

Ameliorative effect of sitagliptin and its cardinal mechanisms in methionine induced- hepatotoxicity in rats

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Abstract

Background/ aim: High methionine (Met) (a precursor for homocysteine) diet is a risk factor for non-alcoholic fatty liver disease (NAFLD). This work demonstrated the hepatic effects of feeding western diet enriched with Methionine. Additionally, we evaluated the anti-oxidative properties of sitagliptin (STG) (an antidiabetic drug) which, counteract the negative effects of high Met diet. **Methodology:** Forty adult male Wister albino rats, divided into 4 group (10 rats/group) normal diet (control and STG groups), or high Methionine enriched diet, 1.5 % Methionine (Met and Met + STG treated groups) for 35 days. Rats were either treated with vehicle (control, Met groups) or Sitagliptin, 100 mg/kg/day (STG, Met + STG groups). Investigations were Lipid profile, liver functions test, serum homocysteine, iron, ferritin, liver reduced glutathione (GSH), serum MDA level and histopathological investigations. **Results:** Met resulted in significant increase in LDL and cholesterol with significant decrease in HDL. Moreover, it had resulted in significant increase ALT and AST, with significant increase serum iron and homocysteine with no effect on serum ferritin, and significant decrease in tissue reduced glutathione as an antioxidant enzyme. STG with normal control diet had positive effects on different parameters. Treatment with STG has resulted in an improvement in most of altered parameters. **Conclusions:** Our findings suggest that Met induced NAFLD could be related to increase serum iron, homocysteine levels as an inflammatory activator factors and antioxidant machinery defects and the ameliorating role of STG in this type of induced hepatotoxicity.

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is believed to be major chronic liver injury locally and the most common liver disease universally [1]. It is characterized by accumulation of lipid in the absence of consumption of alcohol that progress to steatohepatitis, liver fibrosis, and eventually hepatocellular carcinoma (HCC) [2]. The leading hypothesis of disease progression is inspected to the increased susceptibility to inflammatory cytokines and oxidative stress [3]. Although many studies have been done on NAFLD, there are still numerous gaps related to our understanding of the underlying mechanisms.

NAFLD is generally presented asymptotically and commonly companions metabolic syndrome, obesity, type 2 diabetes mellitus (T2DM) and liver tissues pathological alterations [4]. NAFLD is fundamentally characterized by triacylglycerol accumulation in the liver tissue [5]. Ingestion of Western diet is a key inducer of fat accumulation in the liver, leading to dyslipidemia, insulin resistance (IR) and NAFLD [6,7]. Current treatments for NAFLD include lifestyle change (weight reduction), insulin sensitizer agents, lipid-lowering drugs and antioxidants[8]. Anti-diabetic drugs, which improve IR, have an outstanding effect on NAFLD and decelerate the pathology progression [9,10].

Methionine is an essential amino acid that is metabolized by the liver mainly, where it is converted to S-adenosyl Methionine (SAME). Although all mammalian cells synthesize SAME, the liver is main site of formation SAME as it is the organ where metabolism of about 50% of all dietary Methionine occurs. SAME is mainly needed for methylation of a large variety of small

molecules; So, if the concentration of SAME elevated above certain level, the physiological hepatic functions will be also affected. There are many physiological conditions that affect the hepatic content of SAME. In consequence, the control of these fluctuations requires the rate at which the liver synthesizes and catabolizes SAME to be securely regulated. Therefore, maintaining SAME homeostasis may be a therapeutic target in non-alcoholic steatohepatitis, alcoholic- and non-alcoholic liver cirrhosis [11].

Methionine is the precursor of homocysteine (Hcy), plasma sulphur amino acid homocysteine concentration is pointed out as a risk factor for vascular disease [12]. This hypothesis is of great interest because of the possibility that lowering blood homocysteine through nutritional interventions might prove to be safe and effective methods of reducing the disease associated risks.

Sitagliptin (STG), a recently developed dipeptidyl peptidase 4 inhibitor (DPP-4), which has been widely used to treat T2DM and has also been evaluated in diabetic patients with NAFLD symptoms [13]. So, the aim of present study is to investigate the effect of sitagliptin and its cardinal mechanisms in methionine induced- hepatotoxicity in rats.

