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Rafael Chavez-Cartaya
Neville V. Jamieson
Pablo Ramirez
Juana Marin
Gilda Pino-Chavez

Free radical scavengers to prevent reperfusion injury following experimental warm liver ischaemia. Is there a real physiological benefit?

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R. Chavez-Cartaya, M.D., Ph.D (✉)
N.V. Jamieson, FRCS
Department of Surgery,
Addenbrookes Hospital,
Box 202, Hills Road,
Cambridge CB2 2QQ,
United Kingdom

P. Ramirez, M.D.
J. Marin, M.D.
Department of Surgery,
University of Murcia Medical Faculty,
Hospital V. Arrixana, El Palmar,
Murcia, Spain

G. Pino-Chavez, M.D., M.Sc.
Department of Pathology, Imutran,
Papworth Hospital, Cambridge CB3 8RE,
United Kingdom

Abstract Free radical scavengers have been utilized to prevent the consequences of ischemia, however, results do not seem conclusive. In our study we analyzed the blood flow, function, and histology of rat liver tissue after warm liver ischemia, in order to assess the effect of free radicals in liver reperfusion injury. N-acetyl cysteine (NAC), tocopherol, allopurinol, and superoxide dismutase (SOD), pharmacological agents expected to protect from injury mediated by free radicals, were investigated. Laser Doppler flowmetry and photometry were utilized to measure post-ischemic microcirculatory changes as an expression of ischemia-reperfusion injury in a model of segmental liver ischemia in the rat, with an ischemic time of 45 min. Galactose elimination capacity, ALT and histology were used to assess the functional and morphological consequences of ischemia after 24 h of reperfusion. The overall mean blood flow over 1 hour after reperfusion was of 33.9% (SD 11.2) of the normal, non-ischemic control. NAC (31.2% SD 10.9) did not show any protective effect and in some cases the effect seemed to be negative. Tocopherol (41.7% SD 5.1) marginally improved post ischemic liver tissue blood flow. Treatment with allopurinol did not show any beneficial effects (37.5% SD 14.2). Only animals treated with SOD showed an improvement of the post ischemic liver microcirculation (57.9% SD 14.4)($P < 0.001$) and function. Only SOD produced statistically significant differences in galactose elimination capacity, compared with those of the ischemic control group. This moderately protective effect of SOD is encouraging, however, the relevance of all these compounds in a broader pathophysiological setting remains unproven.

Key words Liver ischemia and reperfusion · Free radicals · NAC · N-acetyl cysteine · Tocopherol · Allopurinol Superoxide Dismutase · SOD Laser Doppler

Introduction

It is clear that ischemia-reperfusion tissue injury is primarily due to the ischemia itself; however, the paradox of reperfusion is that the reestablishment of blood supply can produce continued and often intensified tissue injury.

Reactive oxygen intermediates generated after reperfusion seem to be responsible for at least part of this reperfusion injury. In 1981, McCord proposed a mechanism to explain this apparent role of Oxygen in the post-ischemic tissue injury [37]; the key feature of the suggested mechanism is the conversion of the enzyme xanthine dehydrogenase to xanthine oxidase which generates the superoxide radical via univalent reduction of O_2 in the final catabolic route of ATP and ADP [3]. Superoxide is a free radical, a highly reactive, transient

molecule with an odd number of electrons. The oxygen free radicals formed during reperfusion, after ischemia, readily attack the unsaturated bonds of free fatty acids in the phospholipid bilayer of the cell membrane. Peroxidation of lipids spreads through the cell membrane and can result in fragmentation and severe structural and functional alterations, including the activation of endothelial and Küpffer cells, with involvement of adhesion molecules [19], neutrophils [18] and complement [7]. The endothelium is subjected to ischemia and reperfusion injury while it also participates in its escalation. The contribution of free radicals from neutrophils invading post-ischemic tissue aggravates the process and may be the determinant of additional endothelial activation and leukocyte adherence [32, 45].

