

Changes In TNF-Alpha Levels, Microcirculation Status, And Oxidation Products After Liver Ischemia/Reperfusion Injury In Mice

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Citation

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Abstract

Purpose: Experimental study to determine the role of TNF- α in the pathogenesis of oxidative damage in the liver induced by ischaemia - reperfusion.

Methods: 50 Wistar rats in five groups of 10 each, whose livers were subjected to 30 and 60 min of no-flow ischaemia followed by reperfusion for up to 60 min, by occluding blood supply to the left lateral and medial lobes of the liver, and reperfusion was instituted by releasing the occlusion. TNF- α , MPO, PNP, MDA, SGPT and LDH were measured. For the estimation of blood flow in the microcirculation was used the laser-Doppler flowmetry.

Results: Ischaemia of the liver produced an increase two fold in myeloperoxidase activity (MPO), reperfusion produced five fold increase in tumor necrosis factor - α , one and a half fold increase in purine nucleoside phosphorylase activity (P.N.P.). Neutrophils after reperfusion were found three fold increased in the liver tissue and the S.G.P.T. and L.D.H were found five and four fold increased respectively. The mean blood flow in the microcirculation during reperfusion period was decreased by 36.8%. Malonyldialdehyde (MDA) was statistically significant elevated after reperfusion.

Conclusion: The development of delayed perfusion failure in the hepatic microcirculation during reperfusion has been documented by laser-Doppler flowmetry. The longer the ischemic episode, the more severe the microcirculatory disturbance during reperfusion. The PNP, SGPT, LDH are reliable markers of liver function and LDH found to be more sensitive in representing liver injury. Neutrophil accumulation has been documented first by the microscopic findings and also by the elevation of the MPO. Oxidative stress also seems to play an important role in liver cell damage as it is evidence by the elevation of MDA. TNF- α was found to be elevated five fold and seems to be a potent level depended activator of neutrophils and his modulation may serve as a potential target for therapeutic intervention.

INTRODUCTION

A no-reflow phenomenon in ischaemia-reperfusion injury has been reported in the heart, liver, brain, kidney and skeletal muscle.^{1,2,3,4,5} However the mechanisms responsible for this no-reflow phenomenon remain uncertain. There are several reports^{6,7,8} that activated leukocytes accumulate in postischemic liver tissue and adhere to microvascular endothelium and subsequently release superoxide anion or other reactive oxygen products that increase microvascular permeability. An accumulation of neutrophils occurs in the ischaemic reperfused rat liver and TNF- α play a role in

mediating this neutrophil accumulation.⁹ Myeloperoxidase (MPO, EC 1.11.1.7) serve as a marker for tissue neutrophil content. Purine nucleoside phosphorylase (PNP EC 2.4.2.1) is considered to be mainly catabolic enzyme. PNP presents in both hepatocytes and sinusoidal cells and it might be used as a marker of hepatic injury.¹⁰

Tumor necrosis factor - α is a pleiotropic cytokine produced by numerous cell types in response to various inflammatory and immunomodulatori stimuli. TNF- α may mediate direct toxicity to mitochondria, and induce apoptotic or necrotic cell death.^{11,12}

In order to clarify the mechanism of liver injury caused by ischaemia and following reperfusion, the change in the marker enzymes both of hepatocytes and endothelium were investigated. For quantifying the neutrophil accumulation we used biochemical myeloperoxidase (MPO) activity. Tumor necrosis factor- α (TNF- α) was investigated as a representative of cytokines production. Purine nucleoside phosphorylase (PNP) as endothelial marker enzyme and for the overproduction of toxic radicals the activity of malonyldialdehyde (MDA). Microcirculation during ischaemia and reperfusion was measured with laser-Doppler. The hepatic cell damage was estimated by measuring parenchymal marker enzymes, lactate dehydrogenase (LDH) and serum glutamic-pyruvic transaminase (SGPT).

