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Role of linagliptin in preventing the pathological progression of hepatic fibrosis in high fat diet and streptozotocin-induced diabetic obese rats

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ABSTRACT

Liver fibrosis is a common complication of diabetes mellitus, with a major global public health concern. Linagliptin, a dipeptidyl peptidase-4 inhibitor (DPP-4), is classically used to treat type 2 diabetes mellitus and improves insulin resistance. Additional potential influences of linagliptin on liver fibrosis are still unclear. The present study was undertaken to investigate the therapeutic credit of linagliptin in hepatic fibrosis induced by a high-fat diet (HFD) and streptozotocin (STZ) in rats. Moreover, the mechanisms underline its anti-fibrotic effect were explored. To induce liver fibrosis with T2DM; male Sprague-Dawley albino rats were fed on a high-fat high-sucrose diet for 28 days then exposed to a single dose of STZ (30 mg/kg, IP). After two days of STZ injection, a diabetes confirmation test was done and all diabetic rats were constantly fed on HFD for thirty days with or without treatment with linagliptin (6 mg/kg). Hepatotoxicity markers, lipid profile screening, insulin signaling, inflammatory cytokines (TNF-α, IL-6, NF-κB p65), fibrosis markers (Collagen, α-SMA, TGF-β1) and histopathological studies including hematoxylin and eosin (H&E) as well Masson's trichrome stains were performed. In our preliminary study, linagliptin at a dose of 6 mg/kg was chosen as the optimum anti-diabetic dose in rats challenged with STZ. Linagliptin significantly improved insulin sensitivity and lipid profile and reduced inflammatory mediators, and collagen depositions in rats with liver fibrosis and T2DM. In conclusion, above and beyond its anti-diabetic effect, this study introduced linagliptin as a promising option for preventing the pathological progression of liver fibrosis associated with T2DM.

1. Introduction

Type 2 diabetes mellitus (T2DM) is linked to several liver abnormalities, such as non-alcoholic steatohepatitis (NASH), non-alcoholic fatty liver disease (NAFLD), fibrosis, cirrhosis and hepatocellular carcinomas (HCCs) (Saponaro et al., 2015). Liver fibrosis is a common pathological public health concern of T2DM complications with an estimated prevalence of 15.5% (De Lédinghen et al., 2012). Growing evidence links T2DM related liver fibrosis to insulin resistance and chronic inflammatory state (Mohamed et al., 2016). Fatty liver and hyperglycemia seen among diabetic patients may worsen insulin resistance thus lead to severe inflammatory responses and destruction of hepatocytes (Arkan et al., 2005).

Unfortunately, Inflammation recruits a plethora of proinflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and transforming growth factor-β1 (TGF-β1) leading to tissue damage and loss of cellular function (Rutledge and Adeli, 2007). Noteworthy, TGF-β1 induces morphologic and functional changes in activated hepatic stellate cells (HSCs) and transforms them into myofibroblastic ones. These cells are characterized by an elevated content of Alpha-smooth muscle actin (α-SMA) microfilament which plays an important role in fibrogenesis (Puche et al., 2013). Moreover, Extracellular matrix (ECM) proteins (e.g., collagen) will gradually substitute liver parenchyma leading to distortion of hepatic architecture and development of end-stage liver fibrosis (Hernandez-Gea and Friedman, 2011).

Linagliptin is a clinically available dipeptidyl peptidase-4 inhibitor (DPP-4) inhibitor that is well tolerated in type 2 diabetic patients (Russell-jones and Gough, 2012). Besides improving the glycemic index and insulin sensitivity, linagliptin demonstrated a reduction in fat accumulation, cytokines infiltration, and slows the progression of liver steatosis (Klein et al., 2014, 2012). These outcomes suggest that linagliptin may have a protective role against hepatic fibrosis but the exact mechanism remains to be elucidated. Moreover, linagliptin stands among other gliptins by being only excreted through the enteric system

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thus offering an option for diabetic patients with renal impairment (Sortino et al., 2013).

Thus, the present study aimed to explore the role of linagliptin in preventing the pathological progression of liver fibrosis in a high-fat diet (HFD) and streptozotocin (STZ) induced diabetic obese rats. Additionally, we explored the molecular mechanisms underlying this potential anti-fibrotic effect focusing on inflammation, fibrosis, and insulin resistance signaling pathways.

2. Material and methods

2.1. Animals

Male Sprague-Dawley albino rats, weighing 200–250 g, 4–5 months old were obtained from Nile Co., for Pharmaceuticals and Chemical Industries, Cairo, Egypt. Rats were housed at controlled conditions; temperature at 25 ± 1 °C, humidity 55%, light-dark regime: 12 h dark/12 h light, and access to food and water throughout the whole experimental period. Rats were housed in fiber cages with straw bedding and left for an initial adaptation period of one week before any experiment at the animal facility of the Faculty of Pharmacy, Heliopolis University for sustainable development (Cairo, Egypt). The rats were fed either normal chow diet or HFD according to the experimental design and the method of Qiang et al. (2012). Animal handling and experimental protocols were approved by the Research Ethical Committee of the Faculty of Pharmacy, Heliopolis University for sustainable development (Cairo, Egypt) with the serial number (HU.REC 4–2018) following the guide for the care and use of laboratory animals published by the US National Institutes of Health (NIH publication No.85-23, revised 1996) (Clark et al., 1997).

2.2. Chemicals and drugs

Linagliptin (Boehringer Ingelheim, Rhein, Germany) was dissolved in 1% carboxymethyl cellulose (CMC) (Al Gomhorya, Cairo, Egypt). STZ was obtained as a pure powder (Sigma-Aldrich Chemical Co., St. Louis, USA) and suspended in sodium citrate buffer (pH 4.5) using high-grade chemicals; trisodium citrate, saline and citric acid (Al Gomhorya, Cairo, Egypt). High-fat diet (HFD) was prepared using standard chow supplemented with sucrose, lard stearin, cholesterol, and cholic acid (Buchem B.V., Apeldoorn, Netherlands).

2.3. Experimental design

The rat model of liver fibrosis with T2DM was established as follows; rats were fed on high-fat high sucrose diet (10% sucrose, 10% lard stearin, 2% cholesterin, and 0.5% bile acid) for 28 days then injected with a single dose of STZ (30 mg/kg, IP). After two days of STZ injection, a diabetes confirmation test was done and all diabetic rats were constantly fed on HFD (10% lard stearin, 2% cholesterin, and 0.5% bile acid) till the end of the experiment (Qiang et al., 2012).

Sixty rats were randomly divided into four groups, each group containing fifteen rats and treated for 60 day as follows (Fig. 1): The first group was considered as a control group and was fed by a normal chow diet (5% fat, 53% carbohydrate, 23% protein) for sixty days. Starting from day 31, rats were given CMC 1% (the vehicle of linagliptin) daily till the end of the experiment. The second group was considered as liver fibrosis with the T2DM group and was fed by HFD-sucrose diet for the first month then by HFD for the second month. On day 28, rats were exposed to a single injection of STZ (30 mg/kg; IP). Then, starting from day 31, rats were given CMC 1% daily till the end of the experiment. The third group was considered as liver fibrosis with T2DM + linagliptin group and rats were fed by HFD and exposed to STZ as the previous group, then starting from day 31, rats were treated with linagliptin (6 mg/kg, by oral gavage) daily till the end of the experiment. The dose of linagliptin was chosen according to our preliminary study. The last group was given linagliptin alone (6 mg/kg, by oral gavage), daily starting from day 31, and fed by normal chew diet through the whole experiment of two months.

