

## REVIEW

# ISCHAEMIA-REPERFUSION INJURY IN THE LIVER THE ROLE OF HEPATIC MICROVASCULATURE

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**ABSTRACT:** Research into ischaemia and reperfusion injury especially in liver transplantation has been aimed primarily at preventing deterioration of organ function before harvest and at improving organ preservation techniques. Recent studies however, suggest that postischaemic organ function and viability can be improved not only through improved organ protection before ischaemia, but also with therapy aimed at ameliorating the organ reperfusion injury. In order to develop successful therapeutic interventions against ischaemia-reperfusion induced liver injury, it is necessary to consider the primary site of injury as well as to explore the mechanism(s) and possible factors which may contribute to the injury during ischaemia and reperfusion. Studies on hepatic ischaemia-reperfusion injury have focused mainly at hepatocellular level. Until recently, more attention has been drawn to the important role of hepatic microcirculation on the pathophysiology of the above injury. The argument that hepatic microvasculature is the primary site of ischaemia-reperfusion injury and possible factors which cause this injury are among the issues reviewed in this article. (*JUMMEC 1996 1(1): 9-16*)

**KEY WORDS:** ischaemia, reperfusion, liver, microvasculature, oxygen radicals

Hepatic ischaemia-reperfusion is one of the major causes of liver injury resulting in liver necrosis. Severe reductions in hepatic perfusion are common in several different clinical settings such as shock (1), sepsis (2), surgical resection of the liver (3) and in liver transplantation (4,5). In the latter situation, an understanding of the effects of a potentially prolonged period of temporary ischaemia on restoration of hepatic functions after organ reperfusion is particularly important.

## Concepts of ischaemia and reperfusion injury

Ischaemia, either partial or complete is defined as a reduction of blood flow to tissues. If the duration of ischaemia is short, all of the metabolic alterations associated with the ischaemia are reversible (6,7). If the ischaemia persists for longer periods of time, structural deterioration, metabolic dysfunction and microcirculatory derangements occur and the affected cells become irreversibly injured.

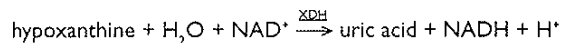
Liver biopsies taken during a period of reversible ischaemia demonstrate significant cellular alterations with the primary changes occurring in the centrilobular

region of the liver. After irreversible hepatic ischaemia, severe light microscopic alterations are evident. The liver shows signs of fatty changes and focal necrosis. The sinusoids are dilated and in severe cases, the nuclei become pyknotic and large intracytoplasmic inclusions, "residual bodies", can be found. Severe lesions are usually concentrated in the centrilobular areas and seldom extend into the periportal regions (8).

Metabolically, a complex series of events occur during liver ischaemia which involve virtually every organelle and subcellular system in the affected cells. Reductions in hepatic blood flow decrease tissue  $pO_2$  (9). As cells become anoxic, oxidative phosphorylation ceases. ATP is rapidly hydrolysed into AMP which is then degraded to hypoxanthine. ATP stores become depleted (10) and virtually all energy dependent functions such as RNA and protein synthesis cease. Aerobic metabolism is shifted to anaerobic metabolism which results in an initial acceleration in glycolysis, leading to increased production of tissue lactic acid and thus lower tissue pH (8). When the ischaemic period is prolonged, glycogen stores become depleted and glycolysis slows down.

Another important process occurring in the cells during ischaemia is the conversion of xanthine dehydrogenase (XDH) to xanthine oxidase (XO) (11). In the normal

situation, approximately 90% of the enzyme xanthine oxidase is present in its dehydrogenase form (12). The dehydrogenase form utilises  $\text{NAD}^+$  as an electron acceptor in the degradation of hypoxanthine to uric acid:

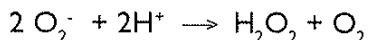
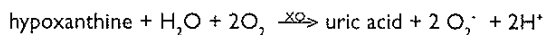


A drop in cellular ATP levels due to limited oxygen availability during ischaemia causes poor maintenance of ion gradients across cellular membranes, increasing the capillary permeability and resulting in oedema. This also allows the influx of calcium ion into the cells. McCord (11) hypothesized that this high concentration of calcium activates a protease which is capable of irreversibly converting the xanthine dehydrogenase enzyme to the oxidase form. McKelvey et al. (13) proposed a second mechanism of conversion which involves sulfhydryl modification and can be reversed by treatment with dithiothreitol (DTT). This DTT-reversible xanthine oxidase was suggested to be an important intermediate occurring prior to irreversible conversion of XDH to XO. The conversion of XDH to XO during ischaemia has important consequences for the tissue during reperfusion as will be discussed below.

