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ORIGINAL ARTICLE

PROTECTIVE EFFECTS OF GEMFIBROZIL, SILYMARIN, AND THEIR COMBINATION ON LIVER ISCHEMIC/REPERFUSION INSULT IN RATS.

Sohair H.S. El-Menshawy [1], Ameen M. Sekina [1], Soad L. Kabil [1], Hayam E.H. Rashed [2], and Amira M. Abd El-Fattah [1]

[1] Clinical Pharmacology Department, Faculty of Medicine, Zagazig University. [2] Pathology Department, Faculty of Medicine, Zagazig University.

Corresponding Author Details
Amira Mohamed Abd El-Fattah

- Assistant lecturer of clinical pharmacology, clinical pharmacology Department, Zagazig University, Zagazig , Egypt, E mail: maryemnour234@gmail.com

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ABSTRACT

Background: Hepatic ischemic reperfusion (I/R) injury is an inevitable critical problem occurs during resection of liver tumors, and liver transplantation. Silymarin is a herbal product widely used for its hepatoprotective effect. Gemfibrozil is a FDA-approved fibrate drug, commonly prescribed for management of dyslipidaemia. ***Objective:*** The aim of this work is to study the possible hepatoprotective effect of Silymarin, Gemfibrozil, and their combination against hepatic ischemic reperfusion injury in rats.

Materials & Methods: Rats were divided into six groups: Group1: normal control; Group2: sham-operated; Group3: I/R (Rats subjected to partial hepatic ischemia for 30 minute followed by 6 h reperfusion), Group4: Silymarin pretreated group (100 mg/kg orally once daily for 14 days); Group5: Gemfibrozil pretreated group (100 mg/kg orally once daily for 14 days), and Group 6: Silymarin and Gemfibrozil- pretreated group. Serum AST and ALT, hepatic tissue MDA, SOD, GSH-Px, TNF-α, MPO, caspase-3 levels, and real-time qPCR for gene expression of IκB-α, and PI3K, and its subunits (P85: p110 α /p110 β) were measured besides liver histopathology and 8-OHdG, NF-κB (p65), and caspase immunohistochemistry.

Results: I/R insult deteriorated the liver function and evoked oxidative stress, inflammation, and apoptosis. Pretreatment with Silymarin and /or Gemfibrozil improved the deteriorated liver function and the histopathological changes as well as attenuating oxidation, apoptosis and inflammatory processes.

Conclusion: The combination of Gemfibrozil and Silymarin has protective effects against liver I/R in rats better than each of these drugs alone due to antioxidant, anti-inflammatory and anti-apoptotic properties of the used drugs.

Key words: Ischemic-reperfusion, Silymarin, Gemfibrozil.

INTRODUCTION

Hepatic I/R injury is an inevitable critical problem frequently occurs during resection of liver tumors, and liver transplantation [1]. It is a complex process, a large number of factors and mediators contribute to hepatic I/R injury including, oxidative stress, up regulation of pro-inflammatory cytokines as tumor necrosis factor-α (TNF-α), which induces neutrophil recruitment. The cellular damage occurs via a combination of apoptosis and necrosis [2].

Nuclear factor kappa B (NF-κB) is pivotal transcription factor that plays a cardinal role in liver I/R insult. During oxidative stress, the activated NF-κB upregulates expression of cell adhesion molecules, and cytokines [2].

The PI3K pathway, a well-known cell survival pathway, exerts a strong protective effect on I/R injury through the inhibition of intrinsic pathway of apoptosis [1]. The majority of research has focused on the class-IA PI3Ks, arguably the most important PI3K signaling pathway. Class-1A PI3-kinases activated by receptor tyrosine kinases (RTKs), are heterodimers consisting of a catalytic subunit p110 (p110α, p110β, or p110 δ) in complex with regulatory subunit p85. PI3K isoforms p110α and p110β are commonly expressed in all tissues including liver [3].

Silymarin, a flavonoid extract from the milk thistle, is used clinically as "hepato protective" agent. In addition, recent studies demonstrated that Silymarin possesses also anti-inflammatory, and anti-apoptotic properties [4].

Gemfibrozil is a FDA-approved fibrate drug, commonly prescribed for management of dyslipidaemia. The most important mechanism of fibrates is considered as agonists of PPAR-α receptor, but, has recently reported that Gemfibrozil has antioxidant and anti-inflammatory properties in diverse situations [5].

So, the aim of this work is to study the possible hepatoprotective effect of Silymarin, Gemfibrozil, and their combination against hepatic ischemia reperfusion injury in rats.

MATERIALS & METHODS

Animals

Adult, male albino rats weighting, 200±10g were used in this study. They were purchased from the animal house of Faculty of Veterinary Medicine, Zagazig University, Egypt and kept in the animal house of faculty of Medicine, Zagazig University under constant temperature and humidity. Animals had free access to standard laboratory diet and water. One week prior to experimentation, rats were left for acclimatization. The care and handling of the animals used in the present study were in accordance with the guidelines of the local institutional review board (IRB), (ZU-IRB# 2620-30-3-2016). Experiments complied with the ARRIVE guidelines and was carried out in accordance with the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978) .

Drugs and chemicals

Silymarin powder (Sedico Pharmaceutical Co., Egypt), Gemfibrozil powder, and Sodium carboxymethyl cellulose [CMC-Na] (Sigma Aldrich., Egypt).

Induction of partial hepatic IR injury

Under complete aseptic conditions, the rats were anesthetized with thiopental sodium (60 mg/kg, intraperitoneal). A midline abdominal laparotomy was performed. Ischemia was induced by clamping the hepatic pedicle which supplies the left and median liver lobes (70% of liver mass) according to the method described by ***Abe et al*** [6] for 30 min followed by 6 h of reperfusion .Sham operated animals underwent the same procedure and anesthesia without vascular occlusion.