MATERIALS & METHODS

Ethics statement: The experimental protocol was approved by the local ethics committee of Faculty of Medicine, Menoufia University. Animals were housed (Medical Experimental Research lab, Faculty of Medicine, Menoufia University ,Egypt) Animals were treated in accordance with Guide for the Care and Use of Laboratory Animals (eighth edition, National Academies Press) (NIH Publication No 85–23, Revised 1996)

Animals: 40 adults male Wistar albino rats, matched for weight and age (between 150- 200 gm) were recruited for the study. Rats were purchased from a local providing facility. Rats were left to be acclimatized for 10 days. During the study, each of the 2 rats was kept in one cage at room temperature on standard rat chow and fresh water.

Experimental design.

Following acclimatization, animals were randomly allocated into four main groups (10 rats each): 1. Control group: fed control diet which is normal rodent diet with 0.5% cholic acid and 2% maltose dextrin and gavaged with vehicle (water) for 35 days [14].

2. methionine (Met)- treated group: high Methionine diet was made by enriching the control diet with 1.5% Methionine (Sigma-Aldrich) and gavaged with vehicle (water) for 35 days [15,16].

3. Sitagliptin (STG)- treated group: fed control diet and gavaged with an aqueous solution of Sitagliptin (Sigma-Aldrich) 100 mg/kg/day orally for 35 days [14].

4. Met + STG treated group: high Methionine diet was made by enriching the Control diet with 1.5% Methionine for 35 days and treated by gavage with an aqueous solution of Sitagliptin. At the end of the experiment (after 35 days), animals were fasted overnight, and blood samples were collected via cardiac puncture after rats being anaesthetized using Na⁺ thiopental (12 mg/Kg i.p.). Blood samples were left for clotting for 10 min and centrifuged at 4000 rpm for another 10 min to isolate the serum and kept at -20°C for further analysis of lipid profile, liver function test, homocysteine, iron and serum ferritin. Lastly, rats

of all groups were sacrificed by cervical decapitation and the livers were excised and divided into two halves. One half was used for estimation of reduced GSH. The other half was used for histopathological and immunohistochemical investigations.

Serum Biochemical Analysis

Lipid profile

Total cholesterol (mg/dl) and HDL-C (mg/dl) levels were determined following their hydrolysis and oxidation to yield colored quinonimine derivatives using test reagent kits (Biodiagnostics, Egypt). Triglycerides (TGs) level (mg/dl) was estimated by a reagent kit (EMAPOL, Poland), in which TGs were hydrolyzed with lipoprotein lipase to form glycerol, which forms a complex with H₂O₂ giving a colored derivative. The obtained levels of total cholesterol, HDL and TGs were then used to calculate the serum level of LDL-C as that described by Friedewald et al. [17].
$$\text{LDL} = \text{Total Cholesterol} - (\text{HDL} + \text{Triglycerides}/5).$$

Assessment of Liver Function

Blood was left to stand for 3 h in a centrifuge tube without anti-coagulant before the samples were centrifuged at 3000 rpm at 4 °C for 10 min to separate the serum. For the liver function test, liver aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities were evaluated using the method by Reitman & Frankel [18]. Serum and liver total protein were analyzed according to the method suggested by Bradford [19].

Determination of serum iron and ferritin.

Additional blood was collected into tubes with clot activator and gel separator. Cobas 8000 c701 (Roche) analyzer was used to determine iron concentration and unsaturated iron-binding capacity (UIBC). Transferrin saturation (TSAT) was calculated using formula: $TSAT (\%) = (\text{Serum Iron} / \text{Total Iron Binding Capacity}) \times 100\%$. Total iron binding capacity (TIBC) was calculated as the sum of serum iron and UIBC values. Concentrations of serum ferritin and soluble transferrin receptor were measured using commercially available Elisa assays (Ferritin Rat Elisa Kit, Abnova and sTfR Immunoassay, R&D Systems) according to the manufacturer's instructions [20].

Determination of serum homocysteine (Hcy)

Serum total Hcy concentrations were determined by HPLC (high performance liquid chromatography) equipped with a fluorometric detection system [21].

