

Effects of Glucagon-like Peptide-1 Analogues and Dipeptidyl Peptidase-IV Inhibitors on Hepatic Oxidative Stress and Non-Alcoholic Steatohepatitis in Male Albino Rats

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ABSTRACT

Background and Aim: Due to their importance in glucose metabolism and lipid accumulation, in addition to their prolonged action, overall safety and efficacy; glucagon-like peptide-1 receptor agonists (GLP-1 RAs) and dipeptidyl peptidase-IV inhibitors (DPP-IV inhibitors) are becoming one of the cornerstones for treatment of obesity, T2DM and NASH. This study aimed to distinguish between the effect of both *Liraglutide* (a long-acting GLP-1 analog) and *Sitagliptin* (a DPP-IV inhibitor) on nutritional steatohepatitis in adult male rats. **Methods:** A total of 40 male Sprague-Dawley rats were divided into four groups; the normal control group and the steatohepatitis placebo group; steatohepatitis liraglutide-treated and steatohepatitis sitagliptin-treated groups. Liver weight and functions, lipid profile, glycemic status, hepatic oxidant-antioxidant parameters and histopathological analysis were assessed in comparison with the control rats. **Results:** Treatment of steatohepatitis in rats with either liraglutide or sitagliptin significantly reduced the levels of blood sugar, insulin, HOMA-IR, hepatic MDA and nitric oxide (NO). Moreover, they significantly increased the antioxidant enzyme activities, improved the histopathological changes compared to the control rats, with slightly superior effects for Liraglutide. **Conclusion:** Treatment with either GLP-1 RAs or DPP-IV inhibitors improved the hepatocyte viability. Treatment improved the hepatic fatty deposition, attenuated the progression of hepatic fibrosis and reduced the hepatic oxidative stress in male steatohepatitis rats, with slightly superior effects for GLP-1 RAs.

Key words: GLP-1 analogues, DPP-IV Inhibitors, Hepatic Oxidative Stress, NASH, Liver function.

INTRODUCTION

Non-alcoholic steatohepatitis (NASH), although it is one of the most important liver diseases worldwide, there is no available effective treatment. Also, its pathogenesis is not fully understood.^[1] Glucagon-like peptide-1 (GLP-1) is a gastrointestinal incretin peptide found initially to improve glucose homeostasis. It seems that GLP-1 affects glucose metabolism via stimulation of insulin synthesis and secretion in a glucose-dependent manner, repression of postprandial glucagon secretion and delaying stomach emptying.^[2] Enteroendocrine L-cells in the small intestine (duodenum, distal ileum and colon) synthesize GLP-1 from proglucagon in the in response to oral nutrient ingestion.^[3] Once in circulation, it is rapidly degraded by endogenous ubiquitous enzyme dipeptidyl peptidase-IV (DPP-IV), this makes its half-life too short (1 to 2 min).^[4] That's why, GLP-1 receptor agonists such as (exenatide, liraglutide, CJC-1131, CJC-1134 and albugon) with increased resistance to dipeptidyl peptidase-IV, or DPP-IV inhibitors such as (sitagliptin, saxagliptin, vildagliptin, alogliptin and linagliptin) have been used in human and

animal studies to overcome this obstacle.^[5] Recent studies have shown an effect of GLP-1 on glucose metabolism and lipid accumulation, along with its overall safety and efficacy, all of which make GLP-1, one of the critical pillars of the treatment of NASH associated type 2 diabetes and obesity.^[6] The present study aimed to compare the therapeutic effects of *Liraglutide* "Victoza" (a long-acting GLP-1 analog) and *Sitagliptin* "Januvia" (a DPP-IV inhibitor) on nutritional steatohepatitis in adult male rats.

MATERIALS AND METHODS

The ethics committee of Taif University, Saudi Arabia, approved this study. Liraglutide, Sitagliptin and enzymes were purchased from Novo Nordisk (Mill Rd, Princeton, NJ). Other chemicals were obtained from the purest grade available.

