Pharmacological Reports 71 (2019) 1044–1049

Contents lists available at ScienceDirect

Pharmacological Reports

journal homepage: www.elsevier.com/locate/pharep

Original article

The hepatoprotective effect of sitagliptin against hepatic ischemia reperfusion-induced injury in rats involves Nrf-2/HO-1 pathway

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ARTICLE INFO

Article history:
Received 20 March 2019
Received in revised form 23 May 2019
Accepted 13 June 2019
Available online 14 June 2019

Keywords:
Liver
Ischemia reperfusion
Sitagliptin
Nrf-2
Heme oxygenase

ABSTRACT

Background: Oxidative stress and inflammation play a key role in the development of hepatic ischemia reperfusion (HIR)-induced injury. Nuclear factor-erythroid 2-related factor-2 (Nrf-2) is a main regulator of numerous genes, encoding cytoprotective molecules including heme oxygenase-1 (HO-1). Sitagliptin (Sit) is an incretin enhancer acting *via* inhibition of dipeptidyl peptidase-4 (DPP-4) enzyme. This study was undertaken to investigate the ability of Sit to prevent the hepatic pathological changes of HIR induced injury and to modify Nrf-2 and its target HO-1.

Methods: Pringle's maneuver was used to induce total HIR in adult male rats that were randomly assigned into 4 groups. Group1 (sham-operated control), Group 2 (sham-operated + Sit-control group), Group 3 (HIR non-treated), and Group 4 (HIR + Sit). Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities together with hepatic contents of malondialdhyde (MDA), nitric oxide (NO) and reduced glutathione (GSH) and superoxide dismutase (SOD) activity were evaluated. Hepatic tissue mRNA of Nrf-2 and protein content of HO-1 along with histopathological examination and scoring of hepatic injury were performed.

Results: Sit caused a significant reduction in ALT and AST activities together with attenuation of HIR-induced histopathological liver injury. Effect of Sit was associated with decreased hepatic level of MDA and NO with increased GSH level and SOD activity. Non-treated rats with HIR showed an increase in Nrf-2 mRNA expression and HO-1 content in hepatic tissue which was further increased by Sit treatment.

Conclusions: These results indicate that hepatoprotective activity of Sit against HIR is attributed at least in part to modulation of Nrf-2/ HO-1 signaling pathway.

Introduction

Hepatic ischemia reperfusion (HIR) injury may be associated with many situations such as liver resection, liver transplantation, biliary tract operations that need temporary ligation of the hepatoduodenal ligament, hepatic trauma, or even hemorrhagic shock [1]. The HIR injury is a complex cascade of events mediated by numerous inflammatory and molecular mediators with oxidative stress resulting in hepatocyte death [2,3], so searching for an effective preventive and treating method is mandatory.

Nuclear factor erythroid 2-related factor 2 (Nrf-2) is a transcription factor, widely expressed in many organs and is considered as a multiorgan protector [4]. The Nrf-2 regulates expression of many genes encoding antioxidant activities including heme oxygenase-1 (HO-1), by binding to the antioxidant response element (ARE) in the promoters segments of the corresponding genes [5]. Therefore, Nrf-2 plays a central role in cellular redox system and has a protective role against HIR injury [6]. There are three isoforms of HO enzyme system serving as one of the most critical cytoprotective mechanisms activated during cellular stress and exerting antioxidant and anti-inflammatory functions. Of the different HO enzyme isoforms, the inducible HO-1 is arguably the most well-known of all Nrf-2-regulated genes and it plays a role in pathophysiological responses including the HIR-induced injury [3,7].

Sitagliptin (Sit) is one of the most well-known gliptins or incretin enhancers, which increases the insulinotropic incretin contents due to the inhibition of dipeptidyl peptidase type-4 (DPP-4) enzyme activity [8] without producing hypoglycemia [9].

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http://dx.doi.org/10.1016/j.pharep.2019.06.006
1734-1140/

Previous studies have revealed that incretin-based therapies like Sit, a DDP-4 inhibitor that accumulates GLP-1, and liraglutide, a GLP-1 analogue, may offer cardiovascular and hepatic protection [10–12]. Sit is recently reported to have a protective effect on HIR [13]; however, its involvement in modulation of Nrf-2/HO-1 in HIR is not completely studied. This study was undertaken to investigate the ability of Sit to prevent the hepatic pathological changes of HIR induced injury and to modify Nrf-2 and its target HO-1.