Despite the structural and metabolic changes occurring during ischaemia, much of the injury that had been attributed to ischaemia itself actually takes place when molecular oxygen is reintroduced to the tissue. This type of injury is more properly termed reperfusion injury (14). Increasing evidence now clearly shows that oxygen-derived free radicals or reactive oxygen species (ROS), play an important role in mediating injury to reperfused tissues after ischaemia (11,14-18).

### ROS and hepatic ischaemia-reperfusion injury

Siems et al. (19) proposed that XO may be a major source of ROS in the liver. The conversion of XDH to XO during ischaemia renders the enzyme unable to use  $\text{NAD}^+$  as a normal electron acceptor. However,  $\text{O}_2$  can act as an electron acceptor for XO during reperfusion, giving rise to superoxide ( $\text{O}_2^-$ ) and  $\text{H}_2\text{O}_2$ :



$\text{H}_2\text{O}_2$  can break down further particularly in the presence of transitional metal ions to produce the most reactive and damaging of the ROS, the hydroxyl radical (OH).

To date, it is generally well accepted that the production of ROS is involved in ischaemia-reperfusion injury in the liver (20-24). This conclusion is largely based on the observation that a competitive inhibitor of XO, allopurinol (20,24,25) or other antioxidants such as superoxide dismutase (SOD) (21,22,25) and catalase

(25-27) are effective in attenuating the injury associated with hepatic ischaemia.

### Studies with allopurinol, SOD and catalase

The protective effects of allopurinol pretreatment have been reported in many studies in hepatic ischaemia-reperfusion injury *in vivo* (20,26,28,29) and in the isolated perfused liver (15,24,23). Allopurinol increased hepatic tolerance to ischaemia and improved hepatic protein synthesis, tissue water accumulation, and adenine nucleotide regeneration during hepatic ischaemia-reperfusion in rats (30). The beneficial effects of SOD (26,29) and catalase (26,27) pretreatment have also been reported in studies of hepatic ischaemia-reperfusion injury. Administration of either SOD or catalase appeared to attenuate the release of enzymatic indicators of hepatocellular injury (15,27) and improve the function of hepatic grafts exposed to ischaemia (27). The results of these studies, together with the fact that hepatocytes contain high concentrations of XO and XDH (31), have been interpreted as evidence for the causal involvement of a hepatocellular XO-induced oxidant stress in the pathogenesis of the injury.

There are studies, however, which showed that XO may play a very limited role in ROS production during reperfusion. Most studies that have reported a protective effect of allopurinol used ischaemic times of only 20 to 90 min. Using *in vivo* and *in vitro* models, recent studies showed that the reversible conversion of XDH to XO begins after 2 hr of ischaemia and an irreversible conversion begins after more than 3 hr of ischaemia (13,32). Marubayashi et al. (33) also reported that hepatic XO activity remained unchanged after 90 min of hepatic ischaemia although there was already a twofold increase in malonaldehyde level (a product of lipid peroxidation) at this time point. Even after 2 hr of ischaemia followed by reperfusion, the quantity of total XDH and XO remained relatively steady (33). A marked conversion of XDH to XO was achieved only if the ischaemic period was prolonged to 6 hr (33).

Furthermore, there is evidence showing that the beneficial effects of allopurinol are not related to the inhibition of XO (34,35). A number of nonspecific actions of allopurinol have been reported, including induction of antioxidant enzymes (36) and direct scavenging of ROS (37,38). Doses as low as 2 - 5 mg/kg have been shown to be sufficient to inhibit hepatic activities of XO and XDH (39). However, all of the experiments which showed a beneficial effect of allopurinol used very high doses (1 to 2 times 50 mg/kg) (20,25,26,28,29). Even with these high doses, there are studies reporting the involvement of ROS during reperfusion of the ischaemic liver and with no beneficial effect of allopurinol pretreatment (40).

