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EMPAGLIFLOZIN MITIGATES ISCHEMIA/REPERFUSION-INDUCED LIVER INJURY IN RATS: MODULATION OF NF-KB, SMAD-4, VEGF, AND FIBRINOGEN PROTEIN EXPRESSIONS

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Liver ischemia/reperfusion (IR) injury is a critical cause of mortality in patients undergoing liver transplantation. Our objective was to assess the protective impact of empagliflozin (EGZ) on IR-induced liver damage in rats and to investigate its potential protective mechanism. A total of 24 male Wistar albino rats were randomly divided into four groups: sham; IR; EGZ (10 mg/kg/day; p.o) and IR + EGZ. Serum levels of aminotransferase ALT and AST, gamma glutamyl transferase GGT and total bilirubin TB were evaluated. Liver samples were used to evaluate oxidative stress indicators, reduced glutathione GSH, malondialdehyde MDA, total nitic oxide NOx and myeloperoxidase MPO, inflammatory mediators as tumor necrosis factor alpha TNF-α and interleukin -33 IL-33, and apoptosis markers, Bcl-2-associated X protein Bax, galactine-9 Gal9 and B-cell lymphoma 2 Bcl2. Hepatic vascular endothelial growth factor VEGF and fibrinogen expressions were determined by Western blot analysis. Immunohistochemical analysis for hepatic SMAD-4 and NF-κB was performed along with histopathological examination. Pretreatment with EGZ significantly ameliorated liver functions and oxidative stress compared to IR injury model. Similarly, the inflammatory cytokines were significantly decreased along with marked decline of the apoptosis biomarkers and a significant decrease of VEGF and fibrinogen expressions. Additionally, SMAD-4 and NF-κB protein expressions were significantly decreased compared to IR group. These results were further supported by improvement liver architecture. Our findings suggested that EGZ has the potentiality to be protective against hepatic IRI

***Keywords**: Empagliflozin, Fibrinogen, Ischemia reperfusion, Liver, SMAD-4 and VEGF*

INTRODUCTION

Hepatic injury induced by I/R, is a clinical problem that appears with certain liver conditions, such as major liver surgery, hemorrhagic shock and liver transplantation, leading to cellular injury and severe organ damage[1]. Ischemia causes shortage and cutting of oxygen and blood supply to this organ, and when the blood returns by reperfusion multiple effects of I/R injury results[1-3]. The degree of the liver damage based primarily on ischemia duration and the existence of dependent hepatic diseases[4]. The major participating factors in IR injury start by elevation in reactive oxygen species (ROS) production in the mitochondria after reperfusion. ROS furthermore activates pro-inflammatory mediators, cytokines and chemokines starting an inflammatory cascade that eventually triggers apoptosis, activating immune cells and ultimately promoting hepatocytes and endothelial cell death[1,5].

Additionally, the nuclear factor kappa B (NF-κB) pathway modulates pro-inflammatory

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mediators expression that include cytokines, interleukin 33 (IL-33) and tumor necrosis factor alpha (TNF-α), and chemokines[6,7].

Reperfusion causes extensive pro-inflammatory mediators discharge from kupffer cells, which may be required for apoptosis initiation by changing the equilibrium between the pro-apoptosis proteins, Bcl-2-associated X protein (Bax) and galactine-9 (Gal-9), and the anti-apoptosis proteins, B-cell lymphoma 2 (Bcl-2), thus speeding up the fragmentation of DNA[8]**.**

Moreover, ischemia results in marked elevation in angiogenic factor, vascular endothelial growth factor (VEGF), and its receptors, which causes edema of hepatocytes after liver IR[9]**.** In addition**,** degradation of fibrin after IR results in the development of fibrin-like protein and fibrin D fragments, which play a crucial role in the acute and chronic liver injury[9,10].

SMAD family member 4 (SMAD)-4 protein, factor involved in fibrogenesis and highly expressed following reperfusion, plays a crucial role in initiating liver fibrosis[11]. In the progression of nonalcoholic steatohepatitis (NASH), SMAD-4 deletion may mitigate fibrosis,inflammation and diminish cell apoptosis[12].

Empagliflozin, a sodium-glucose cotransporter 2 (SGLT2) inhibitor, is a novel anti hyperglycemic agent used for the treatment of type 2 diabetes mellitus (T2DM). It reduces blood glucose level by inhibiting glucose renal reabsorption at the S1 segment of the proximal convoluted tubule[13]. EGZ administration has beneficial effects through its antioxidant power, in addition, its anti-inflammatory effects was evidenced through limitation of IL-1β and TNF-α[14,15]. **Ala et al. (2022)**[16] disclosed that EGZ ameliorated the renal I/R injury in non-diabetic rats through its antioxidant effect.

In this work, we aimed to evaluate the possible role of the preconditioning with EGZ in the attenuation of the hepatic damage induced by liver IRI in rats through examining its antioxidant, anti-inflammatory, anti-fibrotic and anti-apoptotic effects.

MATERIALS AND METHODS

Chemicals, drugs, diagnostic kits and antibodies

Empagliflozin was obtained from Sigma-Aldrich (St. Louis, MO, USA). It was dispersed in 1% freshly prepared carboxymethyl cellulose (CMC). Colorimetric test kits of alanine amino transferase (ALT) (CAT. NO. AL 1031), aspartate amino transferase (AST) (CAT. NO. AS 1061), gamma glutamyl transferase (GGT) and total bilirubin (TB) (CAT. NO. BR 1111) were acquired from Bio-diagnostic (Giza, Egypt). (GR 2511), malondialdehyde (MDA) (MD 2529), and total nitrate/nitrite (NOx) (NO 2533) assay kits were obtained from Bio-diagnostic (Giza, Egypt) and the myeloperoxidase (MPO) assay kit (MAK 068) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Mice TNF-α and IL-33 enzyme-linked immunosorbent assay (ELISA) kits (ab 100747) (ab 213475) respectively were conducted from Abcam company (Waltham, Boston). ELISA tests for hepatic Bax (MBS 9136494), Gal-9 (MBS 7255192) and Bcl-2 (MBS 2515143) were purchased from My BioSource (SD, USA). We purchased the anti-rat SMAD-4 monoclonal rabbit antibody and anti-rabbit IgG from Sigma-Aldrich (SKU: ZR1981). The Anti-rat NF-kB polyclonal rabbit antibody was (SKU: A002835337-1) bought from Wuhan Booster Biological Technology Co., Ltd., Wuhan, China. Bio-diagnostic Company was contacted to obtain polyvinylidene fluoride (PVDF) membrane and tris lysis buffer. Purchased from Sigma-Aldrich were polyclonal anti-rat VEGF rabbit antibody (SKU: SAB5700716) and polyclonal anti-rat fibrinogen rabbit antibody (SKU: SAB5701325). All of the remaining solvents and compounds were of the best available on the market.