Material and methods

Animals and grouping

Adult male albino rats (220–250 g) which obtained from National Research Institute (Giza, Egypt) were included in this research. Rats were fed a standard rat experimental chow diet and tap water. Animals were left to acclimatize for one week before inclusion to the experiment. The experiment was conducted according to the ethical standards approved by the faculty board committee of faculty of medicine, Minia University, Egypt.

Rats were randomly divided into 4 experimental groups (n = 6). Sham-operated control group: received the vehicle orally for 14 days. Sham operated Sit control group received (Sit 10 mg/kg orally for 14 days suspended in 0.5% carboxymethylcellulose). HIR non-treated group: received the vehicle and subjected to total HIR. Sit-treated HIR group: received (Sit as mentioned above 10 mg/kg orally for 14 days suspended in 0.5% carboxymethylcellulose), and subjected to total HIR [14].

Chemicals and drugs

Sitagliptin (Sit) tablets were purchased from MSD medical company. ALT and AST colorimetric kits were obtained from Biodiagnostic (Giza, Egypt). HO-1 ELISA kit (catalog number GBS-30356) was obtained from Glory Science Co., Ltd (Taichung, Taiwan). All other analytical chemicals were obtained from the commercial sources. Oral treatments were administered by gavage.

Surgical procedure

The rats were anesthetized with 1 g/kg *ip* injection of urethane hydrochloride. A mid line abdominal incision was done. The porta hepatis was exposed then clamping of portal triad was done to produce total hepatic ischemia using bulldog microvascular clamp according to Pringle's maneuver [15].

Ischemia was maintained for 45 min. then reperfusion was allowed by removal of the microvascular clamp then the abdomen was closed by the silk sutures till the end of the reperfusion period 2 h. Body temperature was maintained at 37 °C allover the time of the experiment using a heating bad. Sham operated group was subjected to the same conditions of anesthesia and surgery but without portal triad clamping and served as a normal control group.

Sample collection and preparation

At the end of the reperfusion period rats were sacrificed by decapitation after cleaning the cut area then blood samples were collected and centrifuged to attain clear sera for evaluation of the serum parameters. The liver was dissected out, cleaned then liver samples for histopathology were fixed in 10% formalin solution and the remaining hepatic tissue was kept in −80 °C for evaluation of tissue biochemical parameters.

Liver samples were washed with cold normal saline then were homogenized in ice cold potassium phosphate buffer (pH 7.4) by Glas-Col homogenizer (Cole-Parmer, IL, USA). The homogenate was centrifuged at 4000 rpm for 20 min at 4 °C in cooling centrifuge (Ray Wild TGL-16, Germany) then supernatant was used for evaluation of HO-1, malondialdhyde (MDA), nitric oxide (NO) and reduced glutathione (GSH) levels and SOD activity.

Biochemical analysis

Evaluation of the hepatic oxidative stress parameters

Oxidative stress parameters in the form of MDA, SOD activity, GSH, and NO were screened. MDA was evaluated *via* a process based on the reaction of MDA with thiobarbituric acid under high temperature and acidic conditions to form MDA-thiobarbituric acid pink colored Schiff base adduct that was measured colorimetrically at 535 nm [16]. SOD activity was measured chemically *via* a method that depends on SOD inhibition of pyrogallol autoxidation [17] while determination of GSH is based on the reduction of Ellman's reagent by thiol groups of GSH [18]. NO evaluation is based on estimation of nitrite ($NO2^-$) and nitrate ($NO3^-$) levels which are the stable oxidation end products of NO. Levels of $NO2^-$/$NO3^-$ as the indicators of NO production were estimated by the Griess method [19].

Evaluation of hepatic HO-1 level

Enzyme-linked immunosorbent assay (ELISA)-kit was used for quantitative assay of HO-1 protein level according to the kit instructions.

