Role of Heme Oxygenase -1 Induction and Type 5 Phosphodiesterase Inhibition in Hepatic Ischemia Reperfusion Injury in Male Albino Rats

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Abstract: ***Objective****:* Ischemia and reperfusion (I/R) injury is a pathophysiologic process whereby hypoxic organ damage is accentuated following return of blood flow and oxygen delivery to the compromised tissue. Both heme oxygenase producing carbon monoxide and nitric oxide synthase producing nitric oxide are involved in cytoprotection against ischemia and reperfusion. The aim of the present study was to investigate the possible hepatic cytoprotective effects of pretreatment with cobalt (III) protoporphyrin IX chloride (Copp) and sildenafil citrate during ischemia, separately and in combination on hepatic I/R injury assessed by serum alanine transaminase (ALT), a marker of hepatic IR injury, and necrotic index. ***Materials and methods:*** the study was carried out using fifty male albino rats belonging to the local strain aged eight weeks with body weight 165 to 200 g*m.* Rat were divided randomly into five groups, each included 10 rats: group I(control sham-operated), group II(hepatic I/R, ischemia for 45 minutes followed by reperfusion for 2 hours), group III(Copp pretreatment and I/R), group IV (I/R with sildenafil injection during ischemia), and group V(Copp pretreatment and sildenafil injection during ischemia). After two hours of reperfusion following ischemia, animals were killed and blood is collected for serum ALT determination and hepatic tissues were used for determining histological evidence of hepatocellular injury assessed by necrotic index. Liver samples are also used for determining HO-1 gene expression and total hepatic nitrite content. ***Results:*** Hepatic ischemia and reperfusion (group II) resulted in hepatocellular injury as revealed by significant increases ($p< 0.05$) in mean value of serum levels of ALT and necrotic index. This was accompanied by significant ($p < 0.05$) increases in the mean values of hepatic HO-1 gene expression and total hepatic nitrite content compared to the control group. Induction of HO-1, by pretreatment of rats with Copp (group III) resulted in hepatocellular protection as evident by significant decreases ($p < 0.05$) in mean values of serum level of ALT and necrotic index. This was accompanied by significant increases in the mean values of hepatic HO-1 gene expression and insignificant change ($p >0.05$) in total hepatic nitrite content compared to group II. Sildenafil citrate injection during ischemia (group IV) also resulted in hepatocellular protection as evident by significant decreases ($p < 0.05$) in mean values of serum levels of ALT and necrotic index accompanied by significant increases ($p < 0.05$) in the mean values of hepatic HO-1 gene expression and total hepatic nitrite content compared to group II. Compared to group III, sildenafil injection during ischemia produced insignificant changes ($p > 0.05$) in the mean value of serum level of ALT and necrotic index. However, HO-1 gene expressions was significantly ($p < 0.05$) decreased while total nitrite content was significantly ($p < 0.05$) increased. Compared to group II pretreatment of rats with Copp and Sildenafil injection during ischemia(group V) produced significant decreases ($p < 0.05$) in the mean value of serum levels of ALT, necrotic index while hepatic HO-1 gene expression and total nitrite content were significantly($p < 0.05$) increasd. Compared to group III and IV by pretreatment of rats with Copp and Sildenafil injection during ischemia produced significant decreases ($p < 0.05$) in the mean value of serum levels of ALT and necrotic index while hepatic HO-1 gene expression and total nitrite content were significantly($p < 0.05$) increased. *Conclusion*: Induction of HO-1 gene expression and inhibition of phosphodiesterase type 5 could have synergistic hepatoprotective effects against I/R injury. Further investigations are recommended for using agents that are not hepatotoxic and can protect the liver and other organs from I/R injury.

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1. Introduction

Ischemia and reperfusion (I/R) injury is a pathophysiologic process whereby hypoxic organ damage is accentuated following return of blood flow and oxygen delivery to the compromised tissue. Transient episodes of hepatic ischemia occur during solid organ transplantation, trauma, hypovolemic shock, and elective liver resection, when inflow occlusion or total vascular exclusion is used to minimize blood loss. The pathophysiology of liver I/R injury includes both direct cellular damage as the result of the ischemic insult as well as delayed dysfunction and damage resulting from activation of inflammatory pathways. Histopathologic changes include cellular swelling, vacuolization, endothelial cell disruption, neutrophil infiltration, and hepatocellular necrosis [1, 2].

The heme oxygenase (HO) system is the rate-limiting step in oxidative degradation of heme into bilivirdin, carbon monoxide (CO) and free iron [3]. Upregulation of Inducible HO-1 is known to be a protective response from cellular stress following I/R injury, radiation, and inflammation. Overexpression of HO-1 exerts a cytoprotective function in a number of I/R injury and liver transplants models [4]. HO-1 is an attractive target for anti-inflammatory therapies and potential candidate responsible for cell injury [5].

Nitric oxide (NO) has been shown to be released by the conversion of L-arginine to L-citrulline, a reaction catalyzed by one of three NO syntheses (NOS): neuronal NOS, endothelial NOS, and inducible NOS [6]. NO has been shown to be a mediator/protector of ischemia and reperfusion (I/R) tissue-mediated injury [7]. NO has been shown to be tissue-protective through its physiologic regulation of vascular tone, inhibition of platelet aggregation, attenuation of leukocyte adherence to the endothelium, scavenging of oxygen-derived free radicals, maintenance of normal vascular permeability, inhibition of smooth muscle proliferation, immune defenses, and stimulation of endothelial cell regeneration [8]. Delivery of NO during an ischemic insult has been shown to limit the extent of reperfusion damage to the heart [9], liver [10], lungs [11], and kidneys [12].

