

Leucocyte Infiltration in Experimental Warm Hepatic Ischemia Reperfusion; Effect of Ischemic Pre and Post Conditioning; Implications of Adhesion Molecules

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Abstract: Background: Very few studies described the potential effect of ischemic post conditioning (IPO) in warm hepatic ischemia. The present study hypothesizes that IPO might attenuate leucocyte infiltration. Furthermore, we aimed to compare such effect with that produced by ischemic pre conditioning (IPC), and to study the implications of vascular cell adhesion molecule-1 (VCAM-1) and E selectin. Methods: 40 male Wistar rats were divided into 4 groups; group I, control group, group II ischemia reperfusion group I/R, group III underwent IPC, group IV underwent IPO. Serum levels of ALT, albumin, VCAM-1 and E selectin were determined. Liver tissue homogenate was used to assess levels of VCAM-1, E selectin and myeloperoxidase activity. Results: IPO significantly reduced serum ALT, liver VCAM-1 and E selectin levels and myeloperoxidase activity compared to I/R group, but insignificantly decreased VCAM-1 and E selectin in serum. IPO protective effects were less significant when compared to IPC group. Significant negative correlation was observed between serum E selectin and myeloperoxidase in the I/R group. Conclusion: Our results point to the protective effect of IPO in warm hepatic I/R injury through attenuation of leucocyte infiltration, and postulate that serum E selectin can be used as a marker to anticipate the degree of leucocyte infiltration in hepatic I/R injury.

[Zienab Alrefaie and Laila Rashed. **Leucocyte Infiltration in Experimental Warm Hepatic Ischemia Reperfusion; Effect of Ischemic Pre and Post Conditioning; Implications of Adhesion Molecules.** *Life Sci J* 2012;9(3):2290-2295] (ISSN:1097-8135). <http://www.lifesciencesite.com>. 328

Key words: E selectin, ischemia-reperfusion, liver, preconditioning, post conditioning, VCAM-1.

1. Introduction:

Warm hepatic ischemia is a feature of severe liver trauma, hypovolemic and endotoxic shock or inflow occlusion during liver surgery (Rougemont *et al.*, 2009). Activation of neutrophils have been implicated in the hepatic microvascular dysfunction and parenchymal damage associated with ischemia reperfusion (I/R) (Jaeschke and Farhahod, 1990). Activated neutrophils can cause endothelial and hepatocellular damage through the worsening of flow hindrance and the release of oxidants, proteases and hydrolytic enzymes. The concomitant release of myeloperoxidase by the activated neutrophils results in the formation of hypochlorous acid as the major oxidant (Jaeschke and Smith, 1997).

Leucocyte recruitment is a multistep process; it involves initial contact and adhesion to the endothelium, transendothelial migration and subsequent parenchymal cell adherence and damage (Teoh and Farrell, 2003).

Several studies have observed the endothelial cell expression of E selectin, intercellular adhesion molecule (ICAM) and vascular cell adhesion molecule-1 (VCAM-1) which mediate leucocyte-endothelial cell adhesion *in vitro* and *in vivo* (Kim *et al.*, 2001).

After I/R, cellular adhesion molecules are activated and/or upregulated on the surface of neutrophils and sinusoidal endothelial cells (SECs).

This is induced by a variety of inflammatory molecules namely cytokines and chemokines (Martinez-Mier, 2000).

Selectins are expressed on endothelial cells, platelets and leucocytes. E selectin expressed on endothelial cells mediates the initial capture and tethering of leucocytes on (SECs) (Adams *et al.*, 1996).

VCAM-1 is a member of the immunoglobulin like family. It is transcriptionally induced on SECs and its interaction with integrins induces signals in the endothelial cells that trigger changes in their morphology allowing leucocyte transmigration and consecutive tissue injury (Matheny *et al.*, 2000).

Several strategies have been described to ameliorate I/R injury. Ischemic preconditioning which involves short periods of ischemia separated by intermittent reperfusion prior to sustained ischemia has shown many protective effects against I/R injury (Centurion *et al.*, 2007).

Recently very few studies described the ischemic postconditioning strategy in liver I/R injury, it is the application of several brief cycles of ischemia and reperfusion at the onset of sustained reperfusion after a prolonged period of ischemia (Sun *et al.*, 2004).