Real-time reverse transcription polymerase chain reaction

Total RNA was extracted from hepatic tissue homogenate using RiboZol reagent (Amresco, Solon, USA) following the manufacturer's instructions. Real-time polymerase chain reaction (RT- PCR) was performed with 50 ng RNA template per reaction using Thermo Scientific one step kits in 25 μL reaction volume containing 70 nM of specific primers in the Real-Time PCR Detection System (Kappa Biosystems, USA). The SYBR green data were analyzed with a relative quantification to GAPDH as reference gene. The primer Nrf-2 gene used was as follows: sense: 5′-CCCAGCACATCCAGACAGAC -3′ anti sense: 5′- TATCCAGGGCAAGC-GACTC -3′. Then the samples were placed in a thermal cycler (Applied Biosyst 7500 fast, Techne (Cambridge) LTD., UK). The relative expression level of Nrf-2 gene was calculated using the formula (2-ΔΔCT) according to the previously published method [20]. They were scaled relative to controls where control samples were set at a value of 1. Thus, results for all experimental samples were graphed as relative expression compared with the control.

Histopathological study

Parts of the right lobe of the liver were fixed, dehydrated, embedded in paraffin bolcks, and cut into sections of 5 μm thickness. Sections were stained with hematoxylin and eosin dye for histolopathological examination under the light microscope and examined by a pathologist for the presence of lesions and scored according to the following scoring system; grade 0: minimal or no evidence of injury; grade 1: mild injury consisting of cytoplasmic vaculation to focal nuclear pyknosis, grade 2: moderate to severe injury with extensive nuclear pyknosis, cytoplasmic hyperesinophilia and loss of intercellular borders; grade 3: sever necrosis with disintegration of hepatic cords, hemorrhage and neutrophil infiltration [21].

Statistical analysis

Data was analyzed by one way ANOVA followed by Tukey's post-test. The values are represented as means ± SEM. Statistical

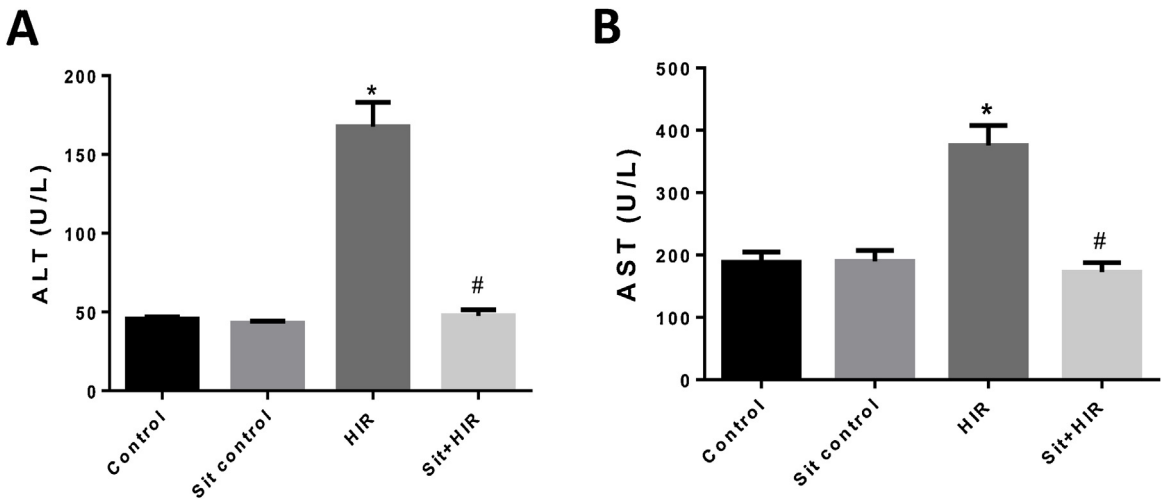

Fig. 1. Effect of HIR with and without Sit-pretreatment on serum aminotransferases.
All results are expressed as mean ± SEM (n = 6). $^{*,\#}$ significantly different (at $p < 0.05$) from control and HIR groups respectively. Sit: sitagliptin; HIR hepatic ischemia reperfusion. ALT: Alanine aminotransferase; AST: aspartate aminotransferase.

analysis was done using GraphPad Prism software (version 6) and the differences were considered significant when the calculated $p < 0.05$.