CO and NO share apparent similarities in structure, molecular weight and solubility [13]. Both NO and CO interact with iron (Fe) to form 5 or 6 coordinated haem complexes, which result in conformational changes and activation of the cyclic guanosine monophosphate (cGMP) / soluble guanylyl cyclase (sGC) [sGC/cGMP] pathway. Thus, many of the biological effects of CO are similar to NO, including its anti-apoptotic, anti-proliferative and anti-inflammatory mechanisms [14]. CO influences cell survival by blocking cytokine-mediated mitochondrial release of cytochrome *C* [15] and has been shown to influence hepatoprotection through the transcriptional upregulation of iNOS in the liver. Both exogenously administered and endogenously released NO stimulates HO-1 gene expression and CO production [15, 16]. Furthermore, CO and NO have been shown to participate in vasoactive cross talk, influencing growth factors, anti-inflammatory mediators, angiogenesis, and vascular remodeling [17].

Sildenafil citrate (Viagra), the first orally active and highly selective inhibitor of cGMP-specific phosphodiesterase type 5 (PDE-5), was approved for treating erectile dysfunction. It exhibited cardioprotective action against ischemia-reperfusion injury in both in situ and isolated hearts ([18, 19, and 20]). Sildenafil induced acute and delayed cardioprotection against ischemia-reperfusion injury through enhancement of nitric oxide (NO) generation by increased expression of endothelial NO synthase (eNOS)/inducible NO synthase (iNOS), activation of PKC, and opening of mitochondrial ATP-sensitive K^+ channels [21].

The aim of the present work is to study the effects of induction of HO-1, inhibiting phosphodiesterase type 5, and combined induction of HO-1 and inhibiting phosphodiesterase type 5 on ischemic reperfusion injury of the liver assessed by changes in serum alanine transaminase (ALT) and necrotic index and their effects on hepatic nitrite production and HO-1 gene expression. Also study the effect of ischemic reperfusion on hepatic NO production, indicated by hepatic nitrite production and on HO-1 gene expression.

2. Material and Methods

This study was carried out in Physiology, Histology, and Biochemistry departments, Faculty of Medicine, Cairo University. Fifty male albino rats aged eight weeks with body weight 165 to 200 gm were used in the study. Rats were placed in animal house under ordinary living conditions and were housed in wire mesh cages, 5 rats per cage, at room temperature. Rats were fed rat chow with free access to water. Rats were fasted for 12 hours before experiments and were randomly classified into the following five groups each contained 10 rats.

Group I: control group (sham operated group):

Rats were injected with 1 ml/kg isotonic saline intraperitoneally (I.P) 24 hours before sham operation. At time of sham operation, they were anesthetized then laparotomy and liver exposure were performed. 22.5 minutes later, isotonic saline (1ml/kg) was injected intravenously in rat tail.

Group II: ischemia reperfusion (I/R) group:

Animals of this group are subjected to hepatic ischemia reperfusion injury as 45 minutes of ischemia followed by 2 hours of reperfusion.

Group III: ischemia reperfusion group (I/R) with pre-operative injection of Cobalt (III) protoporphyrin IX chloride (CoPP):

Rats of this group underwent the same procedure as group II, but they were injected I.P with Cobalt (III) protoporphyrin IX chloride (CoPP) at a dose of 5mg/kg 24 hours before operation [5]. CoPP, an activator of HO-1, was purchased from (Sigma –Aldrich Egypt No. 1900) in the form of vials, each contains 500 mg. CoPP is dissolved in 0.2 mmol/L NaOH, adjusting its pH to 7.4 with 1mmol/L HCl and diluting it with isotonic saline.

Group IV: ischemia reperfusion (I/R) group with sildenafil citrate injection:

Rats of this group underwent the same procedure as group II, but they were injected intravenously with Sildenafil citrate. Sildenafil citrate (supplied from Pfizer, Egypt in the tablet form, 50 mg) was dissolved in saline to obtain a concentration of 2 µg/ml and

injected I.V at a dose of 2 µg/kg 22.5 minutes after the onset of ischemia [22].

Group V: ischemia reperfusion (I/R) group with Copp and sildenafil injection:

Rats of this group underwent the same procedure as group II and were injected I.P with CoPP at a dose of 5mg/kg 24 hours before operation [5] and intravenously injected with Sildenafil citrate at a dose of 2 µg/kg, 22.5 minutes after the onset of ischemia [22].

Induction of hepatic ischemia reperfusion injury

Anesthesia was induced by intraperitoneal injection of thiopental sodium at a dose of 50 mg/kg (supplied from Eipico Egypt in the form of vials, each contains 500 mg) and maintained by repeat doses (IV) of 25 mg/ kg if necessary, based on animal movement. All the surgical procedures were performed under sterile conditions. Body temperature was maintained using a heating pad placed under the animal. I/R was produced by temporarily occluding the blood supply to the left lateral and median lobes of the liver, as described by **Zhang *et al.*** [23]. Laparotomy was carried out through a midline incision, and the ligamentous attachments from the liver to the diaphragm were severed and the liver exposed. Ischemia of the median and left lateral lobes of the liver was produced by clamping the corresponding vascular pedicle containing the portal vein and branches of the hepatic artery using an atraumatic microvascular clamp for 45 min. Other hepatic lobes were not handled during the procedure. This method produces ischemia to the left and median lobes of the liver, and leaves the blood supply to the right and caudate lobes uninterrupted. The liver was then placed back in its original position for 45 min and kept moist with sterile gauze dampened with 0.9% saline. Core body temperature was monitored by recording rectal temperature using rectal thermometer, and a heat lamp was utilized to maintain body temperature at 37 ± 0.4°C At the end of the ischemia period, the vascular clamp was removed, and the liver was reperfused for 2 hrs. After reperfusion, 3–5 ml of blood was collected from the vena cava in sterile syringes without anticoagulant and centrifuged to separate the serum. The serum samples were stored at −20°C for later analysis of ALT. Ischemic hepatic tissue samples were collected and part of the ischemic lobes used for histologic examination was fixed in alcohol – formalin – acetic acid and embedded in paraffin blocks. While samples used for HO-1 gene expression and nitrite were weighted and immediately frozen in isopentane and liquid nitrogen, then stored at −80°C for later analysis. The animals were killed by exsanguination. Sham-operated animals were treated in an identical fashion except for the omission of vascular occlusion

Measurement of serum ALT

Serum alanine transaminase (ALT), an established surrogate marker of hepatic IR injury [24] was measured using kits supplied by Lab Biotechnology (USA) catalog (Sup) according to the manufacturer's instructions.