Experimental design

42 healthy adult male albino rats groups were randomly divided into six groups: Group1: **Normal control group;** Group2: **Sham-operated group**(rats received CMC-Na (0.5%) at dose of 10 ml/kg) **;** Group3: **I/R group;** rats received CMC-Na (0.5%) at dose of 10 ml/kg. Group 4: **Silymarin- pretreated group** (100 mg/kg suspended in 0.5% CMC-Na) [7] Group 5: **Gemfibrozil- pretreated group (**100 mg/kg in 0.5% CMC-Na solution) [8]. Group 6: **Silymarin and Gemfibrozil- pretreated group:** rats received combination of Silymarin followed 30 minutes later by Gemfibrozil at the before-mentioned doses. All of the above drugs were administered orally by gavage once daily for 14 days then I/R was induced.

Blood and tissue preparation

At the end of the reperfusion period, blood was collected from the retro-orbital plexus and serum was separated by centrifugation at 4000×g for 15 min. The obtained serum was stored at -20°C until used in estimation of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels. Livers were rapidly excised and divided into three parts; one part was preserved in 10% formalin for histopathological and immunohistochemical examination.Two pieces were snap-frozen in

liquid nitrogen and stored at -80 ˚C then homogenized and divided into fractions for analysis.

Biochemical measurement

1. Assay of serum AST and ALT levels:

Serum AST and ALT levels were measured using commercial available kits (Bio-diagnostic Co, Egypt).

2. Assay of hepatic oxidative stress and anti-oxidant markers:

The hepatic levels of malondialdehyde (MDA), superoxide dismutase (SOD) and Glutathione peroxidase (GSH-Px) were measured in hepatic homogenates using Kit obtained from (Bio-diagnostic Co, Egypt).

3. Assay of hepatic tumor necrosis factor-alpha (TNF-α), and MPO content:

For the quantitative detection of rat TNF-α level in liver tissue homogenates, Sandwich ELISA Kit (Catalogue number # ELR-TNF-α 001, Ray Biotech Inc. USA) was used. Myeloperioxidase (MPO) level was estimated in liver homogenate using commercial available kits (Bio-diagnostic Co, Egypt).

4. Assay of caspase-3 level:

Hepatic caspase-3 level was assayed by using rat caspase-3 ELISA kits (Catalog # EKU02991, Biomatik, USA) according to manufacture instructions.

5. Detection of IκB-α, PI3-K, and its subunits (P85: p110 α/ p110 β) genes expression by real-time quantitative PCR (qPCR):

Total RNA was extracted from liver tissue homogenate by GF-1 Nucleic Acid Extraction Kits (Vivantis Technologies Sdn. Bhd, Malaysia),and then reverse transcribed into cDNA according to the manufacturer's instructions Quantitative real time PCR was used to detect the expression of target genes. Samples were normalized to the expression of B-actin used as house-keeping gene. The relative expression was calculated from the 2-($^{\Delta\Delta}$ Ct) formula. Primers used for genes expression are summarized in **table 1.**

Histology:

Liver specimens were fixed in 10% neutral-buffered formalin solution and then, embedded in paraffin. Sections were cut at 4 mm and stained with hematoxylin and eosin (H&E) and examined under light microscope**(** ***Amin ; and Mahmoud-Ghoneim.,2011)***

Immunohistochemical staining of 8-hydroxy-2'-deoxyguanosine (8-OHdG), NF-κB-p65, and caspase:

Immunostained sections for detection of 8-OHdG, NF-κB-p65, and caspase-3 expression in liver tissue were prepared according to the method described by ***Abdel Hamid et al.,*** [9]***, Kabil***. [2]***, and Liu et al*** [1] respectively. The intensity of the 8-OHdG immunostaining for the nuclei of hepatocytes was evaluated as follow: negative immunostaining (<5% of cells showing nuclear positivity); weak (+) immunostaining (5-20% of cells showing nuclear positivity); moderate (++) immunostaining (21-80% of cells showing nuclear positivity); and strong (+++) immunostaining (>80% of cells showing nuclear positivity) [9].

The staining intensity of NF-κB-p65,within the nuclear was categorized into four grades (i.e. scores of 0, 1+,2+or 3+ corresponding to the presence of negative, weak, moderate and strong brown staining, respectively).The extent score (1, 1-10 % ; 2, 10-50% ; and 3, 50-100%) [2]. The percentage of caspase-3 with immunostaining was graded as follows: 1 (0-25%), 2 (26-50%), 3 (51-75%), or 4 (76-100%) [10].

Statistical analysis

Statistical differences between groups were computed by one-way analysis of variance (ANOVA) followed by the LSD post hoc test for multiple comparison test. In all cases, *P* value < 0.05 was considered to be statistically significant. Data were expressed as means ± S.D. The results were analyzed using the using Statistical Package of Social Services, version 25 (SPSS).

RESULTS

Sham-operated rats did not show any significant alteration from normal control group in all measured parameters. Thus, all comparisons were carried against sham-operated group.

1. Effect of pre-treatment with Silymarin, Gemfibrozil and their combination on serum AST, and ALT levels: (Table 2)

I/R group showed significant ($p<0.05$) increase in serum AST, and ALT levels (from

18.6±1.4 and 19.7±1.4 respectively in sham group to **96.5±9.5 and 81.3±7.5** respectively). However, pretreatment with Silymarin, Gemfibrozil, and their combination significantly reduced serum AST and ALT (from **96.5±9.5** in I/R group to **68.9±6.9, 58.8±5.8, and 25.8±5.1** respectively for AST) and (from **81.3±7.5** in I/R group to **49.6±4.3, 36±3.9, and 29.5±5.4** respectively for ALT).The combination therapy showed a more significant improvement in AST, and ALT levels more than either one of them alone, $p < 0.05$.

2. Effect of pre-treatment with Silymarin, Gemfibrozil and their combination on epatic MDA, SOD and GSH-Px levels: (Table 2)

Hepatic I/R injury induced lipid peroxidation evidenced by significant ($p<0.05$) elevation in hepatic MDA level coupled with significant ($p<0.05$) reduction in hepatic SOD and GSH-Px levels compared with sham group. Pretreatment with Silymarin, or Gemfibrozil succeeded to decrease hepatic MDA levels significantly ($p<0.05$). On other hand, pretreatment with these agents exhibited significant ($p<0.05$) increase in hepatic levels of SOD and GSH-Px compared to I/R. The combination therapy showed a more significant improvement in the hepatic oxidative stress parameters more than either one of them alone, $p < 0.05$.