Animals

The current study was carried out using 24 adults male Wistar rats weighing (230–280) g. Animals were purchased from the animal house of Faculty of Medicine, Sohag University, Egypt. Throughout the duration of the experiment, the animals received humane care in accordance with institutional guidelines. Institutional Animal Care and Use Committee (IACUC) of Faculty of Pharmacy at Beni-Suef University (Egypt) authorized the protocol (Approval no: 022-263). Before beginning the experiment, all rats were acclimated to the experimental environment for seven days. Animals had unlimited access to a standard laboratory diet and *ad libitum* water. They were allocated in four cages 6 rats per each, the experimental conditions for the animals were

kept at room temperature (22–25 °C) and followed a 12-hrs light/12-hrs dark cycle. Additionally, the subjects were deprived of food for a period of 12 hrs before the experiment commenced.

Experimental design

In this experiment, rats were randomly divided into four groups (n=6):

1) Sham control group: rats received daily 0.9% physiological saline by oral gavage for 10 days then subjected to surgical procedure without ischemia.
2) I/R control group: rats received daily 0.9% physiological saline for 10 days by oral gavage, followed by 30 min of partial hepatic ischemia then 60 min of reperfusion.
3) EGZ control group: rats received daily EGZ (10 mg/kg; p.o) for 10 days, then underwent surgery with no ischemia [16].
4) EGZ plus I/R group: rats received orally EGZ at a dose of 10 mg/kg/day for 10 days, then underwent a 30-minute laparotomy with a partial hepatic occlusion and reperfusion for 60 min.

The drug was administered for 10 consecutive days in accordance with the previous work of **Sherif and Al-Shaalan (2018)**[6].

Hepatic IR Induction

On day 10, the surgical procedure was performed after anesthetizing rats with a combined injection of ketamine (100 mg/kg) and xylazine (10 mg/kg) intraperitoneally. After anesthetizing animals, abdominal hair was trimmed, and then cleaned with povidone-iodine, followed by an abdominal incision. By occluding the blood supply to the hepatic artery with a microvascular clamp, ischemia was elicited in the left and middle liver lobes. An immediate change in the color of the afflicted lobes confirmed the successful induction of ischemia. 30 min of ischemia were followed by 1 hr of blood restoration.

Sampling

At the ending of the experiment, animals were humanely sacrificed by decapitation, and blood was collected from the inferior vena cava then centrifuged on 4,000 x g for 15 min. For biochemical analysis, the collected sera were separated and frozen at−80 °C. The livers were separated into three sections, the first one was homogenized in a solution of phosphate buffered saline (PBS), and the supernatant collected and frozen at −80 °C for biochemical analysis. The second section was quickly frozen at −80 °C for Western blotting analysis, and the last portion was preserved in 10% formalin-buffered saline solution for histopathological and immunohistochemical investigation.

Estimation of liver function biomarkers (ALT & AST)

Serum activities of ALT and AST were determined calorimetrically using Bio-diagnostic assay kit (GIZA, Egypt).

Estimation of serum gamma glutamyl transferase (GGT)

Serum GGT activity was measured spectrophotometrically according to the method illustrated using Bio-diagnostic assay kit (GIZA, Egypt).

Estimation of serum total bilirubin (TB)

Serum TB level was estimated utilizing the described Bio-diagnostic assay kit (Giza, Egypt) method.

Assay of hepatic oxidants/antioxidants state

The reduced GSH content of the liver was calculated using the method described by **Beutler et al. (1963)**[17].

The content of liver lipid peroxides was calculated by determining the amount of substances reactive to thiobarbituric acid (TBARS) expressed as MDA using commercial reagents (Bio-diagnostic company) in accordance with the procedures developed by **Ohkawa et al. (1979)**[18].

Using commercial reagents (Bio-diagnostic company, Egypt) and the modified method of **Hortelano et al. (1995)**[19], the hepatic total NOx concentration was determined**.**

Using commercially colorimetric assay reagents (Sigma-Aldrich, USA), hepatic activity of MPO was quantified according to the manufacturer's guide and the method previously described by **Suzuki et al. (1983)[20].**

Hepatic assay of pro inflammatory mediators

Hepatic contents of TNF-α and IL-33 were assessed using ELISA kits (Bio diagnostic, company, Egypt) in accordance with the procedures described by the manufacturers.

Hepatic assay of Bax, GAL9 and Bcl2

Liver content of these apoptosis regulator markers were estimated using ELISA kits My BioSource (SD, USA) according to the procedures of the manufacturers.

Western blotting analysis for VEGF & fibrinogen assays

Western blotting analysis for assays of VEGF & fibrinogen were carried out according to the methods of **Kurien and Scofield (2015)**[21].

Using a Model GS-700 Imaging Densitometer (Bio-Rad, Hercules, CA, USA) and Molecular Analyst Software/Macintosh (Bio-Rad), the optimized bands were analyzed. The band density of either VEGF or fibrinogen was divided into two groups by comparing it to the extracted band density of the accompanied normal control tissue. Actin levels (anti-actin antibody: 1–19, Santa Cruz Biotechnology Inc., 1:1000 dilution) were measured as protein loading regulators.

Immunohistochemical evaluation of SMAD-4 and NF-κB

Immunohistochemical evaluation of SMAD-4 and NF-κB was performed in accordance with the methods described by **Kim et al. (2016)**[22]**.**

The results of immunohistochemistry were quantified semi-quantitatively using freely available software Fiji (ImageJ) (version 1.2; WS Rasband, National Institute of Health, Bethesda, MD).