Morphometric assessment of reperfusion injury.

Histopathology scoring was performed on randomly selected high-power fields by investigators blinded to sample identity Excised Liver samples embedded in paraffin blocks were cut in 6-µm sections and stained with hematoxylin and eosin. Stained sections were evaluated at 1200 magnification by a point-counting method for severity of hepatic injury using an ordinal scale as follows; **grade 0:** minimal or no evidence of injury, **grade 1:** mild injury consisting in cytoplasmic vacuolation and focal nuclear pyknosis, **grade 2**: moderate to severe injury with extensive nuclear pyknosis, cytoplasmic hypereosinophilia, and loss of intercellular borders, and **grade 3:** severe necrosis with disintegration of hepatic cords, hemorrhage, and neutrophil infiltration[25]**.** To assess necrotic index, the mean score between lobular, periportal, and perivenous necrosis was calculated for each specimen [26].

Measurement of total hepatic nitrite

The concentration of nitrites (NO_2^-) and nitrates (NO_3^-) in the liver was determined by the Griess reaction. After deproteination of liver homogenate by a solution of zinc sulfate, samples were incubated with cadmium granules to reduce nitrate to nitrite; the total nitrite was measured at 540 nm absorbance by diazotization with Griess reagent[27]**.**The results are expressed as the sum of the *N*-oxides of NO (NOx).

Measurement of HO-1 gene expression.

According to the method described by **Hoshida *et al.*** [28] Total RNA was extracted from liver tissue sing SV total RNA extraction kit supplied by (Promega Madisson USA) according to manufacturer instruction. Total RNA (5µg) was subjected to reverse transcription for cDNA synthesis at 42°C for 50min, using 1.6mM dNTPs, 10mM DTT, 176nM random hexamers (Invitrogen, Carlsbad, California, USA), 125U reverse transcriptase SuperScript II (Promega, Madisson, USA), and first strand buffer in a final volume of 30µl. The reaction was terminated by heating the samples at 75°C for 10min. For PCR, 4µl cDNA was incubated with 30.5µl water, 4µl 25mMMgCl2, 1µl dNTPs (10mM), 5µl 10×PCR buffer, 0.5µl (2.5 U) Taq polymerase and 2.5µl of each primer containing 10pmol. The oligonucleotide sequences for HO-1 were 5'- GAGCGCCCACAGCTCGACAG -3' (sense) and 5'-GTGGGCCACC AGCAGCTCAG -3' (antisense). The reaction mixture was subjected to 40 cycles of PCR amplification as follows: denaturation at 95°C for 1 min, annealing at67 °C for 1 min and extension at 72°C for 2min. PCR products were electrophoresed on 2% agarose stained with ethidium bromide and visualized by ultraviolet transilluminator. Semiquantitation was performed using gel

documentation system (BioDO, Analyser, Biometra, Gottingen, Germany). According to the amplification procedure, relative expression of each studied gene (R) was calculated according to the following the formula: densitometrical units of each studied gene/densitometrical units of b-actin.

PCR detection of b-actin

Presence of RNA in all samples was assessed by analysis of the 'house-keeping' gene b-actin. Complementary DNA was generated from 1 mg total RNA extracted with avian myeloblastosis virus reverse transcriptase for 60min at 371C. For PCR, 4ul complementary DNA was incubated with 30.5ul water, u ml 25mM MgCl2, 1ml deoxyribonucleotide triphosphates (10mM), 5ul 10x PCR buffer, 0.5ul (2.5U) Taq polymerase and 2.5ul of each primer containing 10pM. b-actin primers (forward 5-TGTTGTCCCTGTATGCCTCT-3; reverse 5-TAATGTCACGCACGATTTCC-3) The reaction mixture was subjected to 40 cycles of PCR amplification, denaturation at 95 ^{0}C for 1min, annealing at 57^{o}C for 1min and extension at 72 ^{0}C for 2mimutes

Statistical analysis

Data were expressed as mean + standard deviation (S.D.). The difference between two groups was assessed by using student t- test for unpaired data. $p < 0.05$ values are considered statistically significant. Correlation was done to show the association between two quantitative variables [(29)].

3. Results

Table 1 demonstrated that, in sham operated control group (group I), the mean value of serum level of ALT was 47.4± 5.45 U/l while the mean value of necrotic index was 0.000 (Fig.1). The mean value of hepatic HO-I gene expression was 0.001 ± 0.0003 arbitrary unit while the mean value total hepatic nitrite was 19.67 ± 2.78 pico (p) mol/mg protein.

In group II, in which rats were subjected to ischemia for 45 minutes followed by reperfusion for 2 hours, the mean value of serum level of ALT was 74.97± 5.18 U/l while the mean value of necrotic index was 2.79 ± 0.35 (Fig.2).The mean value of hepatic HO-I gene expression was 0.06 ± 0.01 arbitrary unit while the mean value total hepatic nitrite was 31.36 ± 4. 9 p mol/mg protein. These results demonstrated that ischemia and reperfusion resulted in significant increases ($p < 0.05$) in the mean values of serum level of ALT, necrotic index, hepatic HO-1 gene expression and total hepatic nitrite content compared to control group.

In group III, in which rats were pretreated with Copp and subjected to ischemia for 45 minutes followed by reperfusion for 2 hours, the mean value of serum level of ALT was 56.42± 6.8 U/l while the mean value of necrotic index was 1.9 + 0.43(Fig.3).The mean value of hepatic HO-I gene expression was 0.18± 0.04 arbitrary unit while the mean value total hepatic nitrite was 33.56 ± 5.9 p mol/ mg protein. These results demonstrated that, compared to group II, induction of HO-1, by pretreatment of rats subjected to I/R with Copp resulted in significant decrease ($p < 0.05$) in the mean value of serum level of ALT and necrotic index while the mean values of hepatic HO-1 gene expression was significantly increased. However, the mean value of total hepatic nitrite content was insignificantly changed ($p>0.05$). These results demonstrated the cytoprotective effect of HO-1 induction against I/R injury as evident from the significant decreases ($p < 0.05$) in mean values of serum levels of ALT and necrotic index compared to group II.

