The Hepatoprotective Effect of Nitric Oxide Modulators and Antioxidants in Hepatic Ischemia/Reperfusion Injury in Albino Rats

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ABSTRACT

Warm ischemia/reperfusion injury (IRI) of the liver is a major determinant of the outcome of a variety of clinical conditions. During the ischemic phase, tissue injury occurs due to oxygen deficiency. However, much injury arises with the restitution of the circulation (reperfusion injury), in which the generation of reactive oxygen species (ROS) plays a fundamental role. Several studies have emphasized the positive effect of antioxidants in attenuating IRI in many tissues. The role of nitric oxide and its action as an oxidant/antioxidant is considered. The aim of the present work was to investigate the potential of NO in modulating warm IRI of the liver and its possible interaction with different antioxidants in a trial to minimize that injury.

Seventy-two albino rats were used in the present study. Rats were classified into the following groups (8 rats each): The 1st group of rats was subjected to 30 min of ischemia followed by 60 min of reperfusion. The 2nd group was Sham-operated rats underwent the same surgical procedure without interruption of the blood flow to the liver and served as controls. 3rd group L-nitro-arginine methyl ester (L-NAME) treated group, 50 mg/kg (Sigma), the 4th, 5th, and 6th groups were the NO-donor, L-Arginine (Sigma) treated groups, in 3 different doses (100, 300, 500 mg/kg). The drugs were administered intravenously 5 min before ischemia-induction. The 7th group allopurinol (Sigma) treated group (50 mg/kg), the 8th group desferrioxamine treated group (desferal) (Sigma) 100 mg/kg and the 9th group vitamin C treated group (Sigma) 100 mg/kg. Allopurinol was administered intraperitoneally 10 min before the induction of ischemia, whereas desferal and vitamin C were administered intravenously 5 min before the induction of ischemia. The left lobe of the liver of albino rats was subjected to 30 min warm ischemia followed by 60 min of reperfusion. Serum liver enzymes (AST, ALT, and LDH) were determined as a measure of tissue injury. Plasma nitrite level was assessed as a measure of nitric oxide production. Hepatic levels of malondialdehyde were quantified to evaluate the oxidative stress, while those of superoxide dismutase, catalase, and glutathione served as a determinant of the antioxidant status. Thirty minutes of warm ischemia followed by reperfusion for 60 min inflicted signs of tissue injury accompanied by oxidative stress and exhaustion of tissue antioxidants and plasma nitrite level. This was exacerbated with the nitric oxide blocker L-NAME. On the other hand, the nitric oxide donor (L-arginine) its lowest and intermediate doses (100, 300 mg/kg) could attenuate that injury; while in its highest dose (500 mg/kg) accentuated the injury.
Treatment with the xanthine oxidase inhibitor (allopurinol) was accompanied with a mild protective effect. Treatment with the iron-chelating agent (desferrioxamine) was accompanied with a substantial protective effect, while the treatment with vitamin C caused a moderate protection. The results clearly demonstrated that nitric oxide supply during IRI is up to a certain limit beneficial in ameliorating hepatic IRI, while its paucity was accompanied with accentuation of the injury. It seems that too high or too low level of NO is detrimental and aggravates IRI and its fine-tuning is a prerequisite to protect at least hepatic tissue. Furthermore, the results revealed that the antioxidant allopurinol did not practically improve the deteriorated livers subjected to IRI. Also, the results proved that iron-chelator (desferrioxamine) exhibited a very strong protective effect. The role of vitamin C warrants further elucidation, as its sole administration could confer protection to the liver against IRI.

INTRODUCTION

Ischemia-reperfusion injury (IRI) is a major determinant in a variety of clinical conditions such as during liver resections and under conditions of hypoperfusion as in cases of hemorrhagic and other types of shock, as well as in liver transplantation. Generation of ROS and exhaustion of oxidative defense mechanisms are the main factors implicated in the pathogenesis of ischemia/reperfusion-induced liver damage \(^{(1,2)}\). Also, several studies have demonstrated the beneficial effect of antioxidant in protecting the liver against IRI \(^{(3,4,5)}\). The present study aimed to elucidate the pathomechanisms of pre-ischemic treatment with nitric oxide modulators (donor and inhibitor) and antioxidant (Allupurinol, iron-chelator (desferrioxamine) and vitamin C.

