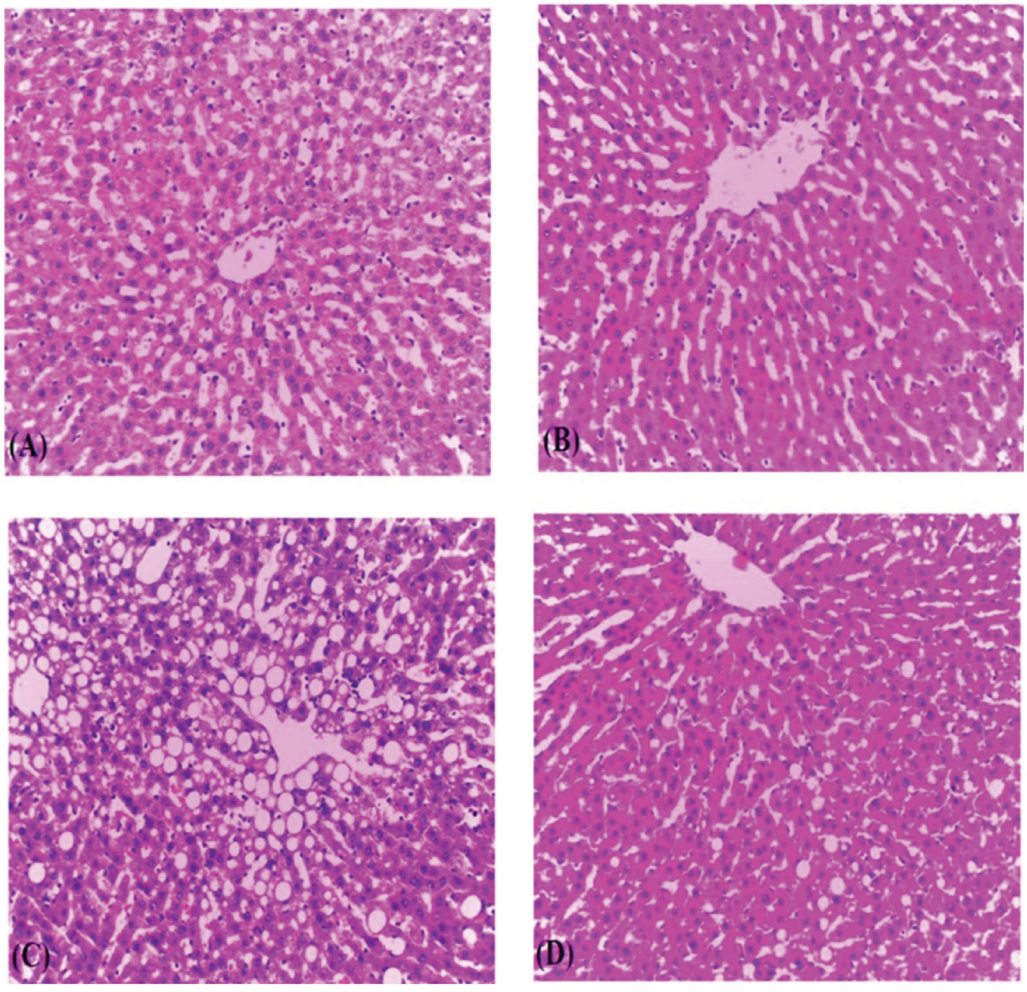

Figure 2. Liver sections of different groups stained with H&E (magnification 200×). Liver section of control group and Sitagliptin group (A, B respectively) showing normal hepatic structure and architecture. Liver section of HFD fed group showing ballooning with obvious inflammatory cell infiltration (C). Administration of Sitagliptin for 8 weeks in HFD + Sitagliptin group resulted in mild ballooning with much less inflammatory cells infiltration.

group resulted in a significant reduction of pathological changes induced by HFD feeding with liver samples showing mild steatosis and ballooning with much less inflammation (Figure 2(D)).

Further analysis revealed that the score of hepatic steatosis in the HFD + Sitagliptin group was significantly reduced, as compared with that in the HFD group, however, they remained significantly higher than that of the control group (Figure 1(D)).