The biochemical methods of reducing the free radical concentration include the deployment of free radical scavengers with low molecular weight and complex enzyme systems. In fact, any molecule that reacts with a free radical can be termed a "scavenger", thus, cell components such as sugars, unsaturated aminoacids, sulfhydryl containing aminoacids and unsaturated fatty acids can also scavenge and be modified by free radicals. In the case of hemoproteins such as oxy-hemoglobin, either $O2^{\bullet}$ or H2O2 can react with the iron to form methemoglobin. Smaller molecules such as glutathione, by enzymatic mechanisms, can reduce H2O2, lipid peroxides, disulfide, ascorbate and free radicals. Glutathione is particularly important in liver ischemia and reperfusion since the hepatocyte is the most important source of this tripeptide [20].

Free radical scavengers have been utilized, alone or in combination with other agents to prevent the consequences of ischemia in different models and situations, however, data do not seem conclusive, and the literature is often contradictory. Important differences between the mechanisms of cold- and warm ischemic injury, and the different sensitivity to ischemia and reperfusion of cells from the various compartments of the liver, may explain in part these contradictory results [4, 13].

In order to comparatively assess the effect of free radical scavengers in liver ischemia, pharmacological agents expected to protect against free radical mediated injury, N-acetyl cysteine (NAC), tocopherol, allopurinol, and superoxide dismutase (SOD were investigated in the same experimental model of rat liver warm ischemia. The post ischemic microcirculatory blood flow and the post reperfusion liver function were analysed in the rat as an expression of ischemia and reperfusion injury to assess a potentially protective effect of these compounds.

Material and methods

All experimental procedures were performed according to the Code of Practice for Scientific Procedures in Animals from the University of Cambridge and the Guidance on the Operation of the Animals (Scientific Procedures) Act 1986, Home Office U.K.

Male Sprague-Dawley rats (Harlan-OLAC Inc. Bicester U.K.), weighing 200 to 250 gr were utilised for this study. The experimental procedure consisted in the temporary interruption of blood flow to the left lateral and medial lobes of the liver [2], and subsequent reperfusion after 45 min of ischemia. Rats were fasted overnight and anaesthetised using fentanyl-fluanysone (Hypnorm^{TM}) 0.4 ml/kg IM, and diazepam 2.5 mg/kg intraperitoneally as an induction dose. Maintenance doses of fentanyl-fluanysone, 0.2 ml/kg and Diazepam, 1.5 mg/kg were injected IM as needed. An arterial line was placed in the carotid artery for blood pressure monitoring during the experiment. Normal saline (1 ml/100 g body weight per h) was administered intravenously during the procedure.

The operation was carried out through a midline abdominal incision. The liver was exposed and mobilised, including portal vein, hepatic artery and common bile duct, and then isolated from any possible collateral blood supply by dividing all of its peritoneal attachments. The hepatic hilum was approached, and all arterial, portal, and biliary branches to the medial and left lateral lobes were clamped with a microsurgical clip [8].

During ischemia the rats remained anaesthetised, and after the pre-defined period of 45 min, the clamp was removed and the liver allowed to reperfuse. Following reperfusion, the rats either remained anaesthetised during the measurements in the immediate reperfusion period, or the laparotomy wounds were sutured and the rats allowed to recover, with free access to water and regular food until the experiment was concluded in a second stage. The waiting period for the second stage was 24 h.

Assessment of the liver tissue blood flow and hemoglobin saturation

To record the liver tissue blood flow, all the peritoneal attachments were thoroughly dissected to move the liver away from the diaphragm and so minimise motion artefacts due to respiratory movements. Laser-Doppler Flowmetry was utilised for the study (Laser-Doppler Perfusion Monitor. Periflux PF3, Perimed Inc. U.K.). The probe was placed in contact with the anterior surface of the medial liver lobe, and a basal recording of liver blood flow was performed before clamping for 5 min after stabilisation of the signal. After clamping, the laser signal from the liver surface was also recorded to gain the physiological zero. After the period of ischemia was completed, the microsurgical clamp was removed, allowing reperfusion of the affected lobes, and continuous recording of post ischemic blood flow for one hour of reperfusion. All the recordings were made from the same area of the medial liver lobe. This method does not measure the tissue blood flow of the liver as a whole, which would be ideal. However, the location of the probe in a fixed point of the liver surface throughout the experiment allows the continuous record of the flow signal to be considered representative of the microcirculatory changes on that point during the different stages of the procedure [9].