MATERIAL & METHODS

Animals

Seventy two Albino rats of both sexes, weighing about 220-320g, were used. Rats were housed one per cage at 22-24°C with a 12-h dark and light cycle, and were kept on water and standard laboratory chow \textit{ad libitum}.

The rats of the present study were subjected to warm \textit{in vivo} ischemia-reperfusion injury

Surgical procedure

A well established surgical technique was followed as described by KOOIJ \textit{et al}.\(^{6}\). Following pentobarbital-anesthesia (50mg/kg i.p.), a mid-line abdominal incision was performed and the liver hilum was gently exposed. A fine a traumatic vascular clip was applied at the pedicle supplying the left lobe of the liver. This allowed for selective interruption of the blood supplying the left lobe of the liver, whilst preserving that to the right lobe, thus avoiding the gastrointestinal congestion and the hemodynamic instability accompanying complete occlusion of the hepatic pedicle. Warm saline was injected in the abdomen, which is temporarily closed. The blood flow to the left hepatic lobe was occluded for 30min (ischemic phase), and the clip was removed -after reopening the abdomen- and this signaled the beginning of the reperfusion phase. The reperfusion lasted for 60min, after
which the animal was sacrificed and sampling was done.

**Methods and Techniques**

1-Sampling and assays

Sampling was done through right atrium puncture and blood was aspirated with a heparin coated syringe. Following blood sampling, the left lobe of the liver was promptly resected and stored at -80°C for subsequent evaluation of the oxidant-antioxidant status. The blood was centrifuged at 3000 rpm for 15min and the plasma was stored at -20°C till biochemical assay.

2-Biochemical assessment of liver integrity

To assess hepatic tissue integrity, plasma samples were analyzed for the activity of the intracellularly located hepatic enzymes aspartate aminotransferase (AST), alanine aminotransferase (ALT), and lactate dehydrogenase (LDH), denoting their release and thereby cellular disintegration. Their activity was spectrophotometrically measured using standard commercial kits according to the manufacturer's instructions.

3-Assessment of nitric oxide production

It is measured using biodiagnostic nitrite assay kit. The concentration was expressed in µmol/L

4-Assessment of the oxidant-antioxidant status

Rat livers were homogenized in 0.05mol/L phosphate buffer. The homogenates were centrifuged at 3500 rpm for 30min at 4°C and the supernatant was taken for the assays of malondialdehyde (MDA) and antioxidant enzymes activities.

To assess the oxidative stress, MDA in liver tissue was measured with OHKAWA'S method using thiobarbituric acid (Sigma) reaction. The unit of measurement was nmol/g liver tissue.

Catalase activity in liver tissue was measured using the method described by BEERS and SIZER. The enzyme activity expressed as U/mg protein.

Tissue Cu-Zn SOD activities were determined by the method of SUN et al., the result expressed as U/g protein.

Total glutathione was determined in liver homogenates according to methods described by ANDERSON. The unit of measurement was µmol/g liver tissue.

**Experimental Protocol**

1-Effect of warm ischemia-reperfusion injury

Rats were classified into 2 main groups (n = 8) according to the surgical procedure applied. The 1st group of rats was subjected to 30min of ischemia followed by 60min of reperfusion. Sham-operated rats underwent the same surgical procedure without interruption of the blood flow to the liver and served as controls.

2-Effect of NO-modulators

Rats were classified into 2 main groups (n = 8) according to the type of the NO-modulator used: the NO-inhibitor, L-nitro-arginine methyl ester (L-NAME), 50mg/kg (Sigma), and the NO-donor, L-Arginine (Sigma), in 3 different doses (100, 300, 500mg/kg). The drugs were administered intravenously 5min before ischemia-induction.
3-Effect of antioxidants

Rats were classified into 3 groups (n = 8) according to the type of the antioxidant used: allopurinol (Sigma) 50mg/kg, desferrioxamine (desferal) (Sigma) 100mg/kg, and vitamin C (Sigma) 100mg/kg. Allopurinol was administered intraperitoneally 10min before the induction of ischemia, whereas, desferal and vitamin C were administered intravenously 5min before the induction of ischemia.