Assay of hepatic reduced (GSH) concentrations

To obtain a 10% (w/v) liver homogenate, 1 g of liver tissue was homogenized with 9 mL of 1.15% KCl. The liver homogenate was then centrifuged at $10,000 \times g$ for 15 min at 4 °C. The resulting supernatant was used to determine the contents of reduced GSH and lipid peroxides as well as the activities of GSH-related enzymes. The GSH content in the liver homogenate was determined by high performance liquid chromatography (HPLC)/mass spectrometer (MS) [22].

Determination of Malondialdehyde Serum Levels

Malondialdehyde was extracted from serum samples by treatment with 20 percent TCA at ratio of 1:1. The serum samples were centrifuge at 8000

rpm and filtered through a 0.2 µm membrane. The supernatant was treated Thiobarbituric acid (20 mM) and boiled in a water bath for 60 min. A 10 µL sample was injected into a high-performance liquid chromatography (HPLC) (Merck Hitachi) and separated with a C-18 phenomenex column, model RP-18, and detected with an HPLC fluorescence detector (Shimadzu, Japan) set at 532 nm excitation and 553 nm emission. The mobile phase consisted of a 35:65 (v/v) mixture of methanol and 0.05 M potassium phosphate buffer, pH 7, and the flow rate was 1 mL/min. MDA standard solutions were used to generate a standard curve. The nM levels of MDA found by us in the tested cohort are in agreement with serum MDA levels previously found in human after several hours of fasting[23]. High MDA levels were defined above the sample median corresponding to 12.87, 12.10, and 13.46 (nM) among the entire sample, men and women, respectively.

Histopathological examination

Liver tissues were fixed in 10% formalin, embedded in paraffin and sectioned at 5 µm thickness. Changes in liver pathology and collagen deposition were observed by hematoxylin-eosin (H and E) staining [24].

Statistical Analysis

Results are expressed as mean ± standard error (SE). Kolmogorov-Smirnov test was performed on all data sets to ensure normal distribution ($p > 0.5$). Student t-test or repeated-measures Analysis of Variances (ANOVA) were used for statistical analysis of the different groups which ever appropriate, using Origin® software and the probability of chance (p values). P values < 0.05 were considered significant.

RESULTS

Serum AST, ALT levels increased significantly in the Met group when compared to the control group (73.6 ± 1.4 vs 41.5 ± 0.9 mg/dl) (65.9 ± 0.9 vs 31.4 ± 1.03 mg/dl, $P < 0.05$). Their levels were significantly lower in the Met +STG treated group than that in the Met group (46.1 ± 2 mg/dl vs 73.6 ± 1.4 , $P < 0.05$) (35.4 ± 1.9 mg/dl vs 65.9 ± 0.9 , $P < 0.05$). There was no statistically significant difference in serum AST between STG treated, Met +STG and control groups (43.8 ± 0 . mg/dl vs 46.1 ± 2 and 41.5 ± 0.9 mg/dl, respectively, $P > 0.05$). There was no statistically significant difference in serum ALT between Met +STG and control groups (35.4 ± 1.9 vs 31.4 ± 1.03 , $P > 0.05$), however serum ALT levels in Met +STG treated group were significantly higher compared to STG treated group (35.4 ± 1.9 vs 25.5 ± 1.3 , $P < 0.05$) (Fig.1.a).

Serum cholesterol levels were significantly higher in the Met group when compared to the control group (263.8 ± 2.7 vs 168.6 ± 2.2 mg/dl, $P < 0.05$). Serum cholesterol levels were significantly lower in Met + STG treated group when compared to the Met group (177.6 ± 2.8 mg/dl $P < 0.05$), and insignificant to corresponding value in the control group, while serum cholesterol levels were significantly higher in Met +STG treated group compared to STG treated group (123.9 ± 3.1 , $P < 0.05$) (Fig. 1.b).

Serum TG level was insignificantly different in control, Met, STG and Met + STG groups (66.5 ± 2.6 , 65.7 ± 1.8 , 70.9 ± 1.5 and 75 ± 1.3 mg/dl, respectively ($P > 0.05$) (Fig. 1.b).

Serum HDL level decreased significantly in the Met group when compared to the control group (18.7 ± 0.8 vs 41.6 ± 0.6 mg/dl, $P < 0.05$). it was

significantly higher in the Met +STG treated group than that in the Met group (37.4 ± 2 mg/dl, $P < 0.05$). There was no statistically significant difference between Met +STG and control groups, while it was still significantly lower in Met +STG treated group compared to STG treated group (50.5 ± 2.2 $P < 0.05$) (Fig.1. b).