Histopathological investigation

Liver sections from all rats were fixed in 10% neutral formalin buffered saline for 72 hrs, then tissues were washed using tap water for 12 hrs followed by dehydration with alcohol, then - embedded in paraffin and sliced to 4-µm thickness with a rotary microtone[23]. Hematoxylin and eosin (H&E) were used to stain liver sections, which were then observed using a Nikon Eclipse 80i microscope (Nikon, Tokyo, Japan) attached to a DXM1200F digital camera. The extent of liver injury was determined using the updated Suzuki liver injury categorization[24]**.**

Statistical analysis

The Graph Pad Prism (Graph Pad Computer software, California, version 6.0, USA) was utilized to conduct statistical analysis. The data were presented as means ± standard deviation (S.D). A one-way analysis of variance (ANOVA) test was used to compare groups, followed by the Tukey-Kramer test for multiple comparisons. When $p<0.05$, differences were considered statistically significant.

RESULTS AND DISCUSSION

Results

EGZ attenuates I/R-induced hepatic impairment

Hepatic I/R markedly elevated serum levels of ALT, AST, GGT and TB as compared to sham control rats. Meanwhile, pre-treatment with EGZ significantly improved hepatic injury by decreasing serum ALT, AST, GGT and TB levels compared to I/R rats **(Table 1)**.

EGZ counteracts I/R-induced oxidative stress injury

Marked depletion of hepatic GSH along with marked elevation of contents of hepatic MDA and NOx as well as hepatic MPO activity were obvious in I/R rats compared with sham control rats. Conversely, EGZ pre-treatment resulted in a significant elevation in hepatic reduced GSH parallel to a remarkable reduction in MDA, NOx and MPO compared with I/R animals **(Fig. 1)**.

Table 1: Impact of EGZ on liver function indicators in I/R injury.

Group/ parameters	Sham	I/R	EGZ	EGZ+ I/R
ALT (U/L)	23.3 ±2.66	241.6±7.88*	16.36 ±2.34@	131 ±8.82*@
AST (U/L)	36.82 ±2.74	242.6 ±8.89*	35.16 ±4.22@	123 ±4.69*@
GGT (U/L)	44.4 ±3.60	122.1 ±7.59*	35.92 ± 2.60@	75.48 ±7.88*@
TB (mg/L)	0.061 ±0.008	0.196 ±0.048*	0.056 ±0.005@	0.132 ±0.021*@

ALT: alanine aminotransaminase; AST: aspartate aminotransaminase; GGT: gamma glutamyl transferase; TB: total bilirubin.

EGZ: empagliflozin; I/R: ischemia reperfusion.

Results are presented as means ± S.D. (n = 6).

*Significantly different versus sham control rats at $p < 0.05$.

@ Significantly different versus I/R control rats at $P < 0.05$.

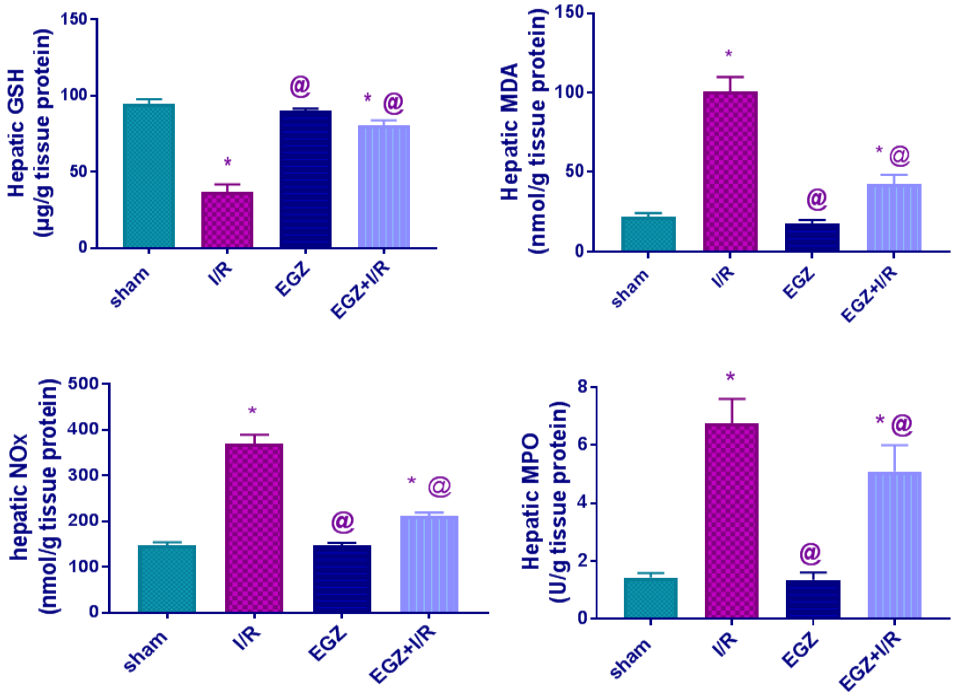

Fig. 1: Impact of EGZ on oxidative stress indicators against I/R- induced liver injury**.**

EGZ ameliorates inflammation induced by I/R

Compared to sham group, hepatic TNF-α and IL-33 contents were considerably enhanced by hepatic I/R induction. In Contrast, pre-treatment of rats with EGZ markedly lowered TNF-α and IL-33 hepatic contents with respect to I/R rats **(Fig. 2).**