Animals

Forty adult male Sprague-Dawley rats (age, 9-11 weeks; body weight, 100-150 g) were obtained from Nile Pharmaceuticals Co (Cairo, Egypt). Animals were

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treated following the Guide for the Care and Use of Laboratory Animals (8th edition, National Academies Press). The animals were acclimatized for two weeks before inclusion in the experiment.

Experimental Design

Induction of non-alcoholic steatohepatitis (NASH)

After acclimatization, thirty rats were exposed to a high-fat diet (HFD) (D12451, Research Diets, NJ) containing 45% fat (kcal %), 35% carbohydrate and 20% protein for four weeks to develop NASH that was confirmed by liver biopsy.^[7] Rats had higher liver enzymes, blood sugar, insulin, HOMA-IR, total cholesterol, LDL and triglycerides than the normal control rats.

Grouping

After induction of NASH, rats were randomly divided into four equal groups ($n=10$) treated as follows: normal control group (NG); administered water; steatohepatitis placebo-treated group (SPG); steatohepatitis liraglutide-treated group (SLG); treated with freshly prepared liraglutide, given by subcutaneous injection at a dose of 200µg/kg/day,^[8] and steatohepatitis sitagliptin-treated group (SSG); treated with freshly prepared sitagliptin given by gastric tube at a dose of 2mg/kg/day as a bolus every afternoon.^[9] Animals were treated for 12 weeks.

Biochemical Analysis

The next day after treatment, blood samples were obtained from the rat tail veins and immediately separated via centrifugation at 2000 rpm for 15 min at 4°C and were stored at -80°C until further use. The separated serum was used for the determination of fasting and postprandial blood sugar, insulin, insulin resistance index (HOMA-IR), liver enzymes, alkaline phosphatase and lipid profile.

Evaluation of Oxidative Stress

Reduced glutathione (GSH) was assessed by treating 0.5mL of the supernatant with 0.5 mL Ellman's reagent (19.8 mg of 5,5'-dithio-2-nitrobenzoic acid in 100 mL of 0.1% sodium nitrate) and 3 mL of phosphate buffer (0.2 M, pH 8).^[10] Ellman's reagent was reduced by -SH group of (GSH) to form 5,5'-dithio-2-nitrobenzoic acid in phosphate buffer producing a yellow color, which was read at 412 nm.^[11] Lipid peroxide was determined colorimetrically by determining the tissue MDA content in the form of thiobarbituric acid reactive substances using 1,1,3,3-tetramethoxypropane as a standard.^[12] Catalase (CAT) activity in the supernatant was measured spectrophotometrically by adding 50µl of the supernatant to 2.95 mL of 19mM H₂O₂ solution (in 50mM potassium phosphate buffer pH 7.05). The changes in the absorbance at 240 nm were monitored spectrophotometrically over 5 min periods. Each unit of CAT activity represents the amount of CAT required to decompose 1µmol of H₂O₂/min at pH seven at 25°C.^[11] The activity of superoxide dismutase (SOD) was determined by assessing the inhibition of pyrogallol autooxidation. The changes in the absorbance were recorded at 420 nm for 5 min at a 1-min interval.^[12] Glutathione peroxidase (GPx) activity was assessed using a Glutathione Peroxidase Assay Kit (ab102530; Abcam) and the results were expressed as units/mg protein.^[13] For measurement of the plasma total antioxidant capacity (TAC), a TAC kit (Abcam, Cambridge, UK) was used, where the plasma was allowed to reduce Cu²⁺ at room temperature for 1.5hr according to the manufacturer's instructions. With a colorimetric probe, the reduced Cu⁺ was chelated and the absorbance was measured at 570 nm. According to a Trolox standard curve, the results were expressed as Trolox equivalent.

Histopathological Study

After the collection of blood samples, the animals of different groups were sacrificed under light anesthesia (diethyl ether). A small piece of hepatic tissue was removed for histological analysis. The sample was fixed by immersing it in 10% neutral-buffered formalin. The sample was then embedded in paraffin, sliced into 5 µm sections and stained with hematoxylin-eosin and Masson's trichrome stain for histological assessment. The degree of liver tissue injury was evaluated. The stained 5 µm sections were graded as follows: 0, absent; 1, minimal; 2, mild; 3, modest; 4, severe.^[14] The histological changes were evaluated in nonconsecutive, randomly chosen ×100, 200 and 400 histological fields using a light microscope, Nikon Eclipse E200.