Statistical Analysis

All data were expressed as mean ± SEM. Differences between groups were assessed using the Student’s t-test. Overall statistical significance was set at p<0.05.

RESULTS

1-Effect of warm IRI on measured parameters (figure 1, 2)

Plasma activities of liver enzymes: Following 30min warm ischemia and after the 1-h period of reperfusion, the liver enzyme activities increased from a base-line activity to reach high levels at the end of the reperfusion period. Analysis of the enzymatic activity in plasma revealed significantly higher levels after the 1-h reperfusion following the 30-min of ischemia in comparison to sham-operated animals.

Plasma nitrite level: Sham-operated animals had a plasma NO2_-level of 310±12µmol/l. On the other hand, rats subjected to ischemia-reperfusion had significantly lower level of plasma nitrite of 203±8µmol/L.

Hepatic lipid peroxidation: Thirty minutes of hepatic ischemia followed by 60min of reperfusion significantly induced lipid peroxidation in the liver, as manifested by the nearly 2-fold increase in the hepatic MDA-level from 152±7nmol/g in sham-operated livers to 320±3nmol/g.

Hepatic antioxidants: Ischemia-reperfusion caused exhaustion of the hepatic antioxidant defense, that is mirrored by the fall in the activity of the antioxidant enzymes catalase and superoxide dismutase on one hand and the decline in the content of the antioxidant substance glutathione on the other hand.

2-Effect of NO-modulation on warm ischemia/reperfusion injury (figure 3, 4)

A- Inhibition of NO-production

Plasma activities of liver enzymes: Treatment of rats with L-NAME while subjecting them to 30min warm ischemia followed by 1h of reperfusion caused a significant increase in the release of liver enzymes.

Plasma nitrite level: Estimation of the plasma NO2_-level revealed that the treatment with the NO-inhibitor, L-NAME, at the time of ischemia-reperfusion significantly suppressed the production of NO2_ (161±8µmol/l) in comparison to the I/R alone (203±8µmol/l).

Hepatic lipid peroxidation: A significant augmentation of hepatic lipid peroxidation was observed as a result of suppression of the NO-generation using L-NAME (400±11nmol/g), when compared to 320±3nmol/g in I/R-group.

Hepatic antioxidants: Suppression of the NO-production was accompanied with a manifest depletion and exhaustion of the
hepatic antioxidant defense as a result of ischemia-reperfusion. This attained significant level only in case of SOD, although the same trend was, also, observed in glutathione level and to a lesser degree in the catalase activity.

B- Stimulation of NO-production

Plasma activities of liver enzymes: Thirty minutes warm ischemia followed by 1h reperfusion induced the release of liver enzymes. Treatment of the animals with the NO-precursor (L-arginine) resulted in attenuation of the liver enzyme activities in a dose dependent manner. This protective effect was abolished applying the highest dose (500mg/kg), which even inflicted cellular injury.

Plasma nitrite level: Rats treated with L-arginine had a higher plasma nitrite level than those subjected to ischemia-reperfusion. It should be noted that the highest dose used was accompanied with a lower level of plasma nitrite compared to the intermediate (300mg/kg) and even the lowest (100mg/kg) dose.

Hepatic lipid peroxidation: both the lowest and intermediate doses of the nitric oxide generator (L-arginine) significantly counteracted the ischemia-reperfusion injury and suppressed the lipid peroxidation. On the other hand, using that agent at the highest dose level failed to protect the liver.

Hepatic antioxidants: L-arginine did not practically influence catalase activity, while in its lowest and intermediate doses induced significant elevation of SOD. The same was observed in case of glutathione, although it did not reach statistical significance.

3-Effect of antioxidants on warm ischemia/reperfusion injury (figure 5, 6)

Plasma activities of liver enzymes: Subjecting rats to antioxidant treatment while exposed to 30min warm ischemia followed by 1h of reperfusion diminished significantly the release of liver enzymes. That reduction was mostly noticeable with desferrioxamine treatment, and least with allopurinol.