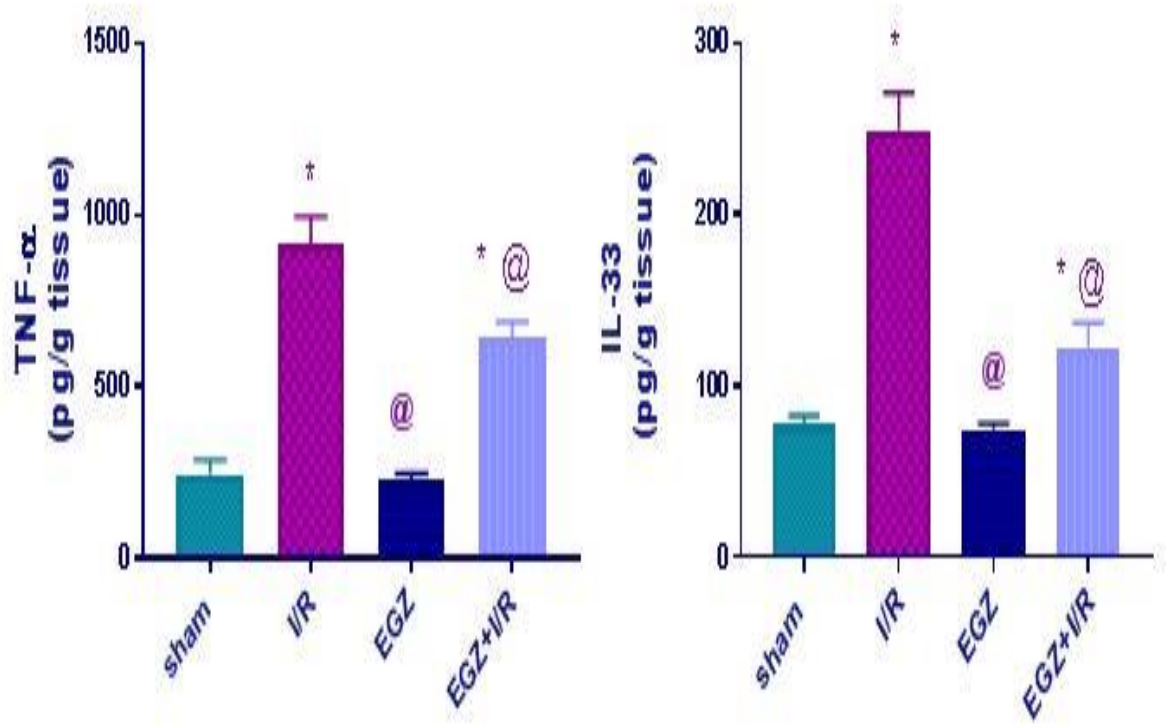

Fig. 2: Influence of EGZ on hepatic TNF-α and IL-33 contents against I/R induced hepatic injury.

EGZ modulates I/R-provoked apoptosis

Liver I/R induction resulted in apoptosis as shown by elevated apoptosis markers, Bax and Gal-9, and diminished anti-apoptosis marker (BcL2) in hepatic tissues. On the contrary, EGZ administration diminished apoptosis as evidenced by significant reduced hepatic Bax and Gal-9 contents together with markedly elevated BcL2 hepatic content **(Table 2).**

EGZ alleviates I/R induced alteration in the expressions of VEGF and fibrinogen

Hepatic triggering of I/R provoked significant enhancement of VEGF and fibrinogen protein expression levels. However, rats pretreated with EGZ displayed normalization in hepatocytes as evidenced by considerable decline in VEGF and fibrinogen protein expression levels in comparison to I/R animals **(Fig. 3&4).**

Table 2: Impact of EGZ on liver apoptotic biomarkers in I/R- provoked liver injury.

Group/ parameters.	Sham	I/R	EGZ	EGZ+I/R
Bax (ng/mg tissue protein)	48.6 ± 5.55	111.2 ± 8.55*	44 ± 3.74@	80 ± 11.48*@
GAL-9 (pg/mg tissue protein)	8.8 ± 1.30	386 ± 26.08*	7.8 ± 1.64@	273 ±21.68*@
Bcl2 (ng/mg tissue protein)	126.8 ± 6.89	73.4 ± 4.98*	148.6 ± 14.81@	105.6 ± 9.71*@

BAX: BCL2 associated X protein; Bcl-2: B-cell lymphoma 2; Gal-9: galactine-9 protein.
EGZ: empagliflozin; I/R: ischemia reperfusion.
Results are presented as means ± S.D. (n = 6).
* Significantly different versus sham control rats at $p < 0.05$.
@ Significantly different versus I/R control rats at $P < 0.05$.

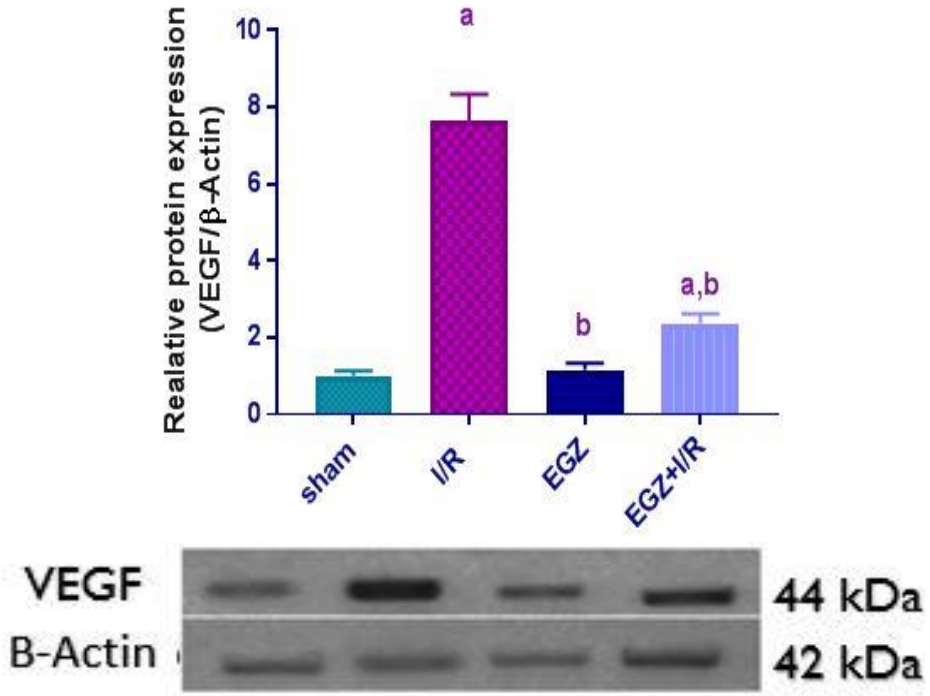

Fig. 3: Impact of EGZ on hepatic VEGF protein expression against I/R-induced hepatic injury.

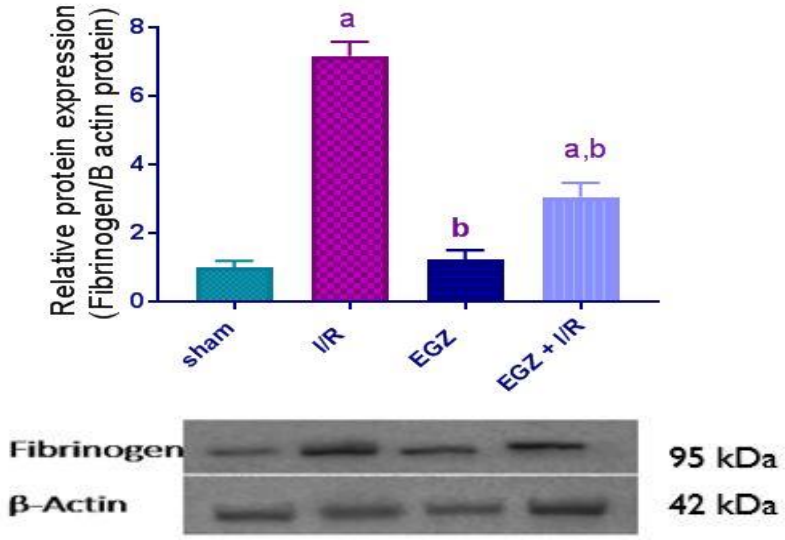

Fig. 4: EGZ influence on fibrinogen protein expression against I/R-induced liver injury.