Reflectance photometry allows continuous measurement of changes of hemoglobin saturation in liver tissue, and is useful in the study of oxygen delivery to the post ischemic liver. The procedure is performed simultaneously by laser Doppler flowmetry, using the equipment already described, as demonstrated elsewhere [10]. The reflectance is measured as total backscattered light level, expressed as arbitrary units on a scale of 0–10. During ischemia

and reperfusion, this system measures relative changes in liver reflectance; the pre- ischemic light level is considered to be 100%.

Post-ischemic liver function

Galactose elimination capacity was assayed 24 h after reperfusion, in order to study the functional hepatocyte mass of the ischemically damaged lobes of the liver. At this stage, the rats were re-anaesthetised, the laparotomy wound was opened, and microvascular clips were applied to the vessels supplying the right and caudate lobes, which were thus excluded from the circulation [8]; the medial and left lateral lobes, previously ischemic, remained perfused for the functional test. Galactose 20% was injected in a saturating dose of 0.5 g/kg in an IV bolus. After injection, blood samples were taken from the tail at 5, 15, 30, 45 and 60 min after injection. Bladder urine was also collected at the end of the experiment to measure urinary excretion of galactose. A colorimetric galactose dehydrogenase (GADH) method was used to measure galactose levels in serum and urine [14]. A standard spectrophotometric method was utilised for ALT assays (Refletron™, Boehringer, Mannheim). Samples of the affected lobes were fixed in 10% formalin and embedded in paraffin. Sections of 3 μm micrometers were made and stained with H&E for histological examination.

The experimental groups

A non-ischemic control group ($n = 8$) was subjected to 30 s of ischemia, in order to mimic the effect of application and removal of the vascular clamp.

All the remaining groups were subjected to 45 min of ischemia.

Untreated ischemic controls ($n = 15$), which were subjected to 45 min of ischemia.

N-Acetyl cysteine (Fluimucil™, Zambon S.A., Spain) was administered in a dose of 300 mg/kg body weight intramuscularly, 30 min before ischemia ($n = 8$).

α-Tocopherol (T-3251, Sigma, Poole, U.K) was administered in three daily intraperitoneal doses of 10 mg/kg body weight, the last 30 min before liver ischemia ($n = 8$).

Allopurinol (A-8003, Sigma, Poole, UK) was administered in two doses of 50 mg/kg body weight, the first dose intraperitoneal, 12 h before ischemia, the second dose intravenously, 5 min before the placement of the clamp and onset of liver ischemia ($n = 8$).

Superoxide dismutase obtained from bovine erythrocytes (S-5395, Sigma, Poole, U.K), was given intravenously in a single dose of 3000 IU per rat, 5 min before the onset of ischemia ($n = 8$).

Statistics

For laser Doppler flowmetry all recordings up to 1 hour post reperfusion were collected and analyzed. The pre-ischemic values of perfusion in all the experiments were normalized to 100%, and the ischemic value was considered zero. The area under the curve was calculated and expressed AS LDF Perfusion units × minute (1 SD). For laser photometry, the areas under the curves of reflectance were calculated in every experiment and expressed as a percentage of the pre ischemic level of total backscattered light (1 SD). Galactose elimination capacity was calculated from the regression line obtained over one hour after injection in each experiment, and was expressed in mg/minute. ANOVA test followed by Scheffé's S test were used for hypothesis testing. The results of ALT were analysed using the Mann-Whitney rank sum test.

Results

N-acetyl cysteine (NAC)

Pre-treatment with NAC did not show any significant difference from the ischemic control group in terms of post ischemic blood flow 31.2% (SD 10.9). Hemoglobin saturation dropped to a mean of 46% at 12 min, and the overall saturation during one hour of recording was of 55.8% (SD 6.5), which is below the values of the correspondent control group (Fig. 1). Galactose elimination or ALT were not significantly different from those of the ischemic control group (Fig. 2).