In group IV, in which rats were subjected to ischemia for 45 minutes followed by reperfusion for 2 hours and intravenously injected with sildenafil citrate during ischemia, the mean value of serum level of ALT was 56.86. + 6.3 U/l while the mean value of necrotic index was 2.06 + 0.33 (Fig.4).The mean value of hepatic HO-I gene expression was 0.09 ± 0.02 arbitrary unit while the mean value total hepatic nitrite was 40.3 ± 4.6 pmol/ mg protein. These results demonstrated that, compared to group II**,** Intravenous injection of sildenafil citrate during ischemia produced significant decreases in the mean values of serum level of ALT and necrotic index while the mean values HO-1 gene expression and total hepatic nitrite were significantly increased ($p < 0.05$). These results demonstrated the cytoprotective effect of sildenafil against I/R injury as evident from the significant decreases($p < 0.05$) in mean values of serum level of ALT and necrotic index. Compared to group III, the mean value of serum level of ALT and necrotic index demonstrated insignificant change ($p >0.05$), HO-1 gene expression was significantly ($p < 0.05$) decreased while total nitrite content was significantly increased ($p < 0.05$).

In group V, in which rats were pretreated with Copp and injected with sildenafil citrate during ischemia, table 1 demonstrated that the mean value of serum level of ALT was 48 84. + 4.9 U/l while the mean value of necrotic index was 0.49 + 0.11(Fig.5).The mean value of hepatic HO-I gene expression was 0.2 ± 0.03 arbitrary unit while the mean value total hepatic nitrite was 46.99 ± 5.4 p mol/ mg protein. These results demonstrated that pretreatment of rats, subjected to I/R with Copp and Intravenous injection of sildenafil citrate during ischemia produced significant decreases in the mean values of serum levels of ALT and necrotic index while the mean values of hepatic HO-1 gene expression and total nitrite content were significantly increased compared to group II. Compared to group III and group IV, data obtained from group V demonstrated significant decreases ($p < 0.05$) in the mean values of serum level of ALT, necrotic index while the mean values HO-1

gene expression and total hepatic nitrite were significantly increased ($p < 0.05$). These results demonstrated that pretreatment with Copp and intravenous injection of sildenafil citrate during ischemia produced significant cytoprotective effect compared to pretreatment with Copp or sildenafil injection individually as evident from the significant ($p < 0.05$) decreases in mean values of serum levels of ALT and necrotic index compared to group III and group IV.

The present work demonstrated significant ($p < 0.05$) positive correlation between the mean values of serum levels of ALT and necrotic index (Fig. 6). Significant positive correlation is also demonstrated between the mean values of hepatic HO-1 gene expression and the mean values of total hepatic nitrite content (Figs. 6, 7).

Table (1): Effects of HO-1 induction and inhibition of phosphodiestarse type5 on serum alanine transaminase (ALT), necrotic index, hepatic heme oxygenase-1 (HO-1) gene expression and total hepatic nitrite contents in male albino rats subjected to hepatic ischemia and reperfusion injury.

	Group I	Ischemia for 45 minutes followed by reperfusion for 2 hours			
	Control group	Group II Ischemia reperfusion	Group III Pretreated with Copp	Group IV Sildenafil injection during ischemia	Group V pretreated with Copp and sildenafil injection during ischemia
Serum ALT (U/l) Mean + SD	47.4+ 5.45	74.97±5.18 @ A	56.4±6.81 * A	56.86+ 6.3 * A Θ B	48.84 + 4.9* A # A
Necrotic index Mean + SD	0.000	2.799 ± 0.35 @ A	1.932± 0.43 * A	2.06+ 0.3 * A Θ B	0.49+ 0.11* A # A
HO-1 (arbitrary unit) Mean + S.D.	0.001±0.0003	0.063± 0.01 @ A	0.18±0.04 * A	0.09+0.02 * A Θ A	0.2+0.03* A # A
Total nitrite (pmol/ mg protein) Mean + S.D.	19.67±2.78	31.36±4.97 @ A	33.65 ± 5.9 * B	40.3+4.61 * A Θ A	46.99+ 5.4* A # A

A Significant changes ($p< 0.05$). B Insignificant changes ($p>0.05$). @ Compared to control group. * Compared to group II. ΘCompared to group III. # Compared to group III and IV

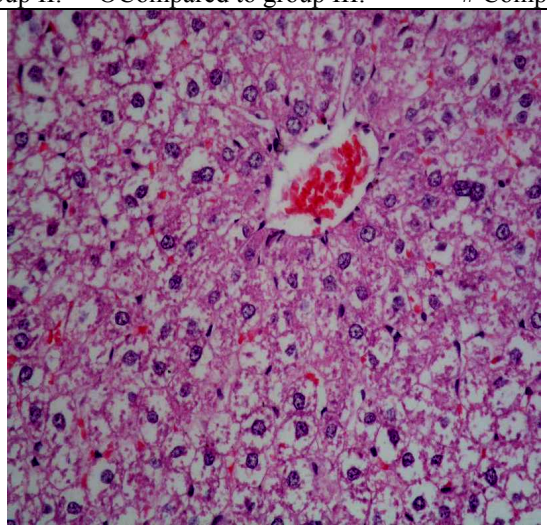


Figure 1:(Group I) This slide shows normal arrangement of cells normal nuclei and cytoplasm (necrotic index = o.oo ± , magnification power = 400).