MATERIALS AND METHODS

Fifty Wistar rats 250-300 g were used in the experiments, divided in 5 groups (License number 1528/25-2-94 Prefectorial Veterinary Department of Thessaloniki). The animals were allowed free access to food and tap water but twelve hours before the experiments had access only to water. They were anesthetized with ketamine and midazolam (50 mg/kg and 1 mg/kg i.m. respectively). A midline laparotomy was performed and the liver was mobilized and isolated by dividing all of its peritoneal attachments. The blood vessels supplying the median and left lateral hepatic lobes were occluded with an arterial clamp for 30 min and 60 min. The incision was closed with 3-0 silk. The testings were carried out in five experimental groups:

Group 1. (n=10): control

Group 2. (n=10): 30 min of ischaemia

Group 3. (n=10): 30 min of ischaemia, then 60 min reperfusion

Group 4. (n=10): 60 min of ischaemia

Group 5. (n=10): 60 min of ischaemia, then 60 min reperfusion

The animal control group undergone the same procedure apart from clamping as the other groups ie. dissection of the portal structures.

At the end of the experiment blood sample was obtained from the vena cava to determine TNF- α , LDH, SGPT. One sample of nonischemic and post-ischemic lobe was freeze-clamped and stored in liquid nitrogen; another sample was fixed in phosphate-buffer formalin. All chemicals were purchases from Sigma Chemical Company (St Louis, Mo).

Hepatic tissue MPO: Homogenized samples of the hepatic tissue in 50mM potassium phosphate buffer 0-0.5%, hexadecyltrimethyl ammonium bromide - 0.416% EDTA, pH 6. Sonicate the samples in ice 10X5 sec and centrifuge at 12.500g for 30 sec. Incubate the supernatants at 60° C for 2 h and assayed the enzyme activity by the method of Bradley and co-authors¹³, as modified by Mullane and colleagues.¹⁴

Hepatic tissue PNP: Fresh rat liver was rinsed with phosphate buffer saline, was homogenized in 4 volumes of 50mM tris-HCL buffer-1mM EDTA - 0.1 mM phenylmethylsulphonyl fluoride, pH 7.5 . The homogenate was centrifuged at 27.000 g for 45 min, assayed the supernatant fluid for enzyme activity and protein.¹⁵

Using the laser-Doppler flow probe (Periflux PF 2B laser Doppler Flowmeter (Perimed, Sweden, laser source 2mW He-Ne, Siemens LGR 7621S, 632,8 nm), a base-line recording of liver blood flow during a 5-min period was obtained. Next there was a period of ischemia varying according to the experimental group: 30 sec (control), 30 min and 60 min. The ischemic lobes were unclamped and recordings obtained of postischemic blood flow during one hour of reperfusion.

Microscopic analysis of the ischaemic lobes of the liver in all groups were studied, especially the accumulation of neutrophils in pathologic paraffin cubes of reperfusion groups.

Statistical analysis with ANOVA (Statistical program: Statview -Brain Power, CA, U.S.A.) was used to examine the statistical significance of the results between all the groups.

RESULTS

The results from study's measurements of SGPT, LDH, MPO, PNP, MDA, mean blood flow are listed on figures 1,2,3,4,5,6. The measurement of S.G.P.T. shows that there is a small elevation during ischaemia from 25 u/l to 36-46 u/l, but the elevation is even bigger during reperfusion up to 122.9 u/l.

