Synergistic Effect of Ischemic Preconditioning, Postcoditioning and Xanthine Oxidase Inhibition on Cardiac Tissue apoptosis of Hepatic Ischemic-Reperfused Male Rats

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Abstract: Accumulating evidences have recently documented that hepatic ischemic mechanical preconditioning, postconditioning or ischemic pharmacological preconditioning had protective effects on the liver, which were associated with a reduction in oxidative stress, inflammation and endogenous antioxidant preservation. However, assessment of cardioprotective effects of remote hepatic ischemic preconditioning, postconditioning or pharmacological preconditioning is unclear and needs further investigations. The aim of this study was to investigate the remote effect of hepatic ischemia/reperfusion (IR) on cardiac tissue. And to investigate whether hepatic ischemic preconditioning (IPC), postconditioning (IPO) and/or pharmacological preconditioning by xanthine oxidase inhibitor (allopurinol) (Allo) may extend a beneficial synergistic effect to protect the cardiac tissue. Forty male Albino rats were divided into 5 experimental groups: group I: sham-operated controls, group II: Hepatic I/R, group III: IPC+ I/R+ IPO, group IV: Allo + I/R, group V: Allo+IPC+I/R+IPO. Serum interleukin-6, cardiac malondialdehyde (MDA), reduced form of glutathione (GSH), Bax and Bcl-2 mRNA expressions were measured at the end of experiment. Results revealed that IPC, IPO and/or Allo treatment significantly reduced the levels of IL-6, MDA, Bax mRNA and Bax/Bcl-2 ratio and significantly increased the levels of GSH and Bcl-2 mRNA. In conclusion: IPC, IPO and Allo treatment may act synergistically to protect cardiac tissue against oxidative stress and mitochondrial injury during hepatic I/R.
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1. Introduction

Warm hepatic ischemia-reperfusion (I/R) injury is a significant medical problem in many clinical conditions such as liver transplantation, hepatic surgery for tumor excision, trauma and hepatic failure after hemorrhagic shock. Partial or, mostly, total interruption of hepatic blood flow is often necessary when liver surgery is performed. This interruption of blood flow is termed "warm ischemia" and upon revascularization, when molecular oxygen is reintroduced, the organ undergoes a process called "reperfusion injury" that causes deterioration of organ function. Ischemia reperfusion results in cellular damage and tissue injury associated with a complex series of events **(1)**.

Ischemia-reperfusion injury is a multifactorial process that results in the accumulation of reactive oxygen species (ROS) that initiate tissue injury and stimulate a cellular cascade leading to inflammation **(2)**. The inflammatory process is secondary to endothelial activation and dysfunction, adherence and activation of neutrophils and platelets **(3)**, and the activation of complement **(4)** and T cells **(5)**. The proinflammatory process results in cell death and in severe cases leads ultimately to organ failure **(2)**.

Ischemic cell death is a consequence of irreversible mitochondrial injury **(6)**. Previous studies reported an association between mitochondrial dysfunction caused by reactive oxygen species (ROS) and both necrotic and apoptotic cell death **(7)**. Oxidative stress and mitochondrial dysfunction are considered key mediators of cardiomyocyte apoptosis associated with post-I/R cardiac damage **(8)**.

Hepatic IR results in proinflammatory process and release of cytokines, chemokines, and adhesion molecules that are identified in peripheral tissues including the heart and kidney resulting in cell death and in severe cases leads ultimately to organ failure **(9,10)**. However, remote effects of hepatic I/R injury on the heart tissue need further clarification.

Accumulating evidences have documented that hepatic ischemic mechanical preconditioning, postconditioning or ischemic pharmacological preconditioning had protective effects on the liver, which were associated with a reduction in oxidative stress, inflammation and endogenous antioxidant preservation **(**11-13**)**. However, assessment of cardioprotective effects of remote hepatic ischemic preconditioning, postconditioning or pharmacological preconditioning, against hepatic ischemia-induced cardiac tissue insult, is unclear and needs further investigations.

Accordingly, the objective of this study was to test the hypothesis that hepatic IR had remote effects on cardiac tissue oxidative/anti-oxidative status and cardiac tissue apoptosis. And to investigate whether hepatic ischemic preconditioning, postconditioning and/or xanthine

oxidase inhibitor (allopurinol) preconditioning may extend a beneficial synergistic effect to protect the cardiac tissue against hepatic ischemia-induced apoptosis.

2. Material and Methods

Experimental Design:

Forty male Albino rats belonging to local strain weighing between 180-250 gm were obtained from the Animal House of Faculty of Medicine, Cairo University and included in this study. The animals were housed in wire mesh cages at room temperature with 12:12h light-dark cycles and maintained on standard rat chow and tap water. Veterinary care was provided by Animal House Unit of Faculty of Medicine, Cairo University. The rats were divided randomly into 5 experimental groups of 8 rats each.

Group I, Control group (sham-operated rats):

Rats were anaesthetized with thiopental sodium (Eipico co., Egypt) 40 mg /kg body weight. A midline laparotomy & liver exposure for 2.5 hours were performed with no further surgical manipulations **(14)**.

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

Rats underwent the same surgical procedure as sham operated rats but with the induction of hepatic ischemia / reperfusion (I/R) injury as follows: 30 minutes (min) of ischemia by clamping the hepatic pedicle using a non traumatic microvascular clip, followed by 2 hours of reperfusion **(15)**.

Group III (IPC+ I/R+ IPO): Hepatic I/R in combination with ischemic pre conditioning (IPC) & postconditioning (IPO):

Rats were subjected first to mechanical IPC by 10 min of ischemia followed by 10 min of reperfusion **(11)**. Then hepatic pedicle was occluded again for 30 min. Then mechanical IPO was performed by 30 seconds of reperfusion followed by 30 seconds of re-occlusion for 3 cycles **(16)**. Finally reperfusion was maintained for 2 hours.

