

Activation of pCREB/Nrf-2 signaling mediates re-positioning of liraglutide as hepato-protective for methotrexate -induced liver injury (MILI)

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ABSTRACT

Methotrexate (MTX) is commonly used to treat several types of cancer and autoimmune diseases. However, there is increasing concern over its organs toxicities particularly liver toxicity. Liraglutide, a glucagon like peptide-1 agonist, possesses antioxidant and anti-inflammatory features. This study aimed to explore the potential protective effect of liraglutide pre-treatment in ameliorating MTX-induced hepatotoxicity and to further investigate the underlying mechanisms. Rats received 1.2 mg/kg liraglutide intraperitoneal twice daily for 7 days before MTX. Results revealed that liraglutide significantly decreased activities of liver enzymes and oxidative stress in hepatocytes. Furthermore, NF- κ B expression and related inflammatory markers (TNF- α , COX-2 and IL-6) were reduced in the pre-treatment group of liraglutide. These data validate the advantageous effects of liraglutide in MTX hepatotoxic animals. In addition, liraglutide increased the expression of the antioxidant transcription factor nuclear factor-erythroid 2-related factor 2 (Nrf-2), along with the transcription of downstream phosphorylated cAMP response element-binding protein (pCREB) which increases the activity of Nrf-2. Additionally, caspase-3 expression/activity and BAX/Bcl-2 ratio were decreased following liraglutide pre-treatment. In conclusion, it was confirmed that liraglutide enhanced the antioxidant activity of liver cells by activating the Nrf-2 and pCREB signaling, thereby, reducing liver cell inflammation and apoptosis induced by MTX.

1. Introduction

More than thousand drugs of the modern pharmacopeia can induce liver injury with different clinical outcomes (Biour et al., 2004; Larrey, 2000). In the most severe cases, drug-induced liver injury (DILI) can require liver transplantation or cause patient's death (Bjornsson, 2009). Methotrexate (MTX) is a cytotoxic anti-folate drug that is considered the first-line drug in the treatment strategies of several malignancies, inflammatory and auto-immune diseases (Al Maruf et al., 2018). Since the toxic effect of MTX on the cells is not selective to cancer cells, it may influence normal cells and thus using MTX for long period has been associated with different organ toxicities (Howard et al., 2016). Hepatotoxicity is one of the crucial toxicities of MTX, which confines its use clinically (Cetiner et al., 2005). Diabetes and obesity are well-known risk factors for MTX-induced liver injury as mentioned in Current American College of Rheumatology (ACR) and American Association of Dermatology (AAD) guidelines (Menter et al., 2009; Singh et al., 2016).

Moreover, previous research has reported that psoriatic patients with type-2diabetes or overweight are at great risk of emerging severe liver fibrosis following MTX treatment compared to patients without same risk factors (Rosenberg et al., 2007).

On the other hand, molecular mechanisms by which MTX induces hepatotoxicity are still unidentified, although they could be related to the drug cellular pathway (Aithal, 2011). The biochemical changes for MTX toxic effects have been reported mainly through the upsurge of oxidative stress and initiation of inflammatory pathways (Mukherjee et al., 2013). Correspondingly, oxidative stress promotes cells towards apoptosis (Kannan and Jain, 2000). In this context, several signaling pathways have been involved in regulating the intrinsic apoptotic pathway. Among them, nuclear factor kappa B (NF- κ B) is considered a substantial intermediate for induction of apoptosis (Abo-Haded et al., 2017; Janssen-Heininger et al., 2000). In contrast, Nrf-2/CREB signaling pathway is considered a protective pathway with regards to decreasing transmission of inflammatory signals in liver cells (Liu et al.,

Abbreviations: MTX, Methotrexate; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GSH, reduced glutathione; MDA, malondialdehyde; SOD, superoxide dismutase; TNF- α , tumor necrosis factor alpha; IL-6, interleukin-6; NF- κ B, Nuclear factor kappa B; COX-2, cyclooxygenase-2; Nrf 2, Nuclear factor erythroid 2-related factor2; HO-1, Heme oxygenase-1; pCREB, phosphorylated cAMP response element-binding protein

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2008; Wardyn et al., 2015). Nuclear factor-E2-related factor 2 (Nrf-2) is a key controller of redox balance and signaling, which promotes the expression of a series of genes that protect against oxidative stress (Niture et al., 2014). Studies reported that activation of Nrf-2 has a protective effect on MTX induced kidney and liver injuries (Abd El-Twab et al., 2016; Mahmoud et al., 2017a). Furthermore, another level of control is modulation of the availability of transcription co-activators, such as cAMP-responsive element-binding protein (CREB) binding protein (CBP), which has been shown to interact with and activate Nrf-2 (Sun et al., 2009). pCREB increases acetylation of Nrf-2 which increases Nrf2 sequence-specific DNA binding capacity and expedite transcription of its downstream target genes (Kawai et al., 2011). Interestingly, a study explained that impaired CREB-1 phosphorylation is related to anti-folate resistance and administration of compounds increasing pCREB can overcome this resistance (Rothen et al., 2004).

Recently, the concept of drug repositioning has been raised. Drug repositioning (also known as drug repurposing), the strategy of finding novel uses for existing drugs, has been gaining popularity in the medical field (Shim and Liu, 2014; Xue et al., 2018). The corresponding benefit is reducing the need for investment in drug discovery and optimization, as well as in safety and pharmacokinetic studies (Duraes et al., 2018). It is generally thought that complex diseases such as cancer and central nervous system diseases may require complex therapeutic approaches. Correspondingly, a drug that “hits” multiple sensitive nodes belonging to a network of interacting targets offers the potential for higher efficacy, and may limit drawbacks generally arising from the use of a single-target drug or a combination of multiple drugs (Anighoro et al., 2014). For instance, vincamine has been examined for its protective effect in MTX-induced nephro and neurotoxicity (Shalaby et al., 2019). Likewise and based on the previously mentioned principles, targeting Nrf-2/CREB pathway may be a novel pathway to exert antioxidant and protective effects on MTX induced liver injury. In this regards, Incretin-based therapy has recently attracted significant attention as a novel treatment for diabetes type-2 with antioxidant and protective effects (Abo-Haded et al., 2017; Li et al., 2015a). As well as reducing poly-pharmacy, this suggests that established diabetes treatments could be repositioned to improve both DILI and diabetes concurrently.

Along with this, Liraglutide is a potent, long-acting synthetic analog of the human glucagon-like peptide 1 (GLP 1) molecule. It shares 97% sequence identity with human GLP 1 and displays a much longer half-life of about 13 h (Jacobsen et al., 2009). In 2010, Liraglutide was approved by the US FDA to treat diabetes mellitus type-2 (Drucker et al., 2010). Recent studies have reported that liraglutide was effective for controlling glucose levels, protection of β -cell function, and decreasing body weight for patients with type 2 diabetes mellitus who are resistant to diet and exercise (Bailey, 2013; Ross and Ballantine, 2013). Another study reported that liraglutide could improve oxidative stress and lipid peroxidation and decrease transforming growth factor β 1 (TGF β 1) and tumor necrosis factor α (TNF α) expression in rats with non-alcohol fatty liver diseases NAFLD (Gao et al., 2015). In a study by Olaywi et al. (2013), it was revealed that liraglutide induced improvements in transaminases in patients with non-alcoholic steatohepatitis (Olaywi et al., 2013). These studies have suggested the potential influence of liraglutide in improving liver diseases.

Recently, a study observed that liraglutide exerts an anti-inflammatory effect on vascular endothelial cells by suppressing NF- κ B activation (Dai et al., 2013). Another report showed a significant improvement in patients with psoriasis and diabetes after treatment with liraglutide. This improvement in psoriasis in liraglutide-treated patients may be due to weight loss, improved glycemic control and the direct effects of GLP-1 receptor activation on immune cells (Ahern et al., 2013). Moreover, liraglutide enhances the activation of Nrf-2 pathway in glucolipototoxicity induced liver injury (Guo et al., 2018). In addition, liraglutide has a neuroprotective effect on mild brain injury via increasing pCREB signaling (Li et al., 2015b). Accordingly, the current

study aimed to address the following research questions: whether liraglutide has potential hepatoprotective value on MTX-induced hepatotoxicity (MILI), and what are the putative underlying mechanisms involved in this protective process.

2. Materials and methods

2.1. Animals

In this study, adult male Sprague-Dawley rats, weighing 160–200 g were obtained from the animal house of the National Organization for Drug Control and Research (NODCAR), Giza, Egypt. The animals were kept in controlled environment ($24 \pm 2^\circ\text{C}$ temperature, 60–70% relative humidity, 12 h light/dark cycle) and were provided with standard pellet diet (containing not less than 20% protein, 5% fiber, 3.5% fat, 6.5% ash and a vitamin mixture) and water *ad libitum*. Animals were assigned a group designation and weighed. Animal pain or suffering was minimized as much as possible during experimentation. All processes of animals experimentation were executed as stated in the Arrive guidelines and in accordance with U.K. Animals Act, 1986 and approved by the Research Ethics Committee, Faculty of Pharmacy, Ain Shams University, Cairo, Egypt under the memorandum No.135.

2.2. Drugs and chemicals

Liraglutide (CAS no. 204656-20-2) was provided from Novo (Nordisk Co., Denmark). MTX injection (50 mg/2 ml) manufactured by Mylan N.V was purchased. All other chemicals used were of highest grade commercially available.

2.3. Induction of liver injury

Acute administration of Methotrexate (20 mg/kg, i.p) in rats was used for experimental induction of hepatic toxicity (Russmann et al., 2009).

2.4. Experimental design

The study was conducted on two phases:

2.4.1. Dose screening for the optimum hepatoprotective dose of liraglutide

After acclimatization for 2 weeks, 48 rats were randomly divided into six groups of eight rats each and treated for 10 consecutive days as follows:

Group 1: served as negative control, receiving saline only during the entire experimental period.

Group 2: received a single intraperitoneal injection of methotrexate at a dose of 20 mg/kg bw (El-Sheikh et al., 2015) on the eighth day.

Group 3, 4, 5: received liraglutide at dose of 0.3, 0.6 and 1.2 mg/kg respectively i.p twice daily and on the eighth day was given single i.p MTX injection at a dose of 20 mg/kg. The dose range of liraglutide is selected based on previous reports (Gao et al., 2015; Shirakawa et al., 2012).

Group 6: received liraglutide only at a dose of 1.2 mg/kg, i.p twice daily for ten days.

Based on our preliminary results, blood samples were collected, following 72 h after MTX injection, from retro-orbital plexus after light anesthesia with sodium pentobarbital (50 mg/kg, i.p.). Centrifugation at 855.27g for 10 min was used to separate serum and was used for the estimation of liver functions. Rats were sacrificed by cervical dislocation and livers were removed and weighed. A Portion was placed in formol-saline for histopathological analysis.

The assessed parameters for phase I to select the optimum liraglutide dose were:

2.4.1.1. Assessment of liver enzymes: serum ALT & AST. Serum concentrations of aspartate aminotransferase (AST), alanine aminotransferase (ALT), were determined colorimetrically using available commercial kits (Spectrum diagnostics, Cairo, Egypt).

2.4.1.2. Histopathological examination. 10% formol saline is used to preserve liver samples from rats of the different experimental groups for 24 h. Tissue washing was done by tap water then serial dilutions of alcohol (methyl, ethyl and absolute ethyl) were used for dehydration. Specimens were cleared in xylene and fixed in paraffin at 56 °C in a hot air oven for 24 h. Sections were embedded in paraffin and sliced into 4 µm thick sections by a sledge microtome. The tissue sections were collected on glass slides, deparaffinized, stained with hematoxylin & eosin stain (H&E) then examined by light microscope (Banchroft et al., 1996).

2.4.2. Studying the hepatoprotective mechanism of liraglutide against MTX induced liver injury

Based on serum ALT & AST and histopathological examination results, liraglutide 1.2 mg/kg dose was selected to be the optimum hepatoprotective dose and used for assessment of oxidative stress and inflammatory markers and studying the signaling hepato-protection pathway.