Figure 1

Figure 1: Histogram that displays the values of S.G.P.T. in all groups

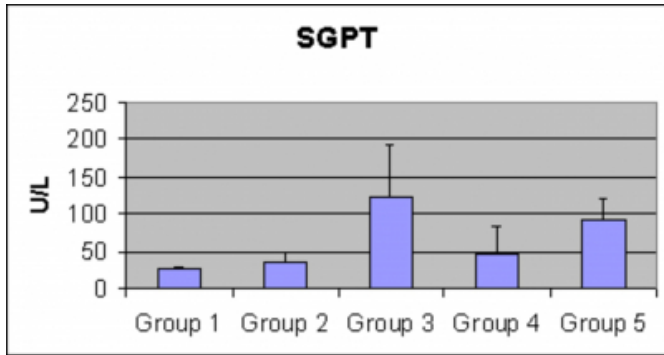


Figure 2

Figure 2: Histogram that displays the values of LDH in all groups

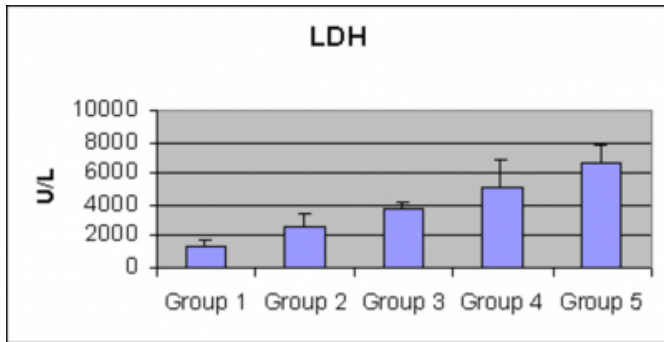


Figure 3

Figure 3: Histogram that displays the values of MPO in all groups

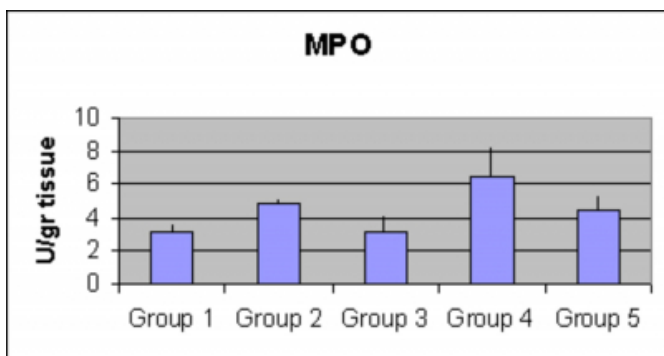


Figure 4

Figure 4: Histogram that displays the values of TNF- α in all groups

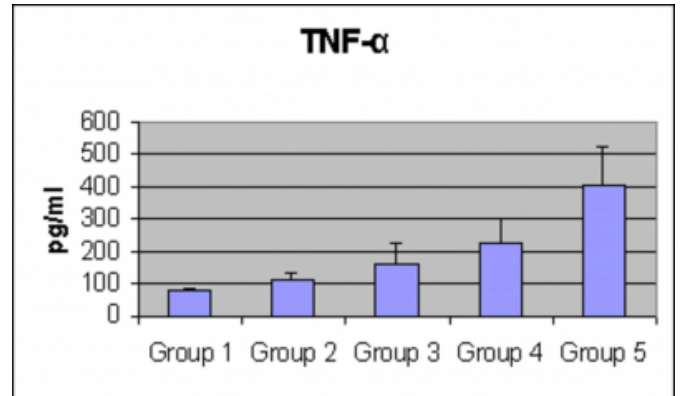
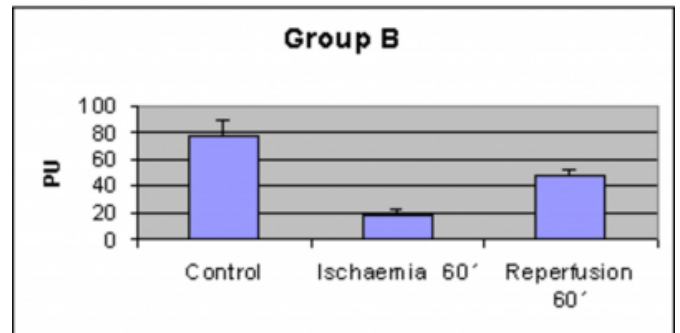
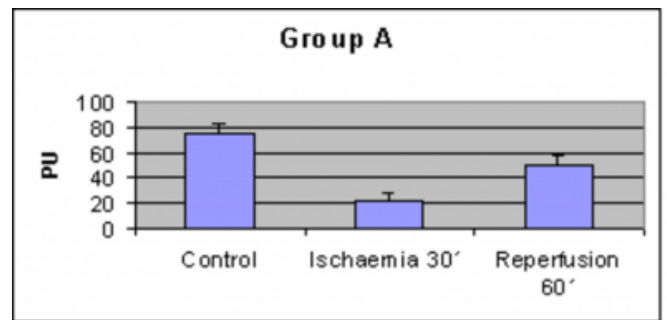


Figure 5

Figure 5: Histogram that displays the values of mean blood flow in all groups



The mean blood flow in the microcirculation was found to be in the reperfusion period decreased 36.8% of the baseline.