Serum LDL levels were significantly higher in the Met group when compared to the control group (231.6 ± 3.2 vs 113.6 ± 2.2 , $P < 0.05$). It was significantly lower in Met+ STG group when compared to the Met group (119.6 ± 3.3 mg/dl, $P < 0.05$). Fortunately, there was insignificant difference between Met+ STG group and control group, while It was significantly higher in Met +STG treated group compared to STG treated group (59.2 ± 2.4 , $P < 0.05$) (Fig. 1.b).

Serum iron level was significantly higher in the Met group when compared to the control group (0.35 ± 0.009 vs 0.22 ± 0.009 mg/dl, $P < 0.001$). Serum iron level was significantly lower in Met + STG treated group when compared to the Met group (0.2 ± 0.018 mg/dl, $P < 0.001$), and insignificantly different, compared to the corresponding value in the control group, while it was significantly lower to the corresponding value in STG treated group (0.14 ± 0.004 mg/dl, $P < 0.05$) (Fig.2.a).

Serum ferritin level was insignificantly different in control, Met, STG and Met + STG groups (118.5 ± 1.8 , 117.8 ± 1.04 , 118 ± 2.6 and 118 ± 2.4 ng/dl, respectively ($P > 0.05$) (Fig. 2.b).

Serum homocysteine level was significantly higher in the Met group when compared to the control group (24.8 ± 1 vs 14.2 ± 0.6 Mmol/l, $P < 0.05$). It was significantly lower in Met + STG treated group when compared to the Met group

(13.8 ± 0.7 vs 24.8 ± 1 Mmol/l, P < 0.05), and insignificantly in Met+ STG to corresponding value in the control group, STG group (13.8 ± 0.7 mg/dl vs 14.2 ± 0.6 and 13.5 ± 0.8 Mmol/l, respectively) (P > 0.05) (Fig. 2.c).

Liver reduced glutathione level was significantly lower in the Met group when compared to the control group (0.5 ± 0.4 vs 1.5 ± 0.06 mmol/mg, P < 0.05). Liver reduced glutathione was significantly lower in Met + STG treated aged group (1.5 ± 0.12 mmol/mg, P < 0.005) when compared to the Met group, and

insignificantly to corresponding value in the control group, STG group (1.7 ± 0.5 mmol/mg, P > 0.05) (Fig. 3.a)

Serum MDA level was significantly higher in the Met group when compared to the control group (157.7 ± 5.5 vs 67.3 ± 2.4 nmol/ml, P < 0.05). It was significantly higher in Met + STG treated aged group (75.1 ± 3.1 P < 0.05) when compared to the Met group, and insignificantly to corresponding value in the control group, STG group (68.4 ± 5.3, P > 0.05) (Fig. 3.b)

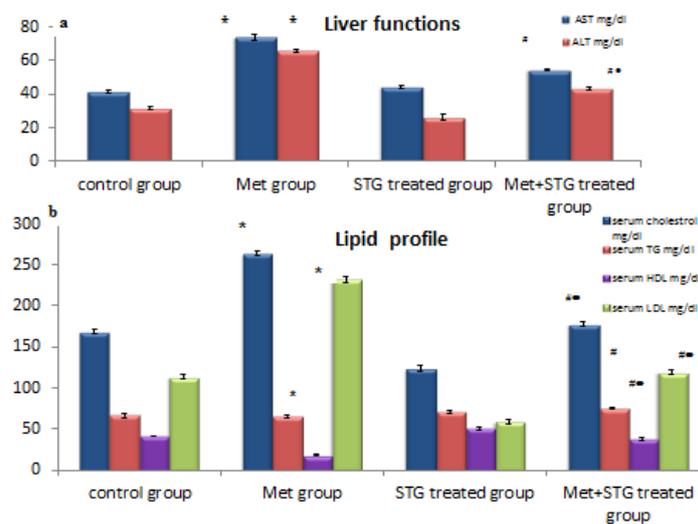


Fig (1): STG attenuates Met-induced hepatotoxic effect. **a.** Effect of STG treatment on serum level of AST and ALT in control, Met, STG and Met+ STG treated rats **b.** Effect of STG treatment on serum level of cholesterol, TG, HDL and LDL in control, Met, STG and Met+ STG treated rats.