2.4.2.1. Hepatic oxidative stress markers. Lipid peroxidation was determined by estimating the level of thiobarbituric acid reactive substances (TBARS); measured as malondialdehyde (MDA). Levels of MDA, reduced glutathione (GSH), and superoxide dismutase (SOD) were measured using commercial kits (Biodiagnostic, Cairo, Egypt). All the procedures were conducted as stated in the manufacturer's instructions.

2.4.2.2. Enzyme-linked immunosorbent assay (ELISA) for interleukin-6 (IL-6), tumor necrosis factor- α (TNF- α), phosphorylated cAMP response element-binding protein (pCREB), nuclear factor erythroid 2-related factor2 (Nrf2), and Heme oxygenase-1 (HO-1). Liver tissues were washed and homogenized in ice-cold PBS (pH = 7.4) to obtain 20% homogenate (w.v-1), which was then centrifuged for 15 min at 5000 rpm and 4 °C. The supernatant obtained was used for measuring TNF- α (Platinum ELISA, eBioscience, Vienna, Austria) and IL-6 (R&D Systems, a biotechnie brand, Quantikine ELISA, Minneapolis, USA) using sandwich ELISA kits as reported by the manufacturer's instructions. They were expressed as pg.mg⁻¹ protein. Protein content was also determined in the supernatant using a commercially available kit (Jenway, Staffordshire, UK). P-CREB (Serine 133), a marker for cAMP response element binding protein (CREB) phosphorylated (S133), Nrf2, a marker for nuclear factor erythroid 2-related factor2, and HO-1 (Heme oxygenase-1) were measured in nuclear protein extract using sandwich ELISA kits (R&D systems, DuoSet IC. Minneapolis, USA), (MyBioSource, Inc, San Diego, USA), and (Elabscience, USA) respectively.

2.4.2.3. Quantitative immunohistochemical analysis of NF- κ B (p65), COX-2, Bax, Bcl-2, and caspase 3. Paraffin fixed tissue sections of 3 µm thickness were rehydrated first in xylene and then in graded ethanol solutions. The slides were embedded with 5% BSA in Tris buffered saline (TBS) for 2 h. Immunohistochemical analyses were performed by a standard streptavidin-biotin-peroxidase procedure (Habib et al., 2019). The sections were immuno-stained with one of the following primary antibodies; rabbit polyclonal anti-rat NF- κ B (p65) (Thermo Fisher Scientific, Cat. No. RB-9034-P), anti-COX-2 (Thermo Fisher Scientific, Cat. No. RB-9072-R7), anti-Bax (Thermo Fisher Scientific, Cat. No. MA5-14003), anti-Bcl2 (Biogenex, Cat.No. AN541), anti-Caspase 3 (Santa Cruz Biotechnology, Cat. No. sc-7272) at a concentration of 1 µg.ml⁻¹ and incubated overnight at 4 °C. After washing the slides with TBS, the sections were incubated with the

corresponding biotinylated secondary antibody for 10–15 min. After that, the horseradish-peroxidase-conjugated streptavidin solution was added and incubated at room temperature for 10–15 min. Sections were then washed with TBS and incubated for 5–10 min in a solution of 0.02% diaminobenzidine containing 0.01% hydrogen peroxide. Counter staining was performed using hematoxylin and the slides were visualized under light microscope (Abdel-Maged et al., 2018). Immunohistochemical quantification was performed by measuring the OD of immunopositive reaction using image analysis software (ImageJ, 1.46a, NIH, USA).

2.4.2.4. Western blot analysis of Bax and Bcl-2. Protein levels of Bax and Bcl-2 in hepatic tissues of different treatment groups were assessed by Western blot technique (Abdel-Maged et al., 2018). Briefly, liver tissues were homogenized with 300 µl RIPA buffer using a T-10 Basic Ultra Turrax Homogenizer (IKA) and protein concentrations of lysates were determined using Bradford Protein Assay Kit (SK3041). The primary antibodies Bax (6A7), Bcl-2 (Bcl-2-100) and β -actin (1:2000 dilution; Thermo fisher, USA) raised in rabbit were used. Following which, The secondary antibody, horseradish peroxidase-conjugated goat anti-rabbit-IgG was used. Detection of proteins bound by the antibody of interest was accomplished by chemiluminescent signals which were captured using a CCD camera-based imager. Image analysis software was used to read the band intensity of the target proteins versus control sample after normalization by β -actin on the Chemi Doc MP imager. Results were expressed as arbitrary units after normalization for β -actin protein (Gallagher, 2006).

2.4.2.5. Caspase-3 activity assay. For measurement of nuclear caspase-3 activity, hepatic nuclei were isolated as described previously (Buckley et al., 1988). 200 mmol/L caspase-3 substrate I (Ac-DEVD-pNA) was used as the substrate for caspase-3 and p-Nitroaniline was used as the standard. Cleavage of the substrate was monitored at 405 nm and specific activity was expressed in pmol of the product, nitroaniline, per minute per mg protein.

2.4.2.6. Cytotoxicity assay

2.4.2.6.1. Mammalian cell lines and chemicals used. HepG-2 cells (human Hepatocellular cancer cell line) were obtained from the American Type Culture Collection (ATCC, Rockville, MD). Dimethyl sulfoxide (DMSO), MTT and trypan blue dye were purchased from Sigma (St. Louis, Mo., USA). Fetal Bovine serum, DMEM, RPMI-1640, HEPES buffer solution, L-glutamine, gentamycin and 0.25% Trypsin-EDTA were purchased from Lonza (Belgium).

2.4.2.6.2. Cytotoxicity evaluation using MTT assay. For cytotoxicity assays, the tumor cell line was suspended in medium at concentration 5×10^4 cell/well in Corning® 96-well tissue culture plates, then incubated for 48 h. The tested compounds were then added into 96-well plates (three replicates). Vehicle controls with either media or 0.5% DMSO were run for each 96 well plate as a control. After incubating for 48 h, the numbers of viable cells were determined by the MTT assay (Mosmann, 1983). The relation between surviving cells and drug concentration is plotted to get the survival curve of each tumor cell line after treatment with the specified drug. The 50% inhibitory concentration (IC₅₀), the concentration required to cause cytotoxic effects in 50% of intact cells, was estimated from graphic plots of the dose response curve for each conc. using GraphPad Prism software (San Diego, CA, USA).

2.4.2.6.3. Evaluation of drug interaction. Dose-response curves for Liraglutide alone was first generated. The extent of the effect of the combined treatment Liraglutide + MTX was analyzed by applying isobologram equation (Berenbaum, 1989): $I = d1/D1 + d2/D2$. Where d1 and d2 are the respective concentrations of MTX and liraglutide used in the combination required to produce a fixed level of inhibition IC₅₀, while D1 and D2 are their concentrations able to produce alone the same magnitude of effect (50% inhibition of cell growth). If "I"

(interaction index) is less than 1, the effect of combination is synergistic, whereas if $I = 1$ or $I > 1$ the effect is additive or antagonistic, respectively (Azab et al., 2005).

2.4.2.7. Statistical analysis. Results are expressed as mean \pm standard deviation (SD). Multiple comparisons were performed using one-way ANOVA followed by Tukey–Kramer as a post-hoc test. A p value of less than 0.05 was considered statistically significant. All analyses were performed using Instat software package (version 3.06). Correlation coefficient was determined by linear regression analysis (Abdel-Daim et al., 2015; Abdel-Maged et al., 2018). Graphs were sketched using GraphPad Prism software (version 5).

3. Results

3.1. Dose screening for the optimum hepatoprotective dose of liraglutide

3.1.1. Hepatotoxicity markers-Serum alanine aminotransferase and aspartate aminotransferase levels

As compared to control group, rats receiving MTX showed significant increase in serum ALT and AST by 103% and 28.4% respectively. Concurrent administration of all the tested doses of liraglutide showed significant decrease in serum aminotransferases as compared to MTX group (Table 1).

3.1.2. Histopathological examination

Microscopic examination of H&E-stained liver sections of control rats showed normal histological structure of the central vein and intact pericentral hepatocytes (Fig. 1A). In contrast, MTX-induced rats revealed focal area of necrosis replaced with inflammatory cells, mild vacuolar degeneration of hepatocytes and activated kupffer cells (Fig. 1B). Different doses of liraglutide pre-treatment showed relatively diverse protective actions. Pre-treatment of 0.3 mg/kg liraglutide showed hepatic sinusoids, necrobiotic changes of hepatocytes (arrow), others showing microvesicular steatosis (arrow head) and activated kupffer cells (Fig. 1C). While, pre-treatment with 0.6 mg/kg liraglutide showed congested central vein necrobiotic changes of some hepatocytes and activated kupffer cells (Fig. 1D). Nevertheless, pre-treatment with 1.2 mg/kg liraglutide restored normal histological structure of central vein with pericentral intact hepatocytes (Fig. 1E). Liraglutide only treated rats showed normal portal vein with intact hepatocyte (Fig. 1F).

As a result of liver transaminases analysis and Histopathological examination of the different groups, we revealed that 1.2 mg/kg liraglutide is the optimum dose to be further studied for the protective mechanism of liraglutide.

Table 1

Effect of different doses of liraglutide on hepatotoxicity indices in rats exposed to methotrexate.

| Group | ALT (U.L ⁻¹) | AST (U.L ⁻¹) |
|-----------------------------|---------------------------------|----------------------------------|
| Control | 69.92 \pm 4.15 | 162.4 \pm 9.073 |
| Methotrexate | 141.95 \pm 13.82 ^a | 208.53 \pm 9.26 ^a |
| 0.3 mg/kg Liraglutide + MTX | 82.04 \pm 14.42 ^b | 184.7 \pm 18.82 ^b |
| 0.6 mg/kg Liraglutide + MTX | 70.25 \pm 12.29 ^b | 174.6 \pm 13.93 ^b |
| 1.2 mg/kg Liraglutide + MTX | 63.14 \pm 3.81 ^{b,c} | 156.2 \pm 3.139 ^{b,c} |
| Liraglutide Only | 74.64 \pm 4.77 ^b | 166.4 \pm 3.87 ^b |

Data are presented as mean \pm SD (n = 8).

a or b or c: significantly different from the control or MTX group or 0.3 mg/kg liraglutide, respectively at $P < 0.001$ using ANOVA followed by Tukey–Kramer as a post-hoc test.

ALT: alanine aminotransferase; AST: aspartate aminotransferase.

3.2. Studying the hepatoprotective mechanism of liraglutide against MTX induced liver injury

3.2.1. Liraglutide mitigates MTX-induced oxidative stress in liver of rats by modulating the activities of antioxidant defense enzymes

The ameliorative outcome of liraglutide on MTX-induced oxidative stress was examined via determination of GSH, SOD, and MDA. MTX-induced rats showed significant deterioration in hepatic GSH content and abolished activity of SOD by 85.7% and 128.7% respectively when compared with control group. MTX-induced rats pre-treated with 1.2 mg/kg liraglutide produced a significant elevation in GSH and SOD by 5.7 folds and 1.5 folds respectively, when compared to MTX-induced group. Contrarily, MDA is increased by 71.5% in MTX-induced rats as compared to control group. Pre-treatment with liraglutide halted MDA content by 45.2% as compared to MTX-induced group. In liraglutide administered group, GSH, SOD and MDA showed non-significant changes compared to control group. Accordingly, liraglutide pre-treatment protects against MTX induced oxidative stress (Table 2).

3.2.2. Liraglutide attenuates MTX-induced inflammation in liver of rats

Immunohistochemical detection of COX-2 and NF- κ B showed almost negative expression in control group (Fig. 2A and 3A). In contrast, MTX group revealed a significant increase in the expression of COX-2 and NF- κ B as shown by intense brown staining as compared to control group (Fig. 2B and 3B). Conversely, Pre-treatment with 1.2 mg/kg liraglutide significantly reduced the expressions of COX-2 and NF- κ B as compared to MTX group (Fig. 2C and 3C). In addition, as shown in (Fig. 2D&3D), liraglutide administered group showed negative levels of expression for COX-2 and NF- κ B as compared to control group. The immunohistochemical staining was counted as optical density (OD) of the stained regions using the image analysis software (Figs. 2E and 3E).