Group IV (Allo + I/R): Allopurinol preconditioning group:

Rats were injected intraperitoneally with xanthine oxidase (XO) inhibitor, allopurinol from Galaxo Smith Kline (S.A.E), in a dose of 50 mg/kg body weight, twice, 18 hrs and one hour before the induction of hepatic I/R procedure **(17)**.

Group V (Allo+IPC+I/R+IPO): Hepatic I/R in combination with allopurinol preconditioning (Allo), ischemic preconditioning (IPC) & postconditioning (IPO):

Rats were pretreated with allopurinol the same as in group IV, then hepatic I/R was performed in combination with ischemic pre & post conditioning the same as described in group III.

At the end of the experimental procedure, blood samples were obtained from retro-orbital vein for detection of serum interleukin-6. Then, all rats were sacrificed and the hearts were rapidly excised for further detection of: Malondialdehyde (MDA), reduced form of glutathione (GSH), Bax and Bcl-2 mRNA expressions.

Measurement of serum IL-6

Serum were examined for IL-6 level by ELISA technique by kit supplied from Quantakine (R&D system) (USA) according to manufacturers instruction **(18)**.

Measurement of MDA in heart tissue

Malondialdehyde was measured by (MDA colorimetric Assay Kit from Oxis International, Inc. Foster City, CA 94404 USA). To measure the MDA concentration **(19)**, 100 mg of heart tissue in 1 mL PBS (phosphate buffered saline) at pH 7.0, was homogenized with micropestle in microtube. 20 % TCA (trichloroacetic acid) was added to heart homogenate to precipitate the protein, and centrifuged. Supernatants were collected and thiobarbituric acid (TBA) solution was added to the supernatants. After boiling for 10 minutes in water bath, the absorbance was measured. Concentration of MDA in supernatants of heart homogenate was calculated using the standard curve of MDA standard solution (0; 0.625; 1.25; 2.5; 5.0 nmol/mL).

Measurement of reduced form of glutathione (GSH) in heart tissue

GSH concentration was measured from heart homogenate in phosphate buffer pH 8.0 and then 5% TCA was added, to precipitate heart protein. After centrifugation, dithiobisnitrobenzoate (DTNB) solution was added to the supernatants of heart homogenate, and incubated for 1hour. The absorbance was measured. Concentration of GSH in heart tissue was calculated using the standard curve of GSH standard solution (0; 10; 20; 40; 50; 100 mg/mL) **(20)**. The heart protein concentration was calculated by using standard curve of bovine serum albumin (BSA) solution.

Polymerase chain reaction (PCR) detection of Bax and Bcl2 gene expression in heart tissue

Total RNA was extracted from heart tissue homogenate using RNeasy purification reagent (Qiagen, Valencia, CA) according to manufacturers instruction then cDNA was generated from 5 µg of total RNA extracted with 1 µl (20 pmol) antisense primer and 0.8 µl superscript AMV reverse transcriptase for 60 min at 37 °C. For PCR, 4 µl cDNA was incubated with 30.5 µl water, 4 µl (25mM) MgCl2, 1 µl (10 mM) dNTPs, 5 µl 10×PCR buffer, 0.5 µl (2.5 U) Taq polymerase and 2.5 µl of each primer containing 10 pmol. Primer

sequences were as follows: Bax forward primer 5 CTGAGCTGACCTTGGAGC-3, reverse primer 5-GACTCCAGCCACAAAGATG-3; Bcl2 forward primer 50GGAGGGCACTTCCTGAG-30 and reverse primer 5-GCCTGGCATCACGACT-3. The reaction mixture was subjected to 40 cycles of PCR amplification as follows: denaturation at 95 °C for 1 min, annealing at 67 °C for 1 min and extension at 72 °C for 2 min. 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 60 min at 37 1C. For PCR, 4 ul complementary DNA was incubated with 30.5 ul water, u ml 25mM MgCl2, 1ml deoxyribonucleotide triphosphates (10mM), 5 ul 10x PCR buffer, 0.5 ul (2.5 U) Taq polymerase and 2.5 ul 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 C for 1min, annealing at 57 C for 1min and extension at 72 C for 2min.

Statistical analysis:

The data were statistically analyzed using the statistical package SPSS (version 15). Values were expressed as mean ± standard error (M±SE). Statistical analysis was performed by ANOVA (analysis of variance) and multiple comparison Post-Hoc Tests to determine significant differences between groups. Correlations were done to test for linear relations between variables using Pearson correlation test. The level of statistical significance was set at $p \leq 0.05$.

3. Results:

This study examined the effects of ischemic mechanical preconditioning (IPC), post conditioning (IPO) and allopurinol (Allo) preconditioning or a combination of them on the extent of cardiac tissue oxidative/anti-oxidative status and apoptosis due to hepatic ischemia (for 30 minutes) followed by two hours reperfusion (I/R).

Hepatic I/R resulted in a significant ($p< 0.05$) increase in serum IL-6 (64.875 ± 4.335 pg/ml), cardiac MDA (3.338±0.404 µmol/mg protein), Bax mRNA (1.163±0.099), and Bax / Bcl-2 ratio (4.808±0.582) and induced a significant ($p< 0.05$) decrease in cardiac GSH (17.325 ±1.514 µmol/mg protein) and Bcl-2 mRNA (0.273±0.042) compared to the corresponding values of sham controls (28.737 ± 2.161 pg/ml, 0.875 ±0.025 µmol/mg protein, 0.116±0.011, 0.075±0.009, 40.075±2.223 µmol/mg protein, , 1.633±0.115 and), respectively (Table 1, 2 and Figure 1).