EGZ reduces I/R induced alteration in the expressions of SMAD-4 and NF-kB

In comparison to sham control rats, I/R-exposed rats exhibited inflammation as well as fibrosis; such inflammation was accompanied by a marked rise in protein expressions of SMAD-4 and NF- kB. On the contrary, EGZ pretreatment produced a significant attenuation in fibrosis and inflammations indicated by significant reduced levels of SMAD-4 **(Fig. 5 A & B)** and NF-kB protein expressions respectively **(Fig. 6 A & B).**

EGZ improves histopathological changes triggered by I/R induction

Sham and EGZ groups showed normal histological structure of liver parenchyma without any detection of abnormality. Rats affected by liver I/R displayed Liver degeneration, hepatic cords atrophy and severe portal vein congestion accompanied by edema, swelling of hepatocytes, periportal infiltration of inflammatory cell and Kupffer cells. Rats pre-treated with EGZ displayed apparent normal hepatic architecture with marked decrease in periportal infiltration of inflammatory cells and sinusoidal and portal venous congestion **(Fig. 7 A & B)**.

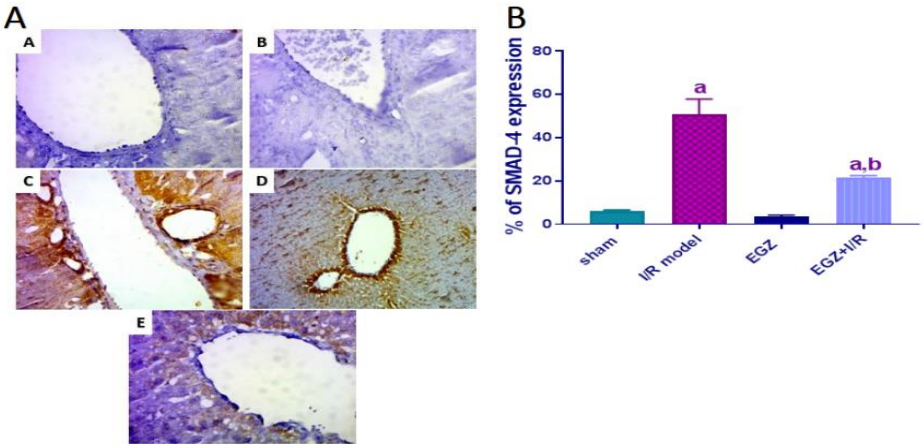

Fig. 5: A; Effect of EGZ on hepatic SMAD-4 protein expression against I/R-induced hepatic injury evaluated by immunohistochemical analysis.

B; Effect of EGZ on percentage of hepatic SMAD-4 protein expression against hepatic injury induced by I/R estimated by immunohistochemical analysis.

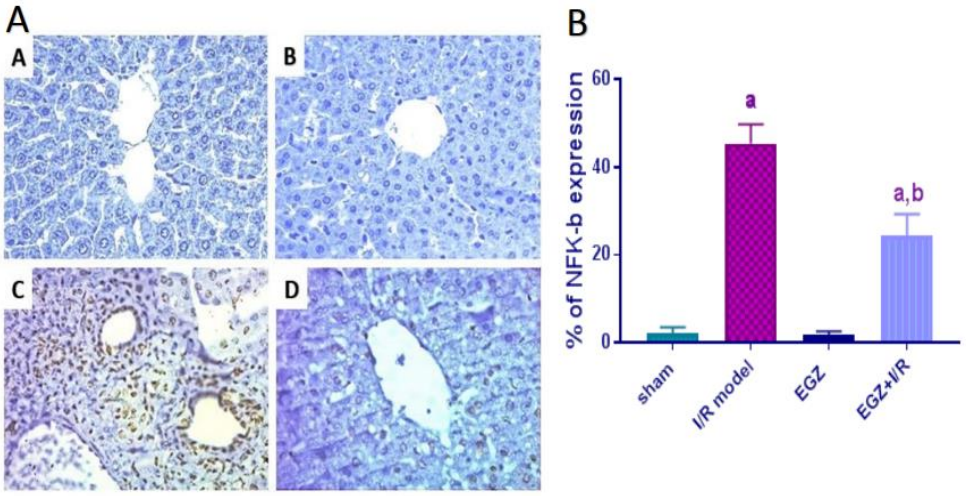

Fig. 6: A; Effect of EGZ on hepatic NF-κB protein expression against I/R-induced hepatic injury evaluated by immunohistochemical analysis.
B; Effect of EGZ on percentage of hepatic NF-κB protein expression against hepatic injury induced by I/R estimated by immunohistochemical analysis.