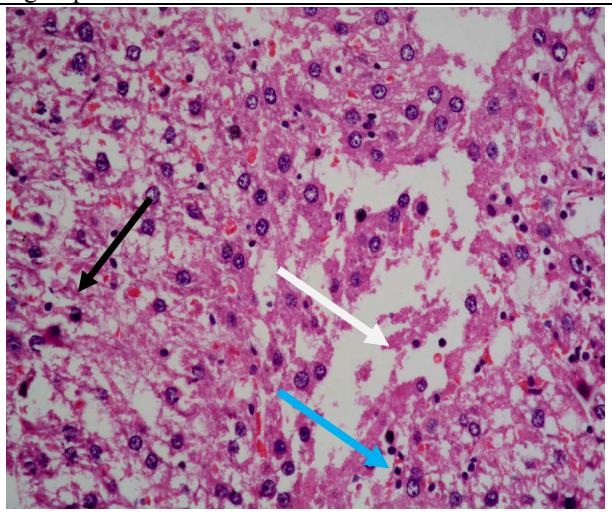


Fig. 2 (Group II): The white arrow represents disintegration of hepatic cords, the black one neutrophil infiltration and the blue arrow shows nuclear pyknosis (necrotic index 2.7±, magnification power = 400).

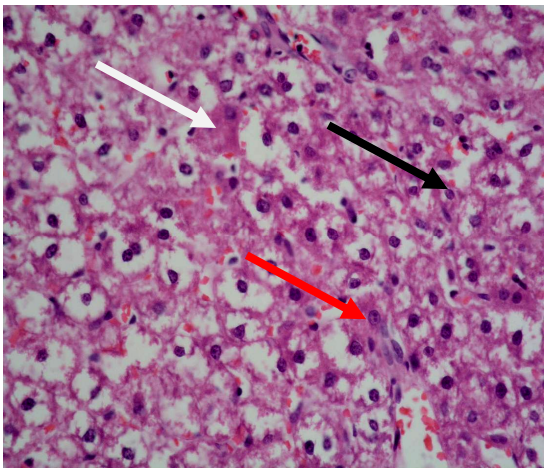


Fig. 3(Group III): The white arrow represents cytoplasmic hypereosinophilia, the blackone shows nuclear pyknosis and the red arrow represents loss of intercellular borders(necrotic index 1.9±, magnification power =400).

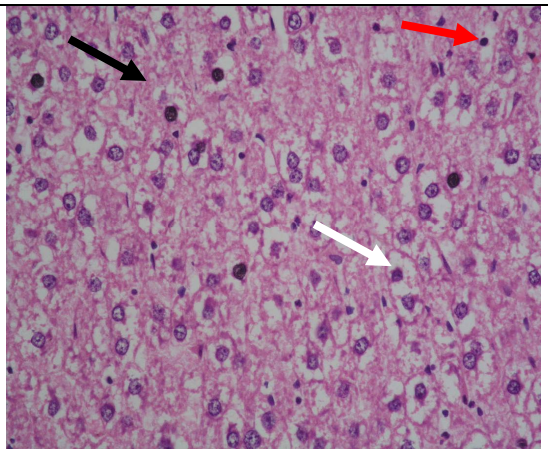


Fig. 4 (Group IV)The white arrow represents cytoplasmic vaculation ,the the red one shows nuclear pyknosis and the black arrow represents loss of intercellular border (necrotic index 2,06±, magnification power = 400)

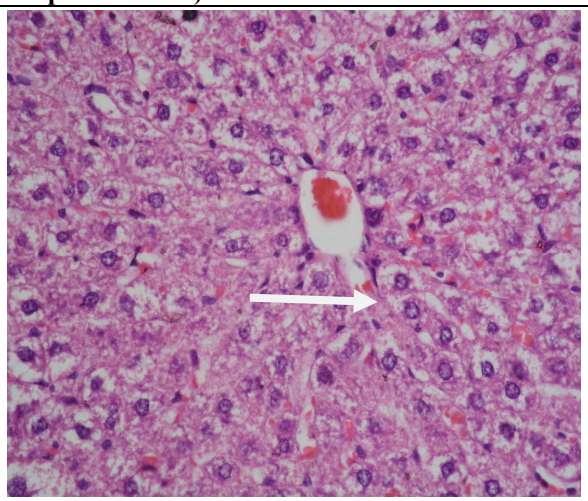
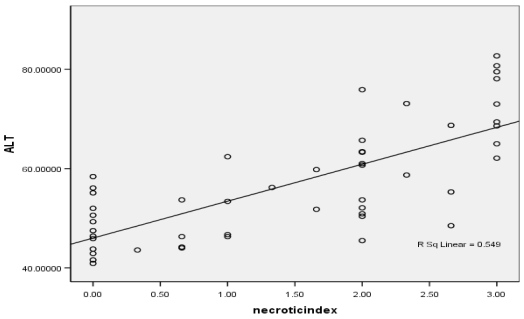
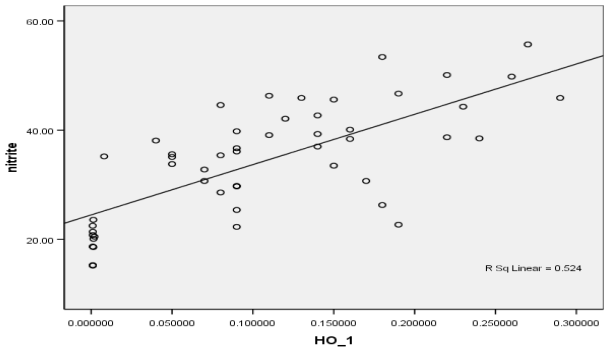


Fig. 5 (Group V)The white arrow represents cytoplasmic vaculation (necrotic index 0.49±, magnification power = 400)



Fig(6): Correlation between the mean level of serum ALT and the mean level of necrotic index.



Fig(7): Correlation between the mean level of HO-1gene expression and the mean level of nitrite in liver tissue

4. Discussion

Ischemia and reperfusion (I/R) injury is a pathophysiologic process whereby hypoxic organ damage is accentuated following return of blood flow and oxygen delivery to the compromised tissue. Transient episodes of hepatic ischemia occur during solid organ transplantation, trauma, hypovolemic shock, and elective liver resection, when inflow occlusion or total vascular exclusion is used to minimize blood loss. The pathophysiology of liver I/R injury includes both direct cellular damage as the result of the ischemic insult as well as delayed dysfunction and damage resulting from activation of inflammatory pathways. Histopathologic changes include cellular swelling, vacuolization, endothelial cell disruption, neutrophil infiltration, and hepatocellular necrosis **([1, 2])**.