3. Effect of pre-treatment with Silymarin, Gemfibrozil and their combination on hepatic TNF-α, MPO, and Caspase-3 levels: (Table 2)

Rats exposed to I/R showed significant ($p<0.05$) increase in hepatic levels of TNF-α, MPO, and caspase-3 compared to sham group. While, pretreatment with Silymarin, or Gemfibrozil, resulted in significant ($p<0.05$) reduction in hepatic levels of aforementioned parameters in respect to I/R group. The effect of the combination is superior to single drugs alone, $p <0.05$.

4. Effect of pre-treatment with Silymarin, Gemfibrozil and their combination on hepatic mRNA expression of Ikβ-α, PI3K, and (p85, p110 α, and p110 β subunits): (Fig. 1).

Real-time qPCR analysis showed significant ($p<0.05$) reduction in hepatic mRNA expression of Ikβ-α , PI3K, and (p85, p110 α, and p110 β subunits) in I/R group relative to sham group. Pretreatment with Silymarin, and /or Gemfibrozil, showed significant ($p<0.05$) up-regulation in hepatic mRNA expression of Ikβ-α , PI3K, p85, and p110 α, subunits in respect to I/R group, Interestingly, the hepatic mRNA expression of p110 β in these pretreated groups showed non-significant difference as compared to I/R group.

5. Liver histology:

By observing the H&E stained slides, the hepatic I/R group showed disorganized hepatic architecture with congestion , and marked degeneration **(Fig.2C)**. The observed liver injuries were improved in Silymarin, or Gemfibrozil group compared with I/R group **(Fig.2D&E)**. However these improvements are best observed in the combination group relative to each drug alone **(Fig.2F)**.

6. The expression level of 8-OHdG in liver tissue:

The immunostaining of hepatic 8-OHdG in I/R group were significantly increased **(Fig.3C)** compared with the sham group **(Fig 3B)**, and were reduced in the Silymarin **(Fig.3D)**, or Gemfibrozil **(Fig.3E)**-pretreated group compared with I/R group. The combination group showed more significant reduction in intensity of hepatic 8-OHdG immunoexpression compared to each agent individually **(Fig.3F)**.

7. The expression level of NF-κB (p65) protein in liver tissue:

The I/R group induced significant increase in NF-κB (p65) hepatic expression levels evidenced by intense brown staining **(Fig.4C)** relative to the staining seen in tissue samples from sham rats **(Fig.4B)**. The immunostained hepatic NF-κB of rats pretreated with Silymarin reveled significant decrease in intensity of hepatic NF-κB(p65) expression with moderate positive nuclear brown staining **(Fig.4D)** in respect to staining seen I/R group. Gemfibrozil pretreated rats had significant reductions in NF-κB- (p65) expression compared to both I/R and Silymarin groups **(Fig.4E)**. Finally, the

greatest significant decrease in abovementioned parameter was observed in combination group compared to individual pretreated groups **(Fig.4F)**.

8. The expression level of caspase in liver tissue:

The immunostained caspase-3 of liver section from I/R group showed significant increase in caspase-3 immunoexpression **(Fig.5C)** compared to intensity of staining seen in tissue samples from sham group **(Fig.5B)**. Pretreatment of rats with Silymarin induced significant reduction in intensity of hepatic caspase-3 immunoexpression evidenced by moderate positive cytoplasmic brown staining **(Fig.5D)**. The previously mentioned parameters significantly decreased in Gemfibrozil-pretreated group **(Fig.5E)** compared to staining seen in both I/R group and Silymarin group. The combination group showed further significant decrease in caspase-3 immunoexpression compared to each agent separately **(Fig. 5F)**.

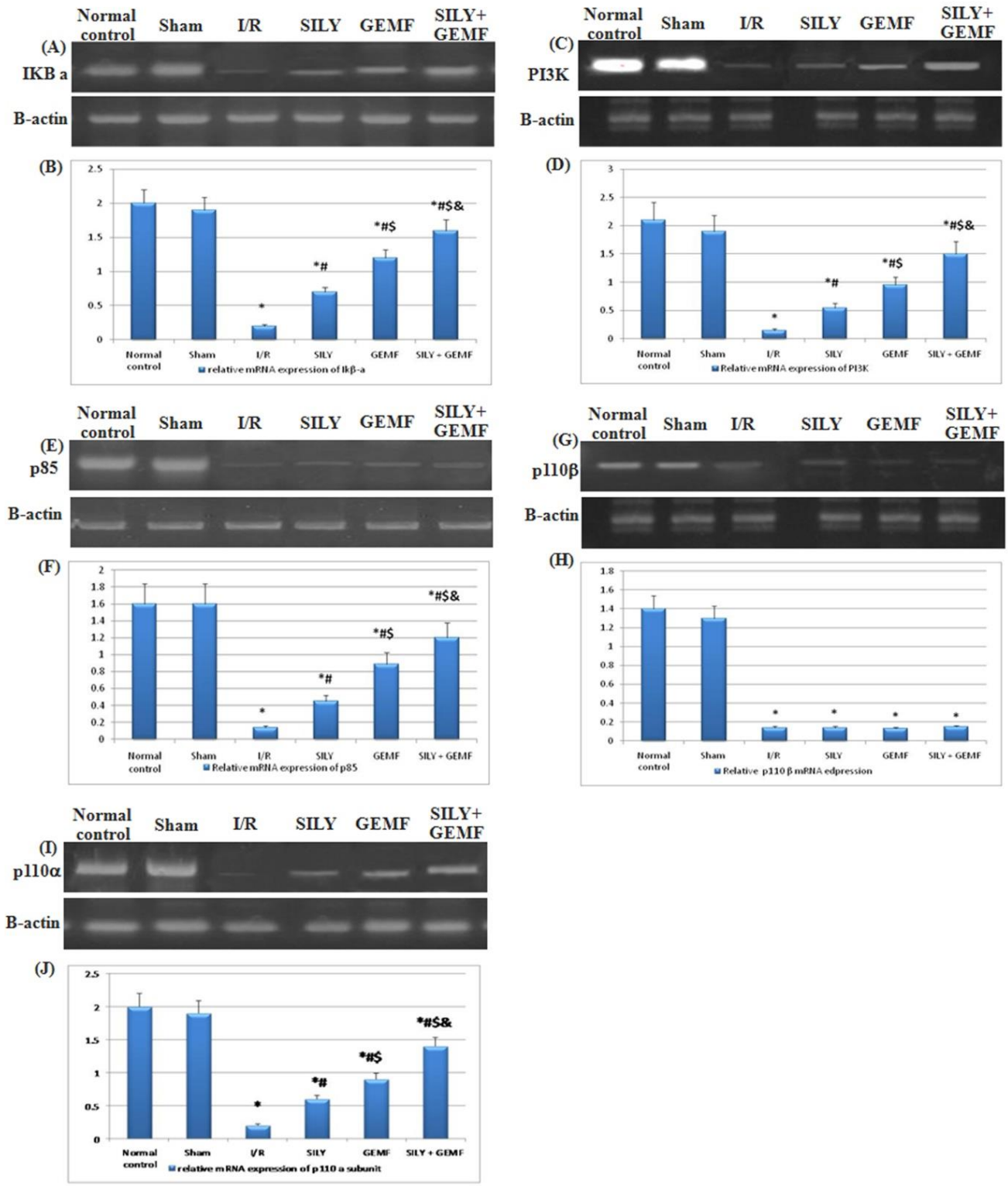
Table (1): Nucleotide sequences of primers used for genes expression