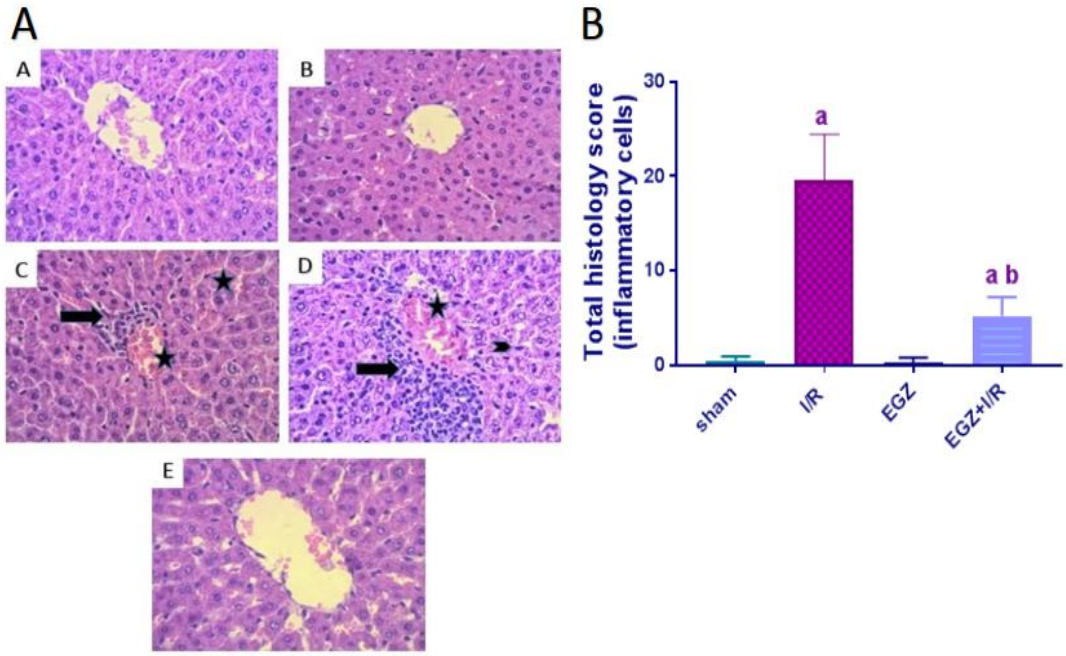

Fig. 7: A; Effects of Empagliflozin on liver histopathological examination in rats subjected to I/R.
B; Total histology of inflammatory cells score is expressed as box plots of the medians of 5 rats.

Discussion

Ischemia-reperfusion injury has a significant threat to the liver during periods of interruption and restoration of blood and oxygen supply. This phenomenon occurs during some clinical scenarios, like shock, sepsis, hepatic arteriolar ligament, hepatic trauma, hepatocellular carcinoma resections, and liver transplantations[25].

Hepatic damage arises by a series of mechanisms, including oxidative stress, which contributes to different degrees of tissue organ inflammation[26].

Empagliflozin, a sodium-glucose cotransporter 2 (SGLT2) inhibitor, belongs to a novel class of oral medications used in the management of type 2 diabetes mellitus (T2DM). It exhibited anti-inflammatory and anti-fibrotic effects[27].

The data of the current study revealed a marked disturbance in liver function following liver I/R injury, as manifested by increased serum levels of ALT, AST and TB in addition to

GGT activity. Similar results were previously reported[28]. Various factors and cells are involved in the hepatic injury processes induced by I/R, such as anaerobic metabolism, mitochondrial dysfunction, intracellular calcium overload, chemokines and cytokines produced by neutrophils and liver Kupffer cells[25, 29].

Furthermore, such result may be attributed to the diffusion of ROS, including hydrogen peroxide and hypochlorous acid, into hepatocytes upon reperfusion, resulting in oxidative stress, mitochondrial malfunction together with calcium buildup, permeable pore formation and cell death[30].

Pre-treatment of rats with EGZ produced a significant decline in serum levels of ALT, AST and TB in addition GGT activity, which is consistent with the finding of other investigators[31]. Based on its anti-oxidant properties, EGZ impact may be attributed to its ability to enhance mitochondrial function by increasing the potentiality of mitochondrial membrane, thereby prevents the development of permeable pores and cell death[32].

The results of the current investigation indicated that liver I/R markedly decreased hepatic GSH while elevated MDA, total NOx and MPO in hepatic tissues. Such outcomes are consistent with earlier studies[33-35], and could be attributed to excessive ROS generation in the rat liver, exceeding endogenous ROS scavengers' capacity[36].

Pre-treatment with EGZ significantly elevated hepatic GSH content along with a significant decrease of MDA, total NOx and MPO contents in hepatic tissues, that is in accordance with previous study[37]. The antioxidant activity of EGZ could be linked to its free radical scavenging ability[38]. In addition, **Iannantuoni *et al*. (2019)** [39] indicated that the antioxidant activity of EGZ may be due to induction of glutathione-disulfide reductase (GSR) gene expression, a regulating enzyme involved in GSH synthesis.

The present investigation revealed that hepatic I/R induction provoked a remarkable increase in the pro-inflammatory mediators, including TNF-α, and IL-33 which is in accordance with earlier investigations demonstrating that I/R can stimulate Kupffer cells, leading to discharge of numerous cytokines[40]. TNF-α has multifaceted effects on hepatic I/R damage owing to complex interactions between ROS, adhesion molecules and nitric oxide, in addition to numerous chemokines and cytokines[41]. It has been revealed that TNF-α induces the hepatic expression of epithelial neutrophil activating protein, a key mediator of neutrophil-dependent hepatic failure linked to liver I/R[42]. In addition, it was demonstrated that IL-33 promote additional inflammation after liver I/R by inducing the formation of neutrophil extracellular traps (NET) formation[43].

Pretreatment with EGZ disclosed to a marked reduction in TNF-α, and IL-33 levels in liver tissues. Such results are in accordance with the finding of **Mohamed et al. (2020)**[44] which indicated that EGZ inhibits TNF-α release, thus enhancing its anti-fibrotic effects in animal models of EGZ resistance. In addition, it has been reported that EGZ suppresses IL-33 production, thereby attributes the expression of genes encoding transforming growth factor beta (TGF-β) and collagen type 1 alpha 1 (COL1α1)[45].

Apoptosis has been demonstrated to be one of the mechanisms of hepatocellular mortality during I/R injury[46]. Data of the present work indicated that rats affected by hepatic I/R showed a significant elevation in pro-apoptotic protein Bax, and significant lowering in anti-apoptotic protein Bcl-2, which is in agreement with previous investigation[47]. According to research of **Lin et al. (2013)** [48], a mitochondrial pathway with a strong link to the Bcl-2 family is involved in the apoptotic process in hepatic I/R. Bcl-2 and Bcl-xL are anti-apoptosis proteins, while Bax, Bad and Bak are pro-apoptosis members of the Bcl-2 family. Moreover, the hepatic I/R injury markedly elevated Gal-9 expression, that causes apoptosis via the calcium-calpain-caspase pathway[49].

On the other hand, rats pre-treated with EGZ showed a significant decline in pro-apoptotic proteins Bax and Gal-9 parallel to considerable increase in the anti-apoptotic protein Bcl-2, both of which play critical roles in EGZ's protective effects. The current findings are supported by those of previous investigation[50]. Moreover, It has been reported that Bax ablation protected transgenic mice from liver I/R injury[51]. It has been revealed that EGZ reduced non-alcoholic fatty liver disease (NAFLD) by increased the Bcl2/Bax ratio, which reduces liver cell apoptosis[50].