α-Tocopherol

Pre-treatment with α-tocopherol resulted in a post ischemic blood flow of 41.7% (SD 5.1). This value was different from that of the ischemic non-treated group and reached statistical significance ($P = 0.046$) (Fig. 1). The saturation of hemoglobin of the liver tissue dropped to 68% at 12 min and then recovered to 92% at the end of the period of observation. The mean hemoglobin saturation was 80.2 (SD 9.5); $P = 0.023$ vs control group of 45 min of ischemia. The elimination capacity of galactose was of 0.12 mg/min (SEM 0.02), which was not significantly different from that of the ischemic control group. ALT levels of 486 IU (SEM 45) were also not significantly different from those of the ischemic control group (Fig. 2).

Allopurinol

Allopurinol showed an average post ischemic blood flow of 37.5% (SD 14.2), which seemed slightly better than that of the ischemic control group, but failed to reach statistical significance. Saturation of hemoglobin of 76.35% (SD 12.1) was not significantly different from that of the control group (Fig. 1). Allopurinol showed an elimination capacity of galactose of 0.12 mg/min (SEM 0.02) which seemed slightly better than that of the ischemic control group, but failed to reach statistical significance. ALT levels were not significantly different from those of the ischemic control group (Fig. 2).

Superoxide dismutase (SOD)

In the group subjected to 45 minutes of hepatic ischemia and treated with SOD, the mean flow level was 57.9% (SD 14.4). The mean value at the end of the experiment was of 58.92% (SD 4.09). This group showed statistically significant differences when compared with the un-