Furthermore, as shown in (Fig. 2F and G), concerning the effect of liraglutide on the pro-inflammatory cytokines, TNF α and IL-6 showed a significant increase in MTX-induced rats by 155% and 46% respectively, when compared to control group. Interestingly, Pre-treatment with 1.2 mg/kg liraglutide resolved TNF α and IL-6 levels elevation by 78% and 28.5% decrease respectively, when compared to MTX-induced rats. In addition, liraglutide administered group showed non-significant changes in the pro-inflammatory cytokines as compared to control group.

3.2.3. Liraglutide attenuates MTX-induced apoptosis in liver of rats

Next, we questioned the status of the apoptotic machinery in MTX induced liver injury and the potential effect of pre-treatment of liraglutide on these markers. To assure the effect of liraglutide pre-treatment on MTX-induced apoptosis, Bax and Bcl-2 proteins expressions were assessed by Western blot. One-way ANOVA showed significant differences among groups in the percent change in liver Bax and Bcl-2 protein expression. Rats receiving MTX showed significant increase in liver Bax expression while Bcl-2 expression was significantly decreased as compared to control group. Pre-treatment with liraglutide showed a significant decrease in Bax expression and a significant increase in Bcl-2 expression as compared to MTX-induced group. In liraglutide alone group, non-significant changes in levels of expression were detected for Bax and Bcl-2 as compared to control group (Fig. 3F & G).

Furthermore, the levels of expression of pro-apoptotic protein Bcl-2 Associated X protein (Bax) and active caspase-3 as well as anti-apoptotic protein B-cell lymphoma 2 (Bcl-2) were assessed using immunohistochemical technique. As shown in Fig. (4 & 5), Caspase-3 and Bax immunoreactivity were increased while Bcl-2 was decreased in livers of MTX-induced rats resulting in apoptotic cell death as compared to control group. Interestingly, pre-treatment with liraglutide significantly suppressed the levels of expression of Caspase-3, Fig. 5C and Bax, Fig. 4C while increased Bcl-2 expression level when compared to MTX group, Fig. 4G. In addition, pre-treatment with liraglutide

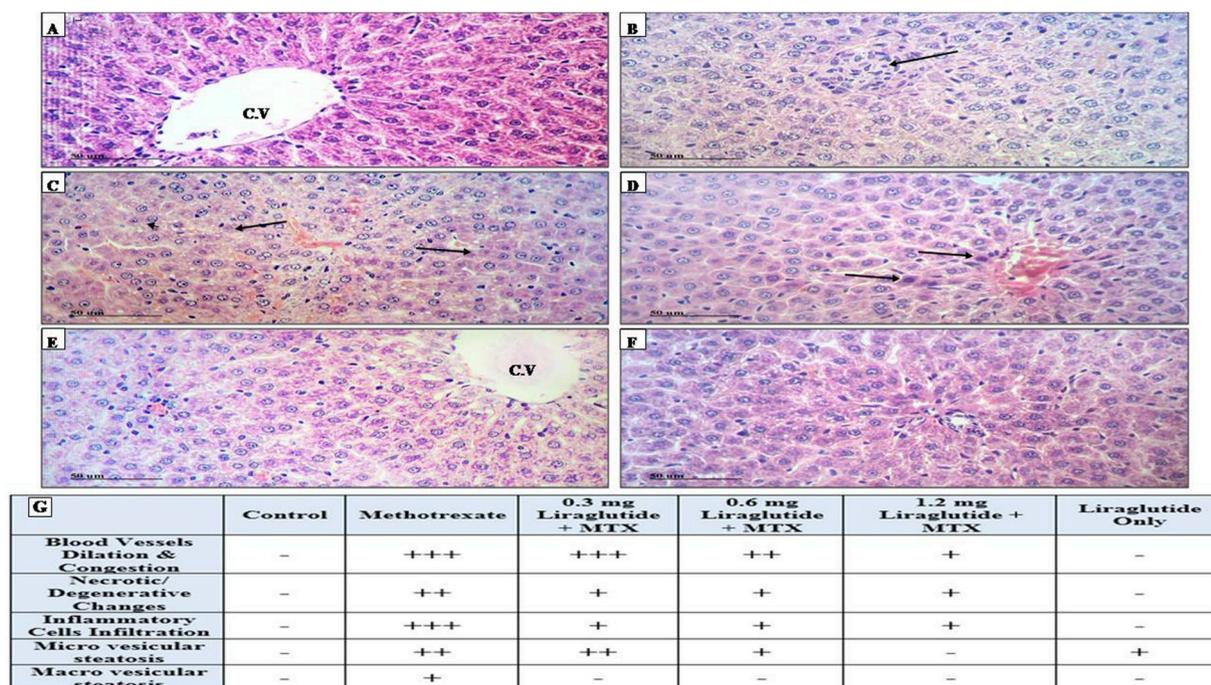


Fig. 1. Different doses of liraglutide pre-treatment ameliorated MTX-induced histopathological deterioration in liver of rats. Histopathological analysis of rat liver sections using H&E staining. A: Control rats showed normal histological structure of the central vein (CV) and intact pericentral hepatocytes. B: MTX-induced rats showed focal area of necrosis replaced with inflammatory cells (black arrow), mild vacuolar degeneration of hepatocytes and activated kupffer cells. C: 0.3 mg liraglutide pre-treatment showed congested central vein and hepatic sinusoids, necrobiotic changes of hepatocytes (arrow), others showed microvesicular steatosis (arrow head) and activated kupffer cells. D: 0.6 mg liraglutide pre-treatment showed congested central vein necrobiotic changes of some hepatocytes and activated kupffer cells. E: 1.2 mg liraglutide pre-treatment showed normal histological structure central vein with pericentral intact hepatocytes. F: Liraglutide only treated rats showing normal portal vein with intact hepatocyte. G: Scoring of histopathological alterations of liver specimens in all treatment groups.

Table 2
Effect of liraglutide on oxidative stress markers in rats exposed to methotrexate.

| Group | GSH ($\mu\text{mol. g}^{-1}$ tissue) | MDA (nmol. g^{-1} tissue) | SOD (U.g^{-1} tissue) |
|----------------------------|---------------------------------------|-------------------------------------|---------------------------------|
| Control | 38.37 ± 2.99^b | 470.3 ± 56.65^b | 3323 ± 52.30^b |
| Methotrexate | 5.493 ± 0.47^a | 806.7 ± 44.6^a | 1453 ± 345.4^a |
| Liraglutide Only | 35.57 ± 2.32^b | 469.6 ± 30.39^b | 3358 ± 132.3^b |
| Liraglutide + Methotrexate | 36.82 ± 3.752^b | 442.1 ± 72.74^b | 3654 ± 142.1^b |

Data are presented as mean \pm SD (n = 8).

a or b: significantly different from the control or MTX group, respectively at $P < 0.001$ using ANOVA followed by Tukey–Kramer as a post-hoc test. GSH: reduced glutathione; MDA: malondialdehyde; SOD: superoxide dismutase.

significantly suppressed Bax/Bcl-2 ratio in comparison with MTX group, Fig. 4K. In liraglutide alone group, non-significant changes in levels of expression were detected for Caspase-3, Bax and Bcl-2 as compared to control group Fig. (4D, 4H & 5D).

Finally, Caspase-3 activity was measured (Fig. 5F). One-way ANOVA showed significant differences among groups on the percent change in liver Caspase-3 activity. Rats receiving MTX showed significant increase in caspase-3 activity while pre-treatment with liraglutide showed a significant decrease in Caspase-3 activity as compared to MTX-induced group. In liraglutide alone group, non-significant changes in levels of expression were detected for activity of Caspase-3 as compared to control group.

3.2.4. Liraglutide protects liver in MTX induced liver injury via activation of Nrf2/HO-1/CREB pathway

To explore the mechanism underlying the effect of liraglutide on protecting MTX induced liver damage, the activation of Nrf2/HO-1/CREB pathway was examined. MTX-induced rats exhibited a significant decline in Nrf2 by 67.4% when compared to control group. On the other hand, liraglutide pre-treatment group significantly increased Nrf2 levels by nearly 2 folds when compared to MTX-induced rats (Fig. 6A). HO-1,

a Nrf2-regulated enzyme, showed significant increase in MTX group by 23.4% when compared to control group. In contrast, liraglutide pre-treated rats showed significant decrease by 26.7% when compared to MTX-induced rats (Fig. 6B).

Likewise, ELISA analysis revealed that pCREB tissue levels were significantly decreased by 40.8% in MTX-induced rats when compared to control group. However, a significant increase in pCREB levels was observed in liraglutide pre-treatment group where its levels reached nearly 79% increase as compared to MTX-induced group. Liraglutide alone group showed non-significant change as compared to control group (Fig. 6C).

3.2.5. Correlation studies

Estimation of liver injury damage by ALT concentration was found to be strongly correlated with the content of GSH, MDA, IL-6, NF-kB, Caspase-3, pCREB and Nrf-2 ($r = -0.907$, $r = 0.8135$, $r = 0.914$, $r = 0.907$, $r = 0.889$, $r = -0.825$ and $r = 0.935$, respectively, $p < 0.0001$) and moderately correlated with the content of HO-1 ($r = 0.598$) as shown in Fig. 7 (A-H).

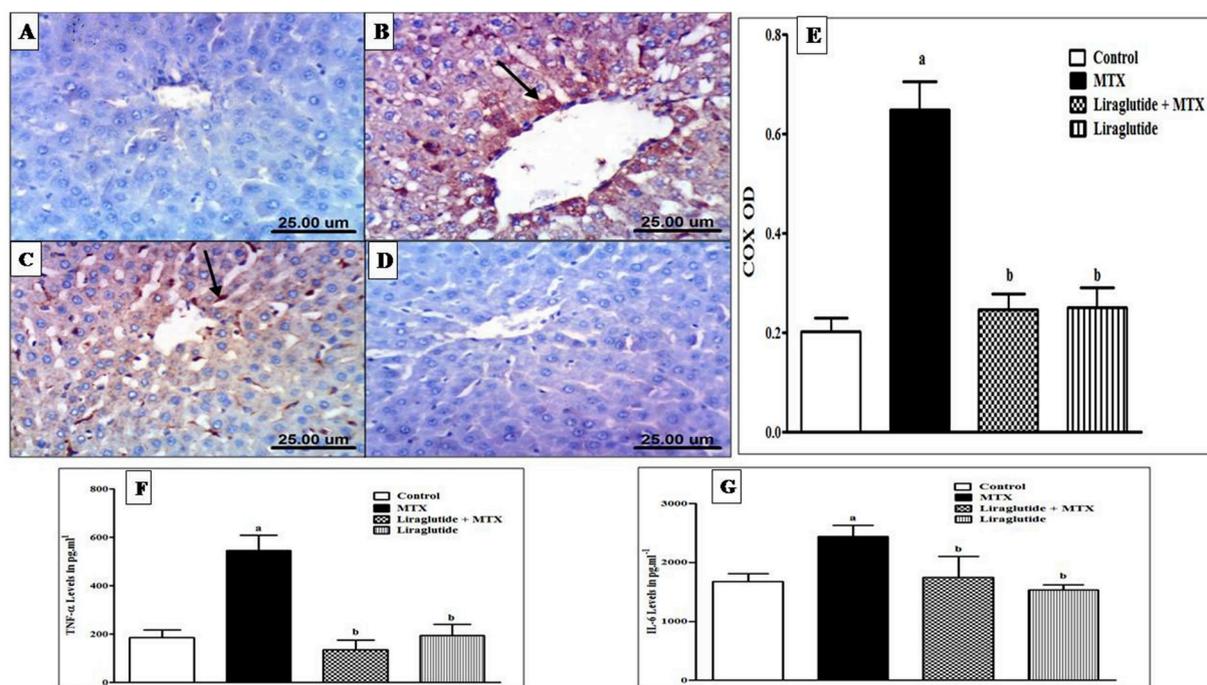


Fig. 2. Liraglutide pre-treatment reduced inflammation associated with MTX-induced liver injury. Immunohistochemical detection of liver sections of **A:** normal rats showed negative expression of COX-2. **B:** MTX-induced liver injury rats showed extensive COX-2 expression (brown stain). **C:** Liraglutide pre-treatment group showed minimum COX-2 expression. **D:** Liraglutide only treated group showed negative expression of COX-2. **E:** Quantitative image analysis for immunohistochemical staining of COX-2 expressed as optical density of stained area in different study groups. **F:** Effect of liraglutide pre-treatment on hepatic TNF- α levels. **G:** Effect of liraglutide pre-treatment on hepatic IL-6 levels. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Each column represents mean \pm SD (n = 6). a or b: statistically significant from the control or MTX group, respectively at $P < 0.001$ using one-way ANOVA followed by Tukey–Kramer as a post hoc test. COX-2: cyclooxygenase-2; IL-6: interleukin-6; TNF- α : tumor necrosis factor alpha.