Plasma nitrite level: Estimation of the plasma NO₃-level revealed that the treatment with the antioxidant, allopurinol, at the time of ischemia-reperfusion could not significantly modulate the production of NO₂ (205±6µmol/l) in comparison to I/R alone (203±8µmol/l). Conversely, both desferrioxamine and vitamin C proved to be effective in boosting the plasma nitrite level (278±3; 260±5µmol/l respectively). That increase was statistically significant.

Hepatic lipid peroxidation: A significant repression of the hepatic lipid peroxidation was observed as a result of the treatment with the antioxidants desferrioxamine and vitamin C (170±11; 183±13nmol/g), when compared to 320±3nmol/g in I/R-group. Suppression of the xanthine oxidase pathway using allopurinol did not influence the lipid peroxidation in the liver.

Hepatic antioxidants: Suppression of the XO-pathway failed to influence the antioxidant defense of the liver. On the contrary, desferrioxamine and vitamin C were accompanied with a manifest repletion of the exhausted hepatic antioxidant defense as a result of ischemia-reperfusion. This reached significant
level only in case of SOD, although the same trend was also observed in glutathione level and to a much lesser degree in the catalase activity. It should be noted that desferrioxamine is efficacious in counteracting the manifestation of I/R.

Figure 1: Effect of ischemia-reperfusion on plasma levels of AST, ALT, LDH, and NO₂ in Rats. Livers were subjected to 30min of warm ischemia followed by 60min of reperfusion. Sham-operated animals served as controls. Data are expressed as mean ± SEM, * p<0.05 vs. sham-operated.
Figure 2: Effect of ischemia-reperfusion on hepatic levels of MDA, catalase, SOD, and glutathione in Rats. Livers were subjected to 30min of warm ischemia followed by 60min of reperfusion. Sham-operated animals served as controls. Data are expressed as mean ± SEM, * p<0.05 vs. sham-operated.
Figure 3: Effect of NO-modulation on plasma levels of AST, ALT, LDH, and NO₂ in Rats. Livers were subjected to 30min of warm ischemia followed by 60min of reperfusion. Data are expressed as mean ± SEM, * p<0.05 vs. I/R.
Figure 4: Effect of NO-modulation on hepatic levels of MDA, catalase, SOD, and glutathione in Rats. Livers were subjected to 30min of warm ischemia followed by 60min of reperfusion. Data are expressed as mean ± SEM, * p<0.05 vs. I/R.
Figure 5: Effect of antioxidant-strategy on plasma levels of AST, ALT, LDH, and NO$_2$ in Rats. Livers were subjected to 30min of warm ischemia followed by 60min of reperfusion. Data are expressed as mean ± SEM, * p<0.05 vs. I/R.
DISCUSSION

Reperfusion following a certain period of ischemia imposes cellular injury and tissue damage. It is one of the most important reasons of aggravation of organ injury following a certain period of ischemia. The model of the partial hepatic ischemia/reperfusion is an appropriate model for in vivo studying of warm IRI. As the blood flow only to the left
hepatic lobe is interrupted, more blood
can flow to the right lobe and thus
obviating intestinal congestion,
 systemic hypotension, and the higher
mortality recorded with total clamping
of the hepatic pedicle\textsuperscript{(13)}. So, 30 min
ischemia and 60 min reperfusion was
made as this has been proven to yield
a good and reproducible pattern of
injury. Furthermore, ischemia for 30-
minutes is considered to be the
maximal duration permitted in clinical
settings for clamping the hepatic
pedicle, beyond which protective
measures have to be taken to
minimize reperfusion injury\textsuperscript{(14)}.

Various liver diseases are
associated with typical ranges of AST
and ALT levels. ALT levels often rise
to several thousand U/L in patients
with acute viral hepatitis. The highest
ALT levels – often more than
10,000 U/L – are usually found in
patients with acute toxic injury
subsequent to, for example, acetaminophen overdose or acute
ischemic insult to the liver. AST and
ALT levels usually fall rapidly after
an acute insult. Lactate dehydrogenase
is less specific than AST and ALT as
a marker of hepatocyte injury.
However, it is worth noting that LDH
is disproportionately elevated after an
ischemic liver injury\textsuperscript{(15)}.