* Significant when compared to control group, # Significant, when compared to Met group, • Significant, when compared to STG treated group (n = 10).

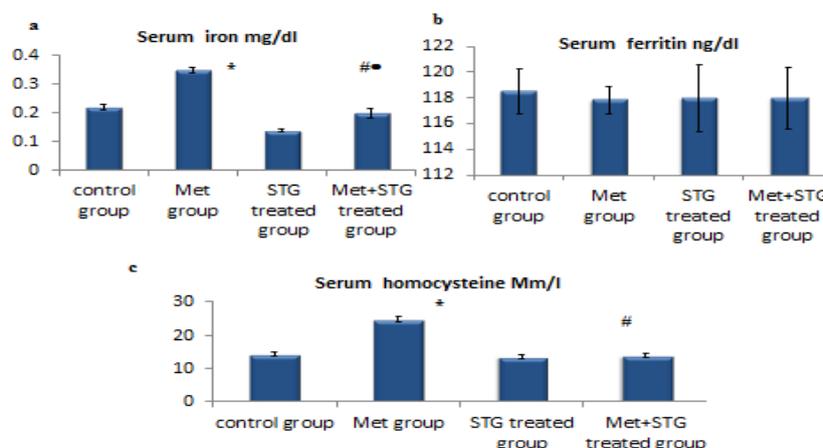


Fig (2): **a.** Effect of STG treatment on level of serum iron in control, Met, STG and Met+ STG treated rats **b.** Effect of STG treatment on level of serum ferritin in control, Met, STG and Met+ STG treated rats **c.** Effect of STG treatment on level of serum homocysteine in control, Met, STG and Met+ STG treated rats.

*Significant when compared to control group, # Significant when compared to Met group. • Significant when compared to STG treated group (n = 10).

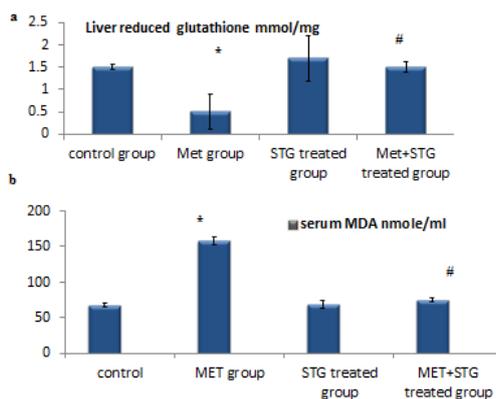


Fig (3): STG attenuates Met-inflammatory and oxidant effect. **a.** Effect of STG treatment on liver reduced glutathione in control, Met, STG and Met+ STG treated rats. **b.** Effect of STG treatment on serum level of MDA in control, Met, STG and Met+ STG treated rats

Fig (4): histopathological examination of rat liver showing:

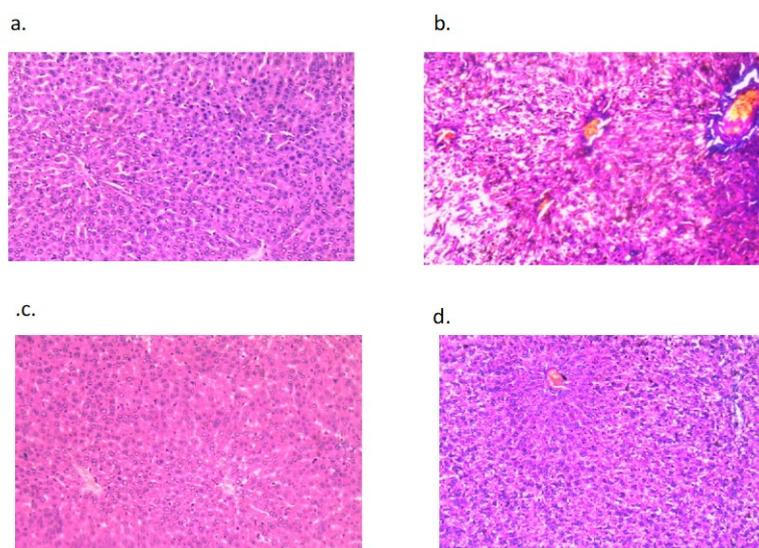


Fig (4): **a.** control group showed normal liver showing hexagonal structure of normal hepatocytes with intervening portal tracts (H&E X 100), **b.** Met group showed Fatty liver disease showing signet ring appearance of the liver cells, fibrosis and inflammatory infiltrate at the portal tract, (H&E X 100) **c.** STG group showed no significant changes in liver tissue showing hepatic lobules formed of cords of hepatocytes (H&Ex100) , **d.** Met + STG showed Liver restored its normal architecture after sitagliptin treatment (H&E X 100).