At the end of the experiment, rats were anesthetized by thiopental (Sandoz, Holzkirchen, Germany) injection, blood samples were collected from the heart in a serum separator tube then samples were allowed to clot for 30 min before centrifugation (Centurion, West Sussex, United Kingdom) at 373 × *g* for 15 min. The serum was then collected and stored at (−80 °C) until further processing. Specimens of the liver were dissected out in full depth and kept in 10% formalin for immunohistochemical and histopathological examination, other specimens were snap-frozen in liquid nitrogen and stored at (−80 °C) for gene expression, western blot, and biochemical analysis.

Liver and serum samples within each group were sub-classified into 3 subsets as follows; **The first subset (n = 6):** Left and the middle lobes of the liver were homogenized in buffer RLT to be prepared for Real-time polymerase chain reaction (RT-PCR) detection for the determination of IRS-1, PI3K, and PKB/Akt. The right and the caudate lobe were homogenized in ice-cold phosphate-buffered saline to be prepared for assessment of TG and NF-kB p-65 using enzyme-linked immunoassay (ELISA) technique and for TC to be detected colorimetrically. Serum was used for assessment of IRS-1, PI3K, PKB/Akt, IL-6, TNF-α, and TGF-β1 using ELISA technique and hydroxyproline using colorimetrical method.

The second subset (n = 6 rats): left and the middle lobes of the liver were fixed in 10% neutral buffered formalin for Masson's trichrome stain for collagen fibers detection and assessment of α-SMA immunohistochemically. The right and the caudate lobe were homogenized in ice-cold RIPA buffer to be prepared for immunoblotting assessment of phospho-IRS (Tyr612), phospho-IRS (Ser307) and phospho-Akt (Ser473). Serum was used for assessment of hepatotoxicity markers and insulin. The same subset was used for assessment of body weight, liver index, HOMA-IR index, and QUIKI.

The Third subset (n = 3): the liver tissues were fixed in 10% neutral formalin and stained by hematoxylin and eosin (H&E) stain for a routine examination.

2.4. Hepatotoxicity markers assessment

2.4.1. Serum parameters

Alanine transaminase (ALT) {Catalog no. 263 002}, aspartate transaminase (AST) {Catalog no. 261 002}, and total bilirubin {Catalog no. 222 001} were determined using test reagent kits (Spectrum, Cairo, Egypt). Fasting blood glucose **(**FBG) {Catalog no. GL 13 20}, total cholesterol (TC) {Catalog no. CH 12 20}, triglycerides (TG) {Catalog no. TR 20 30}, high-density lipoproteins (HDL) {Catalog no. CH 12 30}, and low-density lipoproteins (LDL) {Catalog no. CH 12 31} were measured using test reagent kits (Bio-diagnostic, Cairo, Egypt) according to the manufacturer's instructions.

2.4.2. Tissues parameters

Commercial ELISA kit was used to assess TG liver content {Catalog no. MBS726298} (MyBioSource, San Diego, USA), while TC liver content was measured colorimetry {Catalog no. STA-384}(Cell Biolabs, San Diego, USA), according to the manufacturer's directions.

2.4.3. Liver index

Liver index was calculated according to the formula; Liver index = (liver weight/body weight) x 100.

2.5. Insulin markers assessment

2.5.1. Real-time polymerase chain reaction analysis

Insulin receptor substrate 1 (IRS-1), phosphatidylinositol-3 kinase (PI3K) and protein kinase B/Akt (PKB/Akt) as insulin signaling markers were determined using RT-PCR. Total RNA was isolated from the liver

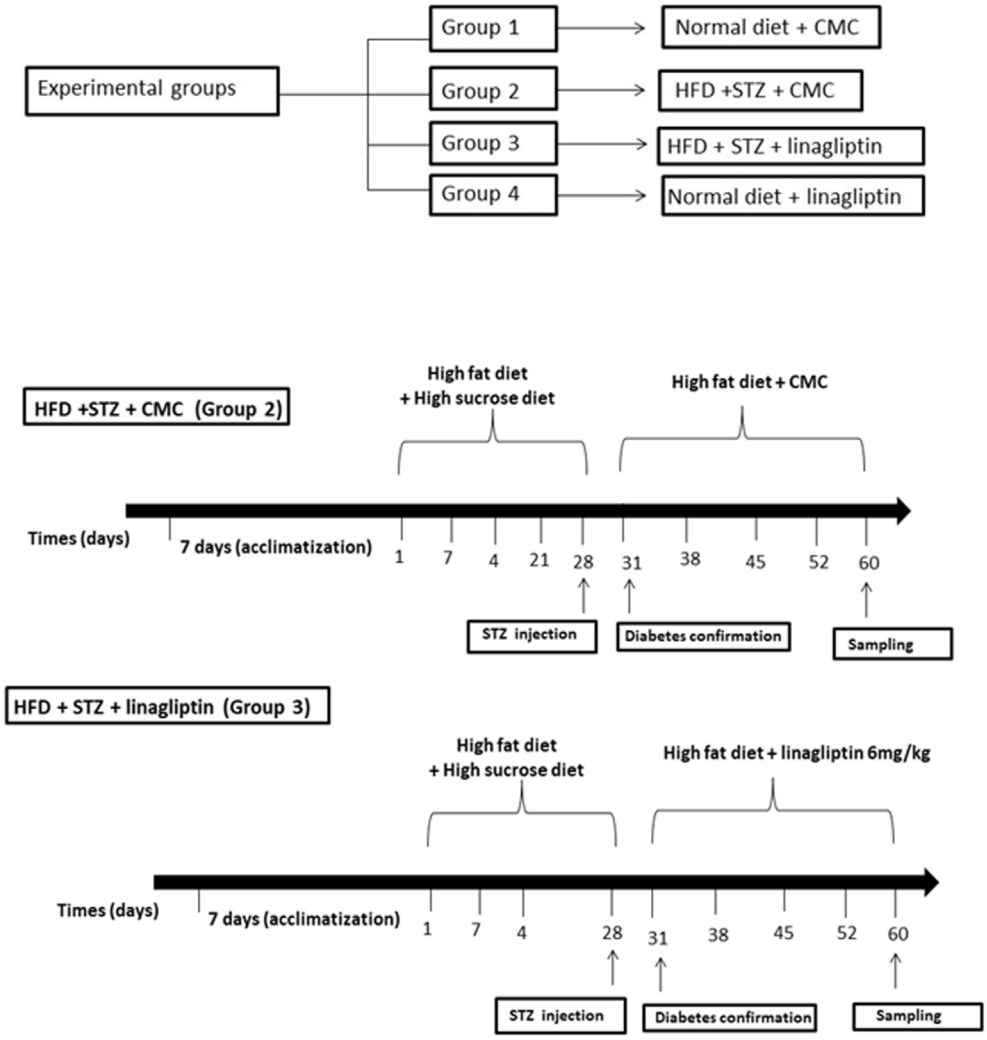

Fig. 1. Timeline representation of the experimental design showing experimental groups and treatments received.

specimens using a total RNA tissue extraction kit {Catalog no. 74104} (Qiagen, Redwood, USA) according to the manufacturer's instructions. Then, reverse-transcribed using RT-PCR kit {Catalog no. QP070} (Fermentas, Waltham, USA) and quantitative RT-PCR were performed using SYBR Green. The sequence of the upstream primer used for IRS-1 was 5′-GGACTTGAGCTATGACACGGG-3′ and the downstream primer sequence was 5′-GCCAATCAGGTTCTTTGTCTGAC-3′, while the sequence of the upstream primer used for PI3K was 5′-AGATGCTTTCAA ACGCTAT-3′ and the downstream primer sequence was 5′-GCTGTCGC TCACTCCA-3′, and the sequence of the upstream primer used for AKT was 5′-TCTATGGCGCTGAGATTGTG-3′ and the downstream primer sequence was 5′-CTTAATGTGCCCGTCCTTGT-3'.