Effects of sitagliptin on HMGB1 expression and macrophage infiltration in hepatic tissues in rats

Immunohistochemical staining of HMGB1 revealed a distinct expression pattern in sections from control and HFD fed rats. HMGB1 was localised in the nucleus of the hepatocytes in the control rats (Figure 3.1(A)). In the HFD fed rats, a relative increase in the expression of HMGB1 was observed, and extranuclear HMGB1-positive staining was visible indicating translocation and extracellular expression of HMGB1 in hepatocytes (Figure 3.1(C)). The expression of HMGB1 and its translocation extracellularly were significantly downregulated in the HFD rats treated with Sitagliptin (Figure 3.1(D)).

As a macrophage-specific marker, CD68 immunohistochemical staining showed much more Kupffer cells infiltration in HFD fed rats (Figure 3.2(C)) than in control rats (Figure 3.2(A)). However, Sitagliptin administration in the HFD + Sitagliptin group decreased CD68 expression (Figure 3.2(D)).

Discussion and conclusions

Our study demonstrates that Sitagliptin attenuates HFD associated hepatic inflammation and steatosis. We have found that Sitagliptin ameliorated the development of NASH by reducing HMGB1 level with down-regulation of TLR4 and NF-κB in the liver of HFD fed rats.

Inflammation is one of the most important factors responsible for the pathogenesis of both NAFLD and obesity-associated hepatic insulin resistance (Perry *et al.* 2015). Interestingly, it is established that innate immune activation provokes such inflammatory responses (Cai *et al.* 2018, Luo *et al.* 2018). It should be noted that till now the response of the innate immune system in the state of NAFLD is still unknown.