Ischemic postconditioning approach could be effective when ischemic preconditioning is not feasible as in non surgical contexts and unscheduled ischemic situations. As far as we know, there are only

few studies suggesting that the liver acquires protection after ischemic postconditioning and the possible mechanisms of protection are still largely unknown.

Aim:

The present study addressed the potential application of ischemic postconditioning in the prevention of hepatic I/R damage specially aimed to fully translate these findings to the clinical arena. We hypothesize that this protective effect might be mediated through attenuating leucocyte infiltration. Furthermore, we aimed to compare such effect of ischemic postconditioning with that produced by ischemic preconditioning and to study their relation to VCAM-1 and E selectin adhesion molecules.

Methods:**Animals:**

This study was approved by the local animal care committee of Kasr-Al Aini Faculty of Medicine, Cairo University, Cairo, Egypt, and carried out in accordance with the international principles of laboratory animal research.

Forty male Wistar rats weighing 200-250 g were included in the present study. The rats were housed in wire-meshed cages at 24°C with constant humidity and 12:12 h light-dark cycle. The animals were fed *ad libitum* with a commercial rat diet consisting of pellet and tap water prior to the studies. All rats were adapted to eating a commercial diet for at least 1 week before the experimental protocol was started.

Experimental groups and study design:

Animals were fasted the night before the experimental procedure. They were randomly divided into four groups 10 rats each; group I (control group), group II (ischemia reperfusion I/R group), group III (ischemic preconditioning IPC group) and group IV (ischemic postconditioning IPO group). Rats were anesthetized by intraperitoneal injection of pentobarbital 40 mg/ kg body weight. Control animals were subjected to midline abdominal incision and sham laparotomy.

Group II I/R, rats were subjected to midline abdominal incision, exposure of the hepatic pedicle and occlusion of the left lateral and median lobes by applying atraumatic microvascular clamp for 60 minutes to induce partial hepatic warm ischemia followed by removal of the clamp and reperfusion for 120 minutes.

Group III IPC, rats underwent the ischemic preconditioning strategy by applying 10 minutes ischemia and 15 minutes reperfusion prior to the 60 minutes ischemia, and 120 minutes reperfusion (Rüdiger *et al.*, 2002).

Group IV IPO, rats underwent the ischemic postconditioning strategy by applying 3 brief cycles of ischemia separated by reperfusion with 30 seconds each at the onset of reperfusion following 60 minutes ischemia (Santos *et al.*, 2010). Reperfusion was completed to 120 minutes.

At the end of reperfusion period, all animals were sacrificed by intraperitoneal injection of pentobarbital 150 mg/ kg body weight. Blood samples were collected from retro-orbital plexus of veins and ischemic liver lobes were harvested.

Hepatocellular function:

Blood samples were centrifuged for 10 minutes at 3000 round per minute. Separated serum was stored at -70°C until analysis. Colorimetric determination of alanine transaminase (ALT) and serum albumin as indicators of hepatocellular injury was carried out according to (Reitman and Frankel, 1957) and (Dumas *et al.*, 1971) respectively.

VCAM-1 and E selectin:

VCAM-1 and E selectin levels were determined in serum and liver tissue homogenate supernatant by enzyme immuno assay using ELISA kit according to the manufacturer's instructions (R&D Systems, Inc USA). Values were expressed as ng/mg tissue in the liver and ng/dl in the serum. Homogenization of liver tissue was performed after the tissue samples had been diluted in 5 vol of homogenate buffer [10 mM HEPES (pH 7.9), 10 mM KCL, 0.1 mM EGTA, 1 mM DTT, and 0.5 mM phenylmethanesulfonyl fluoride] using a vertishear tissue homogenizer. Liver homogenates were centrifuged at 3,000 g for 15 min at 4°C and supernatants were collected.