In the present study, I/R injury of the liver was induced by exposure of the liver to 45 minutes of ischemia followed by 2 hours of reperfusion. Liver injury was assessed by estimation of serum alanine aminotransferase (ALT) and histological examination. Hepatic HO-1 gene expression and total nitrite content in the liver were also estimated after 2 hours of reperfusion.

The present work demonstrated significant increase ($p< 0.05$) in the mean values of serum level of ALT and necrotic index in group II subjected to I/R injury, compared to sham operated control group (group I). Significant positive correlation ($p<0.05$) was reported between mean values of serum ALT and mean values of necrotic index in all studied groups subjected to ischemia and reperfusion .These findings are consistent with the results of **Sepodes *et al.*** [30] who reported that liver ischemia for 30 minutes followed by reperfusion for 2 hours resulted in significant rises in serum levels of ALT. **Wang *et al.*** [31] found increased levels of ALT in mice after 45 minutes of partial hepatic ischemia. **Kim *et al.*** [32] reported that hepatic I/R caused significant hepatocellular damage as demonstrated by elevated plasma ALT level.

Blood perfusion to previously ischemic tissue induces severe tissue injury, which is called ischemia-reperfusion (I/R) injury [33]. Deprivation of oxygen to the liver during ischemia induces severe lesions but much more important ones are originated during reperfusion when oxygen entry to the organ is restored. On this case, an additional liver aggression occurs exacerbating greatly the previous injury induced by ischemia. Both facts lead to the induction of multiple and close related inflammatory processes in liver and extrahepatic organs which define the complex pathophysiology of I/R injury [34]. **Linfert *et al.*** [35] reported that interruption of blood flow to any tissue leads to inadequate tissue oxygenation and an increased cellular anaerobic pathways, and if adequate oxygenation is not restored then disruption of cellular functions and cell death results. On reperfusion, despite restoration of adequate cellular oxygenation, there is further damage caused by direct cytotoxicity from oxygen free radicals and by a secondary immunological assault upon the injured organ involving components of both the innate and adaptive immune system.

I/R injury of the liver is characterized by sinusoidal vasoconstriction, neutrophil accumulation, platelet aggregation and alterations on the capillary permeability leading to a progressive inflammatory reaction with important microcirculatory alterations, which can trigger diffuse cell death and consequent acute organ failure[33]. The progression of I/R injury depends primarily on the presence of pre-existing parenchymal alterations, such as hepatic steatosis and fibrosis, as well as the duration of ischemia period **[36]**. When oxygen supply to hepatocytes becomes insufficient as result of reduced or absent blood flow, there is inhibition of the mitochondrial oxidative phosphorylation with the subsequent reduction in adenosine triphosphate (ATP) synthesis. Depletion of cellular ATP store induces alterations in transmembrane ion transport by inhibition of the ATP-dependent Na+/K+ ATPase, leading to intracellular sodium accumulation, secondary alterations in cellular calcium homeostasis and, particularly, cell swelling and death [37].

Post-ischemic liver injury is biphasic in nature consisting of an acute or early phase and a subacute or late phase **[38]**. The early phase of injury occurs in the absence of leukocyte infiltration and is thought to be initiated by a rapid alteration in the redox state of the tissue in favor of a more oxidative environment. The late phase of injury is dependent upon the production of several different cytokines and chemokines that promote the infiltration of large numbers of polymorph nuclear neutrophils (PMNs) and lymphocytes into the liver interstitium via the up-regulation of endothelial cell adhesion molecules and formation of chemotactic gradients **[39]**. Interstitial PMNs become fully activated and release copius amounts of reactive oxygen species together with extracellular matrix degrading enzymes. The net result of this inflammatory infiltrate is an amplification of the acute injurious response resulting in extensive inflammatory tissue injury **[40]**.

The results of the present work demonstrated that hepatic heme oxygenase (HO-1) gene expression was significantly increased in group II (I/R group) compared to the control group. Induction of HO-1 by Copp (group III) resulted in significant increase in HO-1 gene expression and significant decreases in the mean values of ALT and necrotic index compared to group II. These results demonstrated the cytoprotective effect of HO-1 against I/R injury of the liver.

The heme oxygenase (HO) system is the rate-limiting step in the oxidative degradation of heme into biliverdin, carbon monoxide (CO) and free iron [3]. Three HO isoforms have been identified: inducible HO-1, also known as heat shock protein 32; constitutively expressed HO-2; and a related but less well-characterized HO-3. HO-1 has been shown to be expressed principally in Kupffer cells [4, 41]. HO-1 is induced in a variety of organs during diverse stress-related conditions and is thought to provide cytoprotection [42, 43]. HO-1 is readily induced by heme, oxidants, lipopolysaccharide, cytokines, irradiation, heavy metals, and other stressors [44]. HO-1 presents at low to undetectable levels in Kupffer cells under basal conditions, but it undergoes a rapid transcriptional activation and expresses both in Kupffer cells and hepatocytes as a response to noxious stimuli. Ho-1 induction is considered to be adaptive cellular response to survive on exposure to environmental stress [45].

HO-1 provides an important protective response from cellular stress following ischemia, preventing the deleterious effects of heme as well as mediating anti-inflammatory and antiapoptotic functions via its products [46]. It is unclear whether baseline HO-1 levels before the injury or the degree of HO-1 up-regulation following the injury is important to confer cytoprotection [47]. HO-1 overexpression by pharmacological means or via genetic engineering has been shown to exert potent cytoprotective effects in hepatic I/R injury transplant models, where both proinflammatory and apoptotic responses remain profoundly diminished in HO-1-overexpressing liver transplants [48].