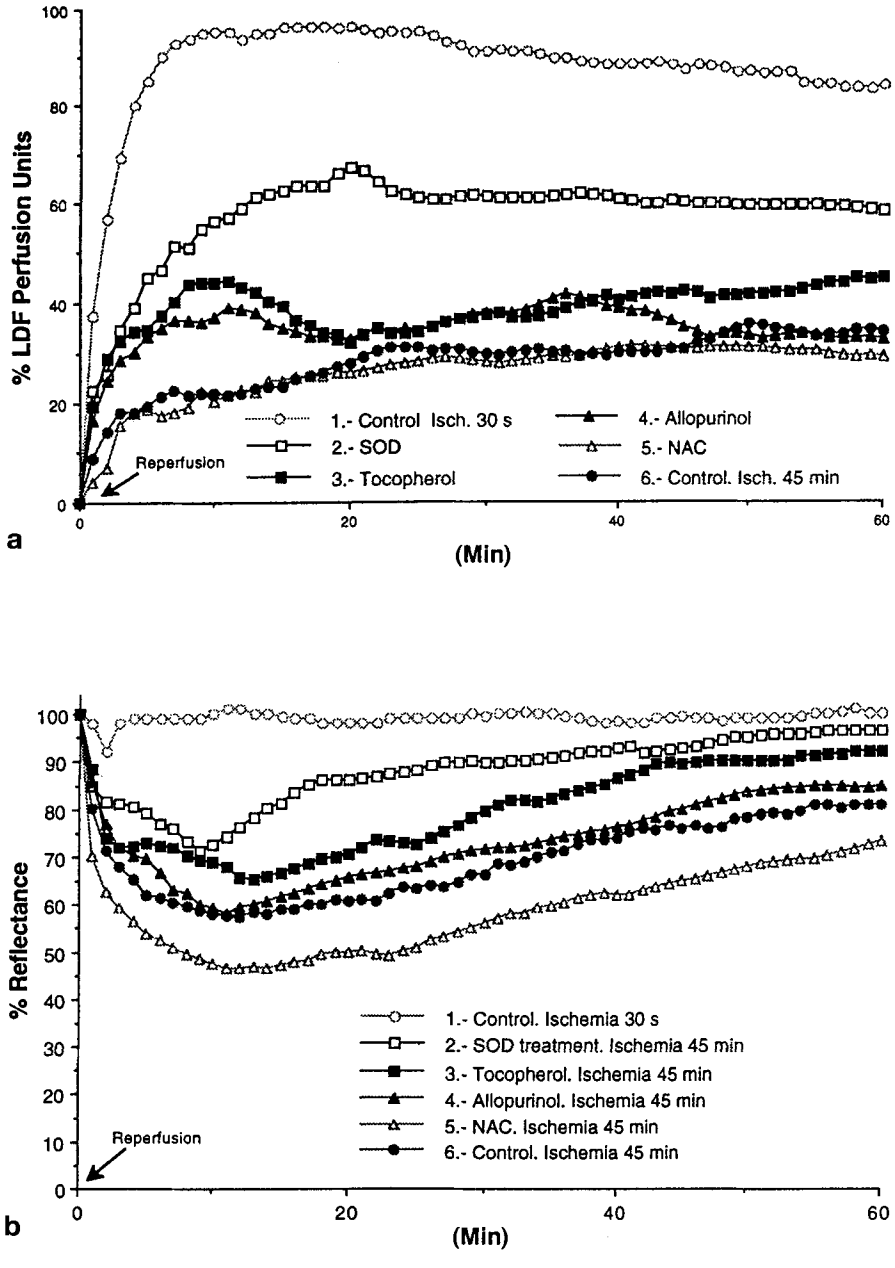

Fig. 1 a, b **a** Effect of various antioxidants on the post ischemic liver blood flow in the rat following 45 min of warm ischemia, measured by laser Doppler flowmetry (LDF) after release of the clamp. 2 vs 6 $P < 0.001$; 3 vs 6 $P = 0.046$. **b** Effect of various antioxidants on the post-ischemic liver hemoglobin saturation in the rat, following 45 min of warm ischemia, measured by laser photometry during one hour after release of the clamp. 2 vs 6 $P < 0.001$; 3 vs 6 $P = 0.023$

treated ischemic control group ($P < 0.001$) and with the non-ischemic control group ($P < 0.001$) (Fig. 1).

Regarding the changes in post ischemic liver hemoglobin saturation, in the group treated with superoxide dismutase, the mean saturation of hemoglobin was 84.7% (SD 9.8) throughout the experiment. The minimal hemoglobin saturation was 71.3% (SD 14.1) at 10 min after reperfusion, and recovered to 96.4% (SD 6.6) after one hour. There were statistically significant differences between this group and the control group of 45 min of ischemia ($P < 0.01$) (Fig. 1). Galactose elimination capacity of 0.38 mg/min (SEM 0.03) showed statistically significant differences compared with the untreated ischemic control group ($P < 0.001$) and with the non-ischemic control group ($P = 0.048$). ALT levels of 391 IU (SEM 72) were significantly lower than the ischemic control group ($P = 0.043$) (Fig. 2).

Histological findings of the post-ischemic rat liver

In the experimental groups treated with tocopherol, allopurinol or NAC, lesions could not be distinguished from those of the untreated ischemic rats; oedema and