Gene	Primer sequence (5'- 3')	Gene bank accession number
PI3K	**Forward** TACTCAGTTGTGTTTCCCGTGC **Reverse** GTTCAAATGGAAGTGGATCCGT	NM_053481.2
P85	**Forward** CTGAAGCAGACACTGAGCAA **Reverse** AATATACCTCATCAGTATTG	D64045.1
p110α	**Forward** TGCCTCTCCTCTGTGGTTACTG **Reverse** AACAAACAACCCTTCGATGGAC	NM_133399.2
p110β	**Forward** TACTCAGTTGTGTTTCCCGTGC **Reverse** GTTCAAATGGAAGTGGATCCGT	NM_053481.2
IKβ- α	**Forward** GATCGCTGGTGCCTAGAGAT **Reverse** TGTTGACTACTGGAGCTTCG	AF115282.2
B-actin	**Forward** TGTTGTCCCTGTATGCCTCT **Reverse** TAATGTCACGCACGATTTCC	J00691

Table (2): Effect of pre-treatment with Silymarin (100mg/kg/day), Gemfibrozil (100 mg/kg /day) and their combination for 14 days on serum AST & ALT, hepatic MDA SOD, GSH-Px, TNF-á, MPO, and Caspase-3 levels in liver ischemic-reperfusion injury in rats.

Groups Parameter	Normal control group	Sham group	I/R group	SILY pretreated group	GEMF pretreated group	SILY + GEMF pretreated group
AST (U/L)	19.2±1.9	18.6±1.4	96.5±9.5 *	68.9±6.9 $^{*\#}$	58.8±5.8 $^{*\#\$}$	25.8±5.1 $^{*\#\$\&}$
ALT (U/L)	20.3±2	19.7±1.4	81.3±7.5 *	49.6±4.3 $^{*\#}$	36±3.9 $^{*\#\$}$	29.5±5.4 $^{*\#\$\&}$
MDA (nmol/g)	8.0±1.0	8.6±1.2	33.7±3.0 *	27.9±2.2 $^{*\#}$	22.9±1.8 $^{*\#\$}$	12.9±1.5 $^{*\#\$\&}$
SOD (U/g tissue)	12.1±0.8	12.6±0.9	3.9±0.3 *	4.9±0.4 $^{*\#}$	7.8±0.7 $^{*\#\$}$	10.6±1.3 $^{*\#\$\&}$
GSH-Px (U/gtissue)	6.1±0.7	5.9±1.2	1.3±0.1 *	1.9±0.1 $^{*\#}$	2.9±0.6 $^{*\#\$}$	4.5±0.6 $^{*\#\$\&}$
TNF-α (pg/g tissue)	16.4±1.2	16.1±3.3	49.5±8.3 *	38.9±2.1 $^{*\#}$	29.1±3.7 $^{*\#\$}$	20.9±2.1 $^{*\#\$\&}$
MPO (U/g tissue)	1.4±0.26	1.4±0.22	9.9±0.7 *	4.7±0.5 $^{*\#}$	3.9±0.3 $^{*\#\$}$	2.2±0.5 $^{*\#\$\&}$
Caspase-3 (ng/gtissue)	3.0±0.3	3.8±0.6	17.2±1.6 *	12.8±1.2 $^{*\#}$	10.0±0.7 $^{*\#\$}$	5±0.7 $^{*\#\$\&}$

Data represented as Mean ± SD.* P< 0.05 compared to sham group, $^{\#}$ P<0.05 compared to I/R group, $^{\$}$ P<0.05 compared to Silymarin-pretreated group $^{\&}$ P<0.05 compared to Gemfibrozil-pretreated group.



1. **Fig (1):** Real-time qPCR showing effect of pretreatment with Silymarin (100mg/kg/day), Gemfibrozil (100 mg/kg /day) and their combination on hepatic mRNA expression of: (A&B) Ikβ-α (C&,D): PI3K, (E&F): p85 subunit, (G&H): p110 β subunit (I&J): p110 α subunit .Data represented as Mean ± SD.* P<0.05 compared to sham group, # P<0.05 compared to I/R group, $ P<0.05 compared to Silymarin-pretreated group & P<0.05 compared to Gemfibrozil-pretreated group.