2.5.2. Quantitative ELISA immunoassay

Commercial ELISA kits were used to assess; Fasting blood insulin (FBI) {Catalog no. E0448r} (EiAab, Wuhan, China), IRS-1{Catalog no. E2889}, PI3K {Catalog no. 30310} and PKB/Akt {Catalog no. G3458} (WKEA, Jilin, China) in the serum according to the manufacturer's instructions.

2.5.3. Estimation of homeostasis model assessment of insulin resistance and quantitative insulin sensitivity check index

The homeostasis model assessment of insulin resistance (HOMA-IR) was calculated according to Matthews et al. (1985) with the formula; HOMA-IR = FBI in mU/l × FBG in mg/dl/405.

While quantitative insulin sensitivity check index (QUICKI) was calculated according to Katz et al. (2000) with the formula; QUICKI = 1/(log [FBI in mU/l] + log [FBG in mg/dl]).

2.5.4. Immunoblotting

Liver tissue lysates were prepared in RIPA buffer, centrifuged and protein concentration was determined using Biovision's BCA Protein Assay kit. Protein samples were mixed with SDS-loading buffer and incubated at 90 °C (10 min). The samples were separated using SDS-PAGE gel electrophoresis then electrically shifted to polyvinylidene fluoride membranes. Afterward, the membrane was blocked with 5% nonfat dry milk in Tris-buffered saline-tween 20 (TBST) for 1 h and incubated overnight at 4 °C with the diluted primary antibody that recognizes phospho-IRS (Tyr612), phospho-IRS (Ser307), phospho-Akt (Ser473) and β-actin [diluted in 1:1 ratio of TBST (pH 7.4) and 5% BSA] (Cell Signaling Technology, Inc., Beverly, MA, USA). After washing with TBST three times, the membranes were incubated with a horseradish peroxidase linked secondary antibody (1:5000) (for 2 h, at room temperature). Finally, the quantitative protein band density was detected and assayed by using ImageJ software, version 1.48 (Rasband, 1997e2015). The signals were captured and normalized with β-actin to assess the fold change concerning respective controls.

2.6. Inflammatory markers assessment

Liver tissue NF-κB p65 {Catalog no. MBS015549} (MyBioSource, San Diego, USA), serum TNF-α {Catalog no. E0133r}, and serum IL-6

{Catalog no. E0079r} (EiAab, Wuhan, China) were measured using commercial ELISA kits.

2.7. Histopathologic examination

Liver tissue samples were fixed in 10% neutral buffered formalin for 72 h. Samples were trimmed and processed and dehydrated by serial grades of alcohol, cleared in Xylene, synthetic wax infiltration and embedding out into paraplast tissue embedding media. 5μ sections were cut by a rotatory microtome. The obtained tissue sections were collected on glass slides, deparaffinized, stained by hematoxylin and eosin (H&E) stain for routine examination (Bancroft and Stevens, 1990) and Masson's trichrome stain for collagen fibers (Sereno et al., 2014).

2.8. Fibrosis markers assessment

The serum level of TGF-β1 was assessed using the ELISA kit {Catalog no. E0124r} (EiAab, Wuhan, China). Liver content of (α-SMA) was examined immunohistochemically with ready-to-use primary antibody: rabbit polyclonal antibody to rat α-SMA {Catalog no. ab5694} (Abcam, Cambridge, UK), which was then visualized using Leica Application Suite attached to full HD microscopic imaging system (Leica Microsystems GmbH, Germany) and represented as the area percentage of the stained sections per field. Besides, liver fibrosis was evaluated biochemically by measuring hydroxyproline content as an index of collagen accumulation (Nemethy and Scheraga, 1986).

2.9. Statistical analysis

Results were expressed as means ± standard deviation. Comparisons between different means were carried out by one-way analysis of variance (ANOVA) followed by Tukey multiple comparison tests using GraphPad Prism (version 5) software at $P < 0.05$ and $n = 6$. Test for normality was carried out for data.

3. Results

3.1. Linagliptin effect on hepatotoxicity markers

Exposure of rats to HFD and STZ caused a significant elevation in serum levels of AST, ALT, and total bilirubin by 98, 85, and 106%, respectively, as compared to the control group. Treatment of rats with linagliptin significantly reduced these hepatotoxicity indices by 33, 36, and 50%, respectively, as compared to non-treated rats with liver fibrosis and T2DM (Table 1). Moreover, a significant increase in the liver index by 26% was observed in the group of liver fibrosis and T2DM as compared to the control group. Treatment of rats with linagliptin showed no significant change in the liver index when compared to non-treated rats with liver fibrosis and T2DM (Table 1).

3.2. Linagliptin effect on lipid profile

Rats exposed to HFD and STZ showed a significant increase in serum levels of TG, TC and LDL-cholesterol by 752, 60%, and 234%, respectively, as compared to the control rats. Treatment of rats with linagliptin induced a significant reduction in serum levels of triglycerides, total cholesterol and LDL-cholesterol levels by 81, 31 and 39%, respectively, as compared to non-treated rats with liver fibrosis and T2DM. No significant change was observed in HDL-cholesterol levels between all groups (Table 1).

Further analysis revealed that exposure to HFD and STZ caused a significant rise in TG and TC liver content by 123 and 116%, respectively, when compared to the control group. On the other hand, the T2DM and liver fibrosis group treated with linagliptin showed a significant decrease in TG and TC by 36 and 37%, respectively, as compared to the liver fibrosis model (Table 1).

3.3. Linagliptin effect on insulin sensitivity and insulin markers

As expected, the group of liver fibrosis and T2DM showed a significant increase in serum levels of FBG and HOMA-IR index by 594 and 96%, respectively, and a significant reduction in serum insulin levels and QUICKI by 74.5 and 11%, respectively, as compared to the control rats. Treatment of rats with linagliptin significantly reduced serum FBG level and HOMA-IR index by 80 and 49%, respectively, and significantly increased serum insulin levels and QUIKI by 166 and 12%, respectively, as compared to non-treated rats with liver fibrosis and T2DM. As well, the treatment of rats with linagliptin alone showed a significant increase in insulin level and HOMA-IR index by 64 and 35%, respectively as compared to the control group (Table 1).

3.4. Linagliptin effect on insulin signaling pathway

The present investigation revealed that rats exposed to HFD and STZ showed a significant reduction in IRS-1, PI3K and Akt/PKB mRNA expression by 85, 73 and 71%, respectively, as compared to the control

Table 1
The effect of linagliptin on hepatotoxicity and insulin markers.