Statistical Analysis

The results were analyzed for statistical significance by Statistical Package in Social Science (SPSS) version 16.0 for Windows (SPSS Inc., Chicago, USA). All data were expressed as mean ± SEM of 10 animals in each group. A value of $P \leq 0.05$ was considered statistically significant.

RESULTS

Effect of Treatment on Liver Weight and Liver Function

Table 1 revealed that the placebo-treated steatohepatitis rats had significantly ($P < 0.05$) higher liver weight as compared with the normal control rats. However, treatment of steatohepatitis rats with liraglutide or sitagliptin revealed a significant ($P < 0.05$) decrease in liver weight when compared with the placebo-treated steatohepatitis rats. Regarding the liver functions, Table 1 also showed a significant ($P < 0.05$) increase in the serum levels of AST, ALT and ALP in placebo-treated steatohepatitis rats when compared with the normal control rats. Whereas treatment with either liraglutide or sitagliptin significantly ($P < 0.05$) reduced the AST, ALT and ALP levels compared with the placebo-treated steatohepatitis rats.

Effect of Treatment on Glycemic Status and Lipid Profile

Table 2 revealed that the placebo-treated steatohepatitis rats had significantly ($P < 0.05$) higher levels of FBG and Postprandial BG compared with the normal control rats. Moreover, treatment with liraglutide or sitagliptin significantly ($P < 0.05$) reduced the levels of FBG and Postprandial BG compared with that in placebo-treated steatohepatitis rats. Concerning the insulin level and HOMA-IR, placebo-treated steatohepatitis rats revealed a significant ($P < 0.05$) increase in insulin level and HOMA-IR compared with the normal rats.

Table 1: Effect of treatment on the liver weight and liver function in HFD-induced steatohepatitic rats.

Parameters	Groups			
	NG	SPG	SLG	SSG
Liver Weight (g)	1.88 ± 18.13	3.33 ± 67.8 ^a	2.02 ± 23.4 ^b	2.13 ± 18.6 ^b
ALT (IU/L)	42.17 ± 16.53	193.4 ± 61.04 ^a	72.26 ± 51 ^b	87.1 ± 71.1 ^b
AST (IU/L)	54.28 ± 0.55	227.9 ± 0.31 ^a	86.1 ± 0.72 ^b	98.3 ± 0.48 ^b
ALP (IU/L)	51.92 ± 0.52	214.9 ± 0.37 ^a	90.1 ± 0.65 ^b	102.1 ± 0.68 ^b

Data are expressed as mean ± standard error of mean, $n = 10$ rats.

NG, normal control; SPG, steatohepatitic placebo-treated group; SLG, steatohepatitic liraglutide-treated group; SSG, steatohepatitic sitagliptin-treated group;

^aSignificantly different from NG; ^bSignificantly different from diabetic control group.

Table 2: Effect of treatment on glycemic status and lipid profile in HFD-Induced steatohepatic rats.

Parameters	Groups			
	NG	SPG	SLG	SSG
FBG (mg/dl)	67.5 ± 11.3	138.4 ± 66.2 ^a	68.9 ± 60.8 ^b	73.5 ± 32.1 ^b
Postprandial BG (mg/dl)	97.11 ± 14.3	187 ± 60.4 ^a	103 ± 41.03 ^b	111.4 ± 51 ^b
Insulin (ng/dl)	2.96 ± 0.25	6.1 ± 0.71 ^a	3.02 ± 0.41 ^b	3.76 ± 0.52 ^b
HOMA-IR	1.95 ± 0.16	3.99 ± 1.28 ^a	2.54 ± 1.18 ^b	2.81 ± 0.49 ^b
Total cholesterol (mmol/L)	93.3 ± 9.55	148.7 ± 7.3 ^a	107.1 ± 5.8 ^b	104 ± 14.2 ^b
HDL-cholesterol (mmol/L)	50.19 ± 3.2	27.33 ± 4.2 ^a	29.32 ± 4.9 ^a	33.98 ± 6.2 ^a
LDL-cholesterol (mmol/L)	70.21 ± 8.6	112 ± 9.5 ^a	68.46 ± 5.3 ^b	71.08 ± 6.2 ^b
Triglycerides (mmol/L)	71.15 ± 5.3	84.4 ± 8.8 ^a	69.5 ± 5.7 ^b	65.99 ± 7.3 ^b

Data are expressed as mean ± standard error of mean, *n* = 10 rats.