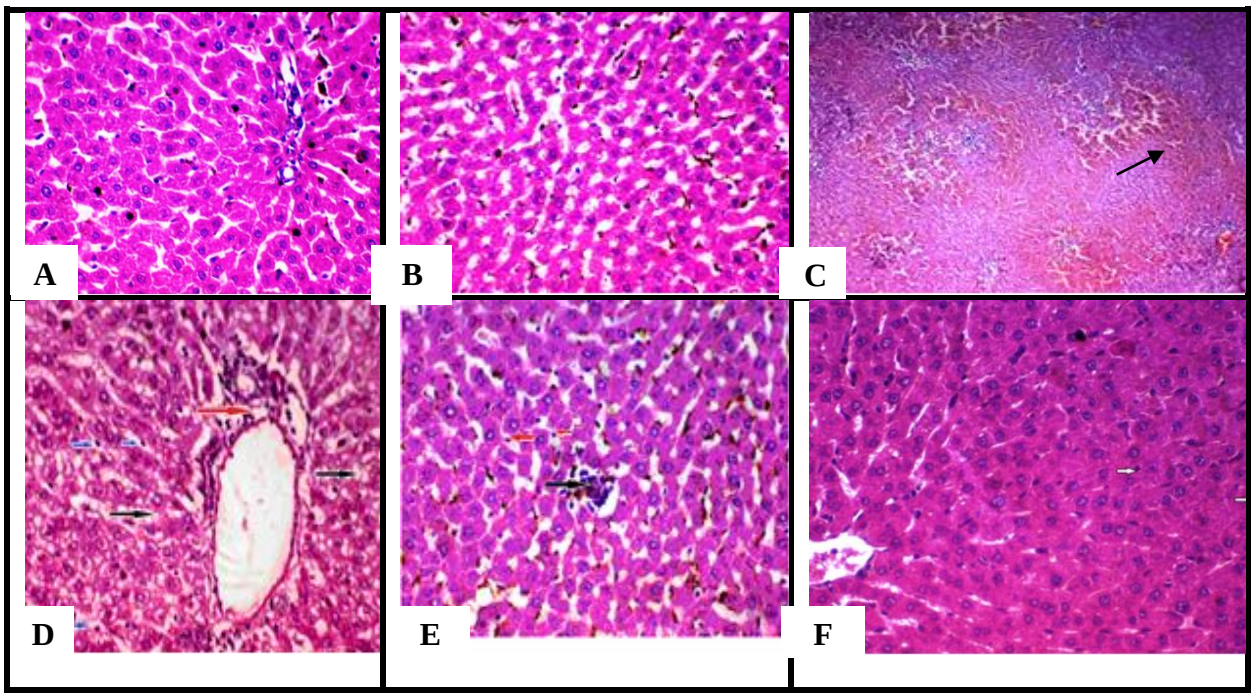


Fig 2: Representative H&E stained sections photomicrographs (magnification 400x) (A) normal control group showed normal hepatic architecture with normal central vein and radiating cords of hepatocytes. Cords of hepatocytes were separated by blood sinusoids. (B) sham group showed showed normal hepatic architecture with normal central vein and radiating cords of hepatocytes. Cords of hepatocytes were separated by blood sinusoids. (C) I/R group showed disorganized hepatic architecture with congestion (black arrow), and marked degeneration (D)Silymarin-pretreated group showed vacuolar degeneration (black arrow) in some hepatocytes, other hepatocytes have small dark nuclei (apoptosis) (blue arrow). Mild focal infiltration around portal triad (red arrow). (E) Gemfibrozil-pretreated group showed mild focal inflammation (black arrow) and mild apoptosis (red arrow). (F) The combination group showed normally appearance of hepatocytes, some hepatocytes contain dark stain nuclei (apoptosis)(white arrow) .

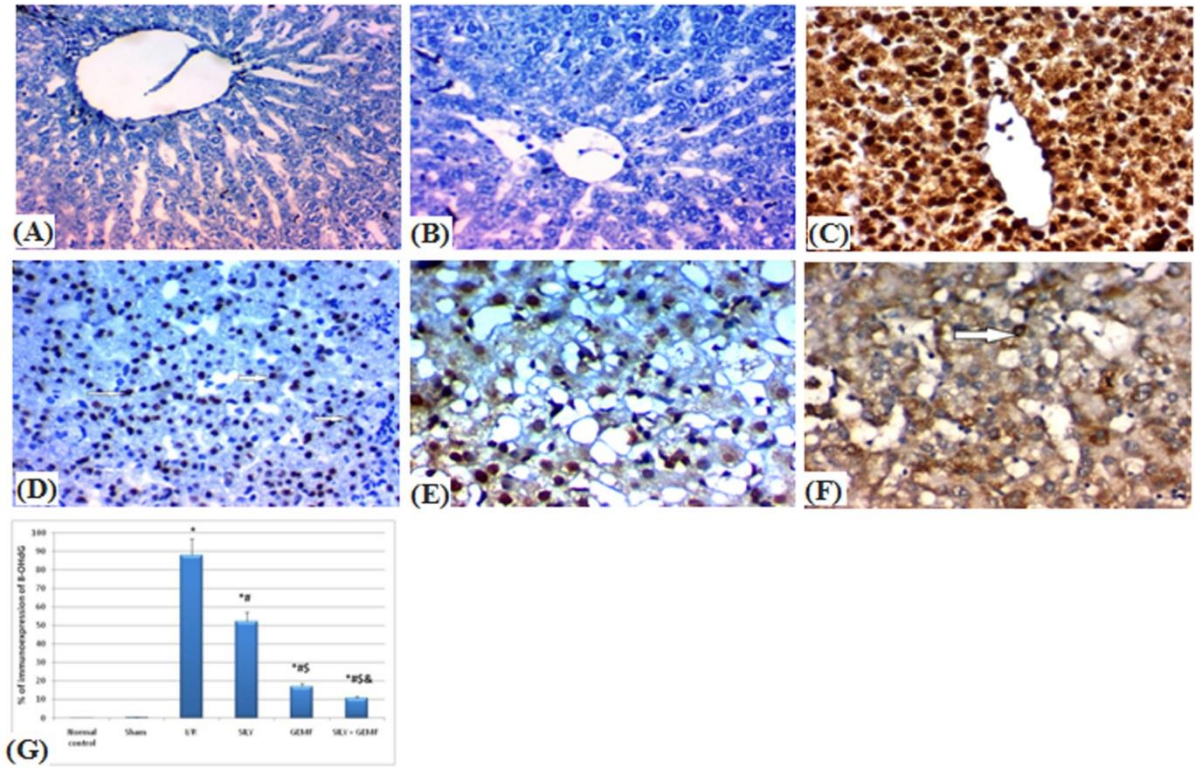


Fig 3: Representative 8-HDOG immunohistochemistry photomicrographs of different groups (magnification 400x). (A) Normal control group (B) Sham group showed negative expression of 8-HDOG (no nuclear brown color). (C) I/R group showed strong positive nuclear brown color. (D) Silymarin-pretreated group showed moderate positive nuclear brown color. (E) Gemfibrozil-pretreated group showed weak positive nuclear brown color. (F) The combination group showed weak positive nuclear brown color. (G) The stained sections when quantified. Data represented as Mean ± SD.* P<0.05 compared to sham group, $^{\#}$ P<0.05 compared to I/R group, $^{\$}$ P<0.05 compared to Silymarin-pretreated group $^{\&}$ P<0.05 compared to Gemfibrozil-pretreated group.