	Control (Group 1)	Liver fibrosis + T2DM (Group 2)	Liver fibrosis + T2DM +linagliptin (Group 3)	Linagliptin (Group 4)
Bodyweight (g)	272.0 ± 6.95	286 ± 9.07[a]	305.5 ± 9.33[ab]	281.0 ± 7.21
Liver weight (mg)	7.07 ± 0.79	9.36 ± 0.977 [a]	8.70 ± 1.31 [a]	5.1 ± 0.56 [a]
Liver index %	2.6 ± 0.29	3.3 ± 0.30 [a]	2.9 ± 0.42	1.8 ± 0.19[ab]
AST (U/L)	50.97 ± 3.30	100.9 ± 6.59 [a]	67.22 ± 6.14 [ab]	51.84 ± 3.54[b]
ALT (U/L)	49.11 ± 3.41	90.75 ± 6.92 [a]	58.29 ± 6.68 [b]	48.53 ± 5.003[b]
Total bilirubin (umol/l)	0.17 ± 0.07	0.36 ± 0.04 [a]	0.18 ± 0.01 [b]	0.19 ± 0.05[b]
TG (mg/dl)	45.83 ± 5.56	390.7 ± 6.12 [a]	74.67 ± 3.386[ab]	59.67 ± 5.82[ab]
TC (mg/dl)	62.67 ± 5.39	100.3 ± 3.32 [a]	68.83 ± 5.74 [b]	71.00 ± 7.403[b]
Liver triglyceride content (mg/g protein)	79.35 ± 10.51	177.2 ± 11.80 [a]	113.9 ± 9.75 [ab]	66.88 ± 10.27 [b]
Liver cholesterol content (mg/g protein)	49.52 ± 11.72	106.8 ± 18.48 [a]	67.76 ± 13.82 [b]	40.52 ± 13.56 [b]
HDL (mg/dl)	19.67 ± 1.63	20.17 ± 1.47	18.33 ± 1.21	20.83 ± 2.041
LDL (mg/dl)	16.83 ± 3.06	56.17 ± 7.98 [a]	34.33 ± 5.27[ab]	32.33 ± 6.97[ab]
FBG (mg/dl)	89.83 ± 5.70	623.0 ± 42.17 [a]	122.3 ± 9.07 [b]	74.17 ± 4.70[b]
Insulin (mlU/L)	2.21 ± 0.15	0.61 ± 0.07 [a]	1.62 ± 0.155 [ab]	3.63 ± 0.35[ab]
HOMA-IR	0.49 ± 0.02	0.964 ± 0.16 [a]	0.49 ± 0.07 [b]	0.66 ± 0.043[ab]
QUIKI	0.43 ± 0.004	0.38 ± 0.01 [a]	0.43 ± 0.016 [b]	0.41 ± 0.004[ab]

Data were expressed as means of 6 rats ± S.D, comparisons between different groups were carried out using one-way Analysis of Variance (ANOVA) followed by Tukey multiple comparisons test. (a) Significantly different from control group at $P < 0.05$, (b) significantly different from hepatic fibrosis model at $P < 0.05$ (Linag.): Linagliptin.

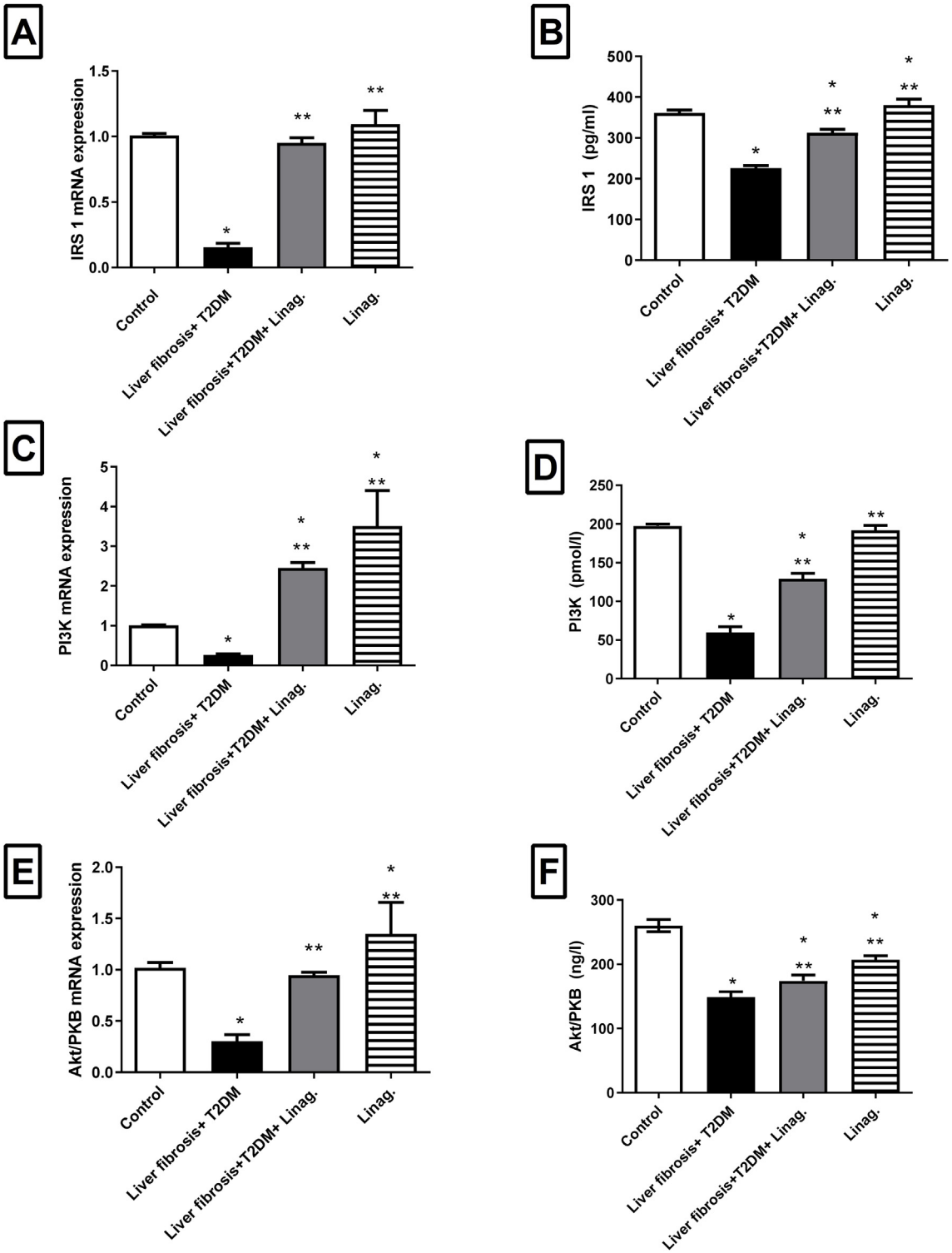

Fig. 2. The effect of linagliptin on (A) IRS-1 mRNA expression, (B) IRS-1 protein serum levels, (C) PI3K mRNA expression, (D) PI3K protein serum levels, (E) Pkb/Akt mRNA expression, (F) Pkb/Akt protein serum levels. Data were expressed as means of 6 rats ± S.D, comparisons between different groups were carried out using one-way Analysis of Variance (ANOVA) followed by Tukey multiple comparisons test. (*) Significantly different from control group at $P < 0.05$, (**) significantly different from hepatic fibrosis model at $P < 0.05$ (Linag.): Linagliptin.

rats. On the other hand, the treatment of rats with linagliptin significantly increased the mRNA expression of IRS-1, PI3K and Akt/PKB by 514, 814 and 210%, respectively, as compared to non-treated rats with liver fibrosis and T2DM (Fig. 2A, C, 2E).