In I/R group the increases in serum IL-6 level & cardiac Bax / Bcl-2 ratio (64.875 ± 4.335 pg/ml, 4.808±0.582 respectively) were suppressed by either IPC+IPO (54.425 ± 3.607 pg/ml, 1.449±0.087 respectively), Allo (41.425 ± 1.764 pg/ml, 0.935±0.113 respectively) or IPC+IPO+Allo (46.613 ±2.049 pg/ml, 0.621±0.041 respectively) without any synergistic effect.

In addition, the increases in cardiac MDA & Bax mRNA (3.338 ± 0.404 µmol/mg protein, 1.163±0.099 respectively) were suppressed by IPC+IPO (2.988 ± 0.175 µmol/mg protein, 0.716±0.033 respectively) or Allo (2.688 ± 0.239 µmol/mg protein, 0.589±0.023 respectively) alone. However, IPC+IPO+Allo appeared to have a synergistic effect in further suppressing the increases in MDA (1.686 ± 0.141 µmol/mg protein) and Bax mRNA (0.479±0.036) (Table 1, 2 and Figure 1).

Meanwhile, the reductions in cardiac GSH and Bcl-2 mRNA observed in I/R group (17.325 ±1.514 µmol/mg protein, 0.273±0.042 respectively) were decreased by IPC+IPO (24.988 ± 1.430 µmol/mg protein, 0.499±0.014 respectively) or Allo (27.400 ± 1.173 µmol/mg protein, 0.678±0.057 respectively) alone. However, IPC+IPO+Allo appeared to have a synergistic effect in further decreasing the reductions and increasing the levels of both GSH (34.438 ± 1.524 µmol/mg protein) and Bcl-2 mRNA (0.778±0.045) (Table 1, 2 and Figure 1).

Correlations between the measured variables were shown in figures (2-7).

Cardiac MDA concentrations showed significant positive correlations with serum IL-6 levels (figure 2), cardiac Bax mRNA and Bax / Bcl-2 ratio (figure 3) ($r = 0.696, 0.785, 0.590$ respectively and $p<0.05$) in the five studied groups. In contrast it showed significant negative correlations with cardiac GSH (figure 4) and Bcl-2 mRNA ($r = -0.721, -0.737$ respectively and $p<0.05$).

Cardiac GSH had significant positive correlation ($r = 0.751$ $p<0.05$) with Bcl-2 mRNA level but significant negative correlations with serum IL-6 (figure 5), cardiac Bax mRNA and Bax / Bcl-2 ratio (figure 6) ($r = -0.722, -0.813, -0.757$ respectively and $p<0.05$) in the five studied groups.

Finally, serum IL-6 level was positively correlated with cardiac MDA, Bax mRNA level and Bax / Bcl-2 ratio (figure 7) (r = 0.696, 0.737, 0.746 respectively and p<0.05) and was negatively correlated with cardiac GSH and Bcl-2 mRNA level (r = -0.722, -0.717 respectively and p<0.05) in the five studied groups.

Table-1: Effect of hepatic ischemia (30 min) followed by two hours reperfusion (I/R) on serum interleukin-6 (IL-6 in pg/ml), cardiac malondialdehyde (MDA in µmol/mg protein) and reduced form of glutathione (GSH in µmol/mg protein) in the five studied groups.

Groups n=8 / Parameters	Group I (Sham control rats)	Group II (I/R)	Group III (IPC+I/R+IPO)	Group IV (Allo+I/R)	Group V (Allo+IPC+ I/R+IPO)
Serum IL-6 (pg/ml)	28.737 ± 2.161$^{¤▲□\#}$	64.875 ± 4.335*$^{▲\#}$	54.425 ± 3.607*$^{▲}$	41.425 ± 1.764*$^{¤□}$	46.613 ±2.049*$^{¤}$
Cardiac MDA (µmol/mg protein)	0.875 ±0.025$^{¤▲□}$	3.338 ± 0.404*$^{\#}$	2.988 ± 0.175*$^{\#}$	2.688 ± 0.239*$^{\#}$	1.686 ± 0.141$^{¤▲□}$
Cardiac GSH (µmol/mg protein)	40.075±2.223$^{¤▲□}$	17.325 ±1.514*$^{□▲\#}$	24.988 ± 1.430*$^{¤\#}$	27.400 ± 1.173*$^{¤\#}$	34.438 ± 1.524$^{¤▲□}$

Results are mean ±SE
n: number of male rats in each group
I/R: ischemia reperfusion
IPO: ischemic preconditioning
IPC: ischemic postconditioning
Allo: allopurinol preconditioning
* Significant compared to sham control value (p<0.05)
¤ Significant compared to I/R value (p<0.05)
□ Significant compared to IPC+I/R+IPO value (p<0.05)
▲Significant compared to Allo/IR value (p<0.05)
Significant compared to Allo+IPC+ I/R+IPO value (p<0.05)

Table-2: Effect of hepatic ischemia (30 min) followed by two hours reperfusion (I/R) on cardiac Bax mRNA, Bcl-2 mRA and Bax / Bcl-2 ratio in the five studied groups

Groups n=8 / Parameters	Group I (Sham control rats)	Group II (I/R)	Group III (IPC+I/R+IPO)	Group IV (Allo+I/R)	Group V (Allo+IPC+ I/R+IPO)
Cardiac Bax	0.116±0.011$^{¤▲□\#}$	1.163±0.099*$^{□▲\#}$	0.716±0.033*$^{¤\#}$	0.589±0.023*$^{¤}$	0.479±0.036*$^{¤□}$
Cardiac Bcl-2	1.633±0.115$^{¤▲□\#}$	0.273±0.042*$^{▲\#}$	0.499±0.014*$^{\#}$	0.678±0.057*$^{¤}$	0.778±0.045*$^{¤□}$
Cardiac Bax / Bcl-2 ratio	0.075±0.009$^{¤□}$	4.808±0.582*$^{□▲\#}$	1.449±0.087*$^{¤}$	0.935±0.113$^{¤}$	0.621±0.041$^{¤}$