In the isolated perfused liver, the minimal allopurinol concentration required for scavenging was 500 mM (38). Marotto et al. (23) showed no protective effects when allopurinol levels in the perfusate were less than 200 mM. Only allopurinol at concentrations of 5mM or higher were protective against reperfusion injury (15,23,24). Thus, it is conceivable that the beneficial action of allopurinol in these studies is due to direct scavenging of ROS rather than XO inhibition, suggesting that XO does not play a critical role in the pathogenesis of hepatic ischaemia-reperfusion injury. However, it remains a strong possibility that XO generated oxidants could be involved in reperfusion injury by generating chemotactic factors, contributing to neutrophil accumulation in the liver and preparing the liver for a neutrophil-dependent injury phase.

In most cases where a protective effect of SOD or catalase was observed, it was assumed that these enzymes protected against oxidant stress within the parenchymal cells or hepatocytes. However, in order to effectively detoxify ROS generated within the hepatocytes, the enzymes have to enter the cell intact or alternatively, ROS have to leave the cell to be metabolized extracellularly. It seems highly unlikely that quantitatively enough  $O_2^-$ , with an estimated half-life of microseconds is able to escape the defence by high endogenous SOD concentrations and cross the cell membrane to initiate a sequence of events extracellularly, leading to significant cell injury (41).

Alternatively, the protective effect of SOD could involve a cellular uptake of the enzyme sufficient to increase significantly the already high endogenous levels. In order to achieve significantly higher activities of intracellular SOD, extremely high doses of SOD are needed for *in vivo* experiments based on the results obtained from an *in vitro* study (42). As the half-life of SOD in blood *in vivo* is only approximately 6 min and SOD is a high molecular weight molecule (approximately 150 kD), it is very unlikely that native SOD is taken up in sufficient quantities to enhance the intracellular detoxification capacity in the liver. Moreover, there are studies which have reported that SOD and catalase are ineffective against hepatic reperfusion injury (43-45), creating doubts to the above interpretations.

Using an isolated blood-free perfused liver model, Jaeschke et al. (41) demonstrated that minor quantities of ROS generated within hepatocytes were insufficient to exceed endogenous defense mechanisms during reperfusion after up to 2 hour of ischaemia. When diquat was used to chemically generate high levels of ROS in the hepatocytes during reperfusion, the postischaemic liver was able to detoxify these without additional cell damage (41). Furthermore, other studies reported no evidence of lipid peroxidation in postischaemic livers (45,46). A significant increase in ROS release from

intracellular sources such as mitochondria and xanthine oxidase was only observed during reperfusion after severe hypoxic injury of the liver (47,48). Even under these conditions however, the liver was still able to detoxify those ROS, resulting in a significant loss of hepatocellular glutathione, without causing further cell damage during reperfusion (47,48). These data suggest that hepatocytes have considerable resistance against intracellular oxidant stress (47,48).

In another study (17), the oxidizing species was found to be neither generated nor detoxified within the hepatocytes during reperfusion. In support of this, hepatocytes isolated from livers subjected to ischaemia-reperfusion were not shown to release any detectable superoxide even with additional stimulation (49). The results of these studies have led to the investigations of alternative mechanisms for reperfusion injury, particularly other sources of ROS in the liver.

### **Other potential sources of ROS during reperfusion in the liver**

#### **Endothelial cells**

Endothelial cells have been shown to produce ROS *in vitro* (50) but the amount of ROS produced by endothelial cells is much smaller compared to Kupffer cells when both were stimulated with similar agents (50). As the number of endothelial cells in the liver is several fold higher than the number of Kupffer cells (51), a contribution of endothelial cells to the postischaemic oxidant stress can not be ruled out. However, endothelial cells isolated from liver subjected to ischaemia-reperfusion injury were shown to generate negligible amounts of superoxide compared to Kupffer cells even after stimulation (18).

#### **Kupffer cells**

There is strong evidence showing that Kupffer cells and neutrophils contribute to reperfusion injury in the liver (18,49,52-54). Two phases of reperfusion injury have been identified after hepatic ischaemia: an initial Kupffer cell-induced oxidant stress and injury (18) and a later severe neutrophil-induced injury (52). The conclusion that Kupffer cells and not neutrophils are mainly responsible for the initial injury was based on two experimental findings using oxidised glutathione (GSSG) levels as an index of oxidant stress (55): 1) the drastic increase of plasma GSSG concentrations during reperfusion indicating an oxidant stress occurring outside the hepatocytes (17); 2) the postischaemic increase of plasma GSSG levels could be enhanced or attenuated by respective alterations of Kupffer cell activity in the liver, while no correlation was found between plasma GSSG levels and the initial accumulation of neutrophils in the postischaemic liver (52).