We subsequently tracked the end product of mRNA insulin signaling genes through quantitative protein expression. Results revealed that the group of liver fibrosis and T2DM showed a significant reduction of IRS-1, PI3K, and Akt/PKB serum protein levels by 37.5, 70 and 43%, respectively, as compared to the control rats. Treatment of rats with linagliptin caused a significant increase in serum levels of IRS-1, PI3K and Akt/PKB by 38, 116 and 17%, respectively, as compared to non-treated rats with liver fibrosis and T2DM (Fig. 2B, D, 2F).

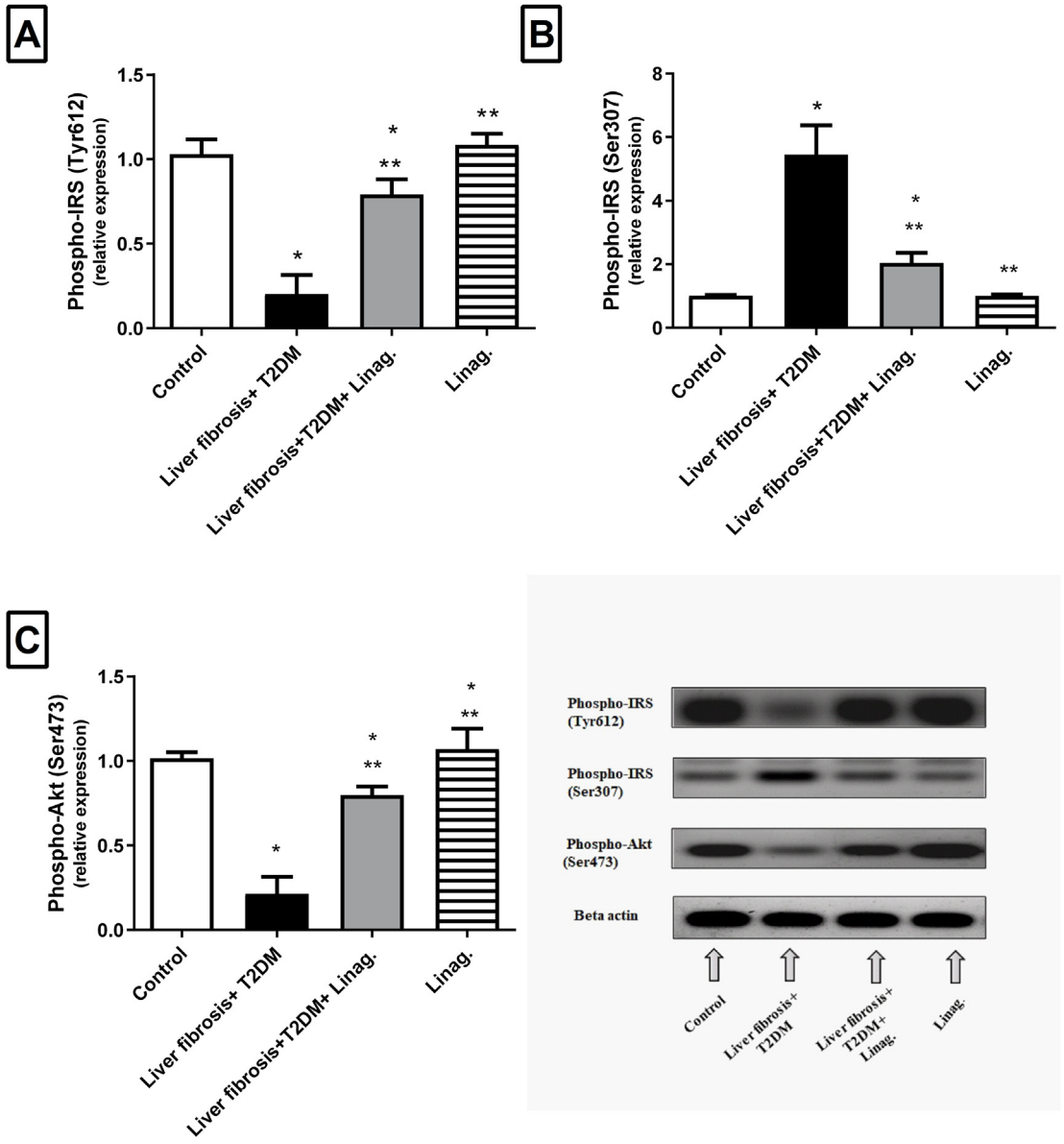

Fig. 3. The effect of linagliptin on protein levels of (A) phospho-IRS (Tyr612), (B) phospho-IRS (Ser307), (C) phospho-Akt (Ser473). Data were expressed as means of 6 rats ± S.D, comparisons between different groups were carried out using one-way Analysis of Variance (ANOVA) followed by Tukey multiple comparisons test. (*) Significantly different from control group at $P < 0.05$, (**) significantly different from hepatic fibrosis model at $P < 0.05$ (Linag.): Linagliptin.

As shown in Fig. 3, rat's exposure to HFD and STZ showed a significant increase in phospho-IRS (Ser307) by 440% and a marked reduction in phospho-IRS (Tyr612) and phospho-Akt (Ser473) by 80 and 79%, respectively when compared to control group. However, the treatment of rats with linagliptin induced a significant reduction in the level of phospho-IRS (Ser307) by 63% and a significant rise in phospho-IRS (Tyr612) and phospho-Akt (Ser473) by 290 and 275%, respectively as compared to non-treated rats with liver fibrosis and T2DM.

3.5. Linagliptin effect on inflammatory markers

Rats exposed to HFD and STZ showed significant rise in NF-κB p65 fraction in the liver tissues, TNF-α, and IL-6 serum levels by 142, 200 and 221%, respectively as compared to the control group. However, treatment of HFD and STZ feeding rats with linagliptin significantly counteracted this elevation by 36, 48 and 40.5%, respectively as compared to non-treated rats with liver fibrosis and T2DM (Fig. 4).

3.6. Macroscopic inspection and histopathological alterations

As can be seen from macroscopic and histological examination of liver sections displayed in Fig. 5, the control and linagliptin treated group fed on normal chow diet showed normal morphology and histological architecture of the liver. On the other hand, macroscopic assessment for the liver sections obtained from the group exposed to HFD and STZ showed enlarged weight and rough surface with many nodules. Beside, histopathological examination revealed severe hepatocellular granular and vacuolar degeneration accompanied by necrotic cells and apoptotic ones with congested central veins. Additionally, marked pericentral hepatic steatosis and fibrous proliferation with the micro and macrovesicular type of fatty changes including fat cysts formation was also observed. Treatment of with linagliptin showed good restoration of the hepatic parenchyma and a mild degree of hepatocellular degeneration. Most of the liver sections were free from steatosis and scattered focal areas of necrosis were replaced with inflammatory cells.