Results

Serum aminotransferases

The current study showed that HIR was associated with impairment of liver function in the form of elevated serum levels of ALT and AST activities in comparison to the sham operated control group. Pretreatment with Sit was able to reverse the elevated liver enzymes compared to the HIR rats. Sit administration did not affect the aminotransferases activities of the control rats (Fig. 1).

Hepatic oxidative stress parameters

Induction of HIR significantly increased levels of oxidative stress parameters in the form of liver tissue MDA and NO; however pretreatment with Sit significantly decreased them nearly to the normal level. On the other hand hepatic antioxidant parameters as SOD activity and GSH level were decreased by HIR in comparison to sham operated group, the effect which was significantly ameliorated by Sit pretreatment. Sit treatment has no effect on oxidative stress parameters of the control rats (Table 1).

Hepatic HO-1 level and Nrf-2 mRNA expression

Rats with HIR had elevated HO-1 protein level which was insignificant from sham operated control group, though sitagliptin pretreatment significantly elevated hepatic HO-1 level compared to HIR induced rats (Fig. 2A). Regarding Nrf-2 mRNA expression, HIR significantly elevated the hepatic Nrf-2 mRNA level compared to sham operated control group. Sit pretreatment significantly increased the Nrf-2 mRNA expression compared to HIR non treated rats (Fig. 2B). Sit has no effect on either Nrf-2 mRNA expression or HO-1 protein level of control rats (Fig. 2A and B).

Table 1
Effect of HIR with and without Sit-pretreatment on hepatic tissue oxidative stress parameters.

	MDA (nmol/g tissue)	NO (nmol/g tissue)	SOD (U/mg tissue)	GSH (nmol/g tissue)
Control	36.35 ± 3.08	80.19 ± 6.21	15.31 ± 1.27	365.6 ± 26.52
Sit control	34.14 ± 2.11	83.55 ± 5.254	16.47 ± 0.82	407.5 ± 19.05
HIR	91.34 ± 7.55*	180.50 ± 5.62*	5.36 ± 0.53*	225.9 ± 20.90*
Sit+HIR	48.63 ± 4.65#	63.70 ± 5.88#	38.98 ± 3.05*#	397.8 ± 30.23#

All results are expressed as mean ± SEM (n = 6). $^{*,\#}$ significantly different (at $p < 0.05$) from control and HIR groups respectively. Sit: sitagliptin; HIR hepatic ischemia reperfusion. MDA: malondialdehyde; NO: nitric oxide; SOD: superoxide dismutase; GSH: reduced glutathione.

Liver histopathology

The sham operated control rats showed normal liver architecture with hepatocytes arranged in cords and separated with sinusoids (Fig. 3A). The Sit treated control rats showed also normal hepatic architecture with no seen pathological changes (Fig. 3B). The HIR rats had severe sinusoidal congestion associated with marked hepatic vacuolation and degeneration with significant increase in histopathological score of hepatic injury in comparison to control rats (Fig. 3C and E). Sit pretreatment resulted in significant attenuation of the histopathological changes in compared to HIR rats with only perinuclear vacuolation of some hepatocytes and multiple cells showing single cell necrosis (Fig. 3D and E).

Discussion

Hepatic vascular control is used to prevent hemorrhage during liver resection or transplantation and provide a bloodless operative field which may results in HIR injury, the main cause of primary graft dysfunction after liver transplantation [22]. Many factors are involved in the pathogenesis of HIR, including anaerobic metabolism, oxidative/nitrosative stress, mitochondrial damage, intracellular Ca^{2+} overload, cytokines, and chemokines produced by Kupffer cells and neutrophils [23].

In the present study, clamping of the portal triad for 45 min followed by reperfusion for two hours resulted in HIR injury manifested by elevation of serum ALT and AST activities as markers of liver damage released to the circulation from injured hepatocytes [24]. The histopathological changes confirmed the liver injury which ranged from sinusoidal congestion to hepatic cell necrosis. The current results are in accordance with the previously published data [15,24,25]. Pretreatment with Sit for two weeks protected the liver from HIR injury manifested by normalization of liver enzymes and confirmed by histopathological improvement. The hepatoprotective effect of Sit was previously reported in different models including diabetes induced