Thirty minutes of ischemia
followed by 60 minutes of reperfusion
could inflict signs of reperfusion
injury, manifested by release of
hepatic enzymes into the circulation,
depletion of the presumably protective
NO, and exhaustion of the antioxidant
defense. This proves that 30 minutes
of warm hepatic ischemia are
sufficient to induce hepatic cellular
injury, and that injury manifests itself
as early as 60 minutes following the
ischemia. This is in accordance with
the resul of Moriisue \textit{et al.}\textsuperscript{(16)} who
reported the same observation.

The present results have
demonstrated that NO-depletion using
L-NAME was accompanied by
deterioration of the liver condition and
augmentation of the hepatic injury;
while its repletion, up to a certain
limit, was associated with
amelioration of that injury and
restitution of the hepatic antioxidant
defense. This could be ascribed to the
well known functions of NO as a
vasodilator and anti-platelet agent as
well as its antioxidant properties, thus
affording cellular protection\textsuperscript{(17)}.

The enzymatic inhibition of
cellular nitric oxide synthase with L-
NAME elicited a greater damage,
presumably due to other vascular
effects of L-NAME on blood flow\textsuperscript{(18)}.
Because neutrophils are not involved
in early hypoxia-reoxygenation injury,
we can rule out an effect of L-NAME
on polymorphonuclear leukocytes.
Protection of hepatocytes by
endogenous NO against hypoxic
damage may be related to the
conservation of cellular bioenergetics
for maintenance of tissue homeostasis
during hypoxia via NO-mediated
inhibition of the use of
mitochondrially derived ATP for
nonessential metabolic processes\textsuperscript{(19)}.
The finding that an exogenous NO
donor afforded protection against that
injury during reoxygenation indicates
that NO is, in fact, cytoprotective in
the reoxygenation phase.

It has, also, been reported that
NO has anti-apoptotic and anti-
necrotic actions \textit{via} many
mechanisms, one of which is through
blocking the mitochondrial permeability transition, a very early step in the death pathway\textsuperscript{(20)}. Moreover, NO has a proven inhibitory action on the deleterious effects of Kupffer cells in the setting of hypoxia and reoxygenation\textsuperscript{(19)}.

The observation that L-arginine when administered in a high dose induced cellular injury rather than protection, could be explained by the formation of the highly injurious per-oxo-nitrite radical, which is responsible for cellular injury. This assumption is supported by the finding that plasma nitrite level is relatively reduced, denoting its consumption, thus being unavailable to detection. It is worthy to mention that Ferraz et al.\textsuperscript{21} reported a paradoxical effect of a high dose of L-arginine (500mg/kg) in a model of ethanol-induced gastric mucosal injury. This would substantiate the herein reported findings.

The current results showed that the allopurinol seemed to be ineffective in reducing hepatic injury. This would cast doubt on the mission of xanthine oxidase in liver injury and the cellular defence mechanism in hepatic tissue. In fact, Metzger et al.\textsuperscript{13} reported the same observation and suggested that xanthine oxidase plays a limited role in the generation of reactive oxygen species in the liver.

Another evidence comes from the work of Fredriks and BOSCH\textsuperscript{22}, who studied the role of xanthine oxidase in the induction of liver cell damage during reperfusion after ischemia and the potential of the antioxidant system in a model of in vivo ischemia of rat liver followed by 1h reperfusion by the use of enzyme histochemistry. Based on decreased lactate dehydrogenase activity in certain areas of liver parenchyma, cell damage could already be detected at 1h reperfusion after ischemia. Incubations performed on serial sections showed that the same areas contained decreased activities of xanthine oxidoreductase, xanthine oxidase, catalase and glucose-6-phosphate dehydrogenase. Some individual cells in the undamaged liver parenchyma expressed a very high glucose-6-phosphate dehydrogenase, which suggests that these cells have a good defence against oxidative stress. It was concluded that oxygen radicals derived from xanthine oxidase do not play a decisive role in the induction of cell damage immediately at reperfusion after ischemia. However, it cannot be excluded that xanthine oxidase present in the blood stream could give rise to the development of additional damage later on. This would support the present finding regarding the ineffectiveness of allopurinol administration in reducing IRI in the present model.