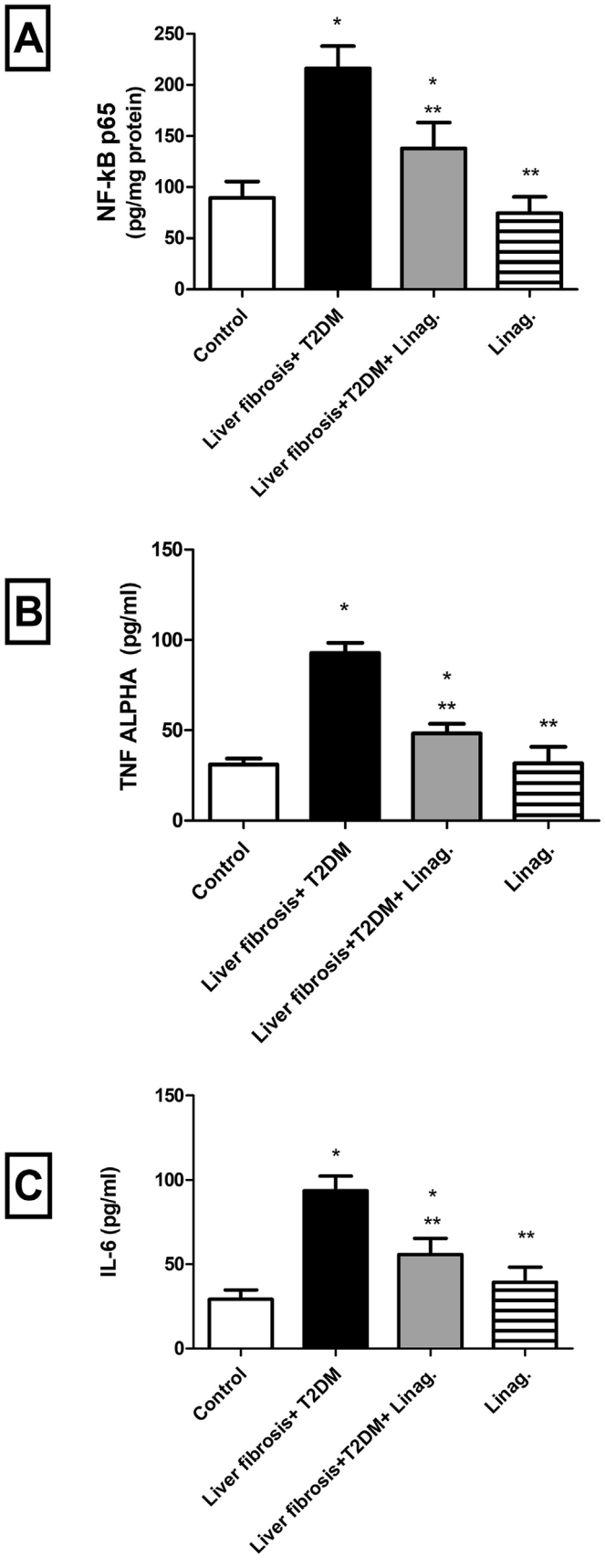

Fig. 4. The effect of linagliptin on (A) NF-κB p65 tissue levels (B) TNF-α serum levels, (C) IL-6 serum levels. Data were expressed as means of 6 rats ± S.D, comparisons between different groups were carried out using one-way Analysis of Variance (ANOVA) followed by Tukey multiple comparisons test. (*) Significantly different from control group at $P < 0.05$, (**) significantly different from hepatic fibrosis model at $P < 0.05$ (Linag.): Linagliptin.

3.7. Linagliptin effect on fibrosis markers

In the current study, immunohistochemical staining of liver sections showed a significant increase in α-SMA expression by 967% in rats exposed to HFD and STZ as compared to the control rats. On the other hand, the treatment of rats with linagliptin showed a significant decrease in α-SMA expression by 66% as compared to untreated rats with liver fibrosis and T2DM (Fig. 6A and B). Furthermore, rats exposed to HFD and STZ showed a significant elevation in serum TGF-β1 by 116% as compared to the control group. Treatment of rats with linagliptin induced a significant reduction in serum level of TGF-β1 by 37% as compared to non-treated rats with liver fibrosis and T2DM (Fig. 6C). Collagen accumulation was evaluated in the hepatic tissue both biochemically and histologically. Biochemically, liver hydroxyproline level was significantly increased upon exposure to HFD and STZ reaching 157% as compared to the control group. Rats co-treated with linagliptin significantly decreased hydroxyproline level back to the normal level (Fig. 6D). Histological examination of collagen accumulation using Masson's trichrome stain showed excessive deposition of collagen fibers visualized as blue fibrous tissue bands around portal tracts and central veins in rats with liver fibrosis and T2DM. On the other hand, treatment with linagliptin averted the deposition of collagen fibers (Fig. 6E and F). No collagen accumulation was observed neither biochemically nor histologically in linagliptin-only treated rats.

4. Discussion

Liver fibrosis is one of the metabolic serious neglected complications (Orasanu and Plutzky, 2009) strongly linked to insulin resistance and inflammation, reaching epidemic significances particularly in diabetic patients (Ong and Younossi, 2007). Due to the limitation, it is challenging to precisely appraise the antifibrotic effect of T2DM medications in clinical trials. Thus, in the present study, we aimed to explore the role of linagliptin as an FDA approved antidiabetic drug in preventing the pathological progression of liver fibrosis in experimentally induced diabetic obese rats. Further, we explored the molecular mechanisms underlying this potential anti-fibrotic effect focusing on inflammation and insulin resistance signaling pathway. In the beginning and according to our pilot study, linagliptin at a dose of 6 mg/kg was chosen as the optimum anti-diabetic dose in Sprague Dawley albino rats challenged with STZ. Consequently, the antifibrotic effect of linagliptin was examined in liver fibrosis model induced by utilizing HFD and STZ. To our knowledge, this investigation is the first of its kind to report the potential value of linagliptin in preventing the pathological progression of liver fibrosis allied to T2DM.

Liver fibrosis is a progression of a wide series of biochemical changes that arise in T2DM. The initial indicators are the increased serum levels of ALT, AST, and total bilirubin (Nannipieri et al., 2005). Furthermore, TG, TC and LDL lipoprotein significantly increased in liver diseases linked to T2DM (Mohamed et al., 2016), all of these biochemical changes are exactly seen in the current study. On the other hand, linagliptin treatment reversed the overall hepatotoxic consequences of the experimentally induced liver fibrosis through the reduction of ALT, AST, and total bilirubin serum levels as compared to the non-treated rats. By the same token, linagliptin significantly decreased TG and TC levels as compared to non-treated rats when assessed in both serum and liver tissues. All the aforementioned results support the hepatoprotective effects of linagliptin.