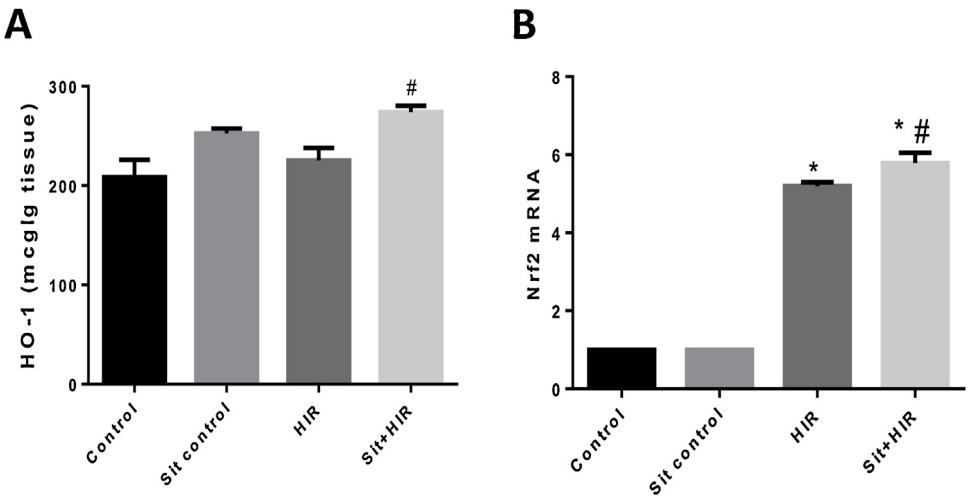

Fig. 2. Effect of HIR with and without Sit-pretreatment on hepatic HO-1 content and Nrf-2 mRNA expression.
All results are expressed as mean ± SEM (n = 6). $^{*,\#}$ significantly different (at $p < 0.05$) from control and HIR groups respectively. Sit: sitagliptin; HIR hepatic ischemia reperfusion. HO-1: heme oxygenase-1; Nrf-2; nuclear factor-erythroid 2-related factor-2.

hepatotoxicity and carbon tetrachloride-induced liver fibrosis [26,27] and recently against HIR [13].

The most important pathways of HIR are initiated by oxidative stress which has a significant role in the progression of hepatic injury and apoptosis. Hepatic reperfusion is associated with reactive oxygen species (ROS)-induced mitochondrial dysfunction, lipid peroxidation, damage of endothelial cells and loss of the integrity of the microvasculature [1]. The overproduction of ROS in perfused organs precedes the appearance of tissue damage in liver [28]. It has been previously reported that inducible NO synthase (iNOS) expression was enhanced with HIR-induced injury [24] with subsequent increase in iNOS-derived NO level. The reactions of ROS such as superoxide anion ($O2^{-\bullet}$) with NO yield products such as peroxynitrite ($ONOO^{-}$) a reactive nitrogen species which can be an extremely aggressive oxidant [29].

In the present study HIR increased oxidative stress parameters in the form of MDA which is the lipid peroxidation end product [30] and NO which is the nitrosative biomarker [31]. Moreover the antioxidant biomarkers in the form of SOD activity and GSH level are decreased in response to HIR. SOD and GSH exist in all normal cells and have an important scavenging activity against reactive oxygen radicals to reduce the oxidative stress and the inflammation [26]. It has been previously demonstrated that excess oxidative stress can kill the cells by either necrosis or apoptosis [32]. In the current study, the levels of MDA and NO were significantly decreased while the hepatic SOD activity and GSH content were significantly increased in response to Sit medication which reflects the antioxidant activity of Sit that was previously reported. Sit was able to modulate the antioxidant response in the diabetic kidney [33], reduce $O2^{-\bullet}$ generation by directly scavenging ROS in ex vivo studies in arteries [34]. Furthermore, Sit decreased iNOS expression and oxidative stress parameters in isoprotrotenerol induced cardiac damage [12] and increased the total antioxidant capacity with decreased oxidative stress biomarkers in methotrexate induced liver injury [35].