Determination of myeloperoxidase (MPO) activity in liver homogenate:

According to (Mizutani *et al.*, 2003), liver tissue (0.5 gm) was homogenized in 10 ml of homogenization buffer pH (4.7) [0.1 mol/L NaCl, 0.02 mol/L NaPO₄ and 0.015 mol/L sodium ethylenediamine tetracetic acid (EDTA)], centrifuged at 260 x g for 10 minutes and the pellet underwent hypotonic lysis using (0.2% NaCl) solution. This was followed 1 minute later by addition of an equal volume of solution containing (1.6% NaCl and 5% glucose). After further centrifugation, the pellet was then suspended in resuspension buffer pH 5.4 (0.05 mol/L NaPO₄ containing 0.5% hexadecyltrimethylammonium bromide) and rehomogenized. One milliliter aliquots of the suspension were frozen and thawed three cycles in liquid nitrogen, then centrifuged for 15 minutes at 3000 g. the pellet was discarded. MPO activity was assayed by measuring the change in optical density at 450 nm using tetramethylbenzidine, as substrate (1.5 mmol/L) and H₂O₂ (0.5 mmol/L). Results were expressed as MPO relative milli units/ mg tissue. One unit of MPO activity was defined as the quantity of

enzyme degrading one mmol peroxide at 25°C. The activity of purified known human neutrophil MPO was used as the standard (Sigma Chemical Co, USA).

Statistical analysis:

Data are presented as mean \pm standard deviation. Comparison between groups was calculated using one way analysis of variance, and association of variables was calculated using Pearson correlation (SPSS package version 12; SPSS, Inc, Chicago, Illinois).

P values < 0.05 were considered as statistically significant.

3. Results:

Serum ALT and albumin, Table I and

Serum level of ALT was significantly elevated following I/R (group II) when compared to sham operated control group ($P < 0.001$). Ischemic pre and post conditioning significantly reduced the ALT level ($P < 0.01$, $P < 0.05$ respectively). As regards albumin level, it decreased significantly in the I/R group when compared to control group ($P < 0.001$). Ischemic preconditioning attenuated the liver functional damage with resultant higher albumin level when compared with I/R group ($P < 0.001$). Ischemic postconditioning strategy led to mild improvement in albumin level which failed to reach statistical significance.

Leucocyte infiltration, Table I

To assess mononuclear leucocyte infiltration, myeloperoxidase activity was investigated. Our results showed that I/R significantly increased leucocyte infiltration (0.59 ± 0.055 mU/mg versus 0.39 ± 0.043 in sham operated group, $P < 0.001$). Both conditioning strategies attenuated leucocyte infiltration as they significantly reduced MPO activity although this effect was less marked in IPO group, (0.4 ± 0.068 and

0.51 ± 0.075 in IPC and IPO groups respectively, $P < 0.001$ and $P < 0.01$ when compared with I/R group respectively).

Liver and serum VCAM-1 and E selectin, Table II

To provide an insight on the mechanism of leucocyte infiltration in relation to adhesion molecules, the level of VCAM-1 and E selectin in liver tissue besides to their soluble fractions in serum were assessed. The 60 minute ischemia followed by 120 minute reperfusion remarkably increased liver content of VCAM-1 and E selectin when compared with the control group, 32.82 ± 9.95 ng/mg versus 11.86 ± 2.71 and 2.85 ± 0.65 versus 1.24 ± 0.32 respectively $P < 0.001$ for both adhesion molecules.

Comparing the effects of IPC and IPO strategies on the liver VCAM-1 and E selectin levels revealed suppression of both adhesion molecules by both conditioning strategies significantly. This suppression was insignificantly more marked in the IPC group.

Soluble fractions of VCAM-1 and E selectin got also elevated in I/R group when compared to control group, 9.17 ± 2.3 ng/dl versus 6.4 ± 1.97 ($P < 0.01$) and 1.86 ± 0.27 versus 0.69 ± 0.43 ($P < 0.001$) respectively. The elevation in serum VCAM-1 and E selectin induced by I/R was significantly alleviated by ischemic preconditioning but not by ischemic postconditioning.

Correlation results, Table III

To investigate the relation of VCAM-1 and E selectin levels with leucocyte infiltration, Pearson correlation tests were performed between variables in different groups. Our results demonstrated a positive correlation between liver VCAM-1 and MPO ($r = 0.723$, $P < 0.05$) and a negative correlation between soluble fraction of E selectin and MPO ($r = -0.687$, $P < 0.05$) in the I/R group.