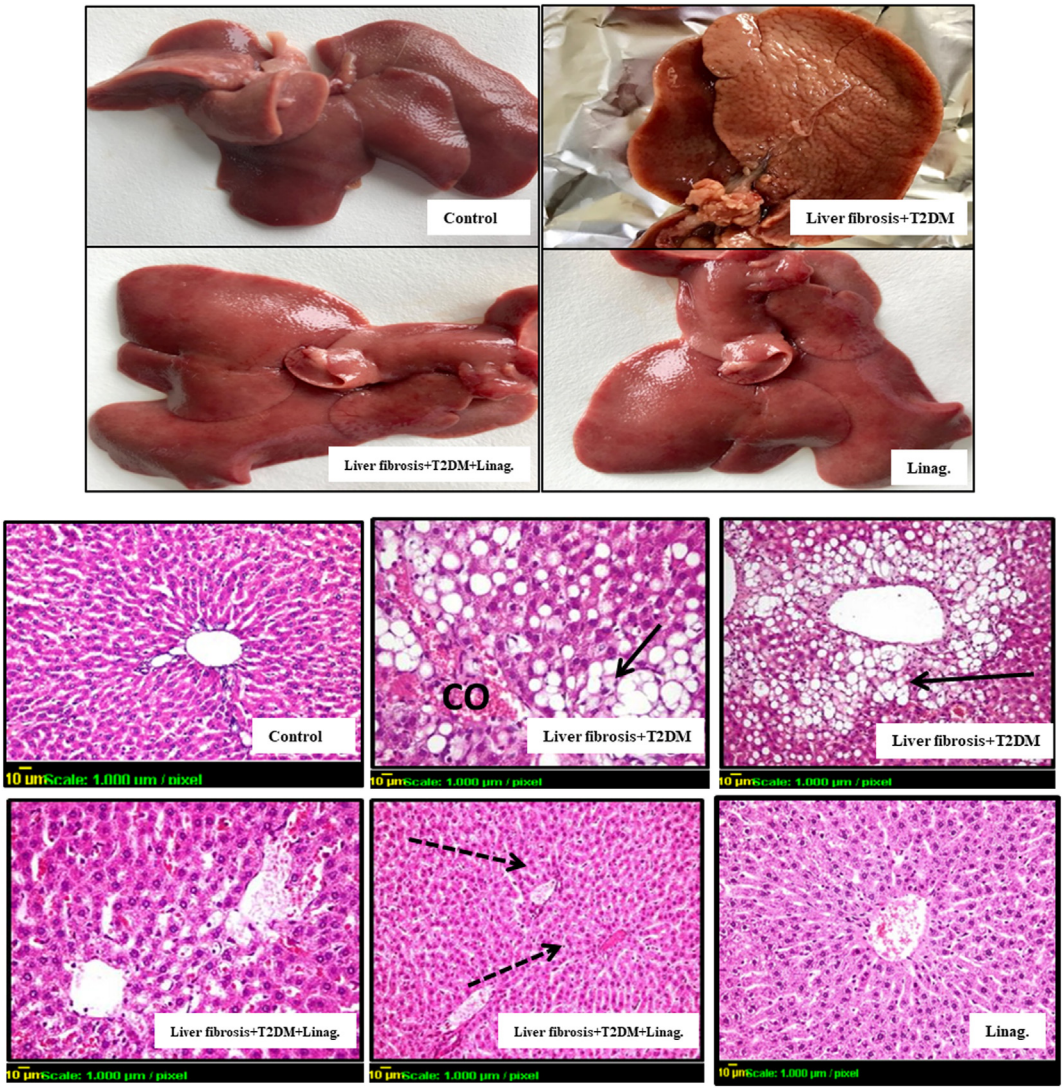

Fig. 5. Macroscopic and histopathological examination of rat livers (Linag.): Linagliptin, (CO): congested central veins, (arrow): Fat cyst, (dashed arrow): activation of Kupffer cells (H&E, x400).

At this step, we started to investigate the possible underlying mechanisms of the potential antifibrotic effect of linagliptin. In the present study treatment with linagliptin showed that in vivo DPP-4 inhibition was associated with unique pharmacological anti-inflammatory effects confirmed through the significant reduction in NF-κB p65 activity and its immunoexpression. Moreover, the results of this study showed that TNF-α and IL-6 serum levels were markedly decreased among linagliptin treated rats with liver fibrosis and T2DM. It is worth noting that the aberrant signal of glucose production in the liver of T2DM enhances fatty acid oxidation due to the lack of fuel demand. Subsequently, free fatty acids are converted to triglycerides which accumulate in the liver (Wisse, 2004). Such lipotoxicity leads to the activation of the inhibitor of nuclear factor kappa-B kinase. This enzyme has a master role in the induced translocation of NF-κB p65 which is a significant messenger for inflammatory cytokine signaling that enters the nucleus and provokes the transcription of genes encoding for TNF-α and IL-6 that trigger insulin resistance (Itani et al., 2005).

The current study sheds the light on the relationship between the anti-inflammatory effects of linagliptin, hypoglycemic and lipid-lowering potential. This association may partially be explained by the fact that linagliptin demonstrated a glucose-lowering profile manifested by the observed significant decrease in FBG levels and HOMA-IR index in addition to the significant elevation of QUIKI, as previously reported by Del Prato et al. (2011) and, Deacon and Holst (2010).

Another promising avenue of our investigation was the connection established between linagliptin's antifibrotic effect and PKB/Akt signaling pathway. Linagliptin demonstrated an increase in IRS-1, PI3K, and PKB/Akt gene expression and protein levels. A good deal of preclinical evidence suggests that linagliptin abovementioned improvements of glucose utilization will cause a reduction of free fatty acids, prevention of NF-κB p65 and pro-inflammatory cytokines activation. Moreover, rats with T2DM and fibrosis treated with linagliptin showed a marked elevation in phospho-IRS (Tyr612) rather than phospho-IRS (Ser307) and a significant increase of phospho-Akt (Ser473) when assessed in the liver tissues. This outcome contributes to our understanding of the interaction of tyrosine-phosphorylated IRS-1 with the regulatory subunit of PI3K which will promote the activation of PI3K. Activated PI3K will speed the activation of phospho-Akt (Ser473) that will potentially mediate the translocation of glucose transporter 4 (GLUT4) to the plasma membrane which is responsible for intracellular uptake of glucose, therefore, regulation of the whole-body glucose homeostasis (Pessin et al., 1999).

Liver fibrosis related to T2DM is characterized by various progressive phases through a complexity of cellular crosstalk displayed as insulin resistance and inflammatory stress. Notably, tissue formation and wound healing process are orchestrated by TGF-β1, an extracellular