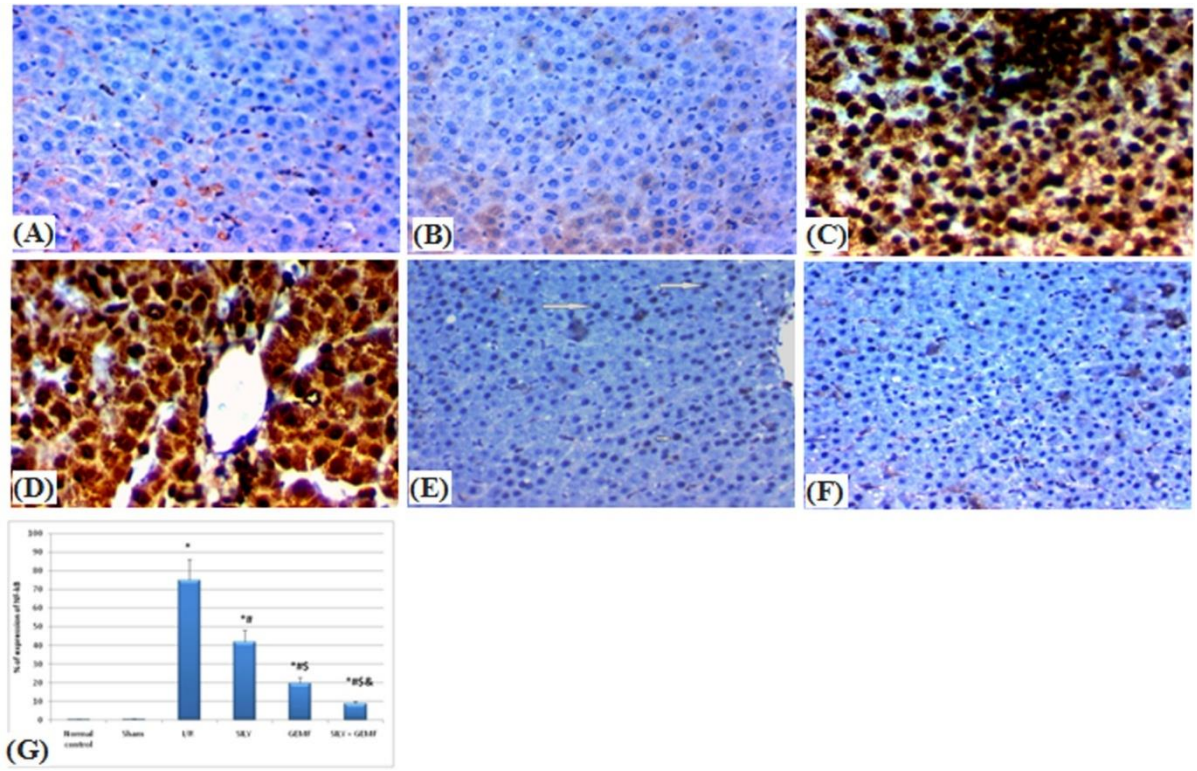


Fig 4: Immunostained liver sections of NF-κB (p65) (magnification ×400). (A) Normal control group (B) Sham group showed negative expression of NF-κB (no nuclear brown staining). (C) I/R group showed strong nuclear brown staining. (D) Silymarin-pretreated group showed moderate positive nuclear brown staining. (E) Gemfibrozil-pretreated group showed moderate positive nuclear brown staining. (F) The combination group showed weak positive nuclear brown staining. (G)The stained sections when quantified. Data represented as Mean ± SD.* P< 0.05 compared to sham group, $^{\#}$ P<0.05 compared to I/R group, $^{\$}$ P<0.05 compared to Silymarin-pretreated group $^{\&}$ P<0.05 compared to Gemfibrozil-pretreated group.