Induction of HO-1 and its metabolites is protective in a large number of seemingly unrelated pathologies, including sepsis, malaria, endotoxic shock, I/R injury, organ transplant, and myocardial infarction,. This spectrum of protection is attributed to multi-level mechanisms of cytoprotection and inflammatory modulation [49]. The cobalt protoporphyrin induction of HO-1 has been shown to improve liver function and histologic characteristics [46]. Using cobalt protoporphyrin and the HO-1 antagonist zinc protoporphyrin demonstrated the protective effects of HO-1 induction during the prolonged storage of liver transplants [48]. Hypertonic saline prevented ischemia-reperfusion injury by promoting the expression of heme oxygenase-1 [50]. Induction with simvastatin preconditioning also had a protective result [51].

The beneficial effects of HO-1 are presumably mediated by the degradation of pro-oxidative heme and production of biologically active HO reaction products [52].Biliverdin and bilirubin are powerful antioxidants [53]. CO mediates the antiapoptotic, anti-inflammatory, antiproliferative and vasodilatory properties of HO-1 [54] and iron induces the synthesis of ferritin, which is also a cytoprotective molecule and sequesters free iron [55].

Overexpression of HO-1 exerts a cytoprotective function in a number of I/R injury and liver transplant models [56]. Thus, HO-1 is an attractive target for anti-inflammatory therapies and potential candidate responsible for cell injury [5].There is evidence that treatment with the products or related molecules of the HO-1 reaction is protective, including biliverdin [57], bilirubin, and carbon monoxide [58]. Specifically, exogenous carbon monoxide was protective in liver transplants [59, 60]. There is also evidence that heme oxygenase-1 mediated cytoprotection depends on and can be substituted by carbon monoxide generation [61].

The results of the present work demonstrated significant increases ($p< 0.05$) in the mean values of total hepatic nitrite in group II and group III compared to the control group. Integrated Nitric oxide production can be estimated from determining the concentrations of nitrite and nitrate end products. The measurement of total nitrate and nitrite concentration (NOx) is used as an index of NO production [62].

Nitric oxide is produced from L-arginine by nitric oxide synthase enzymes (NOS) [63] .Three NOS have been identified: two constitutive (cNOS: type 1 or neuronal and type 3 or endothelial) and one inducible (iNOS: type 2). In the liver, cNOS activity is normally detectable in Kupffer cells, whereas no cNOS is ever encoded in hepatocytes. However, hepatocytes, Kupffer and stellate cells (the three main types of liver cells) are prompted to express an intense iNOS activity once exposed to effective stimuli such as bacterial lipopolysaccharide and cytokines [64].

The process of ischemia and reperfusion is known to cause inducible nitric oxide synthase induction and activation, and there is evidence that interleukin (IL)-1β [65], IL-1 receptor [66], and IL-1 receptor along with nuclear factor-kappa beta (NF-kappa β) [67] may have an important role in that induction. During ischemia and reperfusion, both helpful and harmful effects of nitric oxide have been reported, and the nitric oxide molecule has been described as having a “janus face” [68]These conflicting results about the role of nitric oxide during ischemia reperfusion, with some studies showing beneficial results and others harmful results, have been attributed to the use of nonspecific inhibitors of nitric oxide synthase [69]. Whether nitric oxide has a helpful or harmful effect depends on several factors in the liver [70].

Lhuillier *et al.* [71] measured nitric oxide generated in the liver parenchyma during ischemia and reported that NO concentrations increased after the onset of ischemia to reach a plateau by 10 minutes. The short delay existing between the onset of ischemia and the increase in NO signal is consistent with the involvement of endothelial nitric oxide synthase (eNOS). However, nonselective NOS inhibitors

administered before ischemia failed to inhibit the increase in NO, whereas NO production remained inhibited in the control group treated with nonselective NOS inhibitors, thereby suggesting the presence of enzyme-independent sources of NO. NO stores are available in tissues and could be mobilized during ischemia**.** Another enzyme-independent source for NO might be the endogenous reduction of nitrite and nitrate resulting in the appearance of NO. This mechanism has been demonstrated in ischemic heart and could be the source of NO in the ischemic liver [(72)]. However, this NO production was dependent on the amount of nitrite–nitrate available in the tissue before ischemia. [(73)].

De Caterina *et al.* [(74)] provide evidence that NO enhances the *de novo* synthesis and/or stabilization of the natural inhibitor Ikβ-α.Furthermore, NO is known to interact with and decompose O_2^- or other reactive radicals or oxidants thereby limiting the formation of O_2^- derived H_2O_2 and preventing the downstream oxidant-induced pathways for NFkB activation [(75)]. Another possible mechanism may be that NO-dependent activation of soluble guanylyl cyclase (sGC) with the subsequent production of the vasorelaxant cyclic guanosine 5'-monophosphate (cGMP) may protect against reperfusion injury by enhancing blood flow, thereby limiting the degree of ischemia to the liver. It has been proposed that NO-mediated activation of protein kinase G via the sGC/ cGMP pathway opens mitochondrial K_{ATP} channels which reduces calcium accumulation within the mitochondria and prevents the loss of cytochrome c from the mitochondrial intermembranal space [(76)]. Alternatively or, in addition to, NO may reversibly inhibit mitochondrial respiration via interaction with complex I and/or cytochrome c oxidase. This would inhibit apoptosis, maintain small but significant amounts of O_2 during ischemia and allow for a more controlled resumption of respiration following reperfusion. Similar observations have been made with NO-dependent S-nitrosation of caspase-3 resulting in inactivation of this enzyme and inhibition of apoptosis. This would minimize free radical-mediated damage to the mitochondrial membrane and preserve cellular function [(77)]. NO may attenuate the later stages of post-ischemic tissue damage by inhibiting platelet/leukocyte-endothelial cell interactions [(78)]**.**

The results of the present work demonstrated cytoprotective effect of sildenafil citrate on liver subjected to I/R injury as evident from the significant decrease in mean value of serum level of ALT and necrotic index compared to I/R group .

Sildenafil is a potent and selective inhibitor of cGMP-specific phosphodiesterase type 5A (PDE-5). PDE-5 is responsible for the degradation of cGMP by hydrolysis to guanosine 5'-monophosphate (5'-GMP). Thereby, inhibition of PDE-5 by sildenafil preserves the cGMP pool and potentiates downstream signaling [(79)].