3.2.6. Combined MTX and liraglutide displays additive cytotoxicity against HepG-2 cells

To elucidate the cytotoxic activities of different treatments, HepG-2 cells was treated with liraglutide alone, MTX alone and their combination. Liraglutide was able to induce growth inhibition after 48 h of incubation and the IC_{50} was $50 \pm 1.8 \mu\text{g/ml}$ (Fig. 8A). The IC_{50} of MTX was $369 \pm 7.9 \mu\text{g/ml}$ after 48 h (Fig. 8B). Combination of MTX with $10 \mu\text{g/ml}$ and $20 \mu\text{g/ml}$ of liraglutide resulted in significant reduction of IC_{50} ; where the IC_{50} of MTX with $10 \mu\text{g/ml}$ liraglutide was decreased to $322 \pm 5.8 \mu\text{g/ml}$. Moreover, The IC_{50} of MTX with $20 \mu\text{g/ml}$ liraglutide significantly decreased to $242 \pm 3.9 \mu\text{g/ml}$. To verify the type of interaction between MTX and liraglutide, isobologram analysis was carried out and revealed that the interaction between MTX and liraglutide was additive in HepG-2 cell line ($I = 1.07$ for $10 \mu\text{g/ml}$ liraglutide and $I = 1.05$ for $20 \mu\text{g/ml}$ liraglutide; respectively) Table .3.

4. Discussion

MTX is a disease-modifying anti-rheumatic drug which decreases inflammation in autoimmune diseases by suppressing the immune system (Mahmoud et al., 2017b). High doses of MTX, as used for acute leukemia or severe psoriasis have been related to acute hepatotoxicity, hepatic fibrosis and cirrhosis (Jung et al., 2014). Despite remarkable extensive research about MTX hepatotoxicity, the incidence of adverse liver events in methotrexate-treated patients on clinical trials is 11.2% (Conway and Carey, 2017). A glucagon like peptide-1 agonist, liraglutide, is an approved treatment for type-2 diabetes with demonstrated anti-oxidant and anti-inflammatory effects on clinical trials (Kahal et al., 2014; Rizzo et al., 2015). Besides, liraglutide has been reported to ameliorate liver microvascular dysfunction in cirrhosis and inhibit endothelial inflammation through activation of AMPK pathway (de Mesquita et al., 2017; Krasner et al., 2014). Accordingly, the current

study sheds light on the possible protective effect of liraglutide in MILI and elucidate the underlying molecular mechanisms.

To address our aim, first a dose screening phase was conducted to choose the most effective dose of liraglutide. Liver enzymes along with histopathological examination revealed that 1.2 mg/kg is the best choice for hepato-protection in MILI. Results of the current study observed that rats treated with MTX showed pronounced liver damage as indicated by significant increase in liver transaminases; AST and ALT in agreement with previous reports (Hafez et al., 2015; Mukherjee et al., 2013). The current biochemical findings were supported by histopathological examination results which revealed marked hepatic injury in MTX group. Several studies supported the deleterious effect of MTX on the liver (Erdogan et al., 2015; Moghadam et al., 2015). On the other hand, pre-treatment of MTX-administered rats with liraglutide in the present study significantly ameliorated the circulating liver function markers, AST and ALT. Also, liraglutide markedly alleviated the liver tissue architecture, where it was able to restore back the normal liver histology. In agreement with several studies, the current results confirm the effect liraglutide on reducing liver enzymes (Abdelsameea et al., 2017; Gaballah et al., 2017). These findings demonstrate the potent hepatoprotective activity of liraglutide.

Previous reports have demonstrated the key role of oxidative stress in MTX hepatotoxicity (Finkel, 2003; Mukherjee et al., 2013). The current study showed that a single dose of MTX (20 mg/kg) caused significant hepatotoxicity compared to control by disruption of the balance between oxidant and antioxidant status in a rat model. Likewise, MTX was shown to raise the level of MDA activity as well as to decline the levels of GSH and SOD activities in liver. These results are similar to those from previous studies of MTX effects in liver tissue (Hadi et al., 2012; Kurokawa et al., 1992; Mukherjee et al., 2013; Tunali-Akbay et al., 2010). Herein, liraglutide pre-treatment provided anti-oxidant effects not only on the non-enzymatic defense system

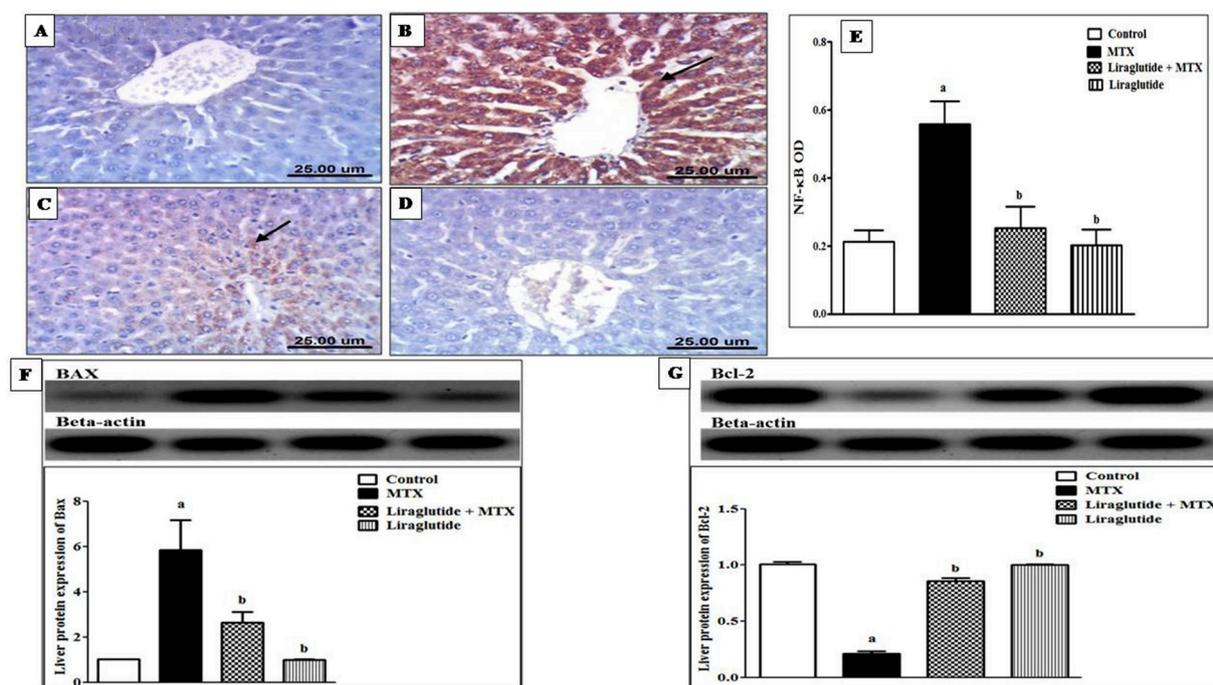


Fig. 3. Liraglutide pre-treatment suppressed NF-κB and BAX in liver tissue and increased Bcl-2 expression.

Each column represents mean \pm SD (n = 6). a or b: statistically significant from the control or MTX group, respectively at $P < 0.001$ using one-way ANOVA followed by Tukey–Kramer as a post hoc test. NF-κB: Nuclear factor kappa B.

Immunohistochemical detection of NF-κB. **A:** Control group showed negative expression of NF-κB. **B:** MTX group showed extensive NF-κB expression (brown stain). **C:** Liraglutide pre-treatment group showed less NF-κB expression. **D:** Liraglutide only treated group showed negative expression of NF-κB. **E:** Quantitative image analysis for immunohistochemical staining of NF-κB expressed as optical density of stained area in different study groups. **F:** Western blot analysis of Bax expression showed significant increase in MTX induced rats and decrease after pre-treatment of liraglutide. **G:** Western blot analysis of Bcl-2 expression showed significant decrease in MTX induced rats and returned to normal level after pre-treatment of liraglutide. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

(GSH), but also on the enzymatic one such as (SOD). Similar antioxidant effects of liraglutide were recently reported in many inflammatory conditions including inflammation on human endothelial cells (Shiraki et al., 2012), cardiotoxicity (Abbas and Kabil, 2017), type 2 diabetes (Rizzo et al., 2015), autoimmune encephalitis (DellaValle et al., 2016), and liver ischemia/reperfusion injury (Abdelsameea et al., 2017). In addition, liraglutide pre-treatment decreases the level of MDA and this finding is supported by a study of liraglutide on high-fat diet/streptozotocin-induced type 2 diabetes in rats (Gaballah et al., 2017).

Another main event involved in MTX liver injury is the inflammatory process initiated by kupffer cell activation (El-Sheikh et al., 2015; Racanelli and Rehmann, 2006). NF-κB is a transcription factor that up-regulates the expression of various pro-inflammatory cytokines such as TNF- α and IL-6 as well as pro-inflammatory enzymes such as COX-2. TNF- α and IL-6 cause further activation of NF-κB, hence, perpetuating the inflammatory cascade (Tacke et al., 2009). Consistent with previous studies, the present study showed that MTX increased NF-κB expression with a subsequent rise in liver TNF- α , IL-6 levels as well as hepatic COX-2 expression, thus, worsening the hepatic inflammation (Abd El-Twab et al., 2016; Mukherjee et al., 2013). Contrarily, it was previously reported that liraglutide counteracted lipopolysaccharide-induced release of different cytokines (Krasner et al., 2014). Another study has demonstrated that liraglutide attenuated the expression of TNF- α and inhibits NF-κB (Zhang et al., 2013). Moreover, liraglutide decreases IL-6 level, thus improves endothelial function (Dai et al., 2013). In line with this, we observed that pre-treatment with liraglutide resulted in marked decrease in COX-2 expression. This finding was supported by a study that reveals neuro-protective effect of liraglutide in diabetic peripheral neuropathy by marked decrease in IL-6 content as well as COX-2 expression (Moustafa et al., 2018).

Impairment of cytokine actions after liver damage can give rise to

excessive apoptosis, a key finding in various acute and chronic liver diseases, e.g., viral and autoimmune hepatitis, cholestatic disease, and alcohol or drug/toxin-induced liver injury (Neuman, 2001). Thus, oxidative stress and inflammation are two main mechanisms working in concert to induce apoptosis of hepatocytes (Jaeschke, 2011). Several studies have demonstrated the activation of proapoptotic proteins in MTX hepatotoxicity (Ali et al., 2014; Mahmoud et al., 2017b; Mukherjee et al., 2013). In the present study, MTX treated rats showed significantly increased liver Bax and caspase-3. These findings were in accordance with other studies which stated that MTX increased the expression of liver caspase-3 and Bax (El-Sheikh et al., 2015; Mehrzadi et al., 2018). Moreover, in the current study, liraglutide significantly decreased both Bax and caspase-3 in the liver of MTX-induced rats. In addition, liraglutide increased Bcl-2 anti-apoptotic factor. Due to the well-acknowledged role of oxidative stress and inflammation in provoking apoptosis, the anti-apoptotic effect of liraglutide could be directly related to its antioxidant and anti-inflammatory efficacies (Abdelsameea et al., 2017; Sharma et al., 2014). Previous studies have demonstrated liraglutide's ability to prevent apoptosis. In a model of ischemia/reperfusion injury in rat livers, liraglutide attenuated hepatocytes apoptosis via declined caspase-3 and augmented Bcl-2 expression (Abdelsameea et al., 2017). Another model of ischemic stroke reported that liraglutide could promote the expression of pro-survival proteins and simultaneously inhibit pro-apoptotic proteins and promote the overall anti-apoptotic effects on ischemic neurons (Zhu et al., 2016).