The statistical analysis of the group A and B :

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Figure 6

Group A.				
Comparison:	Mean Diff.:	Fisher PLSD:	Scheffe F-test:	Dunnett t:
Control vs. Ischaemia 30'	52.028	15.42*	140.351*	16.754
Control vs. Reperfusion 60'	24.266	15.42*	30.531*	7.814
Ischaemia 30' vs. Rep. 60'	-27.762	15.42*	39.962*	8.94

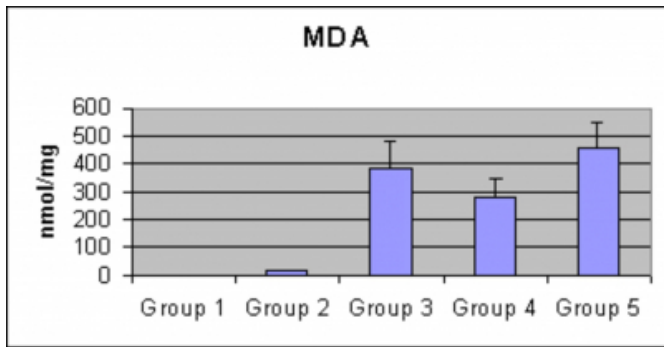
* Significant at 99.99% p < 0.0001

Group B				
Comparison:	Mean Diff.:	Fisher PLSD:	Scheffe F-test:	Dunnett t:
Control vs. Ischaemia 60'	60.342	14.331**	136.33*	16.512
Control vs. Reperfusion 60'	31.237	14.331**	36.533*	8.548
Ischaemia 60' vs. Reperf. 60'	-29.105	14.331**	31.717*	7.964

* Significant at 99.9% p < 0.001
 ** Significant at 99.99% p < 0.0001

Figure 7

Figure 6: Histogram that displays the values of MDA in all groups



The statistical analysis between all the groups for the S.G.P.T. LDH, MPO, PNP, MDA, is given under the figure legends.

The measurement of LDH shows that the ischaemia causes liver damage depending more from the time of ischaemia than the reperfusion, because in the group of 30' ischaemia the LDH elevation was 2.612 u/l and after 60' was 5.168 u/l, while in the reperfusion group 3 was 3.719 u/l.

The measurement of P.N.P. confirms that the reperfusion induces greatest liver injury. The PNP levels increase from 0.27 u/gr in the control group to 0.45 - 0.49 u/gr in the reperfusion groups while in ischaemia groups is elevated till 0.30-0.40 u/gr.

The MPO as an indicator of activated neutrophil showed an elevation only during ischaemia, from 3.2 u/gr to 4.87-6.5 u/gr and the levels were falling after reperfusion.

While the MPO levels are falling during reperfusion the TNF-alpha levels are increasing even more during reperfusion.

Ischaemia of the liver produced an two fold increase in

myeloperoxidase activity (MPO), reperfusion produced five fold increase in tumor necrosis factor- α , one and a half fold increase in purine nucleoside phosphorylase activity (P.N.P.). Neutrophils after reperfusion found three fold increased in the liver tissue and the S.G.P.T. and L.D.H was found five and four fold increased respectively. Malonyldialdehyde (MDA) was statistically significant elevated after reperfusion.

MICROSCOPIC ASSESSMENT OF HEPATOCELLULAR INJURY

The accumulation of neutrophils in pathologic paraffin cubes of reperfusion groups, representative section of specimens after reperfusion in-group 3 and 5. (Picture 1,2)

Figure 8

Picture 1: Liver microscopic findings - group 3 (30 min ischaemia - 60 min reperfusion). Minimal hepatocellular damage and small accumulation of neutrophils in some areas.

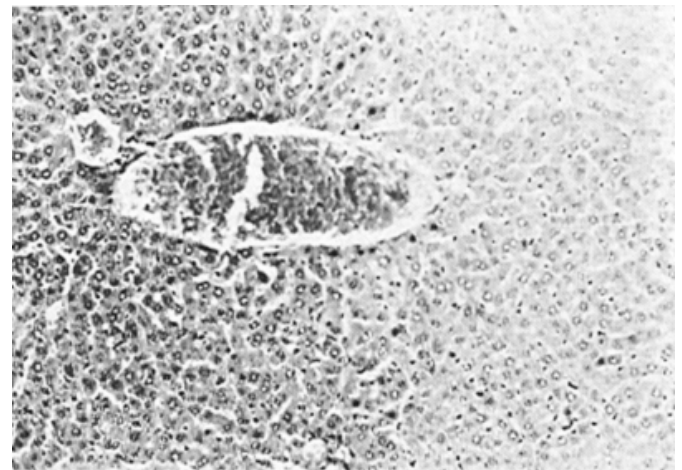
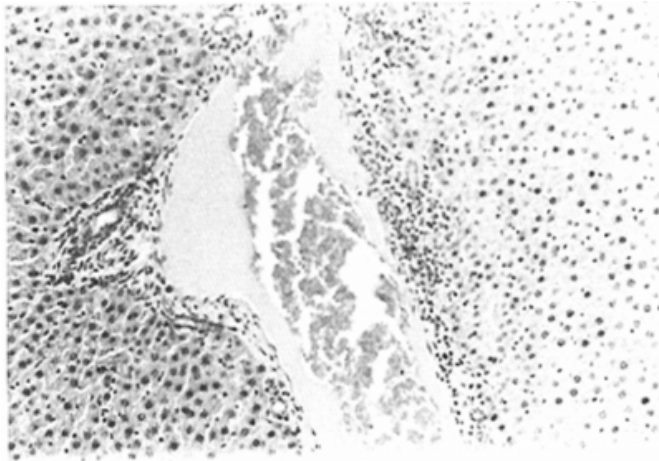