The present results revealed that hepatic I/R produced a significant elevation in hepatic VEGF expression. Such findings are confirmed by previous studies and could be due to I/R-

induced hypoxia, which may lead to enhanced VEGF expression. Elevated VEGF levels can promote microscopic tumor growth through receptors of VEGF on tumor cells, therefore promoting hepatic metastasis[52].

Administration of EGZ prior to I/R resulted in a considerable decline in VEGF expression in hepatic tissues, which is along with the study by **Abdelhamid et al. (2022)**[53]. The modulatory effect of EGZ is primarily mediated via the inhibition of the VEGF-A factor, which stimulates the evolution of tumor-associated blood vessels and provides the way for invasion of cancer cells by increasing endothelial cells permeability through upregulation of endothelial caveolin-1 and plasmalemma vesicle associated protein-1 (PV-1) expression[54]**.**

Hepatic I/R induction led to significant fibrinogen disposition, which is in line with the work of **Khandoga et al. (2002)**[55]. According to previous research, platelet adhesion occurs via fibrinogen accumulation on the intercellular adhesion molecule 1 (ICAM-1) that is expressed on post-ischemic liver micro vessels endothelium. This can lead to microvascular damage and cellular death in hepatic tissues following liver I/R and during early reperfusion**[55].**

On the other hand, EGZ administration showed a significant reduction in fibrinogen expression, which decreases platelet aggregation and clot formation in the micro vessels of the liver. The investigation of **Niitani et al. (2020)**[56] reported that EGZ reverse hypo fibrinolysis in T2DM and reduces fibrinogen activity through decreasing the concentration of plasminogen activator inhibitor-1 (PAI-1) protein**.**

In the current study, hepatic I/R induction led to up-regulation of SMAD-4 protein, which is a major transcription factor for different miRNAs and participates in I/R injury. The obtained result is in line with the work of other investigators[57,58]. **Xu et al. (2016)**[59] also demonstrated that the increased extracellular matrix distribution and fibrosis development have both been linked to the pro-fibrosis protein SMAD-4, which functions as an intracellular controller of transforming growth factor beta 1 (TGF-β1)**.** Moreover, it has been reported that regulation of TGF-β/SMAD pathway by the gliflozin group in cardiac tissues resulted in inhibition of cardiac fibrosis[60].

In the current investigation, pre-treatment with EGZ displayed a significant decrease in the expression of SMAD-4, resulting in inhibited TGF-β expression. The obtained results were supported by the study of **Shentu et al. (2021)** and could be explained through amelioration of fibrosis via suppressing of the TGF-β/SMAD signaling pathway[61]**.**

The data of the present study revealed that liver I/R lead to activation of NF-κB signaling cascade. Such finding is confirmed by the study of other researchers and could be explained via the overproduction of ROS[62]. Stimulation of NF-κB results in increased production of cytokines, enzymes related to oxidative stress and growth factors. Additionally, it influences physiologic or pathologic events like immune and inflammatory responses. Furthermore, NF-κB translocation from cytoplasm to nucleus and its binding to DNA causes the release of inflammatory mediators as TNF-α, IL-6 and IL-1β[63]**.**

In the present investigation, pre-treatment of rats with EGZ displayed marked inhibition of NF-κB expression. Such result is compatible with the work of **Abdelhamid et al. (2020)** [64]**,** which demonstrated that pretreatment with EGZ resulted in a remarkable suppression of NF-κB protein expression and attenuation of inflammation in hepatocytes, thus enhanced the protective anti-inflammatory effect of EGZ against hepatic I/R injury**.**

A histopathological investigation of rat livers subjected to hepatic induction of I/R revealed alterations in architecture of the liver , hepatic cord atrophy, high portal vein congestion accompanied by edema and swollen hepatocytes along with periportal infiltration of inflammatory cell and activated Kupffer cells as well as apoptotic structures. Similar results were previously reported**[65]**. Whereas, EGZ pre-treatment displayed apparent normal architecture of the liver and a considerable decrease in congestion of the sinuses and the portal veins, as well as decreased inflammatory cell infiltration around the ports.

Conclusion

According to the outcomes of the current study, the hepatic induction of I/R led to an impairment of the liver function and oxidative damage status, along with elevated pro-inflammatory cytokines and apoptosis indicators, along with increased expression of VEGF, fibrinogen, SMAD-4 and NF-κB. EGZ pre-treatment confers a protective impact on hepatic I/R injury, evidenced by the EGZ's capacity to alleviate liver function impairment,

oxidative damage, inflammation as well as apoptosis in addition to modulating the NF-kB/SMAD-4 signaling pathway. Interestingly, EGZ is considered as a promising agent against hepatic I/R injury and as a result, it has the potential to be extremely beneficial in human liver allograft surgery.

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Authors' Contributions

H.M.M.: Conceptualization, Methodology, Validation, Supervision, and Writing (review & editing). D.E.A.: Resources, Investigation, Formal Analysis, Visualization, and Writing (original draft). A.M.A.: Conceptualization, Methodology, Supervision, Validation, and Writing (review &editing). R.A.M.H.: Conceptualization, Methodology, and Supervision. All authors read and approved the final manuscript.

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نشرة العلوم الصيدليـــــة
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ايمباجليفلوزين يخفف من اصابة الكبد الناتجة عن نقص واعادة التروية فى الجرذان: تعديل تعبير بروتينات نيوكلر فاكتور كابا بى، سماد ٤، فازكولر اندوثيليال جروث فاكتور، و الفيبرينوجن

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إصابة الكبد نتيجة نقص ضخ التروية إليه هى حالة تتزامن مع ظروف تحدث بسبب إنسداد جزئي أو كامل أو قطع فى الدم الوا صل إلى الكبد مع تقليل كمية الأوكسجين التي تصل اليه حيث يؤدى ذللك إلى فشل الأيض الهوائي، ألإصابة بنقص التروية فى الكبد هى مضاعفات خطيرة حقا من نزيف الكبد ، العمليات الجراحية ، زرع الكبد ، تعفن الدم، ربط الشريان الكبدي، وإستئصال الكبد، والصدمات والكدمات وأشكالاً مختلفة من صدمة الدورة الدموية.