Using electron microscopy, morphological changes in Kupffer cells shown by Caldwell-Kenkel et al. (56) during reperfusion provide additional evidence for the postischaemic activation of Kupffer cells. In addition, large Kupffer cells isolated from postischaemic liver lobes were shown to increase spontaneous  $O_2^-$  formation during reperfusion whereas smaller Kupffer cells and neutrophils did not spontaneously produce more  $O_2^-$  during reperfusion (49,53). However, they both generated considerably more  $O_2^-$  upon stimulation with phorbol ester (PMA) or opsonised zymosan (49). These results further support the contention that large Kupffer cells are the main sources of ROS during the first hr of reperfusion whereas neutrophils are only primed for ROS formation during this early reperfusion phase (49).

Kobayashi and Clemens (57) have shown that the activation of Kupffer cells during reperfusion was not triggered by the production of ROS since SOD and catalase did not ameliorate injury caused by Kupffer cells. The exact activating factor for Kupffer cells is still to be elucidated.

### Neutrophils

Suzuki et al. (58) have recently demonstrated the direct participation of neutrophils in ischaemia-reperfusion injury of the liver in rats. Pretreating animals with the immunosuppressants, FK506 and cyclosporine, before ischaemia improved the survival rate, reduced lipid peroxidation as well as diminishing neutrophil infiltration into the liver, suggesting that the presence and the degree of neutrophil infiltration were important components of liver ischaemic-reperfusion injury in the rat (58). The use of neutropenic animals (59) or monoclonal antibodies that prevent neutrophil adhesion (52,54) further substantiate the role of neutrophils in liver ischaemia-reperfusion injury.

Thus, the source of ROS production in the liver during ischaemia-reperfusion injury is very likely to be within the hepatic vasculature. This might explain the beneficial effects of SOD and catalase as these enzymes would not be required to enter the hepatocytes and could effectively remove ROS in the vascular space. This concept is also supported by experiments which demonstrated a beneficial effect against hepatic ischaemia-reperfusion injury of a SOD preparation with a high affinity for serum albumin, a protein that has a half-life of 6 hr and is confined to the vascular space of the liver (21).

### Hepatic microvascular injury induced by ischaemia-reperfusion

The discovery of vascular oxidant stress during reperfusion after hepatic ischaemia has attracted more attention to the role of the hepatic microvasculature in

ischaemia-reperfusion injury of the liver. Using intravital fluorescence microscopy, Clemens et al. (60) have shown that during reperfusion after 90 min of partial hepatic ischaemia, the number of perfused centrilobular areas and perfused sinusoids per unit area on the surface of the livers in rats was decreased to approximately 50 and 40% of that seen in sham controls.

Evidence of hepatic microvascular abnormalities caused by ischaemia-reperfusion has also been shown in studies of liver preservation. Using a rat model, McKeown et al. (61) showed that a short period of cold preservation of the liver had practically no harmful effect on the hepatocytes. However, the majority of the endothelial cells showed a range of degenerative ultrastructural changes including cell swelling and some detachment from the underlying hepatocytes. With longer periods of cold preservation, the hepatocytes were again relatively preserved whereas the sinusoidal endothelial lining was almost completely destroyed. Caldwell-Kenkel et al. (56) reported two components of storage injury in rat livers. The first was seen without reperfusion and was characterised by fenestral widening, retraction of endothelial processes and parenchymal cell blebbing. The second component however, occurred in livers stored longer in cold preservation solutions, with a brief period of reperfusion. Endothelial structure deteriorated rapidly after warm reperfusion and the degree of endothelial cell damage increased with reperfusion time. After 15 min of reperfusion, endothelial necrosis was close to 100%.