Figure 9

Picture 2: Liver microscopic findings - group 5 (60 min ischaemia - 60 min reperfusion). Dilatation of central lobe vein, edema, perivascular inflammation with accumulation of neutrophils.



The number of cells per unit of liver is given with symbol (+) = 0-5 cells, (++) = 5-10 cells, (+++) = 10-15 cells.

Control group 1(+)/10 there is no liver tissue damage.

Group of 30 min ischaemia: 2(+)/10, that means 20% of all specimens have a small accumulation of neutrophils.

Group of 30 min ischaemia and 60 min reperfusion: 4(++)/10, 4(+)/10, 2(-)/10, that means 80% of all specimens have accumulation of neutrophils

Group of 60 min ischaemia: 4(+)/10, 40% of all specimens have a small accumulation of neutrophils

Group of 60 min ischaemia and 60 min reperfusion: 2(+++)/10, 4(++)/10, 3(+)/10, 90% of all specimens have accumulation of neutrophils

DISCUSSION

The mechanism of ischaemic injury is primarily oriented around the pathological changes of the blood vessels supplying the ischaemic liver. Reperfusion of ischaemic liver tissue, while essential to prevent anoxic cell death, is often associated with additional cellular damage.¹⁶ Paradoxically, reperfusion injury frequently exceeds the original ischaemic insult,¹⁶ as it is profound by our findings (elevated SGPT and PNP). The reperfusion injury is closely linked with the inflammatory response characterized by activation, adhesion and diapedesis of polymorphonuclear neutrophils (PMNs). During the last decade, there has been some information regarding the potential role of PMNs in

the development of ischaemia and reperfusion injury.^{8,17} The subsequent findings that support the involvement of PMNs in hepatic ischaemia and reperfusion injury are the increase of PMNs accumulation in reperfused tissue,¹⁸ and the exacerbation of the reperfusion injury through toxic mediators released by activated PMNs.¹⁹ According to the present study there was an accumulation of PMNs in the microscopic findings and MPO was elevated in both ischaemia groups which is an indicator of PMNs presence in liver tissue.

PMNs-endothelium adherence is, in part, a response to reactive oxygen radicals, as well as cytokines with chemotactic properties.²⁰ This adherence results in microvascular failure, frequently called “no reflow phenomenon” and tissue injury. Kupffer cells also release chemoattractant in response to oxygen radicals reducible by xanthine oxidase inhibition.²¹ In the present study malondialdehyde production rapidly reached values of 458.96 u/gr in the group of 60' isch/60' reperfusion.

The cytokines, a group of polypeptide mediators, are not only an integral part of normal homeostasis, but also have various biologic effects in many pathophysiologic processes.²² Although tumor necrosis factor and interleukin-1 may attract PMNs to the site of inflammation, only TNF may be a direct stimulus to the respiratory burst, which is characterized by generation of reactive oxygen-derived free radicals and their metabolites. In our study, TNF- α levels were found elevated in the groups of ischaemia and statistical significant between control and groups 3, 4, and 5 ($p < 0.05$). Also statistical significant was found between the group of 30 min ischaemia and that of 60 min ($p < 0.05$). Very high TNF- α levels were seen in the 60 min ischaemia groups. These results demonstrate that Kupffer cell activation has an important role in the development of reperfusion injury after hepatic ischaemia through TNF- α release, and PMN activation and infiltration. Other studies^{11,23} also demonstrate that TNF- α and IL-1 promote leukocyte adherence and transendothelial migration.