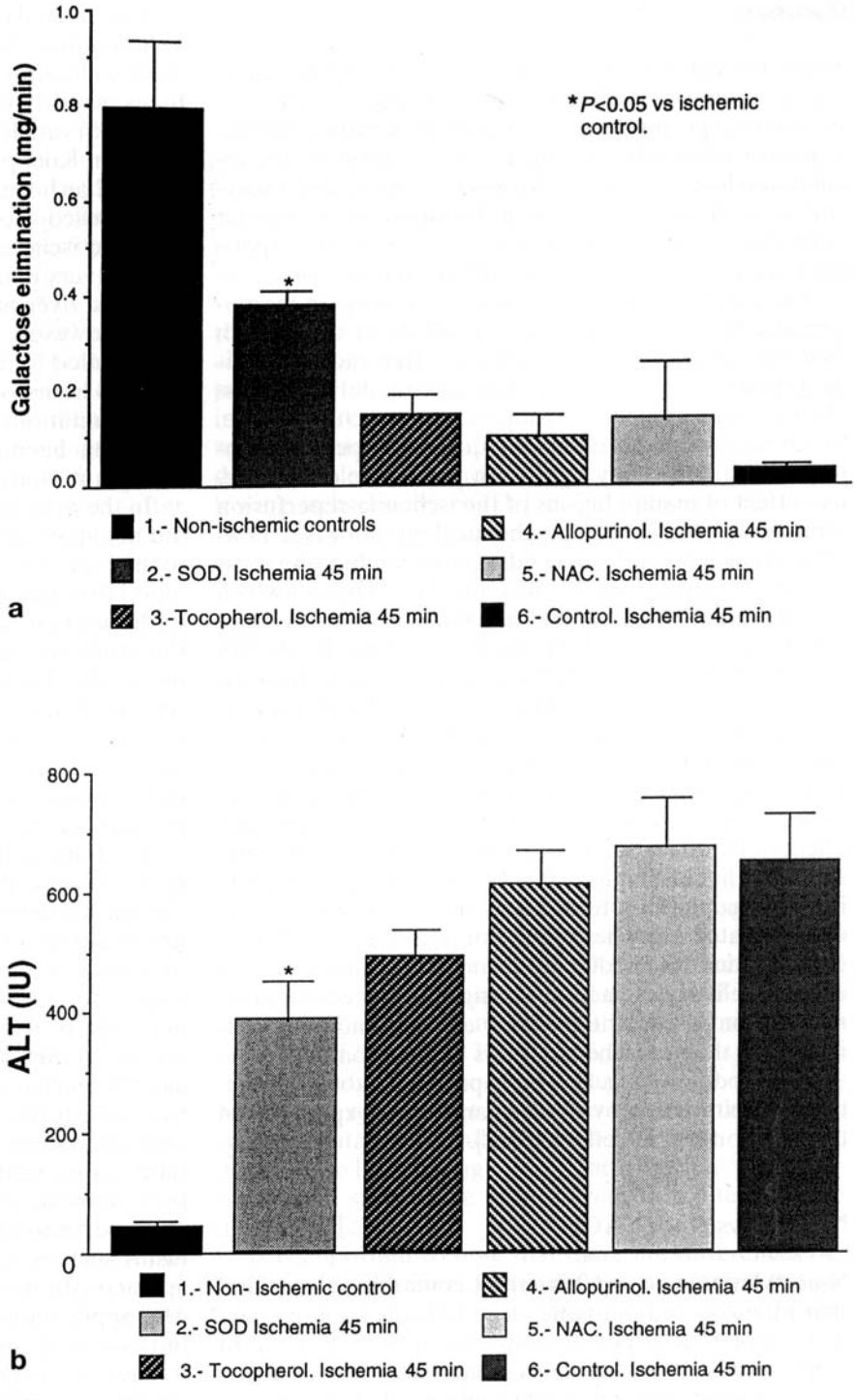

Fig. 2 a,b **a** Effect of various antioxidants on the galactose elimination capacity in the rat after 45 min of liver ischemia and 24 h of reperfusion (SEM). **b** Effect of various antioxidants on the ALT serum levels in the rat after 45 min of liver ischemia and 24 h of reperfusion (* $P < 0.05$ vs ischemic control)

multifocal necrosis with presence of neutrophil infiltration were found, and in some cases, multifocal necrosis was more extensive and confluent. Endothelial cells looked enlarged, and neutrophils were present in the vascular lumen and within the liver parenchyma. In the group treated with SOD, reperfusion injury was much less evident than in the control group that was subjected to the same 45 min of ischemia. Oedema were visible, but most samples showed no necrotic changes. Neutrophils were present in the vascular lumen.

Discussion

There is evidence that reactive oxygen intermediates are generated in ischemia and reperfusion, however, the physiological relevance of these free radicals in the sequence of events of ischemia-reperfusion injury remains unclear. The protective effect of free radical scavengers in the context of organ transplantation has not been definitively clarified, and the numerous experimental and clinical studies are often contradictory.

The purpose of this study was to investigate the potentially protective effect of four different drugs, with different mechanisms of action over free radical-mediated injury, in the same experimental model of liver ischemia and reperfusion, using the same ischemic time, which has proven to effectively quantify reperfusion injury and to detect any significant protective or destructive effect of manipulations of the ischemia-reperfusion sequence [7, 9–12]. The biochemical effect of free radical scavengers in ischemia and reperfusion has previously been demonstrated and was also the setting in which the pharmacological agents utilized in this study were selected [5, 6, 15–17, 22–24, 30, 32–40, 42, 43, 46, 50–54].