NG, normal control; SPG, steatohepatic placebo-treated group; SLG, steatohepatic liraglutide-treated group; SSG, steatohepatic sitagliptin-treated group;

^aSignificantly different from NG; ^bSignificantly different from diabetic control group.

However, treatment with liraglutide and/or sitagliptin significantly (*P* < 0.05) decreased the insulin level and HOMA-IR compared to that in placebo-treated steatohepatitis rats.

Concerning the lipid profile, Table 2 revealed that the placebo-treated steatohepatitis rats had significantly (*P* < 0.05) higher levels of total cholesterol, LDL-cholesterol and triglycerides compared with the normal control rats. Moreover, their levels were significantly (*P* < 0.05) reduced after treatment with liraglutide and/or sitagliptin as compared with the placebo-treated steatohepatitis rats.

Effect of Treatment on Hepatic Oxidant/Antioxidant Parameters

Table 3 revealed that the placebo-treated steatohepatitis rats had significantly (*P* < 0.05) higher levels of MDA and NO compared with normal rats. Treatment with liraglutide or sitagliptin significantly (*P* < 0.05) reduced the level of MDA and NO compared with that in placebo-treated steatohepatitis rats. Placebo-treated steatohepatitis rats showed a significantly (*P* < 0.05) lower GSH, GPx, SOD, CAT and TAC levels as compared with normal rats; whereas, such effects were significantly (*P* < 0.05) improved by either drug treatment.

Effect of Treatment on Histopathological Changes

Microscopic examination of the liver sections of the control animals (NG) showed the normal architecture of hepatic lobules in the form of hepatocytes arranged from the portal vein (Figure 1). Examination of rat livers from the steatohepatitis placebo-treated group showed loss of architecture, hepatocytes ballooning, macro- and microvesicular steatosis, inflammation, apoptotic nuclei, occasional binucleation, cellular inflammatory infiltrate and bridging fibrosis and pericellular/perisinusoidal fibrotic changes (Figure 2-4). These changes were remarkably improved in the steatohepatitis liraglutide-treated group (SLG, Figure 5) than the steatohepatitis sitagliptin-treated group (SSG, Figure 6).

Table 3: Effect of treatment on oxidative stress markers in HFD-Induced steatohepatic rats.

Parameters	Groups			
	NG	SPG	SLG	SSG
MDA (nmol/mg protein)	1.77 ± 0.13.	4.71 ± 0.34 ^a	2.72 ± 0.41 ^b	2.91 ± 0.15 ^b
NO (μmol/L)	4.89 ± 1.73	21.79 ± 4.6 ^a	8.09 ± 1.11 ^b	8.76 ± 2.32 ^b
GSH (μg/mg protein)	7.09 ± 1.05	3.56 ± 0.88 ^a	8.50 ± 0.72 ^b	8.31 ± 1.08 ^b
GPx (Units/mg protein)	28.8 ± 2.82	18.77 ± 1.9 ^a	25.97 ± 3.08 ^b	26.74 ± 2.1 ^b
SOD (Units/mg protein)	5.03 ± 0.43	2.51 ± 0.75 ^a	4.38 ± 1.39 ^b	4.15 ± 1.13 ^b
CAT (μmol H ₂ O ₂ /min/mg protein)	7.11 ± 1.56	3.15 ± 0.56 ^a	4.42 ± 1.07 ^b	4.48 ± 1.16 ^b
TAC (μmol Trolox Eqv/mg protein)	2.41 ± 0.22	1.45 ± 0.46 ^a	2.27 ± 0.19 ^b	2.22 ± 0.13 ^b

Data are expressed as mean ± standard error of mean, *n* = 10 rats.