To delineate the mechanisms concealing the protective effect of liraglutide against MTX-induced oxidative stress and inflammation, Nrf-2 and pCREB expression were determined. Nrf-2 is a redox-sensitive transcription factor that controls the transcription of cytoprotective genes for maintaining cellular homeostasis in harmful stresses (Jaiswal,

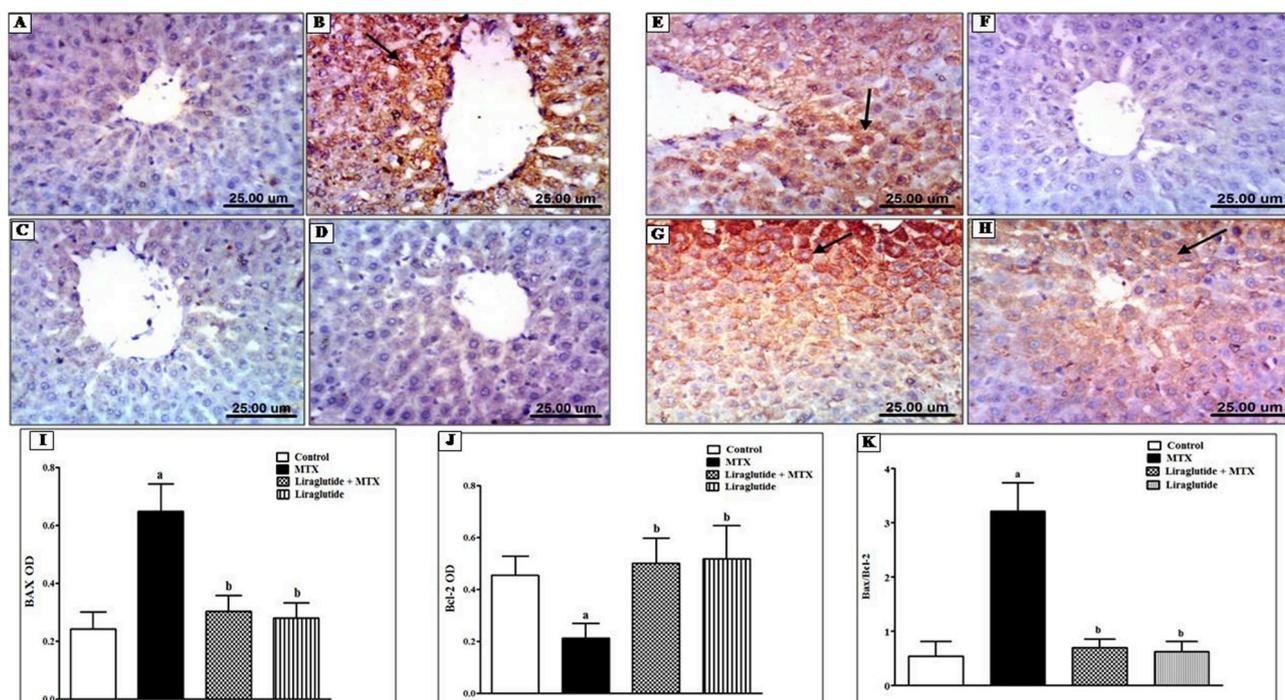


Fig. 4. Liraglutide pre-treatment suppressed apoptosis in liver tissue.

Immunohistochemical detection of Bax and Bcl-2, A: Control group showed negative Bax expression. B: MTX group showed extensive Bax expression (brown stain). C: Liraglutide + MTX group showed less Bax expression. D: Liraglutide only treated group showed minimal Bax expression. E: Control group showed maximal Bcl-2 expression (brown staining). F: MTX group showed less Bcl-2 expression. G: Liraglutide + MTX group showed extensive Bcl-2 expression. H: Liraglutide only treated group showed maximal Bcl-2 expression. I: Quantitative image analysis for immunohistochemical staining of Bax expressed as optical density of stained area of all study groups. J: Quantitative image analysis for immunohistochemical staining of Bcl-2 expressed as optical density of stained area of all study groups. K: Bax/Bcl-2 ratio of all study groups.

Each column represents mean \pm SD (n = 6). a or b: statistically significant from the control or MTX group, respectively at $P < 0.001$ using one-way ANOVA followed by Tukey–Kramer as a post hoc test.

2004). Interestingly, CREB-binding protein (CBP) is required for the transactivation activity of Nrf-2 (Katoh et al., 2001). In the current study, we found that MTX induced rats showed decrease in Nrf-2 and pCREB levels. Accordingly, previous studies presented that Nrf-2 decreased in MTX induced kidney toxicity (Abd El-Twab et al., 2016) and MTX induced liver toxicity (Mahmoud, 2014). On the other hand, previous studies showed that liraglutide has protective and antioxidant effects on hepatic gluco-lipotoxicity-induced liver cell apoptosis through activation of Nrf-2 pathway in diabetic fatty rats (Guo et al., 2018). Another report explained that liraglutide's alleviating effects on diabetes complicated with cerebral ischemia injury may be related to upregulation of Nrf-2 protein expression (Deng et al., 2018). In addition, liraglutide activated Nrf-2 pathway improved survival of mice after experimental myocardial infarction (Noyan-Ashraf et al., 2009). Importantly, liraglutide exerts its action on GLP-1 receptors. It has been demonstrated that activation of GLP-1R pathway activates CREB phosphorylation (Li et al., 2015b). In the current study, liraglutide pre-treated rats showed significant increase in Nrf-2 and pCREB expression. Taken together, these results clearly suggest for the first time that liraglutide hepatoprotective role may be closely linked to its ability to modulate NF- κ B and Nrf2 - pCREB crosstalk.

As a protective and adaptive response, most tissues exhibit robust activation of the highly inducible HO-1 (Agarwal and Bolisetty, 2013). To maintain homeostasis, the human body upregulates HO-1 in traumatic circumstances such as ischemia, atherosclerosis, and inflammation (Lee et al., 1997; Willis et al., 1996). It is reported that the serum level of HO-1 is excessively increased in patients with some inflammatory disorders such as adult-onset Still's disease and hemophagocytic syndrome (Kirino et al., 2005). In agreement with previous studies, the present results showed that MTX induced rats is associated

with increase in HO-1 expression due to oxidative stress and inflammation. This data is supported by previous study that showed increase in HO1 in liver after induced liver injury using heat in old rats (Zhang et al., 2003). Another study observed increased HO-1 activity in the lungs of rodents exposed to hypertoxic conditions (Lee et al., 1996). Liraglutide pretreatment in the current study restores the level of HO-1 in treatment group. This observation is supported by a clinical trial on type-2 diabetic patients which explained that patients with T2DM have abnormally elevated plasma levels of HO-1 and that liraglutide lowers its plasma concentrations (Rizzo et al., 2015).

Finally, the present results documented strong correlation between serum ALT activity and oxidative stress, inflammation, apoptosis markers and pCREB/Nrf-2. Serum ALT activity has been generally used as a major biomarker for liver injury in humans and in preclinical studies (Yang et al., 2014). In consistency with our findings, two studies showed a negative correlation between ALT activity and GSH concentration in patients with acute viral hepatitis and a positive correlation with MDA in women with pre-eclampsia (Atiba et al., 2016; Swietek and Juszczyk, 1997). Furthermore, a correlation was reported between liver damage and elevation of IL-6 and NF- κ B in acute liver injury (Borkham-Kamphorst et al., 2013). Another study reported that high levels of ALT and IL-6 in hepatitis B patients are correlated (Kao et al., 2012). In our study, ALT activity is positively correlated with IL-6 concentration and with NF- κ B expression which supports the association of liver damage and inflammation. Moreover, another report showed a strong correlation between ALT activity and Caspase 3/7 activity in the serum of HCV acute infection and was associated with liver injury in HCV patients (Choi et al., 2016). These data support our result of positive correlation between ALT activity and Caspase-3 expression in liver damage. Interestingly, a study reported that ALT activity increased in genetic

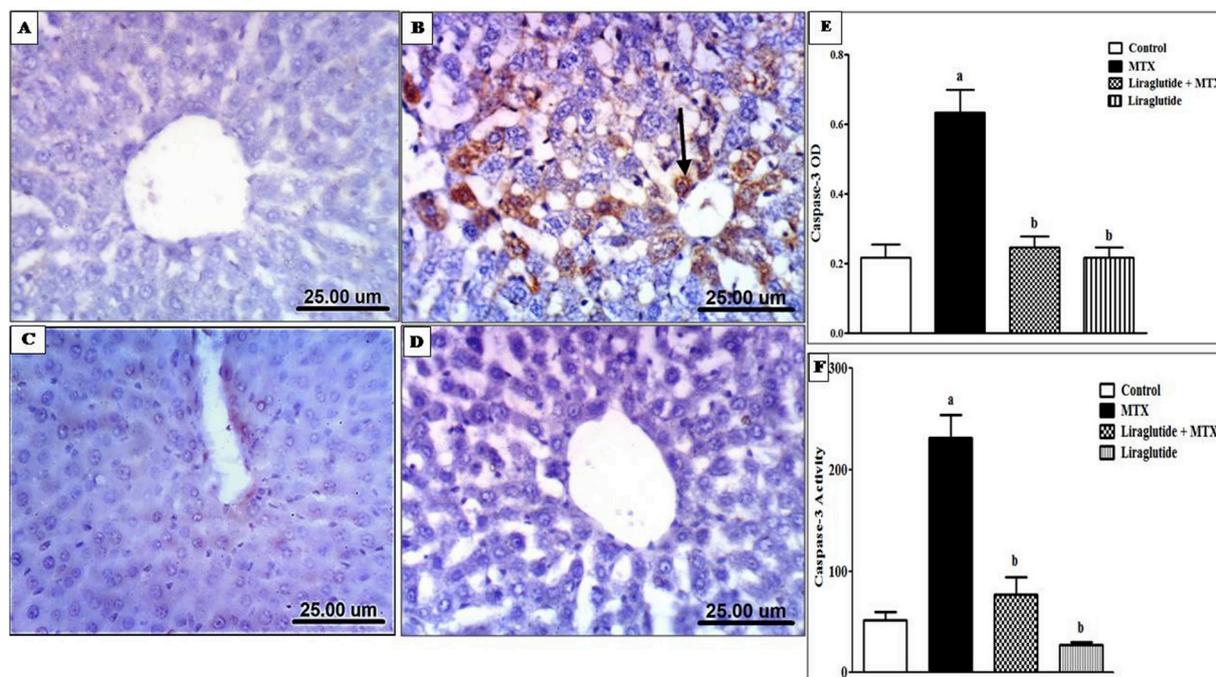


Fig. 5. Liraglutide pre-treatment decreased Caspase-3 expression in liver tissue. Immunohistochemical detection of Caspase-3, **A:** Control group showed negative Caspase-3 expression. **B:** MTX group showed extensive Caspase-3 expression (brown stain). **C:** Liraglutide + MTX group showed less Caspase-3 expression. **D:** Liraglutide only treated group showed minimal Caspase-3 expression. **E:** Quantitative image analysis for immunohistochemical staining of Caspase-3 expressed as optical density of stained area of all study groups. **F:** Effect of liraglutide pre-treatment on Caspase-3 activity. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Each column represents mean ± SD (n = 6). a or b: statistically significant from the control or MTX group, respectively at P < 0.001 using one-way ANOVA followed by Tukey–Kramer as a post hoc test.

disruption of Nrf-2 in a mouse model of hereditary hemochromatosis (Duarte et al., 2017). Here the present study reports a positive correlation between ALT activity and Nrf-2 expression and a negative correlation with HO-1 expression. As explained before that pCREB contributes with Nrf-2 activity for antioxidant effect, herein we reported

for the first time a strong positive correlation between ALT activity and pCREB in liver injury. Consistent with these data, correlative evidence suggests that impaired peroxidative enzyme and Nrf-2/pCREB expression contributed to hepatic oxidative stress, inflammation and apoptosis that may exacerbate drug-induced injury.