In an *in vitro* study, Myagkaya et al. (62) have demonstrated ultrastructural changes in liver sinusoidal cells during normothermic and hypothermic ischaemia. These changes were seen at much earlier stages before the detection of damage in hepatocytes in both types of ischaemia. In support of this, studies have documented an increased permeability of endothelial cell monolayers after ischaemia-reperfusion (63-65).

Using a vascular casting technique, it has been clearly shown that hepatic microvascular injury was an early event and occurred prior to the hepatocellular injury in the rat liver subjected to ischaemia and reperfusion (66). After 90 min of ischaemia in the liver, hepatic microvasculature started to be evident after 30 min of reperfusion whereas the earliest hepatocellular necrosis was only detected after 3 hours of reperfusion (66). This further substantiates the concept that the primary site of ischaemia-reperfusion injury in the liver occurs at the microvascular level.

However, what is the mechanism that causes the microvascular injury? Increasing evidence implicates a role for neutrophils in microvascular injury following ischaemia-reperfusion. Neutrophils have been shown to adhere to the microvascular endothelium, extravasate,

and accumulate in organs subjected to ischaemia-reperfusion (67,68).

The production of  $O_2^-$  during reperfusion has been proposed to induce the accumulation of neutrophils in the intestinal mucosa (69). Using a long-acting SOD which binds to serum albumin, Komatsu et al. (70) reported that  $O_2^-$  also are important mediators in initiating the accumulation of neutrophils in ischaemia-reperfusion in the liver.

During reperfusion, systemic levels of the chemoactivating factors such as leukotriene  $B_4$  ( $LTB_4$ ), thromboxane  $B_2$  ( $TxB_2$ ), platelet activating factor (PAF) and tumour necrosis factor (TNF) are elevated (71-74).  $LTB_4$  is a potent stimulator for  $H_2O_2$  and elastase generation from neutrophils (75). It has also been shown to induce endothelial permeability *in vitro* and *in vivo* (76). Thromboxanes were shown to activate neutrophils and mediate  $H_2O_2$  production following ischaemia (77). These mediators of inflammation are also thought to result in upregulation of adhesion integrins on the neutrophil cell surface, enhancing attachment to the endothelium (78).

It has been proposed that neutrophils may contribute to ischaemia-reperfusion induced microvascular injury through sinusoidal plugging (79,80). *In vivo* microscopy studies by Koo et al. (79,80) show that neutrophils, once recruited to the liver, adhere to the microvascular endothelium of the liver during reperfusion causing obstruction of blood flow in the sinusoids. Capillary obstruction by accumulated granulocytes is thought to be the mechanism responsible for the "no-flow" phenomenon.

Jaeschke et al. (52) however suggest that sinusoidal plugging by neutrophils is unlikely to contribute to reperfusion injury in the liver. They argue that hepatocytes are less susceptible to capillary plugging because each hepatocyte faces two sinusoids and temporary plugging of individual sinusoids seems to occur frequently even under physiological conditions (81-82). In addition, reduction of the number of neutrophils in the postischaemic liver lobes by more than 60% did not essentially improve reperfusion injury and neutrophils also accumulated in the nonischaemic lobes without causing any cell injury in these lobes (52). These observations suggest that neutrophils are unlikely to contribute to reperfusion injury in the liver passively through sinusoidal plugging. A more active role of neutrophils has been proposed. Studies have shown that once adhered to the endothelium, activated neutrophils have the capability to injure vascular endothelial cells by mechanisms independent of sinusoidal plugging. These include oxidant-mediated injury where damage to endothelial cells depends on the production of  $H_2O_2$  and  $\cdot OH$  (83,84), and proteolytic enzyme-mediated

mechanisms where they impair the integrity of endothelial cells by destruction of extracellular matrix components (75,85,86).

In summary, mechanisms of hepatic ischaemia-reperfusion injury are complex. Clinically, it has been an area of intense interest particularly amongst surgeons with the recognition that in some clinical situations, much of the injury that had been attributed to ischaemia itself actually took place at reperfusion and thus is amenable to prevention. Different mechanisms have been suggested to explain why injury to the hepatic tissue is maintained or even aggravated during reperfusion. As increasing studies are now suggesting the hepatic microvasculature as the primary site of early reperfusion injury after a ischaemic insult to the liver, more attention thus research on the effects of therapeutic agents upon the hepatic microvasculature is necessary in order to achieve successful therapeutic intervention.

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