DISCUSSION.

NAFLD is considered the most common cause of liver diseases especially in developed countries [25]. There is a common association between NAFLD, type 2 DM and metabolic syndrome [26]. NAFLD is characterized by hepatic cell damage, inflammation, and liver fibrosis which may progress to cirrhosis [27]. Most Western diets are composed of animal products like meat, dairy and poultry, all of these are rich in Methionine. Although Methionine has been independently

associated with NAFLD, the exact mechanism by which Methionine leads to NAFLD is not completely studied.

Enzyme dipeptidyl peptidase 4 (DPP-4) is widely distributed in many organs throughout the body including liver. There are many evidences suggest that DPP-4 is involved in the pathogenesis of many chronic liver diseases such as hepatitis C virus (HCV) infection, NAFLD, and hepatocellular carcinoma. Sitagliptin, is an oral hypoglycemic agent that act as DPP-4 competitive inhibitor,

inactivates glucagon-like peptide-1 (GLP-1) hormone and glucose-dependent insulinotropic polypeptide (GIP) released in relation to meals [28]. So, it inhibits hyperglycemia and hyperinsulinemia and improves fatty liver [29]. But there is no study examined the effect of Sitagliptin on fatty liver in absence of diabetes. So, the aim of the present study was to investigate the effect of Sitagliptin on Methionine induced fatty liver in non-diabetic albino rats.

As shown in the current results, high Methionine enriched diet increased liver enzymes, lipid profiles, serum iron, and serum homocysteine and decreased tissue reduced glutathione. While treatment by Sitagliptin decreased liver enzymes, lipid profiles, serum iron, serum homocysteine and increased tissue reduced glutathione.

The hepatic damaging effect of high Methionine diet in the current study was in agreement Gomez et al. (2009), who explained it by increased mitochondrial reactive oxygen species (ROS) production and the oxidative damage in rat liver mitochondrial DNA [30]. Additionally, the study by Yamada and coworkers demonstrated that feeding high Methionine diet induces liver cells injury in mice via changing the hepatic lipid metabolism and inducing oxidative stress [31].

As demonstrated in this study, there was significant increase in serum homocysteine in fatty liver group compared to control group. This can be explained by the fact that, the S-adenosylmethionine (SAM), the activated form of Methionine, is converted to S-adenosylhomocysteine (SAH) after donating its methyl group to methyl group acceptor molecules. Then, it is de-adenosylated to homocysteine. After intake of high Methionine diet, this metabolic end

product of the trans-methylation cycle of Methionine metabolism increases resulting in hyperhomocysteinemia (HHcy). It has been demonstrated that excess SAM, is a leading cause for hepatic steatosis and the development of NAFLD [32]. Furthermore, it was reported that hyperhomocysteinemia triggers hepatic steatosis by activating endoplasmic reticulum (ER) stress responses [33]. This ER stress responses lead to producing oxidative stress, inflammatory cell infiltration and fibrosis [34]. Moreover, high levels of Hcy thiolactone (HTL); an intramolecular thioester of Hcy, as a result of hyperhomocysteinemia were demonstrated in many human and animal studies [35]. Interestingly, HTL has been shown to induce oxidative stress [36] which has been involved in many pathological conditions associated with hyperhomocysteinemia [37]. Previous studies had successfully reported the pathophysiological outcome of hyperhomocysteinemia in vivo by using a high-Methionine/low-folate diet or gene-modified animal models [38].