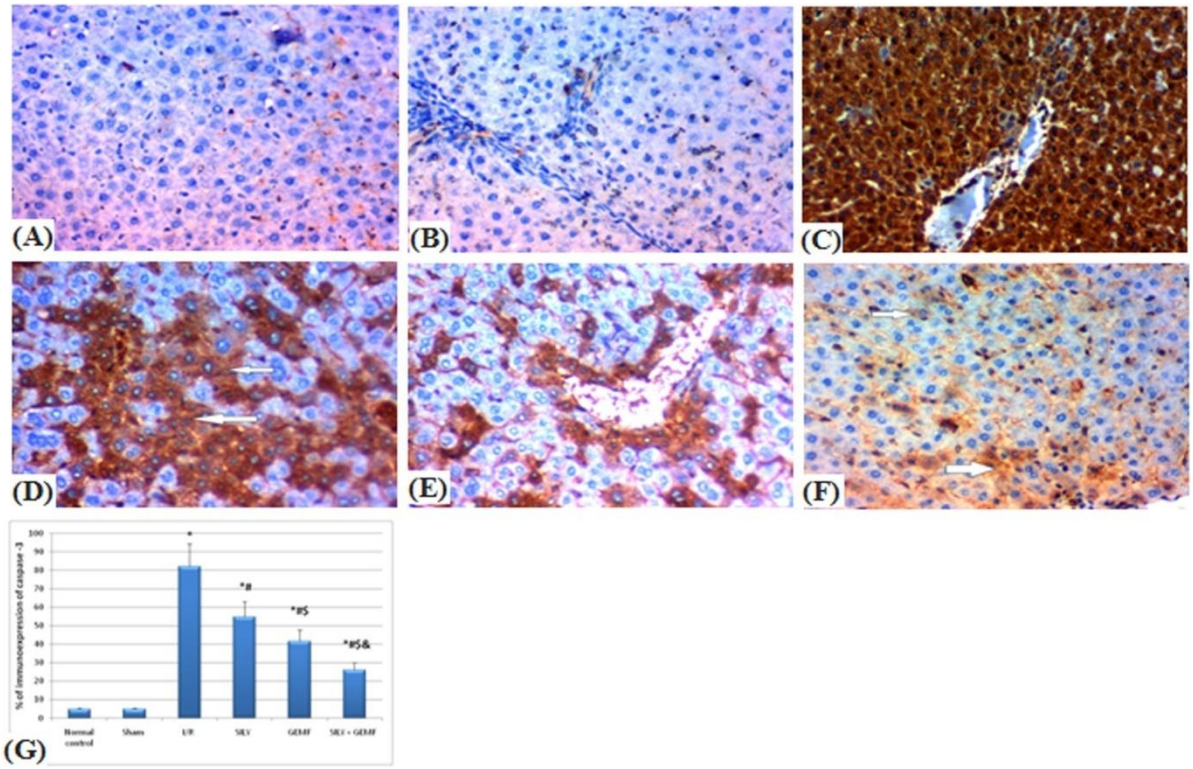


Fig 5: Representative caspase-3 immunohistochemistry photomicrographs of different groups (magnification ×400). (A) Normal control group (B) Sham group showed negative expression of caspase-3 (no cytoplasm brown staining). (C) I/R group showed strong positive cytoplasm brown color. (D) Silymarin- pretreated group showed moderate positive cytoplasm brown color. (E) Gemfibrozil- pretreated group showed weak positive cytoplasm brown color. (F) The combination group showed weak positive cytoplasm brown color. (G)The stained sections when quantified. Data represented as Mean ± SD.* P< 0.05 compared to sham group, $^{\#}$ P<0.05 compared to I/R group, $^{\$}$ P< 0.05 compared to Silymarin-pretreated group $^{\&}$ P<0.05 compared to Gemfibrozil-pretreated group.

DISCUSSION

Hepatic I/R injury has been a subject of intense study in the last decades as it is implicated in numerous clinical practice that threatens the health of patients [1].

I/R injury triggers excessive ROS production which induces hepatocellular injury through breaks the oxidation/anti-oxidation balance, attack polyunsaturated fatty acids and initiate lipid peroxidation, DNA oxidation, and protein and enzyme degradation [11]. Moreover, hepatic I/R injury damage the hepatocellular membrane integrity as a result of membrane lipid peroxidation; this in turn leads to leakage of hepatic enzymes into the circulation because these enzymes are localized to the cytoplasm and mitochondria [12].

The current study showed that exposure to hepatic I/R injury caused marked hepatocellular injury to rats as evidenced by significant elevations of both serum AST and ALT levels. In addition, there were remarkable increase in hepatic MDA level and intensity of hepatic 8-OHdG immunoexpression coupled with significant reduction in levels of hepatic antioxidant protective enzymes (SOD and GSH-Px). Previous investigations showed similar results concerning hepatic I/R injury-induced liver injury where ***Kabil.,*** [2] reported similar increases in liver enzymes after partial hepatic I/R in rat model. In addition, a similar increase in hepatic MDA level together with reduction in levels of hepatic SOD and GSH-Px were recognized by many investigators [2&13]. Additionally, the strong 8-OHdG positive hepatocytes immunoexpression are in harmony with the results reported by ***Zhang et al.,*** [13].

The present work revealed that pretreatment with Silymarin or Gemfibrozil attenuated the increase in transaminases levels induced by hepatic I/R, an effect that correlated with their ability to preserve structural integrity of the hepatocellular membrane. These results of Silymarine or Gemfibrozil are in agreements with other studies [4&5] respectively.

The current work showed that Silymarin has antioxidant activity as evidenced by significant diminution in hepatic MDA level, and 8-OH-dG immunoexpression associated with significant elevation in both hepatic SOD and GSH-Px levels. These results are matched with ***Abouzeinab*** [14], who revealed antioxidant effect of Silymarin against Cisplatin induced renal toxicity in rat model. The anti-oxidant action of Silymarin could be explained on the bases that Silymarin acts as free radical scavenger depending on its phenolic structure. It reduces the free radical load, thus leading to elevation in the cellular antioxidant protective machinery with reduction of lipid peroxidation [14].

The obtained results demonstrated that Gemfibrozil has a powerful antioxidant activity as proved by remarkable reduction in hepatic MDA level coupled with prominent increase in both hepatic SOD and GSH-Px levels. These observations are in line with ***Olorunnisola et al.,*** [15] who illustrated anti-oxidant effect of Gemfibrozil against cholesterol diet-fed rats. Also ***Nikravesh et al.,***[5] confirmed the previous finding in acetaminophen-induced oxidative stress in mice liver.

The antioxidant effect of Gemfibrozil was confirmed also by significant reduction in intensity of 8-OHdG immunoexpression. These findings are in accordance with randomized crossover study of ***Noguchi et al.,*** [16] who observed that treatment of dyslipidemic patients with type 2 diabetes mellitus with PPAR-α agonist (bezafibrate) lowered urine 8-OHdG due to reduction of DNA oxidation. The antioxidant effect mediated by Gemfibrozil could be explained through a multifactorial mechanism that involves increased activity and expression of numerous antioxidant enzymes directly, because PPREs have been found in the promoter regions of several anti-oxidant genes [17]. The antioxidant effects of Gemfibrozil are also based on its direct free radical scavenging ability [18].

During reperfusion, the activated Kuffer cells (KCs) by ROS release pro-inflammatory

mediators (e.g.TNF-α). The increased production of TNF-α induced up-regulation of cellular adhesion molecules with subsequent neutrophils infiltration [2]. TNF-α as well as oxidative stress generated following I/R injury play an important roles in activation of NF-κB, causing IκB-α phosphorylation and degradation, giving the chance for NF-κB to translocate from cytoplasm to the nucleus [19]. Upon nuclear translocation of NF-κB (p65), it bind to DNA and trigger induction of pro-inflammatory cytokines (TNF-α), and adhesion molecules with neutrophils infiltration. This positive feedback is believed to serve to amplify inflammatory signals and exacerbate liver injury [20].