Results are mean ±SE
n: number of male rats in each group
I/R: ischemia reperfusion
IPO: ischemic preconditioning
IPC: ischemic postconditioning
Allo: allopurinol preconditioning
* Significant compared to sham control value (p<0.05)
¤ Significant compared to I/R value (p<0.05)
□ Significant compared to IPC+I/R+IPO value (p<0.05)
▲Significant compared to Allo/IR value (p<0.05)
Significant compared to Allo+IPC+ I/R+IPO value (p<0.05)

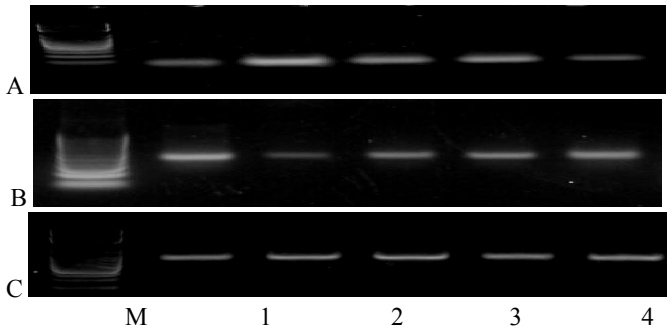

Figure-1: Agarose gel electrophoresis of PCR products Bax (A), Bcl2 (B) and b- actin (C) in the five studied groups.

Lane M, PCR marker with 100 bp ladder Lane 1, control group (sham-operated rats)

Lane 2, hepatic ischemia reperfusion group (I/R) Lane 3, allopurinol preconditioning group (Allo+I/R)
Lane 4, hepatic I/R in combination with ischemic preconditioning (IPC) & postconditioning (IPO) group (IPC+I/R+IPO)
Lane 5, hepatic I/R in combination with allopurinol preconditioning (Allo), ischemic preconditioning (IPC) & postconditioning (IPO) group (Allo + IPC+I/R+IPO)

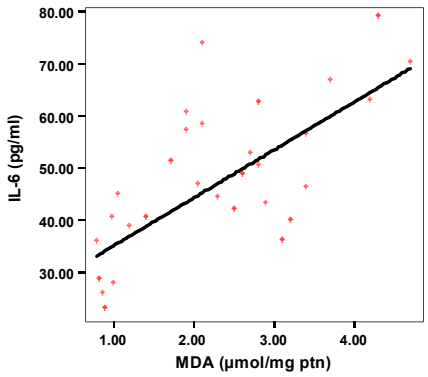

Figure (2): Positive correlation between cardiac malondialdehyde (MDA in µmol/mg protein) and serum interleukin-6 (IL-6 in pg/ml) in all rats of the five studied groups

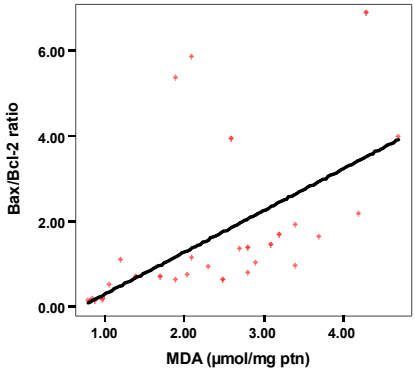

Figure (3): Positive correlation between cardiac malondialdehyde (MDA in µmol/mg protein) and Bax/Bcl-2 ratio in all rats of the five studied groups

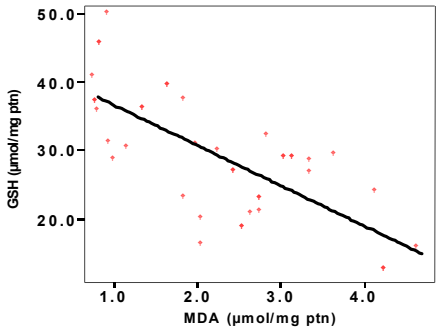

Figure (4): Negative correlation between cardiac malondialdehyde (MDA in µmol/mg protein) and reduced form of glutathione (GSH in µmol/mg protein) in all rats of the five studied groups

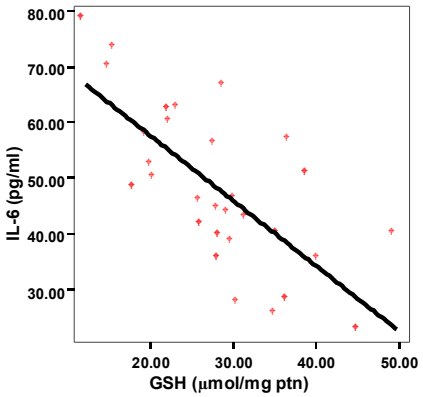

Figure (5): Negative correlation between cardiac reduced forms of glutathione (GSH in µmol/mg protein) and serum interleukin-6 (IL-6 in pg/ml) in all rats of the five studied groups

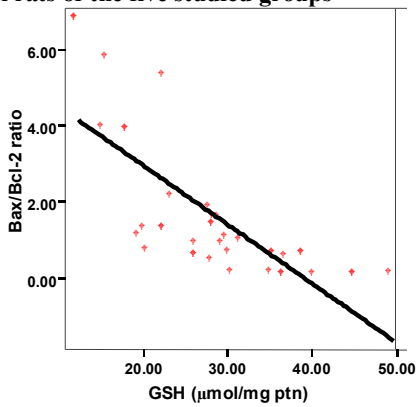

Figure (6): Negative correlation between cardiac reduced forms of glutathione (GSH in µmol/mg protein) and Bax/Bcl-2 ratio in all rats of the five studied groups