The Nrf-2 is a transcription factor with a key role in cytoprotective genes that acts against oxidative stress injury by mediating the levels of endogenous antioxidants [36]. Keap1 binds to Nrf-2 as a complex to suppress it and keeps it into the cytoplasm under normal conditions, whereas during oxidative damage ROS such as hydrogen peroxide (H2O2) activate Nrf-2 leading to dissociation of the Keap-1-Nrf-2 complex enabling Nrf-2 to translocate into to the nucleus. In the nucleus, it binds to the antioxidant response element (ARE) and activates the transcription of antioxidant enzymes, including the inducible HO-1 [37,38]. The HO-1 has a cytoprotective activity *via* inhibition of inflammation and apoptosis [39] and can exert indirect anti-oxidative and anti-inflammatory effects through degradation of heme to carbon monoxide (CO), iron, and biliverdin. CO has both anti-inflammatory and anti-apoptotic effects and has an important role in regulating basal and constrictor-induced vascular tone as well as biliverdin can be reduced to the antioxidant bilirubin [40,41].

In the present study, Nrf-2 was increased significantly in response to HIR as an adaptive response to oxidative stress [42] caused by HIR. Nrf-2 was reported to have a protective activity against HIR [43] and has the ability to inhibit acute inflammatory liver injury [44]. This result is in line with the previously published results [45,46]. HO-1 is one of the key response genes of Nrf-2 activation, and its activation can protect against oxidative damage in cells [47]. In the current study hepatic content of HO-1 was insignificantly increased in response to HIR. The elevated level of HO-1 with associated significant hepatic injury in HIR induced rats means that stress-induced HO-1 elevation was not adequate to provide protection from the hepatic injury caused by IR and similar findings were previously reported [46].

Sit was able to significantly increase both Nrf-2 mRNA and HO-1 protein which was associated with its hepatic protection found in this study. It was recently reported that Sit protected rats from oxidative stress neuronal damage caused by kainic acid in the induced status epilepticus by increasing Nrf-2/Ho-1 expression [48]. In addition, it protected rat kidneys from induced diabetic nephropathy by increasing HO-1 mRNA, protein level, and enzyme activity in renal tissue and increasing Nrf-2 nuclear translocation [49]. Moreover, the protective effect of Sit in renal and myocardial IR was associated with up-regulation of HO-1 [50,51]. The hepatoprotective effect of Sit in methotrexate induced injury was also mediated by up-regulation of Nrf-2 [35]. It has been previously reported that HO-1 up regulation resulted in increase in SOD, catalase activities, endothelial NO synthase (eNOS), endothelial relaxation and decrease in $O2^{-\bullet}$ level [52]. The decrease in the level of $O2^{-\bullet}$ leads consequently to a decrease in the lipid peroxidation [30].

The antioxidant and anti-inflammatory effects of the DPP-4 inhibitor Sit may be attributed to the increased GLP-1 levels that activate the GLP-1 receptor that present in different organs including liver. It has been reported that GLP-1 receptor activation could protect the liver from IR injury *via* cAMP dependent pathway