The basic mechanisms of NO-dependent cytoprotection are diverse and include direct neutralization of the superoxide radical [(80)] or inhibition of proapoptotic enzymes, such as caspase-3–like proteins, through *S*-nitrosylation [(81)]**.** These actions are cGMP independent and do not require increased activity of soluble guanylyl cyclase, the target enzyme of NO in many biological systems [(82)]. By contrast, endothelial protection afforded by NO against the deleterious effects of proinflammatory cytokines has clearly been shown to be cGMP dependent [(83)]**.**

NO modulates hepatocellular/tissue injury through its participation in neutrophil adhesion, platelet aggregation, and maintenance of normal vascular permeability [(84)]. Nitric oxide binds to the haem moiety of guanylate cyclase and increases its activity by 400-fold, catalyzing the conversion of guanosine triphosphate to cyclic guanosine monophosphate (cGMP). Elevation of cGMP relaxes the smooth muscles in blood vessels, inhibits platelet aggregation and adhesion, and blocks the adhesion of white cells to the blood vessel wall, cellular necrosis and apoptosis [(85,86)]**.**

The cytoprotective effect of sildenafil against hepatic I/R injury, observed in the present work, is supported by the work of **Duranski *et al.*** [(22)] who reported that the soluble isoform of guanylyl cyclase is an important cellular target of NO. Genetic overexpression of eNOS protects against hepatic I-R injury. Direct sGC inhibition increased serum ALT levels in mice after hepatic I-R, suggesting that sGC function plays pivotal role in attenuating I-R injury. Control mice treated with sildenafil were significantly protected against hepatic I-R injury. Taken together, these data suggest that the NO-sGC-cGMP axis plays a critical role in limiting the extent of hepatic I-R injury [(22)]**.**Contrarily to the hepatic protective effect of sildenafil against I/R injury observed in the present work, ***Leão et al.*** [(87)] reported that pre-treatment with sildenafil in rats resulted in increased damage to hepatocytes in a model of hepatic ischemia/reperfusion. These controversy can be explained on the basis of different protocols used as method, dose, and timing of sildenafil administration.

The present work demonstrated a significant increase in the mean value of hepatic nitrite concentration in group IV compared to group II .**Salloum *et al.*** [(19)] reported that sildenafil up regulated both eNOS and iNOS and that inhibition of iNOS completely abolished the protective effect of sildenafil.

The present work demonstrated significant increase in the mean value of hepatic HO-1 expression in sildenafil group compared to group II (I/R group).

In addition to its action as a vasodilator, NO can regulate the expression of a variety of genes. In

particular, there is solid evidence that NO regulates the expression of HO-1 [88]. Treating aortic smooth muscle cells with the NO donor increases HO-1 gene transcription, resulting in increased mRNA and protein expression. This induction of HO-1 by NO occurs in a cGMP-independent manner. The pathways by which NO regulates the expression of HO-1 and other genes are complex and but appear to involve mitogen-activated protein kinase (MAPK) members such as extracellular signal-regulated kinase (ERK) and p38 [88]. On the other hand, the pathway, through which cGMP induces HO-1 could be a direct one, i.e., via cGMP-sensitive transcription factors, such as activator protein-1[89]. Alternatively, cGMP may act through secondary increases in cAMP, which were reported to occur in endothelial cells in response to NO donors and which were possibly due to cGMP-elicited inhibition of cAMP breakdown [90]. Because activator protein-1 and cAMP-responsive elements have been identified in the promoter region of HO-1, [91]. HO-1 induction by NO/cGMP may be regulated through different mechanisms, depending on species and tissue [92].

The results of the present work demonstrated that pretreatment of rats with both Copp and sildenafil during ischemia resulted in significant decreases in the mean values of ALT and necrotic index compared to group III and IV, and resulted in significant increases in the mean values of hepatic HO-1 and nitrites compared to group II, III, and IV. These results demonstrated that combined effects of both Copp and sildenafil have more cytoprotective effect than the effect of either copp or sildenafil alone.

CO, like NO, is a second messenger gas involved in a number of physiological processes [93]. Both CO and NO activate soluble guanylate cyclase to increase cyclic GMP (cGMP) levels. It is becoming increasingly clear that iNOS/NO and HO-1/CO can modulate each other's activity. These two system are linked in that NO can up-regulate HO-1 expression leading to the formation of endogenous CO [94], and CO can bind to the heme group in the iNOS protein and influence the production of NO [95]. This interaction between HO-1/CO and NO can explain the positive correlation between hepatic HO-1 gene expression and nitrite content, an index of NO production [62] observed in the present work

CO and NO share apparent similarities in structure, molecular weight and solubility [96]. Both NO and CO interact with iron (Fe) to form 5 or 6 coordinated haem complexes, which result in conformational changes and activation of the sGC/cGMP pathway. Thus, many of the biological effects of CO are similar to NO, including its anti-apoptotic, anti-proliferative and anti-inflammatory mechanisms [14]. Other studies have confirmed the participation of both NO and CO-mediated signaling cascades in suppression of platelet aggregation. In addition to regulating vascular cell growth, CO influences cell survival by blocking cytokine-mediated mitochondrial release of cytochrome C [15] and has been shown to influence hepatoprotection through the transcriptional upregulation of iNOS in the liver. Both exogenously administered and endogenously released NO stimulates HO-1 gene expression and CO production [95]. Furthermore, CO and NO have been shown to participate in vasoactive cross talk, influencing: growth factors, anti-inflammatory mediators, angiogenesis and vascular remodeling [17].

Conclusion and Recommendations

The results of the present work demonstrated that induction of HO-1 gene expression and inhibition of cGMP-specific phosphodiesterase type 5 (PDE-5) could have synergistic hepatoprotective effect against I/R injury observed in many clinical situations. Further investigations are recommended for using agents that are not hepatotoxic and can protect the liver and other organs from I/R injury.

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