في هذا العمل، تهدف هذه الدراسة إلى دراسة الدور المحتمل لعقاقير ايمباجلفلوزين فى حماية الكبد من الإصابات الكبدية الناتجة عن نقص ضخ التروية في الفئران.

الحيوانات المستخدمة في هذه الدراسة كانت ذكور ألبينو ويستار (٨ أسابيع عمر) وزنها ١٨٠–٢٠٠ جم. وقسمت هذه الحيوانات عشوائياً إلى اربع مجموعات ،التى تمت تغذيتها بالغذاء المناسب للحيوانات من هذا النوع ، ووقسمت على النحو التالي:

١. المجموعة الضابطة وتمت المحافظة على حمية غذائية قياسية طوال التجربة واعطاء المذيب فقط بدون جراحة
٢. مجموعة ضابطة معالجة بالايمباجلفلوزين
٣. مجموعة مصابة I / R ولم يسبق معالجتها باى شئ وتمت المحافظة على حمية غذائية قياسية طوال التجربة
٤. مجموعة مصابة I / R سبق معالجتها بالايمباجلفلوزين (عقار يستخدم فى علاج السكرى من النوع الثانى) (١٠مجم/كجم/يوم).

أثناء الدراسة ، كانت الفئران تتمتع بحرية الوصول إلى الطعام والماء وتم إعطاء جميع الادوية عن طريق الفم مرة واحدة في الصباح. في نهاية فترة العلاج ، تم صيام الفئران بين عشية وضحاها لإجراء عملية جراحية في صباح اليوم التالي.

تم عمل الجراحة للفئران الصائمة وتم جمع الدم لفصل السيرم (المصل) . بالإضافة إلى ذلك ، تم فصل الكبد ، تجفيفه، لتحديد مؤشر الكبد. كما تم تقدير القياسات التالية:

- قياس تركيز انزيمات الكبد فى مصل الدم وتشمل انزيم الألانين أمينو تراندسفيراز والاسبارتات أمينو ترانسفيراز، توتال بيلوروبين والجاما جلوتاميل ترانسفيراز.
- تقدير الاجهاد التاكســـدى فى الكبد من خلال قياس المحتوى الكبدى من المالون داى الدهيد ومستوى ايض اكسيد النيتريك وكذلك انزيم مايلو بيروكسيداز كما تم ايضا تقدير قدرة منع التاكسد من خلال قياس نشاط الغلوتاثيون المختزل فى الكبد.
- قياس تمثيل بروتينات الـ تى. ان. اف. الفا وانترليوكين ٣٣ كمقياس للالتهابات الناتجة
- تم قياس عوامل موت الخلايا المبرمج عن طريق تقدير محتوى الكبد من Bax/Bcl2 and Gal-9
- تك عمل تحليل ويسترن لبروتينات فازكولار اندوثيليال جروث فاكتور وكذلك للفايبرينوجين
- تم قياس سماد ٤ و نيوكلير فاكتور كاببا بى بروتينات عن طريق عمل اميونوهيستوكيميسترى
- تم عمل هيستوباثولوجى لخلايا الكبد

وقد أظهرت نتائج الدراسة الحالية ما يلي:

أظهر نموذج نقص ضخ التروية الى الكبد إصابة الكبد وموت الخلايا الخاصه بالكبد.

الإختلال الوظيفي في الكبد تم الاســـتدلال عليه من خلال إرتفاع ملحوظ في انزيم الألانين أمينو تراندسفيراز والاسبارتات امينو تراندسفيراز، توتال بيلوروبين والجاما جلوتاميل تراندسفيراز في مصل جرذان نموذج نقص التروية بالمقارنة مع المجموعة الضابطة.

تم زيادة الاجهاد التأكسدي بشكل ملحوظ، يتجلى من خلال زيادة محتوى الكبد من المالون داى الدهيد ومستوى ايض اكسيد النيتريك وكذلك انزيم مايلو بيروكسيداز كما تم ايضا اضعاف قدرة منع التاكسد من خلال نقص نشاط الغلوتاثيون المختزل فى الكبد في جرذان نموذج نقص التروية.

تمت ملاحظة الكبد المتليف الذي يتميز بالتهاب الكبد والتليف بعد نموذج نقص التروية ويتضح ذلك من خلال التعبير الكبدى المرتفع لبروتين الـ تى. ان. اف. الفا وبروتين ســـماد ٤ والفايبرينوجين والنيوكلير فاكتور كاببا بى فى محتوى الكبد في جرذان نموذج نقص التروية.

كذلك تم التأكد من وجود موت للخلايا من خلال لخبطة بروتينات باكس/بى ســـى ال ٢ وبروتين جالاكتين ٩.

ووجد تورم فى خلايا الكبد حيث اســـتدل عليه من ارتفاع محتوى الكبد من بروتين فاســـكولار اندوثيليال جروث فاكتور فى جرذان نموذج نقص التروية

إعطاء الايمباجليفلوزين عن طريق الفم يؤدى الى تحســـن كبير فى الكبد والذى يشـــير اليه الانخفاض الملحوظ فى انزيم الألانين أمينو تراندسفيراز والاسبارتات أمينو تراندسفيراز، توتال بيلوروبين والجاما جلوتاميل ترانسفيراز.

علاوة على ذلك فقد تم تخفيض الإجهاد التأكسدي بشكل كبير (المالون داى الدهيد ومستوى ايض اكسيد النيتريك وكذلك انزيم مايلو بيروكسيداز) الغلوتاثيون المختزل فى الكبد. كذلك تم تحسين نسبة التهابات الكبد والتليف فى الجذان المعالجة وتم قياس ذلك من خلاص انخفاض الملحوظ لبوتين الـ تى ان اف الف وبروتين سماد ٤ والفيبرينوجين و النيوكليير فاكتور كاببا بى فى طرق القياسات المختلفة التى تمت.

بالاضـــافة الى ذلك، تم تقليل موت الخلايا المبرمج وذلك اســـتدل عليه من اخلال اعادة اضـــباط بروتينات باكس/بى سى ال ٢ وبروتين جالاكتين ٩ وكذلك انخفاض بروتين فاسكولار اندوثيليال جروث فاكتور فى الجرذان المعالجة مقارنة بالجرذان الغير معالجة.

وهذه النتائج اوضحت ان الايمباجليفلوزين له دور فعال فى حماية الكبد من التلف الناتج من نقص التروية من خلال تأثيره كمضاد للاكسدة ومضاد للالتهابات ومضاد للتليف.