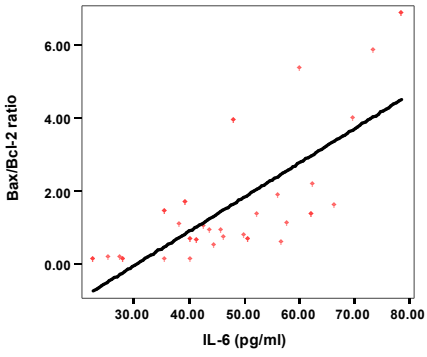

Figure (7): Positive correlation between serum interleukin-6 (IL-6 in pg/ml) and cardiac Bax/Bcl-2 ratio in all rats of the five studied groups

4. Discussion

The results of the present study provide evidence that combined hepatic ischemic preconditioning (IPC); postcoditioning (IPO) and allopurinol preconditioning (Allo) have synergistic protective effect on cardiac tissue oxidative stress and endogenous antioxidant preservation in male albino rats subjected to 30 minutes hepatic ischemia followed by 2 hours reperfusion (I/R). In this experiment, combined IPC+IPO+Allo show statistical significant ($P<0.05$) reduction of cardiac malondialdehyde (MDA) and Bax mRNA and statistical significant increase ($P<0.05$) of cardiac reduced form of glutathione (GSH) and Bcl-2 mRNA compared to IPC+IPO alone. However, that synergism is lost as regards the Bax/Bcl-2 ratio which shows insignificant ($P>0.05$) difference on comparing the three groups IPC + IPO, Allo and IPC+IPO+Allo.

Hepatocytes are important sources of Reactive Oxygen Species (ROS) along with Kupffer cells and neutrophils in hepatic ischemic/reperfusion injury (IRI) **(21)**. **Bhogal *et al.* (22)** suggested that hepatocytes should not be considered bystanders and targets of the injury. They should be seen as active participants in IRI in both the ischemic and reperfusion phases. With ischemia the respiratory cytochromes become redox-reduced, allowing them to directly transfer electrons to oxygen. This will improve the understanding of this complex and multifactorial injury **(23)**.

It is reported that hepatocytes are likely to play at least 2 key roles in hepatic IRI. First, hepatocytes generate significant levels of intracellular ROS during hypoxia and hypoxia/reoxygenation; that augments local tissue damage or affects organs remote from the site of I/R **(24)**. Second, hepatocytes can secrete and release proinflammatory cytokines, chemokines and adhesion molecules that are identified in peripheral tissues including the heart and kidney, and may play an important role in the development of multi-organ failure. **(9,10,25)**. An important function of ROS is the regulation of cytokine gene expression and stimulation of a cellular cascade leading to inflammation **(26)**.

In line with the previous results, the present study shows significant increased level of serum interleukin-6 in rats with hepatic IR, suggesting systemic leakage of proinflammatory cytokines/chemokines released by injured hepatic tissues. Also, there is significant increase of cardiac MDA and significant decrease of cardiac GSH due to hepatic ROS-induced cardiac oxidative stress and due to the systemic inflammatory response. Moreover, positive correlation is found between the serum IL-6 and cardiac MDA.

Accumulating evidences have documented that the cell death observed during the first few hours of myocardial ischemia occurs mainly through apoptosis **(27)**, rather than necrosis, which has long been considered as the predominant form of myocardial damage generated by ischemia **(28)**. Moreover, apoptotic cell death causes considerable cardiomyocyte loss, decreasing contractile function of the heart **(29)**, and eventually acts as a precursor of heart failure **(30)**. Thus, interventions aim at inhibition of mechanisms leading to apoptosis before the process becomes irreversible might therapeutically prevent excessive cell death **(31)**.

Oxidative stress is a major apoptotic stimulus in ischemic heart disease. Reactive oxygen species (ROS) are therefore excessively generated from a likely mitochondria source and then hasten lipid peroxidation, DNA damage, and other direct cellular injuries, consequently initiating apoptosis in cells **(32,33)**. Apoptotic signaling induces apoptosis primarily through three types of complex pathways. They include 1) cytokine/Fas receptor-driven pathway, 2) mitochondrial-driven pathway, and 3) endoplasmic reticulum/Ca2+-driven pathway. Among them, mitochondrial-mediated pathway, including the Bcl-2 family is the best characterized and believed to be critical in regulating apoptosis **(34)**.

An outcome of this study is a significant upregulation of cardiac Bax mRNA expression and a significant decrease in cardiac Bcl-2 mRNA expression with overall significant increase in the Bax/Bcl-2 ratio due to hepatic I/R. Reversal of those results, with lowering the magnitude of increased Bax/Bcl-2 ratio, are observed on inducing hepatic ischemic mechanical preconditioning-postconditioning and/or allopurinol preconditioning.

The Bcl-2 family proteins are key regulators of cell death and survival that can either inhibit or promote apoptosis. Family members include antiapoptotic Bcl-2 and Bcl-xl and proapoptotic Bax, Bad, truncated Bid (tBid), and Bim. Interactions between these antiapoptotic and proapoptotic Bcl-2 proteins exist in a delicate balance at the mitochondrial membrane that determines cell fate. Heterodimerization of antiapoptotic members, such as Bcl-2, with proapoptotic members, such as Bax can inhibit or activate apoptosis depending on the relative levels of each protein **(35)**. The ratio of proapoptotic to antiapoptotic proteins (e.g., Bax/Bcl-2) regulates myonuclei integrity and cell survival by controlling mitochondrial membrane permeability. Decreased mitochondrial membrane stability and pore formation initiates the release of cytochrome c, formation of the apoptosome, and subsequent activation of caspase-9 and caspase-3 leading to mitochondria –mediated apoptosis **(36)**.