NG, normal control; SPG, steatohepatic placebo-treated group; SLG, steatohepatic liraglutide-treated group; SSG, steatohepatic sitagliptin-treated group;

^aSignificantly different from NG; ^bSignificantly different from diabetic control group.

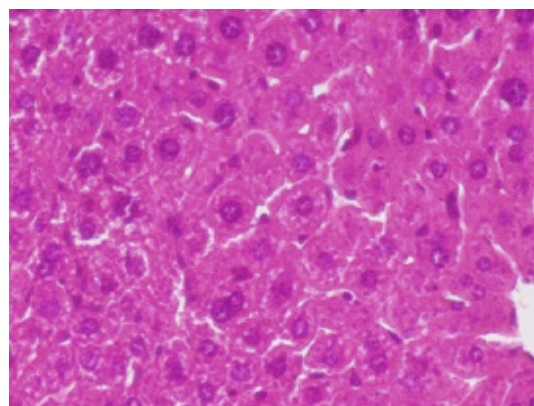


Figure 1: Microscopic examination from the liver tissue of control group showing normal hepatic structure (H&E, 100x).

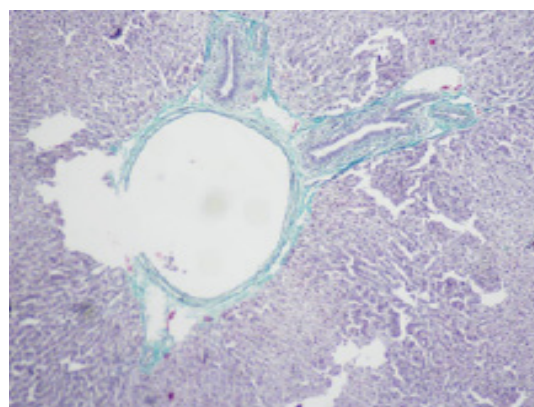


Figure 2: Microscopic examination from the liver tissue of placebo-treated group showing periportal and bridging fibrosis (Masson's trichrome stain, 100x).

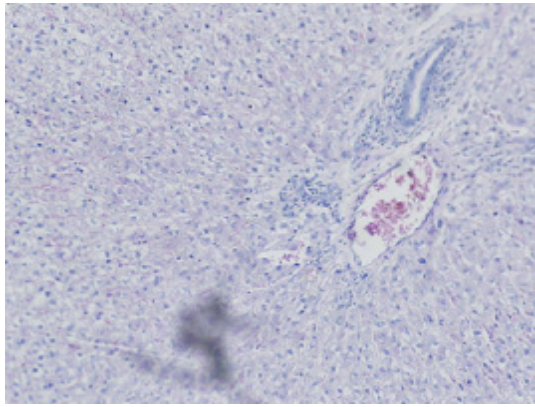


Figure 3: Microscopic examination from the liver tissue of placebo-treated group showing hepatocyte ballooning, steatosis and inflammatory infiltrate (H&E, 100x).

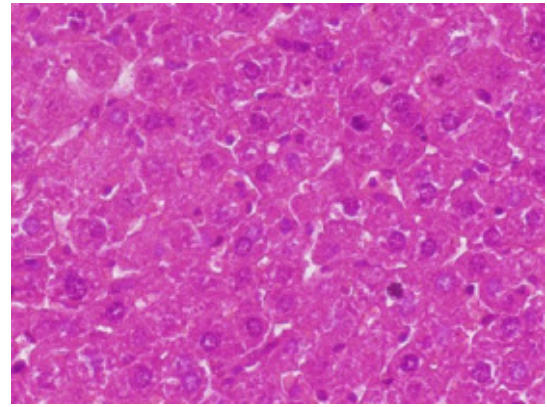


Figure 5: Microscopic examination from the liver tissue of steatohepatic liraglutide-treated group showing restored liver architecture with near normal hepatocytes (H&E, 200x).