Table I: Levels of alanine transaminase (ALT), albumin and myeloperoxidase (MPO) in all experimental groups:

Groups	ALT (U/dl)	Albumin (g/dl)	MPO (mU/mg)
Sham operated control group (Group I)	45.04 ± 3.26	5.12 ± 0.39	0.39 ± 0.043
Ischemia reperfusion group (Group II)	68.28 ± 8.24 $P < 0.001^*$	3.29 ± 0.42 $P < 0.001^*$	0.59 ± 0.055 $P < 0.001^*$
Ischemic preconditioning group (Group III)	61.41 ± 3.31 $P < 0.01^{**}$	4.16 ± 0.25 $P < 0.001^{**}$	0.4 ± 0.068 $P < 0.001^{**}$
Ischemic postconditioning group (Group IV)	61.88 ± 5.26 $P < 0.05^{**}$	3.58 ± 0.45 $P < 0.01^{***}$	0.51 ± 0.075 $P < 0.01^{**}$ $P < 0.001^{***}$

*: Significant when compared to control group

** : Significant when compared to ischemia reperfusion group

***: Significant when compared with ischemic preconditioning group

Table I I: Levels of liver (L) vascular cell adhesion molecule 1 (VCAM-1) and E selectin and soluble (S) VCAM-1 , and E selectin in all experimental groups:

Groups	L- VCAM-1 (ng/mg)	L- E selectin (ng/mg)	S- VCAM (ng/dl)	S- E selectin (ng/dl)
Sham operated control group (Group I)	11.86 ± 2.71	1.24 ± 0.32	6.4 ± 1.97	0.69 ± 0.44
Ischemia reperfusion group (Group II)	32.82 ± 9.94 <i>P</i> < 0.001*	2.85 ± 0.65 <i>P</i> < 0.001*	9.17 ± 2.3 <i>P</i> < 0.01*	1.86 ± 0.27 <i>P</i> < 0.001*
Ischemic preconditioning group (Group III)	18.22 ± 6.33 <i>P</i> < 0.001**	1.96 ± 0.2 <i>P</i> < 0.001**	6.5 ± 1.09 <i>P</i> < 0.01**	1.42 ± 0.59 <i>P</i> < 0.05**
Ischemic postconditioning group (Group IV)	21.86 ± 5.5 <i>P</i> < 0.01**	2.07 ± 0.55 <i>P</i> < 0.01**	10.39 ± 1.05 <i>P</i> < 0.001***	2.05 ± 0.27 <i>P</i> < 0.01***

*: Significant when compared to control group

** : Significant when compared to ischemia reperfusion group

***: Significant when compared with ischemic preconditioning group

Table (III): Correlation results between myeloperoxidase (MPO), liver (L) vascular cell adhesion molecule 1 (VCAM-1) and soluble (S) E selectin in I/R group

Parameter	L- VCAM-1		S- E Selectin	
	r	<i>p</i>	r	<i>p</i>
MPO	0.723	< 0.05	- 0.687	< 0.05

4. Discussion:

Recruitment and activation of neutrophils into liver vasculature, and in particular hepatic sinusoids, have been implicated in microvascular dysfunction and parenchymal damage associated with hepatic I/R injury (Cutrin *et al.*, 2000).

In the present study, results revealed a substantial liver damage at the end of the I/R period as indicated by the significant elevation of serum ALT and concomitant decline of serum albumin.

Ischemic postconditioning strategy significantly contributed to attenuation of hepatic I/R injury leading to significant lowering of ALT level when compared with I/R group and elevation of albumin level which did not reach statistical significance. The degree of liver damage was notably reduced by ischemic preconditioning which decreased the hepatic enzymatic leakage and ameliorated the suppression in plasma protein synthesis. In accord with our results, only the study of (Santos *et al.*, 2010) observed that levels of ALT were increased with lower intensity in ischemic postconditioning when compared with I/R group, while the hepatoprotective efficacy of ischemic preconditioning has been demonstrated in many experimental and clinical studies. (Clavien *et al.*, 2003) and (Bedirli *et al.*, 2005).

Although the protective effect of ischemic postconditioning against I/R injury in the present study was less prominent when compared to ischemic preconditioning, our results provide evidence to the minimizing effect of ischemic postconditioning on hepatocellular damage in warm hepatic I/R injury and

add to the few researches available in the literature about hepatic ischemic postconditioning.