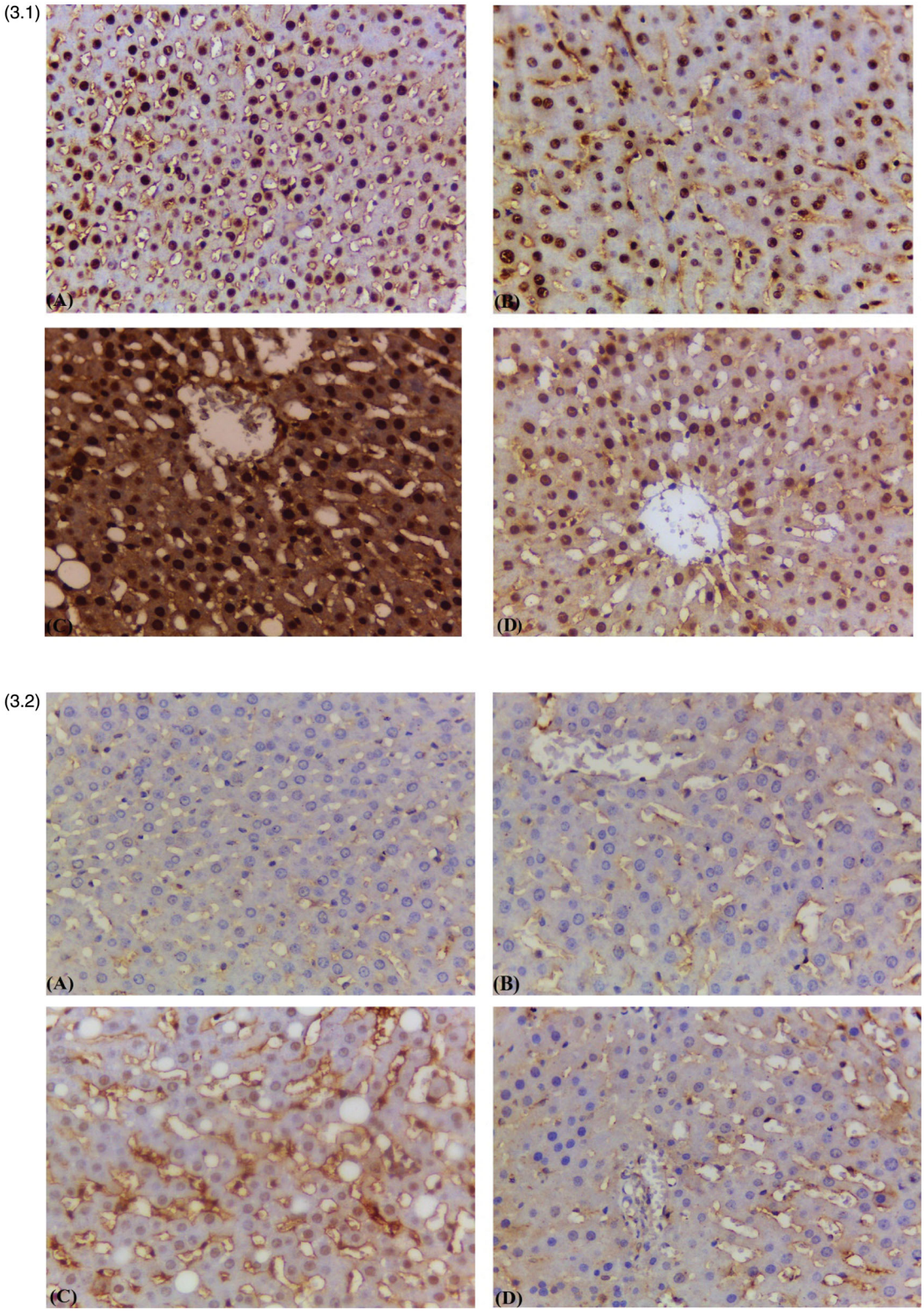

Figure 3. (3.1) Immunohistochemical staining of HMGB1 (magnification 400×). HMGB1 was localised in the nucleus of the hepatocytes in the control rats (A). In the HFD fed rats, a relative increase in the expression of HMGB1 with cytoplasmic translocation and extracellular expression of HMGB1 in hepatocytes (C). The expression of HMGB1 and its translocation extracellularly were significantly reduced in the HFD rats treated with Sitagliptin (D). (3.2) Immunohistochemical staining of CD68 (magnification 400×). More Kupffer cells infiltration was detected in HFD fed rats (C) that was downregulated by Sitagliptin administration (D).

Feeding rats with HFD has been shown to induce T2DM with a hepatic manifestation of NAFLD, mimicking the metabolically obese individuals. According to our results, the HFD stimulated weight gain, dyslipidemia, insulin resistance, as well as steatohepatitis (the main pathological finding) (Hasan *et al*. 2014, Ma *et al*. 2017). Although sitagliptin is known to

exert positive effects on the liver as an insulin sensitiser (Akaslan *et al*. 2013, Jung *et al*. 2014), moreover a beneficial effect of sitagliptin on serum lipid parameters was detected, it was partly explained by an improvement in glycemic control and insulin resistance, weight loss, and delayed gastric emptying (Fan *et al*. 2016). Also, Hsieh *et al*. (2010) suggested that DPP- 4 inhibitors augment the level of GLP-1 receptors thus reduce the secretion of triglycerol, cholesterol, and apolipoprotein B-40 from the intestine.

However, the mechanism by which this drug ameliorates steatohepatitis is still unknown. One study indicates that it may be as a result of hepatic gene expression modulation via the regulation of the SIRT1/AMPKα pathway (Shen *et al*. 2018).

Current evidence pinpoints the role of HMGB1 in triggering inflammation mediated by the innate immune system via its release from liver parenchymal cells into extracellular milieu upon obesity or HFD feeding, as well as endogenous stimuli (cellular stress/trauma or, bacterial endotoxin) (Li *et al*. 2011, Lundbäck *et al*. 2016). Several studies noted that HMGB1 levels are elevated in NAFLD (in both humans and animals) (*et al*Alisi *et al*. 2014, Ganz *et al*. 2015). Also, HMGB1 inhibition causes a significant decrease in the inflammatory response in NAFLD (Li *et al*. 2011)., which suggests that HMGB1 is an essential mediator in NAFLD and its down-regulation or even inhibition may represent a potential approach for anti-inflammatory therapy of NAFLD. After being secreted, extracellular HMGB1 binds to certain receptors, such as TLR4, which are expressed on hepatocytes, cholangiocytes, and hepatic stellate cells (HSCs) to activate innate immune signalling that regulate the inflammatory response (Andersson *et al*. 2018).