In consistent with the present study, the positive correlation between serum IL-6 and cardiac Bax/Bcl-2 ratio due to increased Bax mRNA and decreased Bcl-2 mRNA, Escandell et al. **(37)** reported that Bcl-2 is a negative regulator of interleukin-1b cytokine secretion in murine

macrophages in pharmacological-induced apoptosis. In contrast, **Waxman and Kolliputi (38)** demonstrated that IL-6–mediated protection against hyperoxia is partly mediated by up-regulation of Bcl-2 expression and regulation of Bcl-2 family member interactions. While **Ryazantseva *et al.* (39)** suggested that the inductive and inhibitory effects of IL-2 on apoptotic process depend on the dose of the cytokine and cell micro-environmental conditions.

Ischemic preconditioning (IPC) is an adaptational response of briefly ischemic tissues which serves to protect against subsequent prolonged ischemic insults and reperfusion injury. Ischemic preconditioning can be mechanical or pharmacological. Direct mechanical preconditioning in which the target organ is exposed to brief ischemia prior to prolonged ischemia has the benefit of reducing ischemia-reperfusion injury (IRI) but its main disadvantage is trauma to major vessels and stress to the target organ. Remote (inter organ) preconditioning is a recent observation in which transient non-lethal ischaemia and reperfusion of one organ confers resistance to a subsequent episode of lethal ischaemia reperfusion injury in a remote organ or tissue without direct stress to the organ **(40)**.

Potential mechanistic pathways underlying remote ischemic preconditioning (RIPC): the actual mechanism through which transient ischemia and reperfusion of an organ or tissue confers cardioprotection is currently unknown although several hypotheses have been proposed to reduce oxidative stresss and preserve mitichondrial function: (1) The neural hypothesis proposes that preconditioning of the organ or tissue remote from the heart generates an endogenous substance such as adenosine, bradykinin or calcitonin gene-related peptide (CGRP), which then activates a local afferent neural pathway stimulating an efferent neural pathway, which terminates at the heart and mediates cardioprotection. (2) The humoral hypothesis proposes that the endogenous substance (such as adenosine, bradykinin, opioids, CGRP, endocannabinoids, Angiotensin I) or some other as yet unidentified humoral factor generated in the remote organ or tissue enters the blood stream and activates its respective receptor in the myocardium thereby recruiting the various intracellular pathways of cardioprotection implicated in ischemic preconditioning. (3) The third hypothesis proposes that transient ischemia and reperfusion of an organ or tissue provokes a systemic protective response, which suppresses inflammation and apoptosis. Recent data suggest that the activation of the mitogen-activated protein kinases (MAPKs) within the remote organ may also contribute to RIPC-induced cardioprotection **(41)**.

Tapuria *et al*. (40) reported that some studies demonstrate endothelial NO, kinases, opioids, catecholamines and KATP channels (ATP-sensitive potassium channels) as the candidate mechanism in remote preconditioning. Experiments show suppression of proinflammatory genes, expression of antioxidant genes and modulation of gene expression by RIPC as a novel method of IRI injury prevention.

Although preconditioning is a source of scientific inspiration, its clinical applicability is limited by the inability to predict acute ischemic events. Accordingly, postconditioning may confer benefits similar to preconditioning. Postconditioning is a procedure of repetitive brief cycles of reperfusion performed immediately at the onset of reperfusion to induce intracellular protective reactions **(2,42)**.

Both local and remote postconditioning may ultimately prove to be effective in rodent models of acute myocardial infarction by potentially invasive renal manipulation **(43)** and by limb manipulation **(44)**. The previuos studies show that remote postconditioning (rather than conditioning before reperfusion) by ischemic hindlimb manipulation is safe and effective **(43)**. Contradictory result was reported by **Bretz *et al.* (45)**, that ischemic postconditioning does not attenuate ischemia–reperfusion injury of rabbit small intestine. **Kin *et al.* (46)** demonstrated that, when the initiation of postconditioning is delayed for greater than 1 minute, the cardiac protection from IRI is lost.

Although it is likely that the precise mediators and their time course of action will vary (depending on the organ rendered ischaemic and the temporal aspects of the ischemia) and the mechanisms underlying remote conditioning (preconditioning or postconditioning) remain elusive, it is likely that some similarities exist between the two **(47)**. It is generally accepted that adenosine release and, hence, activation of the A_{2A} and A_3 receptors have a critical role in the reduction in the infarct size **(48)**. As in the case of humoral factors, a neurogenic arc could be one of the triggers easing the release of adenosine in the myocardium in some forms of preconditioning. Other potential triggers include reaction-elaborated reactive oxygen species, endogenous opioids operating through the κ and δ receptors and nitric oxide. Downstream effectors include protein, kinase C, reperfusion injury-signalling kinases, KATP channels and the mitochondrial permeability transition pore **(47)**.

In the present study, blockage of xanthine oxidase (XO) with allopurinol decreases the production of ROS indicating reduced oxidant stress and is reflected by restoration of GSH levels and reversed MDA content in the heart. Decreased ROS and the subsequent inflammatory response (reduction of serum IL-6), results in attenuation of cardiac tissue apoptosis which is reflected by reduced cardiac Bax mRNA and Bax/Bcl-2 ratio.

The mechanism by which allopurinol exerts a protective effect on liver IR injury is still under debate, but strong evidence exists to support that

allopurinol blocks (XO) and aids in resynthesis of ATP by inhibiting the breakdown of its catabolites, inhibiting the formation of ROS, and preventing mitochondrial membrane damage, therefore decreasing anaerobic respiration **(49)**. Contradictory result is reported by **Rajesh *et al.* (50)** as allopurinol pretreatment failed to upregulate Bcl-2 expression in cardiac I/R model.

Interestingly, in a jejunum-segment model the allopurinol pre-treatment expresses the highest level of apoptotic activity, and thus, the ratio between necrosis and apoptosis significantly altered. Since significant oxidative stress is known to induce necrosis, it is supposed that the effect of allopurinol might direct the dominant cell death type from necrosis to apoptosis by decreasing the level of reactive oxygen species, and rather increasing the number of apoptotic cells. However, the effect of allopurinol is dose-dependent and supposedly the way of administration would have an influence on the results too. The authors suggested that, the effect of administration of allopurinol is contradictory **(51)**.