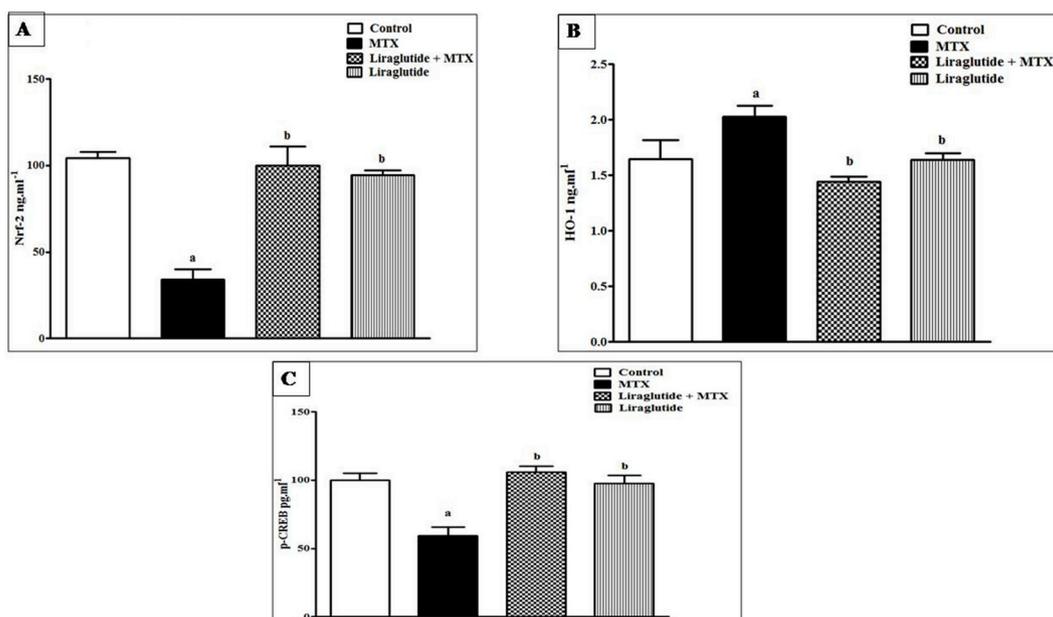


Fig. 6. Liraglutide pre-treatment activates Nrf-2/p-CREB and reduces HO-1 in MTX induced liver injury. Effects of Liraglutide on tissue levels of (A) Nrf-2, (B) HO-1, and (C) p-CREB.

Each column represents mean ± SD (n = 6). a or b: statistically significant from the control or MTX group, respectively at P < 0.001 using ANOVA followed by Tukey–Kramer as a post-hoc test. Nrf 2: Nuclear factor erythroid 2-related factor2; HO-1: Heme oxygenase-1; P-CREB: phosphorylated cAMP response element-binding protein.

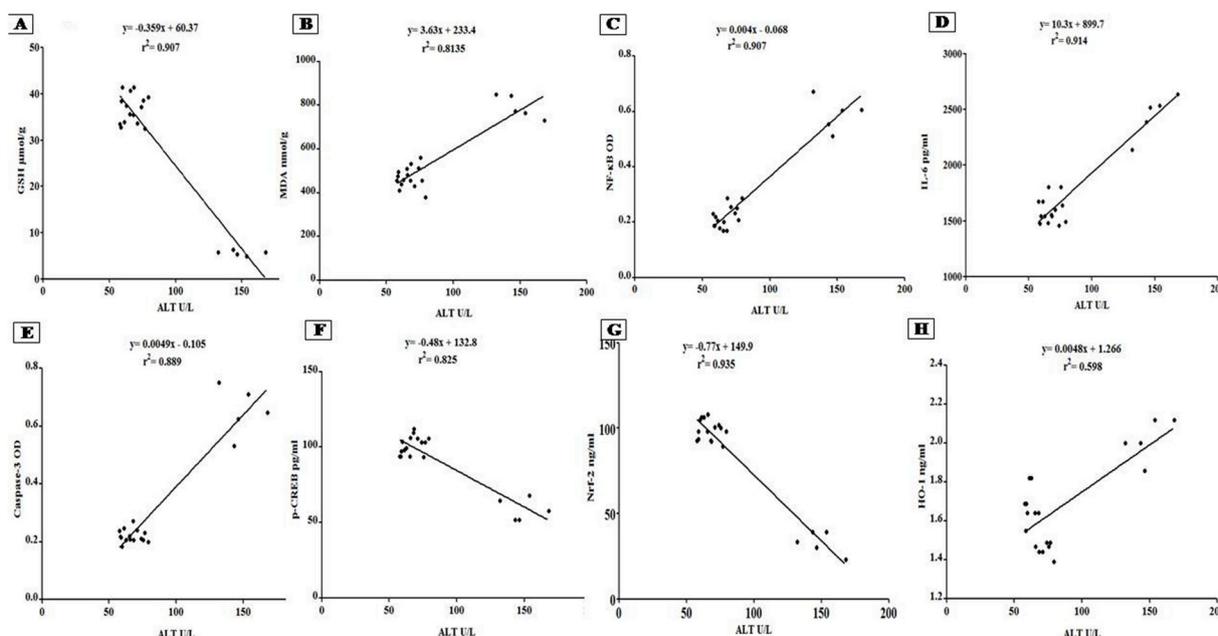


Fig. 7. Correlation analysis. Analysis of the correlation coefficients between ALT concentration and GSH (A), MDA (B),NF-κB (C), IL-6 (D), Caspase-3 (E), pCREB (F), Nrf-2 (G) and HO-1 (H).

Collectively, our experimental and correlational designs suggest a robust association between MTX induced liver injury and hepatic oxidative stress, inflammation and apoptosis. In addition, the correlation between Nrf-2/pCREB and MTX liver injury sheds light on a promising hepato-protective pathway. Furthermore, upon studying the modulatory effects of liraglutide on MTX anti-cancer activity, liraglutide exhibited inhibitory effect on HepG-2 cell line proliferation. These results are in agreement with previous results on HepG-2 cells, showing that liraglutide was able to induce autophagy and has antiproliferative effects via PI3K/Akt/mTOR (Krause et al., 2017). Furthermore, the current investigation led to the conclusion that simultaneous combination of MTX and 20µg/ml liraglutide resulted in additive growth inhibitory effect in HepG-2 cells.

5. Conclusion

Pre-treatment of liraglutide in MTX induced rats reversed toxic effects of MTX by decreasing oxidative stress, inflammation and apoptosis. Results suggest that liraglutide exerts hepatoprotective effects mainly by regulation of the triggering of the transcription factors Nrf-2 and NF-κB. Such protective effects are mediated through up-regulation of Nrf2 and pCREB concomitant with down-regulation of NF-κB with subsequent inhibition of inflammatory, oxidative stress and apoptotic pathways. Based on the current findings, liraglutide as a direct

Table 3

Effect of liraglutide on MTX cytotoxicity in HepG-2 cells.

| Drugs | HepG-2 | |
|----------------------------|------------------------------|-----------------------|
| | IC ₅₀ (µg/ml) | Interaction Index (I) |
| Liraglutide | 50 ± 1.8 µg/ml ^a | |
| Methotrexate | 369 ± 7.9 µg/ml | |
| MTX + 10 µg/ml liraglutide | 322 ± 5.8 µg/ml ^a | 1.07 |
| MTX + 20 µg/ml liraglutide | 242 ± 3.9 µg/ml ^a | 1.05 |

IC₅₀: The concentration of MTX necessary to produce 50% inhibition of cell growth, interaction index: $I = d1/D1 + d2/D2$, where d1 and d2 are the respective concentrations of MTX and liraglutide used in the combination required to produce a fixed level of inhibition IC₅₀, while D1 and D2 are their concentrations able to produce alone the same magnitude of effect. a: significantly different from MTX group, respectively at P < 0.001 using ANOVA followed by Tukey–Kramer as a post-hoc test.

antioxidant may be an effective treatment to preserve normal liver function under conditions of oxidative stress and thus improve outcomes in this significant patient population. A controlled clinical trial is needed to explore the potential of these results in patient care, in both cancer and type 2 diabetic patients.

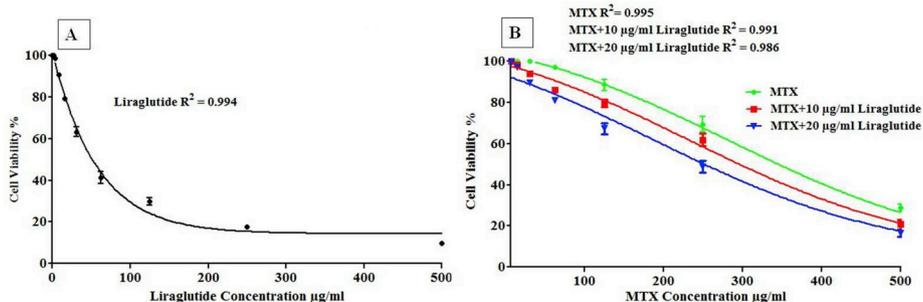


Fig. 8. Cytotoxicity Assay. Inhibition of human hepatic cancer cell line HepG-2 cell growth by liraglutide (A). Cytotoxic effects of Methotrexate (MTX) alone and in combination with 10 & 20 µg/ml of liraglutide on HepG-2 cells (B).

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Declaration of interests

None.

Transparency document

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References

Abbas, N.A.T., Kabil, S.L., 2017. Liraglutide ameliorates cardiotoxicity induced by doxorubicin in rats through the Akt/GSK-3beta signaling pathway. *Naunyn Schmiedeberg's Arch. Pharmacol.* 390, 1145–1153.

Abd El-Twab, S.M., Hozayen, W.G., Hussein, O.E., Mahmoud, A.M., 2016. 18beta-Glycyrrhetic acid protects against methotrexate-induced kidney injury by up-regulating the Nrf2/ARE/HO-1 pathway and endogenous antioxidants. *Ren. Fail.* 38, 1516–1527.

Abdel-Daim, M.M., Farouk, S.M., Madkour, F.F., Azab, S.S., 2015. Anti-inflammatory and immunomodulatory effects of *Spirulina platensis* in comparison to *Dunaliella salina* in acetic acid-induced rat experimental colitis. *Immunopharmacol. Immunotoxicol.* 37, 126–139.

Abdel-Maged, A.E., Gad, A.M., Abdel-Aziz, A.K., Aboulwafa, M.M., Azab, S.S., 2018. Comparative study of anti-VEGF ranibizumab and interleukin-6 receptor antagonist tocilizumab in adjuvant-induced arthritis. *Toxicol. Appl. Pharmacol.* 356, 65–75.

Abdelsamea, A.A., Abbas, N.A., Abdel Raouf, S.M., 2017. Liraglutide attenuates partial warm ischemia-reperfusion injury in rat livers. *Naunyn Schmiedeberg's Arch. Pharmacol.* 390, 311–319.

Abo-Haded, H.M., Elkablawy, M.A., Al-Johani, Z., Al-Ahmadi, O., El-Agamy, D.S., 2017. Hepatoprotective effect of sitagliptin against methotrexate induced liver toxicity. *PLoS One* 12, e0174295.

Agarwal, A., Bolisetty, S., 2013. Adaptive responses to tissue injury: role of heme oxygenase-1. *Trans. Am. Clin. Climatol. Assoc.* 124, 111–122.

Ahern, T., Tobin, A.M., Corrigan, M., Hogan, A., Sweeney, C., Kirby, B., O'Shea, D., 2013. Glucagon-like peptide-1 analogue therapy for psoriasis patients with obesity and type 2 diabetes: a prospective cohort study. *J. Eur. Acad. Dermatol. Venereol.* 27, 1440–1443.

Aithal, G.P., 2011. Hepatotoxicity related to antirheumatic drugs. *Nat. Rev. Rheumatol.* 7, 139–150.

Al Maruf, A., O'Brien, P.J., Naserzadeh, P., Fathian, R., Salimi, A., Pourahmad, J., 2018. Methotrexate induced mitochondrial injury and cytochrome c release in rat liver hepatocytes. *Drug Chem. Toxicol.* 41, 51–61.