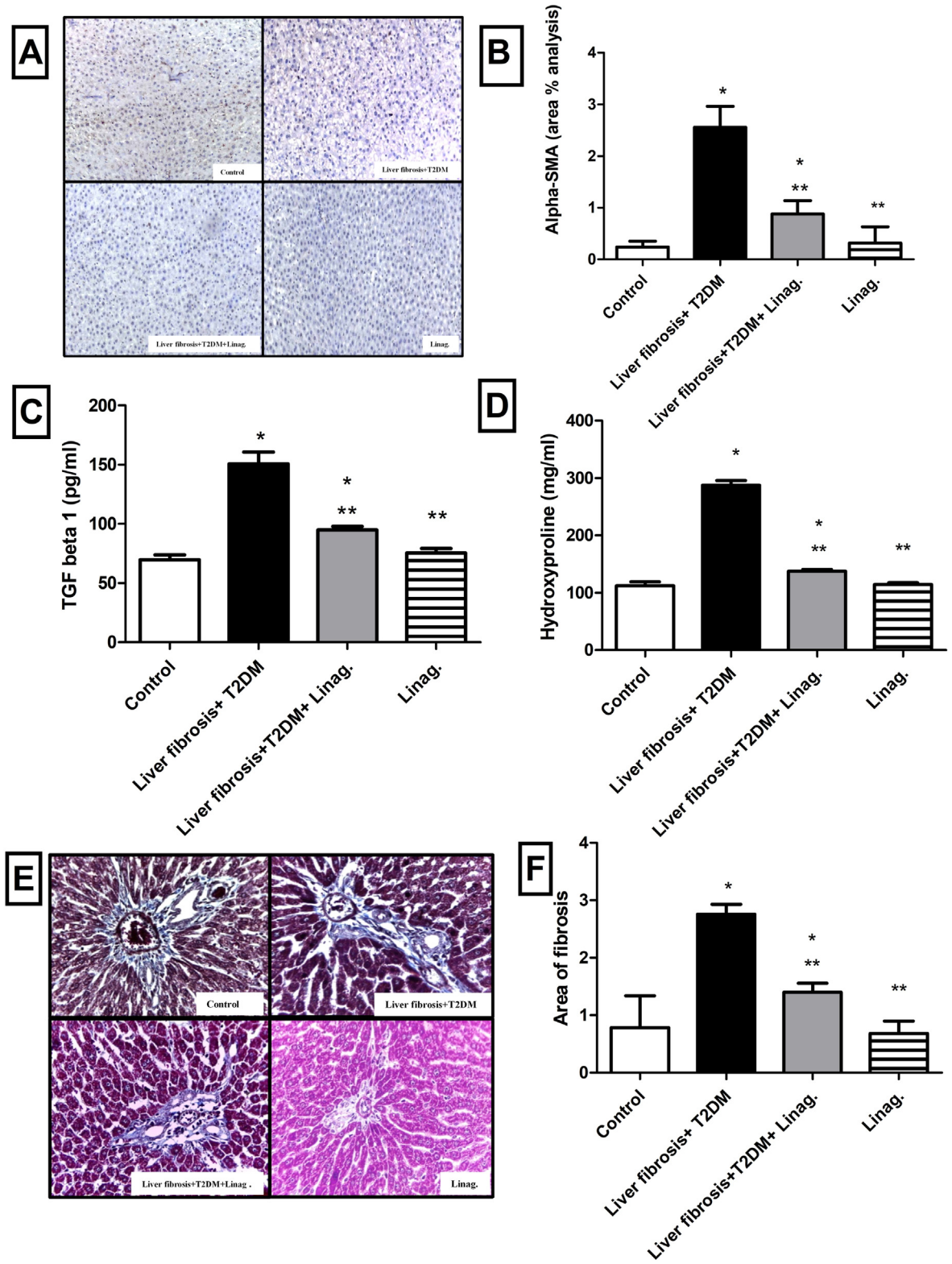

Fig. 6. The effect of linagliptin on (A) α-SMA expression, (B) α-SMA area percent analysis, (C) TGF-β1 serum levels (D) Hydroxyproline serum levels, (E) Masson trichrome staining of different rat livers, (F) Area of fibrosis percent analysis. Data were expressed as means of 6 rats ± S.D, comparisons between different groups were carried out using one-way Analysis of Variance (ANOVA) followed by Tukey multiple comparisons test. (*) Significantly different from control group at $P < 0.05$, (**) significantly different from hepatic fibrosis model at $P < 0.05$ (Linag.): Linagliptin.

protein that regulates cell differentiation and cellular migration. Furthermore, its hyperactivity leads to HSCs activation, which is the critical pathogenetic event in hepatic fibrogenesis (Gressner et al., 2002). Increased proliferation of HSCs and TGF-β1 expression in liver fibrosis is triggered by NF-κB p65 signaling pathways as reported by Shoelson et al. (2006) and, Fehrenbach et al. (2001). Activation of NF-κB p65 in T2DM acts as a key mediator of fibrosis through initiating a cycle of deleterious events. Owing to its elevation HSCs are converted

to myofibroblasts characterized by an elevated content of α-SMA microfilament (Friedman, 2000). Furthermore, the accumulation of ECM proteins mainly collagen and hydroxyproline are among the most noticeable molecular mechanism of stellate cell injury (Iredale et al., 1998). The same exists in the liver fibrosis and T2DM model of the present study.

Indeed, the abovementioned results advocate the inspection of linagliptin antifibrotic effect and further consolidate it by Masson's trichrome, immunohistochemical and histopathological studies. Linagliptin demonstrated a significant reduction in collagen content, α-SMA immunohistochemical expression, hydroxyproline and TGF-β1 serum levels in the hepatic fibrosis model. Histopathological inspection of liver sections under hyperglycemic and normal conditions showed that liver sections of rats with liver fibrosis and T2DM treated with linagliptin were free from steatosis and displayed good restoration of the hepatic parenchyma. Interestingly, focal areas of necrosis were replaced by massive inflammatory cells. By the same token, liver sections of the rats fed on a normal diet and received linagliptin showed that most of the hepatocytes in all samples were normal with few activated kupffer cells.

In the current study, hyperglycemia and hyperlipidemia triggered the proliferation of HSCs, up-regulated the expression of α-SMA, and caused fibrosis. Additionally, NF-κB p65 exerted a fibrogenic effect which provoked hepatic stellate cell activation. Novelty speaking, if we consider hepatic fibrosis as the sum of interconnected pathological components namely inflammation, insulin resistance, and impaired glucose metabolism then our investigation raises the prospect that the antifibrotic potential of linagliptin might be attributed to improving insulin signaling and reversing the inflammatory insults through the restoration of the IRS-1/PI3K/Akt pathway signaling and down-regulation of NF-κB p65.

The most interesting finding observed in our speculation was that the efficiency of linagliptin to inhibit NF-κB p65 when added to the activation of the IRS-1/PI3K/Akt pathway diminished the expression of collagen and HSCs proliferation. This is supported by a growing body of literature that DPP-4 is highly expressed in the liver and the fact that linagliptin is superior when compared to other gliptins due to its extensive interaction with the DPP-4 hydrophobic pocket through its hydrophobic inhibitor moiety. Thus DPP-4 inhibition by linagliptin could have such beneficial effects of the reduction of liver inflammation, and fibrosis (Arulmozhiraja et al., 2016; Jojima et al., 2016).

Another promising avenue in this study was that linagliptin treatment hinders the activation of Kupffer cells besides blunting the pathological signaling of TGF-β1 in the liver cells of rats with liver fibrosis and T2DM. Thus, we assumed that the antifibrotic effect of linagliptin might also be correlated with the down-regulation of active TGF-β1 protein produced from activated HSCs. These outstanding data supported the outcomes of the present study revealing that the significant down-regulation of NF-κB p65, TNF-α and IL-6 serum levels upon linagliptin treatment diminished collagen buildup and TGF-β1 expression in HSCs (Jojima et al., 2016; Waang et al., 2018).

Collectively and in conclusion, the present study is the first to potentially address the outstanding antifibrotic effect of linagliptin in HFD and STZ induced hepatic fibrosis rat model. The most obvious findings emerged from this study was that the inclusion of linagliptin in rats with liver fibrosis and T2DM reduced NF-κB p65, restored phospho-IRS (Tyr612) and phospho-Akt (Ser473), and improved liver markers of fibrosis (α-SMA, TGF-β1, hydroxyproline, and collagen) in the context of improved glycemic control.

Taken together, this study deeper insight into exploring the mechanistic actions of linagliptin on HSCs under hyperglycemic conditions showed that the anti-fibrotic effect of linagliptin in diabetic rats could be achieved by its direct role in suppressing HSCs proliferation with concomitant down-regulation of α-SMA and TGF-β1 overexpression, or indirectly through reduction of NF-κB p65 and stimulation of IRS-1/PI3K/Akt pathway which are the core behind linagliptin insulin-sensitizing and anti-inflammatory consequences. Such a portfolio should indeed create an effective advocate for the use of linagliptin as an innovative therapeutic option to prevent the pathological progression of liver fibrosis linked to T2DM.

CRediT authorship contribution statement

Yara M. Aboulmagd: Formal analysis, Writing - original draft. **Alshaymaa A.Z. El-Bahy:** Supervision, Writing - original draft. **Esther T. Menze:** Formal analysis, Writing - original draft. **Samar S. Azab:** Writing - original draft. **Ebtehal El-Demerdash:** Supervision, Writing - original draft.

Declaration of competing interest

The authors declare that there are no conflict interests in the submitted manuscript.

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