In this work, rats submitted to hepatic I/R associated with noticeable elevations in all measured tissue inflammatory markers, namely hepatic TNF-α and MPO levels. These results coincide with recent reports [1&2]. In addition, the present work showed also dramatic decrease in hepatic IκB-α mRNA expression (an inhibitor of the NF-*κ*B signaling pathway) in I/R group and marked increase in nuclear intensity of hepatic NF-κB (p65) immunoexpression. These results are in line with ***Wang et al.,*** [21] ***and Kabil.,*** [2].

Supplementation of rats with Silymarin minimized the increase in hepatic TNF-α, and MPO level induced by ischemic reperfusion. These findings confirmed work of ***Razavi-Azarkhiavi et al.,*** [22] who recognized similar decrease in serum TNF-α, and lung MPO levels upon administration of Silymarin against bleomycin induced pulmonary toxicity.

The anti-inflammatory effect of Silymarin was confirmed also by significant increase in hepatic mRNA expression level of IκB-α together with decrease in intensity of NF-κB (p65) proteins immunoexpression. Similar observations were reported by ***Wang et al.,*** [23].

According to the results of present work, the pretreatment with Gemfibrozil possessed strong anti-inflammatory effect which was proved by remarkable decrease in hepatic TNF-α, MPO level, and intensity NF-κB (p65) protein immunoexpression. On other hand the hepatic mRNA expression of IκB-α was increased. Similar findings were detected by many studies [8&24&25] which confirmed the ability of Gemfibrozil to reduce oxidative stress parameters and pro-inflammatory cytokines in animal model. The anti-inflammatory effect of Gemfibrozil are also confirmed by investigational studied carried out by ***Yue et al.,*** [26], ***Refaie*** [27], who recorded ability of PPAR-α agonist to induce IκB-α expression accompanied by inhibition of NF-κB activation.

There are several concurring mechanisms may explain the overall inhibitory effect of PPAR-α ligands on NF-*κ*B with subsequently inhibition of expression of their target genes. The most important one is induction the expression of inhibitor of NF-κB, IκB-α, in hepatocyte through PPAR-α depended mechanism. Taken together, PPAR-α dependent transactivation and transrepression provide a molecular mechanism for PPAR-α agonist induced normalization of NF-κB/ DNA binding activity with sharp reduction of the p65-mediated gene activation [28].

In this research, exposure of rats to liver I/R injury was accompanied by apoptosis as evidenced by marked elevation of hepatic caspase-3 level, and strong caspase positive hepatocytes immunoexpression . These results are similar to the results of ***Abdel-Wahab and Al-Harizy.,*** [29] who recorded an increase in serum caspase-3 level in rat model with hepatic I/R, and stated that TNF-α and oxidative stress formed during reperfusion played a crucial role in magnifying the apoptosis in the ischemic liver.

Additionally, remarkable reduction in hepatic mRNA expression of PI3K and its subunits (P85: 110 α / p110 β) were recorded in rat group with hepatic I/R injury. These results are in agreement with work of ***Ren et al.,*** [30] and ***Liu et al.,*** [1] who recorded that reduction in PI3K intracellular signaling pathway aggravates hepatic I/R injury through the aggravating intrinsic pathway of apoptosis with subsequent increased active caspase-3 level.

The decrease in p85 PI3-kinase regulatory subunit and p110 α/ p110 β catalytic subunits might be due to oxidative

stress in which excessive generation of ROS causes oxidation and degradation of various signaling molecules, including enzymes and protein kinases [31].

The obtained result demonstrated that rats pretreated with Silymarin or Gemfibrozil exhibited reduction in hepatic caspase-3 level and intensity of hepatic caspase immunoexpression. Similar observations were reported by ***Younis et al.,*** [32], and ***El-Sisi et al.,*** [24] respectively.

In this study, the anti-apoptotic activity of Silymarin or Gemfibrozil was supported also by noticeable upregulation in hepatic mRNA expression of PI3K, and its subunits (P85: p110 α as key subunit). The findings of Silymarin point to an anti-apoptotic effect, these results coincide with ***Ghosh et al.,*** [33] who observed that Silymarin protected mouse liver and kidney from thioacetamide induced toxicity not only by scavenging ROS but also by inducing PI3K pathway as evidenced by an increase in protein expression level of PI3K in the liver tissues with subsequent inhibition of intrinsic apoptotic pathways. They stated that Silymarin acting as an extracellular ligand which bind to and activate RTKs forming the fully active PI3K enzyme.

The results of Gemfibrozil are confirmed by an earlier study of ***Bouzakri1 et al.,*** [34] who found that PPAR-α agonist (Wy-14643) induced mRNA and protein expression of the PI3K regulatory subunit p85 in human skeletal muscle cells in type 2 diabetic subjects. Many investigator [18&24&35&36] proposed that Gemfibrozil might be mediate its action in part by PPAR-α independent pathway (non-genomic effect) through stimulation of RTKs and forming fully active PI3K enzyme at times too rapid to account for new protein synthesis. Activation of PI3K signaling pathway by Gemfibrozil is not involved only in fibrate anti-apoptotic effect [37], but also involved in its anti-inflammatory action [35& 38].

The increase in p110 α not p110 β as key subunit could be attributed according to previous studies which demonstrated that p110α bind to activated RTKs, and it is mainly responsible for downstream signaling, whereas p110 β seems to signal downstream of GPCRs. In addition, p110 α not p110 β was the principal class IA PI3-K promotes survival and mediating anti-apoptotic actions in several apoptosis-inducing models [39&40].

CONCLUSION

Pretreatment with Silymarin or Gemfibrozil exerted hepatoprotective effect against hepatic I/R injury. Overall, the combination therapy showed hepatoprotective effect in a more significant manner than single drugs alone. The mechanism of this hepatoprotective effect is due to antioxidant, anti-inflammatory and anti-apoptotic properties of the used drugs.

Conflicts of interest

The authors have no conflicts of interest

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