In consistent with the results of this study, **Foly and Chari (2)** suggested that the additive effects of preconditioning (IPO) and postconditioning (IPC) may be beneficial in human transplantation and needed to be studied. In addition, **Lee and Lee (11)** demonstrated that IPC and Allo act synergistically to protect cells against mitochondrial injury and preserve the hepatic energy metabolism during hepatic I/R.

In conclusion, the results of the present study demonstrate that there is an increase in cardiac tissue apoptosis in the hepatic I/R model, which may be, at least partly, due to enhanced mitochondrial pathway resulting possibly from increased oxidative stress. Remote hepatic mechanical IPC, IPO and Allo preconditioning may act synergistically to protect cardiac tissue against oxidative stress and mitochondrial injury during hepatic I/R.

The underlying mechanisms and pathways need further clarification. Future clinical studies using more than one approach to minimize injury at different time points in the transplant process may be needed to achieve significant clinical benefit.

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References:

1. Dogan S and Aslan M. (2011). Hepatic ischemia-reperfusion injury and therapeutic strategies to alleviate cellular damage. Hepatol Res. ; 41(2):103-17.
2. Foley DP and Chari RS (2007). Ischemia-reperfusion injury in transplantation: novel mechanisms and protective strategies. Transplantation Reviews; 21: 43-53
3. Lemasters JJ and Thurman RG (1997). Reperfusion injury after liver preservation for transplantation. Annu Rev Pharmacol Toxicol.; 37: 327- 38.
4. Arumugam TV, Magnus T, Woodruff TM, *et al.* (2006). Complement mediators in ischemia-reperfusion injury. Clin Chim Acta; 374:33 - 45.
5. Zhai Y, Shen XD, Hancock WW, *et al.* (2006). CXCR3+CD4+ T cells mediate innate immune function in the pathophysiology of liver ischemia / reperfusion injury. J Immunol.; 176:6313 - 22.
6. Clemens MG, Mcdonagh PF, Chaudry IH, *et al.* (1985). Hepatic microcirculatory failure after ischemia and reperfusion: improvement with ATP-MgCl2 treatment. Am. J. Physiol.; 248: H804–H811.
7. Zamzami N, Hirsch T, Dallaporta B, *et al.* (1997). Mitochondrial implication in accidental and programmed cell death: apoptosis and necrosis. J. Bioenerg. Biomembr.; 291: 85–193.
8. Kumar D and Jugdutt BI (2003). Apoptosis and oxidants in the heart. J Lab Clin Med.; 142: 288–297.
9. Takada M, Nadeau KC, Hancock WW, *et al.* (1998). Effects of explosive brain death on cytokine activation of peripheral organs in the rat. Transplantation; 65: 1533- 42.
10. Gasser M, Waaga AM, Kist-Van Holthe JE, *et al.* (2002). Normalization of brain death induced injury to rat renal allografts by recombinant soluble P selectin glycoprotein ligand. J Am Soc Nephrol.; 13:1937-45.
11. Lee W-Y and Lee S-M (2006). Synergistic protective effect of ischemic preconditioning and allopurinol on ischemia/reperfusion injury in rat liver. Biochemical and Biophysical Research Communications; 349:1087-1093.
12. Ajamieh HH, Candelario-Jalil E, Fernández OS, *et al.* (2008). Ischaemic and pharmacological preconditionings protect liver via adenosine and redox status following hepatic ischaemia/reperfusion in rats. Clin Sci (Lond). ; 115(2):69-77.
13. De Rougemont O, Lehmann K, Clavien P-A. (2009). Preconditioning, organ preservation, and postconditioning to prevent ischemia-reperfusion injury to the liver. Liver Transplantation; 15(10):1172-1182
14. Tsung A, Stang MT, Ikeda A , *et al.* (2006).The transcription factor interferon regulatory factor-1 mediates liver damage during ischemia-reperfusion injury Am J Physiol Gastrointest Liver Physiol; 290: 1261- 1268.
15. Sepodes B, Maio R, Pinto R, *et al.* (2006). Recombinant human erythropoietin protects the liver from hepatic ischemia reperfusion injury in