In our study, we observed that sitagliptin administration significantly reduced translocation and extracellular expression of HMGB1 in hepatocytes with subsequent reduction of the serum levels of HMGB1. Moreover, sitagliptin treatment significantly downregulated hepatic TLR4 protein expression, these results were accompanied by reduced hepatic TG accumulation and improved histopathological architecture with a significant reduction of the steatotic score. These results are in accordance with our hypothesis that the anti-inflammatory and anti-NAFLD activities of Sitagliptin occur even in part along the HMGB1/TLR4 axis. Similar to our results, previous studies reported that Sitagliptin could decrease both the HMGB1 levels (Lin *et al*. 2020) and TLR4 expression in animal models (Mehal 2014, El-Kashef and Serrya 2019) Stimulation of the TLR4 via HMGB1 will stimulate downstream adaptor molecules like Myeloid differentiation factor 88 (MyD88), which will, in turn, activate NF-κB; a transcription factor responsible for macrophage infiltration and the expression of proinflammatory cytokines. This would further stimulate an inflammatory response which may result in the activation of hepatocyte lipid accumulation and lipogenesis. Moreover, these cytokines could stimulate NF-κB activity in order to form a positive feedback mechanism which would further promote the inflammatory response. Our results revealed that Sitagliptin treatment significantly down-regulated hepatic NF-κB, with subsequent reduction of macrophage infiltration which suggests an underlying mechanism of action associated with the TLR4/NF-κB axis. In accordance with these results Abo-Haded *et al*. (2017) and El-Kashef and Serrya (2019) reported that Sitagliptin down-regulates the NF-κB expression in hepatic toxicity rat models.

IL-1β and TNF-α are responsible for the downstream of the TLR4/NF-κB axis (Wang *et al*. 2013). In animal models, both of these cytokines have been found to enhance the development and progression of NAFLD (Wang *et al*. 2013). It should be noted that, in humans, the expression of these cytokines is up-regulated in patients with NAFLD (Salmenniemi *et al*. 2004), which may suggest that, these cytokines are essential mediators of NAFLD. TNF-α has the ability to promote the aggregation of TG within hepatocytes via mechanisms related to impaired insulin signalling. States of insulin resistance cause a rise in serum-free fatty acid (FFA) levels. Elevated concentrations of insulin facilitate FFA influx into hepatocytes, with subsequent stimulation of hepatic lipogenesis (Cawthorn and Sethi 2008). Additionally, TNF-α stimulates hepatocyte cholesterol accumulation (Ma *et al*. 2008), IL-1β enhances hepatic TG accumulation via inhibiting peroxisome proliferator-activated receptor – α (PPARα) as well as stimulating diacylglycerol acyltransferase 2 expression, which converts diglycerides into TG that enhances the progression of NAFLD (Miura *et al*. 2010). As per our results, Sitagliptin administration decreased hepatic TNF-α and IL-1β, which subsequently decrease hepatic insulin resistance, TG buildup, and inflammation. This was confirmed with the improvement of liver function tests and restoration of the histopathological architecture of the liver.

In conclusion, we found that sitagliptin effectively ameliorated HFD-induced hepatic steatohepatitis in rats. Sitagliptin, which is used as an insulin sensitiser in T2DM treatment significantly reduced HFD-induced steatohepatitis by reducing HMGB1 and liver TLR4/NF-κB pathway. Currently, this is the first in vivo study which identified the mechanisms by which Sitagliptin ameliorates hepatic steatohepatitis (via modulating the HMGB1/TLR4/NF-κB axis).

Acknowledgements

The research group shows gratitude to all participants and the entire staff of physiology and biochemistry departments, Benha faculty of medicine, Benha University for their dedicated and conscientious collaboration and assistance.

Ethical approval

The animal study was approved by the Local Ethical Committee, Benha Faculty of Medicine, Egypt.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Author contributions

All authors of this research paper have contributed equally to the planning, execution, or analysis of this study.

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Data availability statement

All relevant raw data will be freely available by the authors.

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