The effect of ischemic postconditioning strategy on leucocyte infiltration and its correlation to cell adhesion molecules hasn't been previously investigated in experimental models of warm hepatic I/R.

The results of the present study showed a significant increase in the MPO activity in the livers subjected to 60 minutes ischemia and two hours reperfusion compared to sham operated livers. The myeloperoxidase is used as an index of hepatic leucocyte accumulation and activation (Shen *et al.*, 2003). Our observation accords with that of (Tsuchihashi *et al.*, 2006) and (Wu *et al.*, 2009), who demonstrated a significant increase in MPO activity in warm I/R models in mice, but no previous work directly assessed the effect of ischemic pre or postconditioning on MPO activity. In the present work, both strategies of conditioning significantly attenuated the elevation in MPO activity indicating inhibition of leucocyte accumulation and consequent hepatocellular damage which was evident by the decrease in ALT level and the increase in albumin level.

The hepatic VCAM-1 and E selectin was significantly elevated in I/R group. Our results accords with (Teoh *et al.*, 2007) who observed significant increase in VCAM-1 in mice livers following I/R, and (Hafez *et al.*, 2007) who revealed elevation of E selectin in canine hepatic I/R. Both conditioning

strategies in our model significantly down regulated the liver levels of VCAM-1 and E selectin.

Serum levels of E selectin and VCAM-1 were significantly increased in the rats of I/R group, this increase was ameliorated by ischemic preconditioning strategy, but not by ischemic postconditioning.

Our results showed that ischemic postconditioning resulted in less marked reduction of liver levels of VCAM-1 and E selectin than preconditioning and failed to reduce the levels of their soluble fractions in serum. (Tsuchihashi *et al.*, 2006) in their study demonstrated that 90 minutes ischemia significantly increased expression of vascular endothelial growth factor VEGF, and its level peaked at 2 hours of reperfusion. VEGF is known to function via the expression of pro-inflammatory cytokines like tumor necrosis factor α , TNF- α , which in turn together with VEGF induce expression of adhesion molecules including E selectin, ICAM-1 and VCAM-1 (Reinders *et al.*, 2003). This may partially explain our findings, as ischemic preconditioning increases liver tolerance to ischemia prior to starting I/R, this may result in more lowering of VEGF and its resultant cascade more than ischemic postconditioning which is implemented at the onset of reperfusion after establishment of ischemia for 1 hour leading to more expression of VEGF, TNF- α and adhesion molecules. In addition, (Peralta *et al.*, 2001) have suggested that ischemic preconditioning protects against systemic disorders associated with I/R by blocking TNF- α release and increasing activated adenosine monophosphate kinase. Such effect has not been investigated or shown to be produced by ischemic postconditioning; this may also explain the difference between effects of both conditioning strategies on the level of soluble fractions of VCAM-1 and E selectin.

An interesting finding in our results is the correlations between levels of adhesion molecules and activity of MPO, the positive correlation between liver VCAM-1 and MPO can be expected as the higher the level of liver VCAM-1, would result in more tethering, and infiltration by leucocytes. The significant negative correlation between soluble fraction of E selectin and MPO in the present study can be explained by the fact that the soluble fractions of such adhesion molecules are partly released from the activated endothelial cells (Pigott *et al.*, 1992), this means that more endothelial-leucocyte interaction, with increased leucocyte infiltration will lead to less shedding of E selectin, and lower levels of its soluble fractions

Conclusion

Our results revealed that ischemic postconditioning could contribute to liver protection against I/R injury in the initial phase of warm hepatic ischemia by attenuating leucocyte infiltration and

decreasing VCAM-1 and E selectin levels in the liver. Although the protection offered by ischemic preconditioning was more significant when compared to that produced by ischemic postconditioning, yet the present findings endorse the clinical potential of ischemic postconditioning which enables modification of reperfusion after the occurrence of ischemic injury, in situations with unscheduled ischemia.

Our results also postulate that soluble E selectin can be used as a marker to judge the degree of leucocyte infiltration in hepatic warm ischemia reperfusion injury. Further investigations are still needed to elucidate the molecular mechanisms underlying inhibition of leucocyte infiltration observed with ischemic postconditioning.

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