the rat. Transplant International Journal; 19: 919-926.
16. Wang KX, Hu SY, Jiang XS, *et al.* (2008). Protective effects of ischaemic postconditioning on warm/cold ischemic reperfusion injury in rat liver: a comparative study with ischemic preconditioning. Chin Med J; 121(20):2004-2009.
17. Liu PG, He SQ, Zhang YH, *et al.* (2008). Protective effects of apocynin and allopurinol on ischemia/reperfusion-induced liver injury in mice World J Gastroenterol.; 14(18): 2832-2837.
18. Peters M, Müller AM, Rose-John S (1998). Interleukin-6 and soluble interleukin-6 receptor: direct stimulation of gp130 and hematopoiesis. Blood; 92(10): 3495-504.
19. Wills ED. (1987). Evaluation of lipid peroxidation in lipids and biological membranes. In: Snell K, Mullock B, eds. Biochemical toxicology: A practical approach. London: Oxford.
20. Ellman GL (1959). Tissue sulfhydryl groups. Arch of Bioch & Biophys.; 82:70-7.
21. Clarke CN, Kuboki S, Tevar A, *et al.* (2009). CXC chemokines play a critical role in liver injury, recovery, and regeneration. Am J Surg; 198:415-419.
22. Bhogal RH, Sutaria R, Afford SC (2011). Hepatic liver ischemia/reperfusion injury: processes in inflammatory networks – A review. Liver Transplantation; 17(1): 95
23. Nohl H, Kozlov AV, Gille L, *et al.* (2003). Cell respiration and formation of reactive oxygen species: facts and artefacts. Biochem Soc Trans.; 31:1308–1311.
24. Bhogal RH, Curbishley SM, Weston CJ, *et al.* (2010). Reactive oxygen species mediate human hepatocyte injury during hypoxia/ reoxygenation. Liver Transplantation; 16:1303–1313
25. Rowell DL, Eckmann L, Dwinell MB, *et al.* (1997). Human hepatocytes express an array of proinflammatory cytokines after agonist stimulation or bacterial invasion. Am J Physiol.; 273 (2 Pt 1): G322-G332.
26. Remick DG and Villarete L. (1996). Regulation of cytokine gene expression by reactive oxygen and reactive nitrogen intermediates. J Leukoc Biol.; **59**:471–475.
27. Veinot JP, Gattinger DA, Fliss H. (1997). Early apoptosis in human myocardial infarcts. Hum Pathol.; 28:485–492.
28. Freude B, Masters TN, Kostin S, *et al.* (1998). Cardiomyocyte apoptosis in acute and chronic conditions. Basic Res Cardiol.; 93:85–89.
29. Segers VFM, Lee RT. (2008). Stem-cell therapy for cardiac disease. Nature.; 451:937–942.
30. Yeh CH, Wang YC, Wu YC, *et al.* (2003). Continuous tepid blood cardioplegia can preserve coronary endothelium and ameliorate the occurrence of cardiomyocyte apoptosis. Chest.; 123:1647-1654.
31. Saraste A (1999). Morphologic criteria and detection of apoptosis. Herz.; 24:189–195.
32. Halliwell B and Aruoma OI. (1991). DNA damage by oxygen-derived species- its mechanism and measurement in mammalian systems, FEBS Lett.; 281:9–19.
33. Yoshikawa Y, Hizume K, Oda Y, *et al.* (2006). Protective activity of vitamin C against double-strand breaks in reconstituted chromatin revealed by single-molecule observation. Free Radic Res.; 40:S127–S127.
34. Hengartner MO (2000). The biochemistry of apoptosis. Nature; 407: 770–776
35. Waxman AB and Kolliputi N. (2009). IL-6 protects against hyperoxia-induced mitochondrial damage via Bcl-2–induced Bak interactions with mitofusions. American Journal of Respiratory Cell and Molecular Biology; 41: 385-396.
36. Mignotte, B., and Vayssiere, J. L. (1998). Mitochondria and apoptosis. *Eur. J.* Biochem.; 252:1-15.
37. Escandell J, Recio M, Giner R, *et al.* (2010). Bcl-2 is a negative rgulator of interleukin-1b cytokine secretion in murine macrophages in pharmacological-iduced apoptosis. Brit. J. Pharmacol.; 160(7):1844-1856.
38. Waxman AB and Kolliputi N. (2009). IL-6 protects against hyperoxia-induced mitochondrial damage via Bcl-2–induced Bak interactions with mitofusions. Am. J. Respir. Cell Mol. Biol.; 41 (4): 385- 396.
39. Ryazantseva NV, Novitskii VV, Zhukova OB, *et al.* (2010). Molecular mechanisms of the effect of interleukin-2 on apoptosis of blood lymphocytes. Bull Exper Biol Med.; 149(4) :547-550
40. Tapuria N, Kumar Y, Habib MM, *et al.* (2008). Remote ischemic preconditioning: a novel protective method from ischemia reperfusion injury--a review. J Surg Res.; 150(2): 304-30.
41. Hausenloy DJ and Yellon DM. (2008). Remote ischaemic preconditioning: underlying mechanisms and clinical application. Cardiovasc Res., 79 (3):377-386
42. Dal Ponte C, Alchera E, Follenzi A, *et al.* (2011). Pharmacological postconditioning protects against hepatic ischemia/reperfusion injury. Liver Transplantation; 17(4): 474-482.
43. Kerendi F, Kin H, Halkos ME, *et al.* (2005). Remote postconditioning. Brief renal ischemia and reperfusion applied before coronary artery reperfusion reduces myocardial infarct size via endogenous activation of adenosine receptors. Basic Res Cardiol; 100:404–12.
44. Li CM, Zhang XH, Ma XJ, *et al.* (2006). Limb ischemic postconditioning protects myocardium from ischemia-reperfusion injury. Scand Cardiovasc J; 40:312–17.

45. Bretz B, Blaze C, Parry N, *et al*. (2010). ischemic postconditioning does not attenuate ischemia–reperfusion injury of rabbit small intestine. Veterinary Surgery; 39(2): 216- 223.
46. Kin H, Zhao ZQ, Sun HY, *et al*. (2004). Postconditioning attenuates myocardial ischemia- reperfusion injury by inhibiting events in the early minutes of reperfusion. Cardiovasc Res.; 62:74 - 85.
47. Andreka G, Vertesaljai M, Szantho G (2007). Remote ischaemic postconditioning protects the heart during acute myocardial infarction in pigs *Heart*;93:749-752
48. Zhao ZQ, Vinten-Johansen J (2006). Postconditioning: Reduction of reperfusion-induced injury. Cardiovasc Res .; 70:200–11.
49. Peglow S, Toledo AH, Anaya-Prado R, *et al*. (2011). Allopurinol and xanthine oxidase inhibition in liver ischemia reperfusion. J Hepatobiliary Pancreat Sci.; 18(2): 137-46.
50. Rajesh KG, Sasaguri S, Suzuki R, *et al*. (2003). Antioxidant MCI-186 inhibits mitochondrial permeability transition pore and upregulates Bcl-2 expression. AJP-Heart; 285(5): H2171-H2178.
51. Brath E, Miko I, Nemeth N, *et al*. (2011). Effects of allopurinol and preconditioning on apoptosis due to ischemia-reperfusion on a double jejunum-segment canine model. Acta Cir. Bras.; 26(3):186-93.