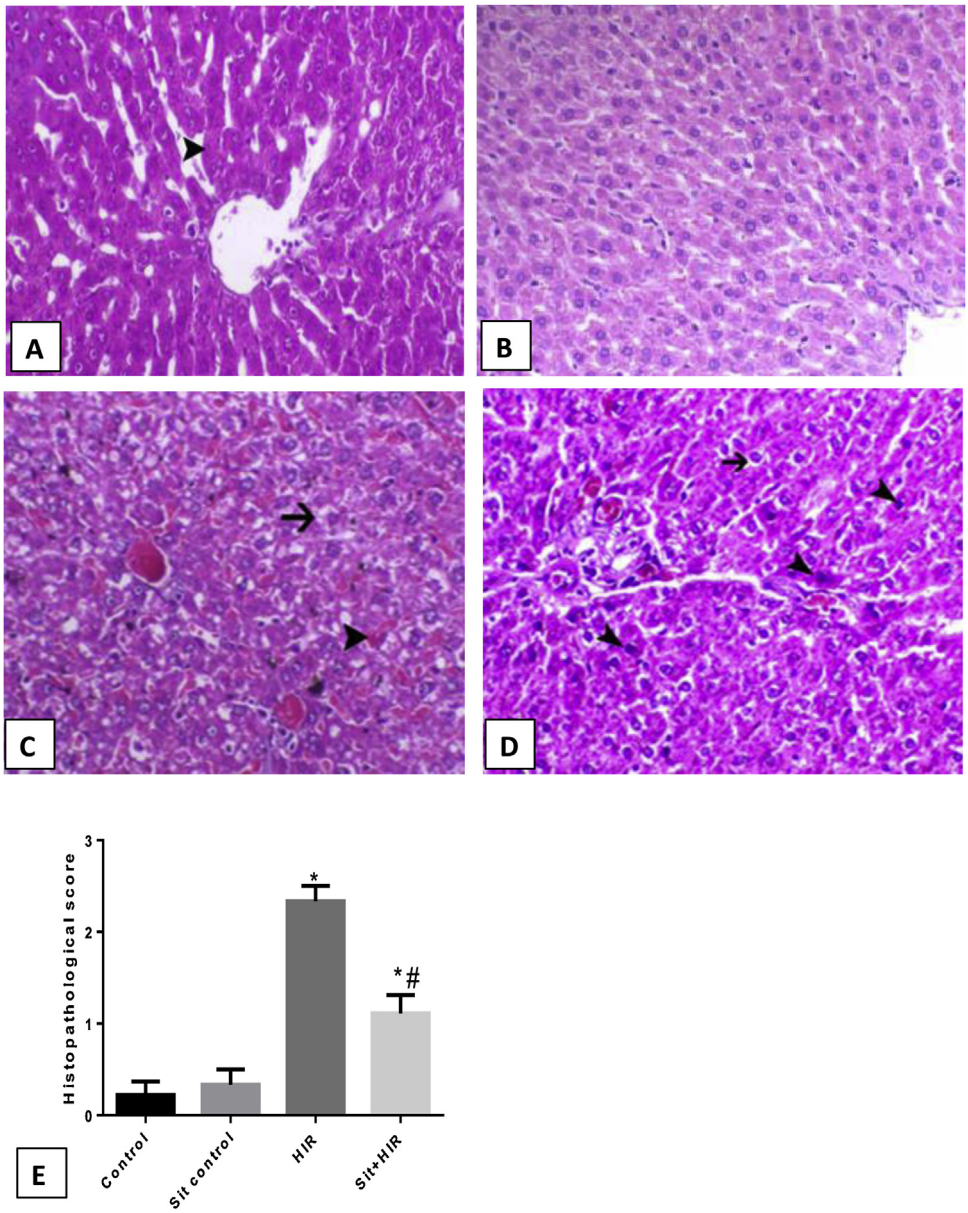

Fig. 3. Effect of HIR with and without Sit-pretreatment on liver histopathology.
Liver sections stained with H&E (200X magnification). A: The control group shows normal hepatocytes arranged in cords (arrow head) and separated with sinusoids; B: Sit control group shows normal hepatic architecture with no seen pathological changes. C: HIR group shows severe sinusoidal congestion (arrow head) associated with marked hepatic vacuolation and degeneration (arrow); D: Sit+HIR group shows perinuclear vacuolation of some hepatocytes (arrow) and multiple cells showing single cell necrosis (arrow head); E: Quantification of histopathological finding. All results are expressed as mean ± SEM (n = 6). $^{*,\#}$ significantly different (at $p < 0.05$) from control and HIR groups respectively. Sit: sitagliptin; HIR hepatic ischemia reperfusion.

[53]. The DPP-4 enzyme, inhibited by Sit, may cleave peptides other than GLP-1 leading to the inactivation and/or generation of new bioactive peptides. As measuring the level of GLP-1 and of other peptide substrates for DPP-4 is beyond the scope of the current experiment, further studies are needed to specifically explore the role of GLP-1 in the effect of Sit on HIR-induced injury.

Results of the present experiment show that induction of Nrf-2/HO-1 signaling pathway is an important and primary mechanism of Sit hepatic protection against HIR with a resulting antioxidant and anti-inflammatory property. The current work is the first study for evaluating the role of sitagliptin in the modulation of Nrf-2/HO-1 signaling in HIR induced injury. In conclusion, Sit has a hepatoprotective effect against HIR and this protection is mediated *via* the activation of Nrf-2/HO-1 pathway with subsequent antioxidant and antiinflmmatory activities.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Conflict of interest

The authors declare that they have no conflict of interest.

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