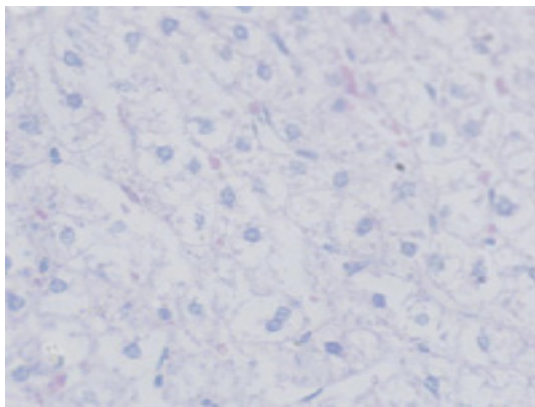


Figure 4: Microscopic examination from the liver tissue of placebo-treated group showing ballooning degeneration and apoptotic bodies in centrilobular portion (H&E, 400x).

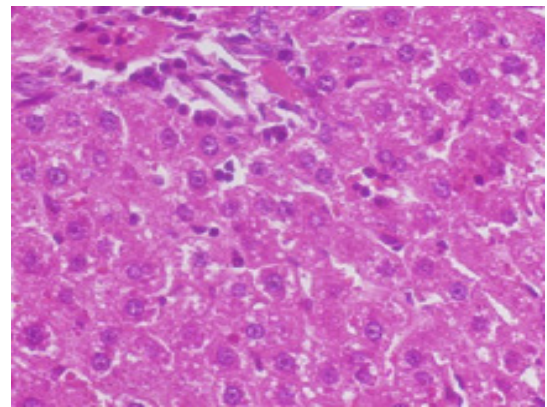


Figure 6: Microscopic examination from the liver tissue of steatohepatic sitagliptin-treated group showing scattered microvesicular steatosis (H&E, 200x).

DISCUSSION

This study compared the therapeutic effects of a long-acting GLP-1 receptor agonist “liraglutide” and a dipeptidyl peptidase-IV inhibitor “sitagliptin,” on nutritional steatohepatitis in adult male rats. Results of the present study revealed that the treatment of steatohepatitis rats with liraglutide or sitagliptin improves the steatohepatitis-induced deterioration of liver functions proved by decreasing the liver enzymes, alkaline phosphatase along with diminishing the liver weight. These effects clearly appeared with GLP-1R agonist as it binds to its specific receptors on hepatocytes,^[15] and significantly reduces the hepatic fatty acid content, improves the hepatic markers and consequently the hepatocyte viability.^[16,17] Recent studies also revealed similar results with sitagliptin which improves the hepatic fatty deposition and inhibits inflammation of hepatocytes in obese male rats.^[18,19]

Regarding the deteriorated glycemic status secondary to NASH induction in adult male rats, our results revealed a significant reduction of fasting, postprandial BG and insulin resistance after treatment with either liraglutide or sitagliptin. Previous studies revealed similar results with GLP-1R agonists,^[20,21] where the authors reported that GLP-1 analogues reduce the blood glucose in obese mice by increasing its hepatic uptake and also by reducing hepatic gluconeogenesis by inhibiting the enzymes (glucose-6-phosphatase and phosphoenolpyruvate carboxykinase). Park *et al.*^[22] also reported that GLP-1R agonists decrease the hepatic glucose output and improve the hepatic insulin signaling in HFD fed diabetic

rats via potentiation of tyrosine phosphorylation of the insulin receptor substrate-2. Similar results also recorded for sitagliptin in a study conducted by Tessari *et al.*^[23] who found that sitagliptin attenuates degradation of GLP-1 and consequently increases insulin and reduces blood glucose levels.