Laser Doppler flowmetry is a useful procedure for the study of liver blood flow in this rat model of warm ischemia and reperfusion. The alteration observed in the blood flow of liver tissue after reperfusion is an expression of the microcirculatory events that occur in the liver following ischemia-reperfusion [9]. The immediate effect of ischemia and reperfusion on the oxygen delivery to the liver is made visible by the continuous recording of hemoglobin saturation by surface photometry, as demonstrated elsewhere [10]. An initial drop of hemoglobin saturation is due to an increased imbalance of oxygen delivery/extraction during the immediate post-reperfusion period; the recovery of the hemoglobin saturation to the pre-ischemic levels depends on the recovery of blood flow (Fig. 1). The hepatic galactose elimination capacity test provides a quantitative expression of the functional liver cell mass [12].

N-acetylcysteine (NAC)

N-acetylcysteine (NAC) is a thiol-containing compound that interacts and detoxifies free radicals by non-enzymatic reactions either by conjugation or reduction. In many tissues, NAC is deacetylated to form cysteine, which supports glutathione biosynthesis. In high concentrations NAC can protect cells against oxidative damage by two mechanisms; by reacting directly with H2O2 extracellularly as a direct antioxidant, and by increasing the cytoplasmic reserve of glutathione [1, 40, 53]. It has also been suggested that sulfhydril donors such as NAC may protect nitric oxide from oxidation, therefore prolonging NO half-life and potentiating its effect [26].

NAC is said to have a strong protective effect on liver ischemia and reperfusion, by its antioxidant effect, by restoring glutathione levels in the hepatic cells [17, 53] and by its protective effect on endothelial dependent relaxation [48] which protects the post ischemic blood flow to the liver. Koeppel et al. reported some protective effect of NAC on hepatic microcirculation, assessed by intravital microscopy on experiments carried out in both warm and cold ischemic conditions [23, 24], and Dunne et al. showed very marginal effects of NAC in the isolated perfused rat liver after sequential cold and warm ischemia [15]. However, Walcher et al. [54] demonstrated that NAC failed to improve early microcirculatory changes, after 20 hours of cold ischemia in a model of rat liver transplantation. NAC also failed to show any beneficial effect on haemodynamics and graft function in liver transplantation in patients with chronic liver disease [47].

In the present study, treatment with N-acetylcysteine did not show any significant difference when compared to the ischemic controls. In some cases the post ischemic blood flow and hemoglobin saturation were worse than in the untreated controls (Fig. 1). There are no data in this study to explain the lack of protection of NAC in our model, however, it seems plausible that an ischemic time of 45 min may not be long enough to deplete the reserves of glutathione of hepatic cells [21]. A protective effect caused by NAC improving those reserves is not obvious. According to reported quantitative comparisons of free radicals and liver injury [29], it seems highly unlikely that free radicals are the primary mechanism of parenchymal cell injury during reperfusion, although it cannot be ruled out that free radicals may be important as a damaging mechanism in a limited compartment of the liver, e.g., endothelial cells, close to sources of reactive oxygen such as Kupffer cells and neutrophils. However, the enhanced release of hepatocellular GSH functions as a defensive mechanism against reactive oxygen species generated by inflammatory cells during ischemia and reperfusion. This internal defense system of the liver may be of general importance in preventing, or at least limiting, liver damage by reactive oxygen [28].

Reperfusion injury is complex, and involves mechanisms such as cell adhesion, neutrophil- and macrophage activation and numerous inflammatory mediators. The application of information obtained from related, but essentially different, situations in which NAC seems to have a beneficial effect, such as paracetamol hepatotoxicity, [49] must be critically evaluated.

α-Tocopherol

α-Tocopherol, a liposoluble, free radical scavenger of low molecular weight, is believed to seep into membranes, reducing lipophilic free radical species to a less

toxic form [16]; it functions in vivo as a free radical scavenger and as an antioxidant. It has been demonstrated in a model of rat liver ischemia and reperfusion that pre-treatment with α-tocopherol is effective in ischemic liver injury [31]. In our study, using the same dosage of the drug, α-tocopherol-treated rats had a mean post ischemic liver blood flow that was slightly better than that of the ischemic control ($P = 0.046$); the changes in hemoglobin saturation also showed a protective effect of α-tocopherol pre-treatment ($P = 0.023$) (Fig. 1). However, galactose elimination capacity, ALT and histology did not show any significant beneficial effect of pre-treatment with α-tocopherol (Fig. 2). These results did not show a strong protective effect of α-tocopherol after 45 min of liver ischemia, compared to the untreated control group that underwent the same length of ischemia. In a study by Marubayashi et al. [31], α-tocopherol improved the 30 days' survival of rats after 90 min of ischemia from 0 to 45%; it is possible that the protective effect of α-tocopherol against free radical injury was less obvious in our study, using shorter periods of ischemia (45 min); hepatocytes are well supplied with antioxidants [21, 55], thus, the periods of ischemia might need to be longer to demonstrate a free radical injury that could be ameliorated by the protective effect of antioxidants.