Furthermore, in the same line with the results of the current study, it has been demonstrated that diet supplemented with Methionine increased plasma hydroperoxides and LDL-cholesterol [39], serum cholesterol due to increased hepatic cholesterol synthesis [40], raised iron and lipid peroxidation, conjugated dienes in rat liver, alters liver antioxidant enzymes and glutathione [41]. Sitagliptin is an oral anti-diabetic drug, has been proven to have antioxidant and anti-inflammatory effects in many body organs [42,43,44]. Regardless the hypoglycemic effect of Sitagliptin, it has been documented to have other beneficial effects in diabetic patients. Among them, it

ameliorates liver steatosis by inhibiting fatty acid synthesis in the liver [45,46]. Additionally, Sitagliptin was shown to improve the oxidative stress responses in diabetic nephropathy [47]. On the opposite side, there is only one study by Kumar et al. [14], stated that sitagliptin increased hepatic markers of oxidative stress in rats fed a high Cholesterol diet. But none of these studies have investigated the possibility of the hepatic protective effect of Sitagliptin after feeding with high Methionine diet only.

In the study performed by Onovama [48], it has been reported that treatment with Sitagliptin enhanced peroxisome proliferator activated receptor (PPAR- α) and attenuated sterol regulatory element-binding protein 1c (SREBP1c) and (fatty acid synthase) FAS. In other words, Sitagliptin decreased hepatic steatosis by attenuating lipogenesis and stimulating lipolysis. Also, it has been shown that DPP4i enhances the hepatic levels of phosphorylated AMP-activated protein kinase, which leads to suppression of de novo lipogenesis. Therefore, our results are in agreement with those of previous studies, and the effect of DPP4i on hepatic lipid metabolism may be the mechanism for improving steatohepatitis.

There is great evidence that excess iron is usually associated with more liver injury in NAFLD, although the exact mechanisms are still unclear [49]. The effect of hepatic iron on the pathogenesis of NAFLD is usually due to production of reactive oxygen species by iron. In non- alcoholic steatohepatitis (NASH), oxidative stress causes cell death through depletion of ATP, NAD and glutathione, and by direct DNA, lipids and proteins damage inside liver cells.

Furthermore, oxidative stress leads to an increase in the production of pro-inflammatory cytokines and a fibro genic response [50]. Therefore, the ability of Sitagliptin to reduce serum iron level, as demonstrated in the current results can be used as an explanation for the associated increase in the hepatic reduced glutathione level, as an antioxidant parameter, and the associated improvement in liver functions parameters.

It is well known that homocysteine is correlated with oxidative stress formation [51]. There are many studies found a significant association between NASH and higher level of HCY [52]. Moreover, some studies found negative correlation between HCY values and reduced glutathione (GSH) [53,54]. This suggests the real role of hyperhomocystinemia in the pathogenesis of NAFLD. In this study, Sitagliptin succeed to reduce serum homocysteine levels and so protect liver cells from the damaging effect of homocysteine. This was in agreement with the studies who reported that Linagliptin, Sitagliptin, and MK-0626 can improve hepatic steatosis [55,56,57]. Additionally, there was a clinical study which demonstrated that Vildagliptin can decrease liver triglyceride content by about 27% in type 2 diabetic patients [58]. Moreover, Vildagliptin was found to decrease ER stress. ER stress occurs when protein maturation in the ER is impaired, which leads to a characteristic stress response named the unfolded protein response (UPR). However, prolonged activation of UPR is correlated with many metabolic diseases, such as insulin resistance, type 2- diabetes, and hepatic steatosis [59]. So, when ER stress is inhibited hepatic steatosis will be decreased [60].

The results of Wang et al. (2018) have reported that treatment of NASH and liver fibrosis with Sitagliptin and Linagliptin significantly decreased parameters of steatosis and inflammation. Gliptins, via suppression of inflammation decrease steatosis, apoptosis, oxidative stress, and vascular dysfunction in murine models of NASH and liver fibrosis, with mild direct anti-fibrotic properties. They reduce the numbers of liver and vascular inflammatory monocytes/macrophages and induce their alternative polarization, with beneficial effect on NASH-associated hepatic fibrosis [61]. Unfortunately, Joy and his coworkers found that Sitagliptin did not improve the fibrotic score or NASH after 24 weeks of therapy. This may be due to the different dose and duration of therapy [62].

Conclusion:

The present study elucidates that sitagliptin as a DDP4i can improve NAFLD, which was induced by high methionine diet and preserves the liver function. The study revealed that oxidative stress, increase serum iron and serum homocysteine could be some of the underlying mechanisms in methionine induced hepatotoxicity.

Conflict of interest:

The authors elucidate that there is no conflict of interest associated with this work.

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