Ali, N., Rashid, S., Nafees, S., Hasan, S.K., Sultana, S., 2014. Beneficial effects of Chrysin against Methotrexate-induced hepatotoxicity via attenuation of oxidative stress and apoptosis. *Mol. Cell. Biochem.* 385, 215–223.

Anighoro, A., Bajorath, J., Rastelli, G., 2014. Polypharmacology: challenges and opportunities in drug discovery. *J. Med. Chem.* 57, 7874–7887.

Atiba, A.S., Abbiyesuku, F.M., Oparinde, D.P., Niran-Atiba, T.A., Akindele, R.A., 2016. Plasma malondialdehyde (MDA): an indication of liver damage in women with pre-eclampsia. *Ethiop. J. Health Sci.* 26, 479–486.

Azab, S.S., El-Demerdash, E., Abdel-Naim, A.B., Youssef, E., El-Sharkawy, N., Osman, A.M., 2005. Modulation of epirubicin cytotoxicity by tamoxifen in human breast cancer cell lines. *Biochem. Pharmacol.* 70, 725–732.

Bailey, T., 2013. Options for combination therapy in type 2 diabetes: comparison of the ADA/EASD position statement and AACE/ACE algorithm. *Am. J. Med.* 126, S10–S20.

Banchroft, J.D., Stevens, A., Turner, D.R., 1996. *Theory and Practice of Histological Techniques*, fourth ed. Churchill Livingstone, New York, London, San Francisco, Tokyo.

Berenbaum, M.C., 1989. What is synergy? *Pharmacol. Rev.* 41, 93–141.

Biour, M., Ben Salem, C., Chazouilleres, O., Grange, J.D., Serfaty, L., Poupon, R., 2004. [Drug-induced liver injury; fourteenth updated edition of the bibliographic database of liver injuries and related drugs]. *Gastroenterol. Clin. Biol.* 28, 720–759.

Bjornsson, E., 2009. The natural history of drug-induced liver injury. *Semin. Liver Dis.* 29, 357–363.

Borkham-Kamphorst, E., van de Leur, E., Zimmermann, H.W., Karlmark, K.R., Tihaa, L., Haas, U., Tacke, F., Berger, T., Mak, T.W., Weiskirchen, R., 2013. Protective effects of lipocalin-2 (LCN2) in acute liver injury suggest a novel function in liver homeostasis. *Biochim. Biophys. Acta* 1832, 660–673.

Buckley, A.R., Crowe, P.D., Russell, D.H., 1988. Rapid activation of protein kinase C in isolated rat liver nuclei by prolactin, a known hepatic mitogen. *Proc. Natl. Acad. Sci. U. S. A.* 85, 8649–8653.

Cetiner, M., Sener, G., Sehirli, A.O., Eksioğlu-Demiralp, E., Ercan, F., Sirvanci, S., Gedik, N., Akpulat, S., Tecimer, T., Yegen, B.C., 2005. Taurine protects against methotrexate-induced toxicity and inhibits leukocyte death. *Toxicol. Appl. Pharmacol.* 209, 39–50.

Choi, Y.H., Jin, N., Kelly, F., Sakhivel, S.K., Yu, T., 2016. Elevation of alanine aminotransferase activity occurs after activation of the cell-death signaling initiated by pattern-recognition receptors but before activation of cytolytic effectors in NK or CD8+ T cells in the liver during acute HCV infection. *PLoS One* 11, e0165533.

Conway, R., Carey, J.J., 2017. Risk of liver disease in methotrexate treated patients. *World J. Hepatol.* 9, 1092–1100.

Dai, Y., Mehta, J.L., Chen, M., 2013. Glucagon-like peptide-1 receptor agonist liraglutide inhibits endothelin-1 in endothelial cell by repressing nuclear factor-kappa B activation. *Cardiovasc. Drugs Ther.* 27, 371–380.

de Mesquita, F.C., Guixé-Muntet, S., Fernandez-Iglesias, A., Maeso-Diaz, R., Vila, S., Híde, D., Ortega-Ribera, M., Rosa, J.L., Garcia-Pagan, J.C., Bosch, J., de Oliveira, J.R., Gracia-Sancho, J., 2017. Liraglutide improves liver microvascular dysfunction in cirrhosis: evidence from translational studies. *Sci. Rep.* 7, 3255.

DellaValle, B., Brix, G.S., Brock, B., Gejl, M., Landau, A.M., Moller, A., Rungby, J., Larsen, A., 2016. Glucagon-like peptide-1 analog, liraglutide, delays onset of experimental autoimmune encephalitis in lewis rats. *Front. Pharmacol.* 7, 433.

Deng, C., Cao, J., Han, J., Li, J., Li, Z., Shi, N., He, J., 2018. Liraglutide activates the Nrf2/HO-1 antioxidant pathway and protects brain nerve cells against cerebral ischemia in diabetic rats. *Comput. Intell. Neurosci.* 3094504 2018.

Drucker, D.J., Dritselis, A., Kirkpatrick, P., 2010. Liraglutide. *Nat. Rev. Drug Discov.* 9, 267–268.

Duarte, T.L., Caldas, C., Santos, A.G., Silva-Gomes, S., Santos-Goncalves, A., Martins, M.J., Porto, G., Lopes, J.M., 2017. Genetic disruption of NRF2 promotes the development of necroinflammation and liver fibrosis in a mouse model of HFE-hereditary hemochromatosis. *Redox Biol.* 11, 157–169.

Duraes, F., Pinto, M., Sousa, E., 2018. Old drugs as new treatments for neurodegenerative diseases. *Pharmaceuticals* 11.

El-Sheikh, A.A., Morsy, M.A., Abdalla, A.M., Hamouda, A.H., Alhaider, I.A., 2015. Mechanisms of thymoquinone hepatorenal protection in methotrexate-induced toxicity in rats. *Mediat. Inflamm.* 2015, 859383.

Erdogan, E., Ilgaz, Y., Gurgor, P.N., Oztas, Y., Topal, T., Oztas, E., 2015. Rutin ameliorates methotrexate induced hepatic injury in rats. *Acta Cir. Bras.* 30, 778–784.

Finkel, T., 2003. Oxidant signals and oxidative stress. *Curr. Opin. Cell Biol.* 15, 247–254.

Gaballah, H.H., Zakaria, S.S., Mwafy, S.E., Tahoon, N.M., Ebeid, A.M., 2017. Mechanistic insights into the effects of quercetin and/or GLP-1 analogue liraglutide on high-fat diet/streptozotocin-induced type 2 diabetes in rats. *Biomed. Pharmacother.* 92, 331–339.

Gallagher, S.R., 2006. One-dimensional SDS gel electrophoresis of proteins. *Curr. Protoc. Mol. Biol.* Chapter 10 Unit 10 12A.

Gao, H., Zeng, Z., Zhang, H., Zhou, X., Guan, L., Deng, W., Xu, L., 2015. The glucagon-like peptide-1 analogue liraglutide inhibits oxidative stress and inflammatory response in the liver of rats with diet-induced non-alcoholic fatty liver disease. *Biol. Pharm. Bull.* 38, 694–702.

Guo, J., Li, C., Yang, C., Li, B., Wei, J., Lin, Y., Ye, P., Hu, G., Li, J., 2018. Liraglutide reduces hepatic glucolipotoxicity-induced liver cell apoptosis through NRF2 signaling in Zucker diabetic fatty rats. *Mol. Med. Rep.* 17, 8316–8324.

Habib, R., Wahdan, S.A., Gad, A.M., Azab, S.S., 2019. Infliximab abrogates cadmium-induced testicular damage and spermotoxicity via enhancement of steroidogenesis and suppression of inflammation and apoptosis mediators. *Ecotoxicol. Environ. Saf.* 182, 109398.

Hadi, N.R., Al-Amran, F.G., Swadi, A., 2012. Metformin ameliorates methotrexate-induced hepatotoxicity. *J. Pharmacol. Pharmacother.* 3, 248–253.

Hafez, H.M., Ibrahim, M.A., Ibrahim, S.A., Amin, E.F., Goma, W., Abdelrahman, A.M., 2015. Potential protective effect of etanercept and aminoguanidine in methotrexate-induced hepatotoxicity and nephrotoxicity in rats. *Eur. J. Pharmacol.* 768, 1–12.

Howard, S.C., McCormick, J., Pui, C.H., Buddington, R.K., Harvey, R.D., 2016. Preventing and managing toxicities of high-dose methotrexate. *The Oncologist* 21, 1471–1482.

Jacobsen, L.V., Hindsberger, C., Robson, R., Zdravkovic, M., 2009. Effect of renal impairment on the pharmacokinetics of the GLP-1 analogue liraglutide. *Br. J. Clin. Pharmacol.* 68, 898–905.

Jaeschke, H., 2011. Reactive oxygen and mechanisms of inflammatory liver injury: present concepts. *J. Gastroenterol. Hepatol.* 26 (Suppl. 1), 173–179.

Jaiswal, A.K., 2004. Nrf2 signaling in coordinated activation of antioxidant gene expression. *Free Radic. Biol. Med.* 36, 1199–1207.

Janssen-Heininger, Y.M., Poynter, M.E., Baeuerle, P.A., 2000. Recent advances towards understanding redox mechanisms in the activation of nuclear factor kappaB. *Free*