Allopurinol

During ischemia, the catabolism of high-energy phosphates generates products such as AMP, inosine, hypoxanthine, xanthine and adenosine that accumulate in the ischemic cell. Alteration of Ca^{++} transport activates a cytosolic protease, presumably calpain [1, 11, 37, 38, 46, 55] which changes the enzyme xanthine dehydrogenase into xanthine oxidase. Hypoxanthine, accummulated during ischemia, reacts with molecular oxygen that appears upon reperfusion, generating a burst of oxygen free radicals. Allopurinol, an inhibitor of xanthine oxidase should therefore limit the production of oxygen-derived free radicals from this source, and thereby protect the tissue against this component of reperfusion injury. Differing reports regarding the efficacy of allopurinol in in-vivo ischemia and reperfusion [35, 46, 50] may be attributed to the dosing strategies employed, since allopurinol inhibits xanthine oxidase in a competitive fashion and therefore may not be effective in the presence of a large amount of substrate [46]. In addition, free radicals are not the sole mechanism of reperfusion injury, hence the effect of allopurinol may be masked by other damaging mechanisms of reperfusion injury. Moreover, hypoxanthine-xanthine oxidase is not the only source of free radicals, allopurinol alone may not be the solution to free radical injury. Treatment with allopurinol in this study did not show beneficial effects on any of the parameters studied; this was not unexpected, since reports show a wide variation of results.

Superoxide dismutase

Superoxide dismutase, a well known free radical scavenger, is a metalloprotein that dismutes the superoxide anion $O2^-$ to H2O2, which in turn is converted to the less harmful products H2O + O2 by catalase, another enzyme whose activity is increased according to the rate of H2O2 production [16]. The utilisation of SOD, either alone or associated with catalase, has been described as protecting the liver against ischemia-reperfusion injury in various systems [25, 41]. Marzi, using intravital fluorescence microscopy, demonstrated a reduction of endothelium-leukocyte adherence in hepatic sinusoids following ischemia by SOD [33, 34]. The mechanism is likely to be the prevention of endothelial activation by free radicals. Another mechanism proposed is the prevention of the inactivation of Nitric Oxide by free radicals by means of SOD [36, 39], in this way, endothelial dependent vasodilation is protected.

In this study, SOD dosage was based on the work of Nauta et al. [41]. The group of rats treated with superoxide dismutase (SOD) had a post ischemic liver blood flow significantly better than the untreated group after 45 min of ischemia (Fig. 1). The recovery of hemoglobin saturation also reached statistical significance. Galactose elimination capacity and ALT were also better than in the untreated control group, although the significance in the case of ALT was rather marginal ($P = 0.043$). These results demonstrated a discrete beneficial effect of pre-treatment with SOD in liver ischemia and reperfusion, and correspond to a number of studies in which SOD was found to protect the liver from reperfusion injury [22, 25, 43]. In conclusion, the data presented by this study are primarily negative. SOD and tocopherol had some effect on the parameters investigated, and the data are significant in some cases. However, the possible long lasting effect of attenuation of reperfusion injury, such as the reduced rejection rate might be of interest [27].

It is important to note that there are differences between cold and warm ischemic injury, and that the results of this study on warm liver ischemia must be applied to the situation of transplantation, which is a mixed model of cold and warm ischemia, with great caution. [4, 13]. The presence of free radicals in reperfusion injury has been well documented, and a protective effect of free radicals scavengers has been demonstrated under specific experimental conditions [44], however, the relevance of highly reactive oxygen compounds in a broader pathophysiological or clinical setting, and the benefits of their eventual correction or regulation, deserve further confirmation.

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