On the other hand, Rhoden et al.\textsuperscript{23} reported a beneficial effect of allopurinol when administered to Wistar rats before subjecting them to 50min of ischemia followed by reperfusion for 1 hour. The severity of ischemia and the difference in strain could account for the observed discrepancy.

A critical point to be considered is the kinetic of conversion of xanthine dehydrogenase to xanthine oxidase. Engerson et al.\textsuperscript{24} reported that in response to global ischemia, tissue xanthine dehydrogenase was
converted to xanthine oxidase in all tissues with half-times of conversion at 37°C of ~3.6, 6, 7, and 14h for the liver, kidney, heart, and lung, respectively. They provided evidence that a proteolytic mechanism is responsible for that conversion in ischemic rat liver. Furthermore, Brass et al. 25 found that in a functionally intact, isolated perfused rat liver model, the mean % xanthine oxidase activity increased as a function of both the duration (25 to 45% in 3h) and degree of hypoxia. This process was markedly accelerated in ischemic liver by an overnight fast (45 vs. 30% at 2h), and by imposing a short period of in vivo ischemia (cardiopulmonary arrest 72%). Moreover, only under these conditions was there a significant rise in the xanthine oxidase activity due to the conformationally altered xanthine dehydrogenase molecule (18%), as well as concomitant morphologic injury. From these two kinetic studies, one can infer that the duration of ischemia and subsequent reperfusion in the present model might be too short to allow for an immense conversion of xanthine dehydrogenase to xanthine oxidase and subsequently for a manifest action of allopurinol.

The results of the current study clearly demonstrated that desferrioxamine is highly effective in attenuating liver injury following warm ischemia and reperfusion. The present results match those of Omar et al. 26 and Colletti et al. 27 who communicated the efficacy of iron-chelation in attenuating hepatic IRI.

Another applied approach was the use of vitamin C in a trial to scavenge the generated ROS. That approach proved successful in ameliorating tissue damage as a result of warm ischemia and reperfusion. In fact, such a beneficial effect of vitamin C was previously described by Seo and Lee 28. Vitamin C exerts an antioxidant action both by itself and by interacting with reduced glutathione or vitamin E. 29-30. It repairs vitamin E, thereby permitting vitamin E to recycle 31. Ascorbic acid scavenges reactive oxygen species such as superoxide radical (O2·−), hydrogen peroxide (H2O2), hydroxyl radical (OH·), singlet oxygen, and hypochlorous acid (HOCl), which is produced from H2O2 and Cl− via myeloperoxidase present in neutrophils, in vitro 32-33.

In addition, vitamin C scavenges peroxynitrite (ONOO−), a highly reactive nitrogen oxide species, which is produced by the reaction of O2·− and nitric oxide in vitro 34-35. It is known that vitamin C scavenges O2·− generated by activated NADPH oxidase in neutrophils and in the hypoxanthine-xanthine oxidase (XO) system in vitro 36. It is, also, known that vitamin C prevents neutrophil adherence to endothelium by scavenging ROS derived from activated neutrophils in vitro 37. Furthermore, a stable derivative of vitamin C, ascorbic acid 2-glucoside, was found to possess antiapoptotic properties after cold ischemia-
reperfusion injury in rat liver transplantation (40).

The lowest dose of L-arginine (100mg/kg) was chosen in order to detect minor changes in response. Additional application of allopurinol did not significantly change the response to L-arginine alone. This would raise the question regarding the role of xanthine oxidase in liver injury.

Vitamin C, a known reducing agent, could reduce nitrites (the end product of NO) back to its original form (NO), which in its excess would react with oxygen free radicals to form the highly injurious peroxynitrite radical. That speculation is reinforced by the detected reduction of nitrite level.

It could be concluded that reperfusion following warm ischemia inflicts cellular damage due to the generation of ROS and the exhaustion of the liver defence mechanisms. Activation of the nitric oxide pathway up to a certain limit ameliorates that injury, while its overactivation augments that injury. Xanthine oxidase seems to play a minor role in the evolution of hepatic IRI, whereas as iron-chelation with desferrioxamine markedly inhibits cellular injury. Vitamin C ameliorates IRI. A judicious multimodal approach seems to be an effective tool to abrogate warm IRI of the liver.

REFERENCES