- Radic. Biol. Med. 28, 1317–1327.
- Jung, Y.A., Choi, Y.K., Jung, G.S., Seo, H.Y., Kim, H.S., Jang, B.K., Kim, J.G., Lee, I.K., Kim, M.K., Park, K.G., 2014. Sitagliptin attenuates methionine/choline-deficient diet-induced steatohepatitis. *Diabetes Res. Clin. Pract.* 105, 47–57.
- Kahal, H., Abouda, G., Rigby, A.S., Coady, A.M., Kilpatrick, E.S., Atkin, S.L., 2014. Glucagon-like peptide-1 analogue, liraglutide, improves liver fibrosis markers in obese women with polycystic ovary syndrome and nonalcoholic fatty liver disease. *Clin. Endocrinol.* 81, 523–528.
- Kannan, K., Jain, S.K., 2000. Oxidative stress and apoptosis. *Pathophysiology* 7, 153–163.
- Kao, J.T., Lai, H.C., Tsai, S.M., Lin, P.C., Chuang, P.H., Yu, C.J., Cheng, K.S., Su, W.P., Hsu, P.N., Peng, C.Y., Wu, Y.Y., 2012. Rather than interleukin-27, interleukin-6 expresses positive correlation with liver severity in naive hepatitis B infection patients. *Liver Int.* 32, 928–936.
- Katoh, Y., Itoh, K., Yoshida, E., Miyagishi, M., Fukamizu, A., Yamamoto, M., 2001. Two domains of Nrf2 cooperatively bind CBP, a CREB binding protein, and synergistically activate transcription. *Genes Cells* 6, 857–868.
- Kawai, Y., Garduno, L., Theodore, M., Yang, J., Arinze, I.J., 2011. Acetylation-deacetylation of the transcription factor Nrf2 (nuclear factor erythroid 2-related factor 2) regulates its transcriptional activity and nucleocytoplasmic localization. *J. Biol. Chem.* 286, 7629–7640.
- Kirino, Y., Takeno, M., Iwasaki, M., Ueda, A., Ohno, S., Shirai, A., Kanamori, H., Tanaka, K., Ishigatsubo, Y., 2005. Increased serum HO-1 in hemophagocytic syndrome and adult-onset Still's disease: use in the differential diagnosis of hyperferritinemia. *Arthritis Res. Ther.* 7, R616–R624.
- Krasner, N.M., Ido, Y., Ruderman, N.B., Cacicedo, J.M., 2014. Glucagon-like peptide-1 (GLP-1) analog liraglutide inhibits endothelial cell inflammation through a calcium and AMPK dependent mechanism. *PLoS One* 9, e97554.
- Krause, G.C., Lima, K.G., Dias, H.B., da Silva, E.F.G., Haute, G.V., Basso, B.S., Gassen, R.B., Marczak, E.S., Nunes, R.S.B., de Oliveira, J.R., 2017. Liraglutide, a glucagon-like peptide-1 analog, induce autophagy and senescence in HepG2 cells. *Eur. J. Pharmacol.* 809, 32–41.
- Kurokawa, T., Kobayashi, H., Nonami, T., Harada, A., Nakao, A., Sugiyama, S., Ozawa, T., Takagi, H., 1992. Beneficial effects of cyclosporine on posts ischemic liver injury in rats. *Transplantation* 53, 308–311.
- Larrey, D., 2000. Drug-induced liver diseases. *J. Hepatol.* 32, 77–88.
- Lee, P.J., Alam, J., Sylvester, S.L., Inamdar, N., Otterbein, L., Choi, A.M., 1996. Regulation of heme oxygenase-1 expression in vivo and in vitro in hyperoxic lung injury. *Am. J. Respir. Cell Mol. Biol.* 14, 556–568.
- Lee, P.J., Jiang, B.H., Chin, B.Y., Iyer, N.V., Alam, J., Semenza, G.L., Choi, A.M., 1997. Hypoxia-inducible factor-1 mediates transcriptional activation of the heme oxygenase-1 gene in response to hypoxia. *J. Biol. Chem.* 272, 5375–5381.
- Li, C.L., Zhao, L.J., Zhou, X.L., Wu, H.X., Zhao, J.J., 2015a. Review on the effect of glucagon-like peptide-1 receptor agonists and dipeptidyl peptidase-4 inhibitors for the treatment of non-alcoholic fatty liver disease. *J. Huazhong Univ. Sci. Technol. Med. Sci.* 35, 333–336.
- Li, Y., Bader, M., Tamargo, I., Rubovitch, V., Tweedie, D., Pick, C.G., Greig, N.H., 2015b. Liraglutide is neurotrophic and neuroprotective in neuronal cultures and mitigates mild traumatic brain injury in mice. *J. Neurochem.* 135, 1203–1217.
- Liu, G.H., Qu, J., Shen, X., 2008. NF-kappaB/p65 antagonizes Nrf2-ARE pathway by depriving CBP from Nrf2 and facilitating recruitment of HDAC3 to MafK. *Biochim. Biophys. Acta* 1783, 713–727.
- Mahmoud, A.M., 2014. Hesperidin protects against cyclophosphamide-induced hepatotoxicity by upregulation of PPARgamma and abrogation of oxidative stress and inflammation. *Can. J. Physiol. Pharmacol.* 92, 717–724.
- Mahmoud, A.M., Hozayen, W.G., Ramadan, S.M., 2017a. Berberine ameliorates methotrexate-induced liver injury by activating Nrf2/HO-1 pathway and PPARgamma, and suppressing oxidative stress and apoptosis in rats. *Biomed. Pharmacother.* 94, 280–291.
- Mahmoud, A.M., Hussein, O.E., Hozayen, W.G., Abd El-Twab, S.M., 2017b. Methotrexate hepatotoxicity is associated with oxidative stress, and down-regulation of PPARgamma and Nrf2: protective effect of 18beta-Glycyrrhetic acid. *Chem. Biol. Interact.* 270, 59–72.
- Mehrzadi, S., Fatemi, I., Esmailizadeh, M., Ghaznavi, H., Kalantar, H., Goudarzi, M., 2018. Hepatoprotective effect of berberine against methotrexate induced liver toxicity in rats. *Biomed. Pharmacother.* 97, 233–239.
- Menter, A., Korman, N.J., Elmetts, C.A., Feldman, S.R., Gelfand, J.M., Gordon, K.B., Gottlieb, A.B., Koo, J.Y., Lebwohl, M., Lim, H.W., Van Voorhees, A.S., Beutner, K.R., Bhushan, R., 2009. Guidelines of care for the management of psoriasis and psoriatic arthritis: section 4. Guidelines of care for the management and treatment of psoriasis with traditional systemic agents. *J. Am. Acad. Dermatol.* 61, 451–485.
- Moghadam, A.R., Tutunchi, S., Namvaran-Abbas-Abad, A., Yazdi, M., Bonyadi, F., Mohajeri, D., Mazani, M., Marzban, H., Los, M.J., Ghavami, S., 2015. Pre-administration of turmeric prevents methotrexate-induced liver toxicity and oxidative stress. *BMC Complement Altern. Med.* 15, 246.
- Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods* 65, 55–63.
- Moustafa, P.E., Abdelkader, N.F., El Awdan, S.A., El-Shabrawy, O.A., Zaki, H.F., 2018. Liraglutide ameliorated peripheral neuropathy in diabetic rats: involvement of oxidative stress, inflammation and extracellular matrix remodeling. *J. Neurochem.* 146, 173–185.
- Mukherjee, S., Ghosh, S., Choudhury, S., Adhikary, A., Manna, K., Dey, S., Sa, G., Das, T., Chattopadhyay, S., 2013. Pomegranate reverses methotrexate-induced oxidative stress and apoptosis in hepatocytes by modulating Nrf2-NF-kappaB pathways. *J. Nutr. Biochem.* 24, 2040–2050.
- Neuman, M.G., 2001. Apoptosis in diseases of the liver. *Crit. Rev. Clin. Lab. Sci.* 38, 109–166.
- Niture, S.K., Khatri, R., Jaiswal, A.K., 2014. Regulation of nrf2-an update. *Free Radic. Biol. Med.* 66, 36–44.
- Noyan-Ashraf, M.H., Momen, M.A., Ban, K., Sadi, A.M., Zhou, Y.Q., Riazi, A.M., Baggio, L.L., Henkelman, R.M., Husain, M., Drucker, D.J., 2009. GLP-1R agonist liraglutide activates cytoprotective pathways and improves outcomes after experimental myocardial infarction in mice. *Diabetes* 58, 975–983.
- Olaywi, M., Bhatia, T., Anand, S., Singhal, S., 2013. Novel anti-diabetic agents in non-alcoholic fatty liver disease: a mini-review. *Hepatobiliary Pancreat. Dis. Int.* 12, 584–588.
- Racaneli, V., Rehermann, B., 2006. The liver as an immunological organ. *Hepatology* 43, S54–S62.
- Rizzo, M., Abate, N., Chandalia, M., Rizvi, A.A., Giglio, R.V., Nikolic, D., Marino Gammazza, A., Barbagallo, I., Isenovic, E.R., Banach, M., Montalto, G., Li Volti, G., 2015. Liraglutide reduces oxidative stress and restores heme oxygenase-1 and ghrelin levels in patients with type 2 diabetes: a prospective pilot study. *J. Clin. Endocrinol. Metab.* 100, 603–606.
- Rosenberg, P., Urwitz, H., Johannesson, A., Ros, A.M., Lindholm, J., Kinnman, N., Hulterantz, R., 2007. Psoriasis patients with diabetes type 2 are at high risk of developing liver fibrosis during methotrexate treatment. *J. Hepatol.* 46, 1111–1118.
- Ross, S.A., Ballantine, J., 2013. Early use of glucagon-like peptide-1 receptor agonists (GLP-1 RAs) in type 2 diabetes. *Curr. Med. Res. Opin.* 29, 1617–1626.
- Rothem, L., Stark, M., Assaraf, Y.G., 2004. Impaired CREB-1 phosphorylation in antifolate-resistant cell lines with down-regulation of the reduced folate carrier gene. *Mol. Pharmacol.* 66, 1536–1543.
- Russmann, S., Kullak-Ublick, G.A., Grattagliano, I., 2009. Current concepts of mechanisms in drug-induced hepatotoxicity. *Curr. Med. Chem.* 16, 3041–3053.
- Shalaby, Y.M., Menze, E.T., Azab, S.S., Awad, A.S., 2019 May. Involvement of Nrf2/HO-1 antioxidant signaling and NF-kappaB inflammatory response in the potential protective effects of vincamine against methotrexate-induced nephrotoxicity in rats: cross talk between nephrotoxicity and neurotoxicity. *Arch. Toxicol.* 93 (5), 1417–1431.
- Sharma, M.K., Jalewa, J., Holscher, C., 2014. Neuroprotective and anti-apoptotic effects of liraglutide on SH-SY5Y cells exposed to methylglyoxal stress. *J. Neurochem.* 128, 459–471.
- Shim, J.S., Liu, J.O., 2014. Recent advances in drug repositioning for the discovery of new anticancer drugs. *Int. J. Biol. Sci.* 10, 654–663.
- Shirakawa, J., Tanami, R., Togashi, Y., Tajima, K., Orime, K., Kubota, N., Kadowaki, T., Goshima, Y., Terauchi, Y., 2012. Effects of liraglutide on beta-cell-specific glucokinase-deficient neonatal mice. *Endocrinology* 153, 3066–3075.
- Shiraki, A., Oyama, J., Komoda, H., Asaka, M., Komatsu, A., Sakuma, M., Kodama, K., Sakamoto, Y., Kotooka, N., Hirase, T., Node, K., 2012. The glucagon-like peptide 1 analog liraglutide reduces TNF-alpha-induced oxidative stress and inflammation in endothelial cells. *Atherosclerosis* 221, 375–382.
- Singh, J.A., Saag, K.G., Bridges Jr., S.L., Akl, E.A., Bannuru, R.R., Sullivan, M.C., Vaysbrot, E., McNaughton, C., Osani, M., Shmerling, R.H., Curtis, J.R., Furst, D.E., Parks, D., Kavanaugh, A., O'Dell, J., King, C., Leong, A., Matteson, E.L., Schousboe, J.T., Drevlow, B., Ginsberg, S., Grober, J., St Clair, E.W., Tindall, E., Miller, A.S., McAlindon, T., 2016. 2015 American College of Rheumatology guideline for the treatment of rheumatoid arthritis. *Arthritis Care Res.* 68, 1–25.
- Sun, Z., Chin, Y.E., Zhang, D.D., 2009. Acetylation of Nrf2 by p300/CBP augments promoter-specific DNA binding of Nrf2 during the antioxidant response. *Mol. Cell. Biol.* 29, 2658–2672.
- Swietek, K., Juszczyk, J., 1997. Reduced glutathione concentration in erythrocytes of patients with acute and chronic viral hepatitis. *J. Viral Hepat.* 4, 139–141.
- Tacke, F., Luedde, T., Trautwein, C., 2009. Inflammatory pathways in liver homeostasis and liver injury. *Clin. Rev. Allergy Immunol.* 36, 4–12.
- Tunali-Akbay, T., Sehirli, O., Ercan, F., Sener, G., 2010. Resveratrol protects against methotrexate-induced hepatic injury in rats. *J. Pharm. Pharm. Sci.* 13, 303–310.
- Wardyn, J.D., Ponsford, A.H., Sanderson, C.M., 2015. Dissecting molecular cross-talk between Nrf2 and NF-kappaB response pathways. *Biochem. Soc. Trans.* 43, 621–626.
- Willis, D., Moore, A.R., Frederick, R., Willoughby, D.A., 1996. Heme oxygenase: a novel target for the modulation of the inflammatory response. *Nat. Med.* 2, 87–90.
- Xue, H., Li, J., Xie, H., Wang, Y., 2018. Review of drug repositioning approaches and resources. *Int. J. Biol. Sci.* 14, 1232–1244.
- Yang, X., S., L., Shi, Q., Salminen, W.F., 2014. Hepatic toxicity biomarkers. In: G.R. (Ed.), *Biomarkers in Toxicology*. Elsevier Inc, NY, USA, pp. 241–259.
- Zhang, H.J., Xu, L., Drake, V.J., Xie, L., Oberley, L.W., Kregel, K.C., 2003. Heat-induced liver injury in old rats is associated with exaggerated oxidative stress and altered transcription factor activation. *FASEB J.* 17, 2293–2295.
- Zhang, L., Yang, M., Ren, H., Hu, H., Boden, G., Li, L., Yang, G., 2013. GLP-1 analogue prevents NAFLD in ApoE KO mice with diet and Acp30 knockdown by inhibiting c-JNK. *Liver Int.* 33, 794–804.
- Zhu, H., Zhang, Y., Shi, Z., Lu, D., Li, T., Ding, Y., Ruan, Y., Xu, A., 2016. The neuroprotection of liraglutide against ischaemia-induced apoptosis through the activation of the PI3K/AKT and MAPK pathways. *Sci. Rep.* 6, 26859.