Concerning the resulting dyslipidemia (increased total cholesterol, LDL-cholesterol and triglyceride levels and decreased HDL-cholesterol level) after NASH induction, results of our study reveals the effectiveness of treatment with either liraglutide or sitagliptin in improving the lipid profile of steatohepatitis rats proved by significant reduction of the total cholesterol, LDL-cholesterol and triglycerides levels with no significant improvement in HDL-cholesterol level after treatment. Sun *et al.*^[24] have previously recorded such conclusions for liraglutide and Scott *et al.*^[25] and Shirakawa *et al.*^[26] for sitagliptin. The lipid-lowering effect of GLP-1 may be attributed to its ability to reduce the intestinal absorption of dietary lipids, either by deceleration of gastric emptying^[27] or inhibition of gastric lipase enzyme and inhibition of lymph flow.^[28] Farr *et al.*^[29] reported that GLP-1 possibly produced its lipid-lowering effect via reduction of chylomicrons formation after fatty meals and/or augmentation of hepatic fatty acid oxidation or autophagy. Suppression of lipolysis secondary to increased insulin and decreased glucagon secretion is an additional mechanism for the lipid-lowering effect of GLP-1.^[30]

Concerning the hepatic antioxidant status, the present study revealed increased tissue concentration of NO, MDA and reduction of antioxidant enzymes activities and TAC secondary to NASH induction. Li *et al.*^[31]

reported that oxidant/antioxidant imbalance greatly disturbs the homeostasis and the resulting oxidative stress enhances liver diseases and other chronic degenerative disorders, including diabetes and its complications, neurological disorders and cancer. After treatment with liraglutide or sitagliptin, our results revealed a significant reduction of NO and MDA levels and restoration of antioxidant enzymes activities and TAC. A recent study by Allam *et al.*^[32] showed that administration of GLP-1 analogs to steatohepatitis mice restores the oxidant/antioxidant balance, which is necessary for proper liver cell functions and various metabolic processes. Ding *et al.*^[33] also recorded a significant reduction in the oxidative stress markers in the liver tissue of mice with hepatic steatosis after treatment with GLP-1 analogs. Activation of GLP-1 receptors activates protein kinase A (PKA) and increases the production of cAMP via increasing intracellular Ca²⁺ and stimulation of adenylate cyclase enzyme.^[34] Both PKA and cAMP are inhibitors of NOX and, accordingly, ROS production.^[35] However, the role of GLP-1 varies in different cell types and has to be investigated depending on various factors, such as stimuli, species and the expression of the GLP-1R.

Finally, in this study, the histopathological evaluation of liver tissues showed loss of architecture, hepatocytes ballooning, macro- and microvesicular steatosis, inflammation, apoptotic nuclei, occasional binucleation, cellular inflammatory infiltrate and bridging fibrosis and pericellular fibrotic changes in steatohepatitis placebo-treated group. These changes were remarkably improved in the liraglutide-treated group than the sitagliptin-treated group. Liraglutide decreased hepatocyte ballooning, steatosis, inflammation, although not reaching statistical significance in comparison with sitagliptin. Bridging fibrosis was observed in most in the steatohepatitis placebo-treated group and was not significantly affected by treatments. Portal inflammation was not significantly different among liraglutide-treated and sitagliptin-treated groups, although a nominal reduction was observed by treatment with liraglutide.

CONCLUSION

Treatment with either GLP-1 RAs or DPP-IV inhibitors improved the hepatocyte viability by inhibiting its inflammation, improving the hepatic fatty deposition and attenuating the progression of hepatic fibrosis. These drugs also correct resulting dyslipidemia, histopathological changes and reduced the hepatic oxidative stress in male steatohepatitis rats, with slightly superior effects for GLP-1 RAs.

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CONFLICT OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

ABBREVIATIONS

GLP-1 RAs: Glucagon-like peptide-1 receptor agonists; **DPP-IV:** Dipeptidyl peptidase-IV; **NO:** Nitric oxide; **MDA:** Malondialdehyde; **NASH:** Non-alcoholic steatohepatitis; **HFD:** High-fat diet; **NG:** Normal control group; **SPG:** Steatohepatitis placebo-treated group; **SLG:** Steatohepatitis liraglutide-treated group; **SSG:** Steatohepatitis sitagliptin-treated group; **GSH:** Reduced glutathione; **CAT:** Catalase; **GPx:** Glutathione peroxidase; **SOD:** Superoxide dismutase; **TAC:** Total antioxidant capacity; **PKA:** Protein kinase A.

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