The MPO is an enzyme found in neutrophils and, in much smaller quantities, in monocytes and macrophages. However there has been no documentation activity in the liver. The cumulative number of neutrophil adherence to the endothelium increased parallel with microcirculatory stasis of blood flow. Plugging of liver sinusoids by adherent neutrophils was observed to cause obstruction to blood flow in the sinusoid.⁷ This adherence results in microvascular

failure, frequently called “no-reflow phenomenon” and tissue injury.²⁴

Moreover, activated PMNs can secrete several enzymes, such as myeloperoxidase and elastase from azurophilic granules.²⁴ Myeloperoxidase is indirectly involved in tissue injury through hypochlorous acid and singlet oxygen that have significant destructive potential.²⁵ In the present study MPO levels were found significantly elevated in the 30 min ischaemia group ($p < 0.05$), and when livers were subjected to 60 min of ischaemia the levels of MPO were even more higher and statistically significant in comparison with control group ($p < 0.001$). The results of statistical analysis for MPO between the groups 3 (30/60) vs 5 (60/60) shows that time of ischaemia is important for activation, adhesion and diapedesis of PMNs ($p < 0.05$).

The effect of ischaemia and reperfusion on purine nucleoside phosphorylase (enzyme that is localized in the cytoplasm of the endothelial and Kupffer cells) was also studied. In the 30 min ischaemia group, we observed a normalization of PNP levels, indicating a reversible endothelial cell injury. This was in contrast to the situation when livers were subjected to 60 min of ischaemia where a slight rise of PNP levels was followed in the end of 60 min reperfusion by a sustained rise indicating an irreversible injury to the endothelial cell. This study demonstrates that PNP levels in the organ effluent is a quick, easy, reproducible and relatively inexpensive assay and may be a reliable index of ischaemic damage to the microvascular endothelial cell confirming others.²⁶

Many different techniques have been used for the estimation of microcirculatory blood flow, including plethysmography, radioactive microspheres, clearance of dyes or markers, fluorescent agents, thermodilution, and more recently video microscopy and laser-Doppler flowmetry.^{27,28,29} Of these, only laser-Doppler flowmetry allows a continuous, *in vivo* recording of the tissue blood flow, without directly affecting the microcirculation. In the present study the degree of microvascular shut-down during reperfusion was modulated by the length of ischaemia. A significant decrease of approximately 20-32.6% was found after 30 min of ischaemia and 60 min of reperfusion, while, after 60 min ischaemia 36.8% of the sinusoids failed to conduct flow.

Pathological findings in-group 5 showed that the neutrophils were increased in association with the group 3. That indicates the activation of neutrophils, which was increased in this group probably due to the longer duration of

ischaemia.

Latest studies show that tumor necrosis factor alpha is implicated in the pathogenesis of hepatic ischemia reperfusion injury but can also prime hepatocytes to enter the cell cycle. Ischemic preconditioning protects against ischaemia-reperfusion liver injury and is associated with activation of nuclear factor kappaB (NF-kappaB) and cell cycle entry. The hepatoprotective effects of “preconditioning” can be stimulated by TNF- α injection, which has identical downstream effects on cell cycle entry.³⁰ The timing, intensity, and context may be all that determines whether TNF- α is protective or injurious to the liver in the context of hepatic IR injury. The suppression of tumor necrosis factor-alpha and the subsequent amelioration of microcirculatory disturbances were observed, suggesting that the mechanism underlying the protective effect of ischemic preconditioning in hepatic ischemia/reperfusion injuries may involve tumor necrosis factor-alpha and microcirculatory regulation.^{31,32,33,34}

CONCLUSIONS

In conclusion the results of this study indicate that reperfusion following ischaemia of short duration 30 min can result in the aggravation of tissue injury. The PNP, SGPT, LDH are reliable markers of liver function and LDH found to be more sensitive in representing liver injury. Reperfusion after a period of ischaemia induces an accumulation of neutrophils in the liver, and cytokines (TNF- α) are important mediators in the mechanism of this neutrophil accumulation. This accumulation was found first in the microscopic findings and also with the elevation of the MPO. The neutrophil adherence influences the microcirculation as this was found with laser-Doppler flowmetry. The oxidative stress also plays an important role in liver cell damage with elevation of MDA. TNF- α is a potent activator of neutrophils and its modulation may serve as a potential target for therapeutic intervention. A better understanding of the basic pathophysiology will reveal potential targets for therapeutic interventions and will show us how to restore circulation avoiding risk factors that may aggravate reperfusion injury.

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