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N-Acetylcysteine failed to improve early microcirculatory alterations of the rat liver after transplantation

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Abstract The application of radical scavengers reduces reperfusion injury of liver grafts despite the natural occurrence of cellular defense mechanisms enabling the cell to tolerate moderate oxidant stress without further cell damage. The glutathione peroxidase mechanism of the liver serves to reduce hydroxyl radical-induced lipid peroxidation by releasing reduced glutathione from intracellular stores. There is evidence that the application of cysteine-providing aminoacids for glutathione synthesis could maintain or even increase liver glutathione. Therefore, the purpose of this study was to evaluate the effect of *N*-acetylcysteine (NAC) on oxidative stress-induced reperfusion injury after liver transplantation. This was done by applying intravital microscopy. Livers from female Sprague-Dawley rats weighing 220–260 g were stored for 20 h in University of Wisconsin (UW) solution and transplanted orthotopically using the cuff technique. Donors were given 150 mg/kg body weight NAC i. v. or placebo in a blind, random fashion 6 h prior to harvesting, followed by two injections of 50 mg/kg body weight, 4 and 2 h before explantation. In additional experimental groups, recipients were given a bolus of 83 mg/kg body weight NAC or placebo at the beginning of the recipient operations, 1 min prior to reperfusion, and 60 min after surgery. Ninety minutes after transplantation, intravital microscopy was applied and five liver lobules were recorded for 30 s after injection of acridine orange, a fluorescent leukocyte marker. Sinusoidal perfusion, sinusoidal width, and leukocyte adhesion, as well as reduced and oxidized glutathione, were determined in all livers. Neither microcirculatory disturbance nor leukocyte adhesion was less, nor was the liver glutathione in the recipient groups pretreated or treated with NAC greater than that in rats receiving the placebo. Moreover, liver glutathione was significantly decreased in livers from donors pretreated with NAC. In conclusion, the application of NAC as a pretreatment for donors and as treatment for recipients, respectively, failed to reduce early microvascular failure after liver transplantation.

Key words Liver transplantation, rat, *N*-acetylcysteine · *N*-acetylcysteine, rat, liver transplantation · Preservation, rat liver, *N*-acetylcysteine · Microcirculation, *N*-acetylcysteine, liver preservation

Introduction

It is generally known that oxygen free radicals (OFR) are associated with reperfusion injury in transplanted livers [7]. During cold ischemia, adenosine triphosphate (ATP) is metabolized to adenosine, inosine, and hypoxanthine, and xanthine dehydrogenase is converted to xanthine oxidase [26]. In the presence of molecular oxygen during reperfusion, reactive superoxide anions and hydrogen peroxides are generated [9]. The subsequent formation of hydroxyl radicals requires the catalytic effect of the Haber Weiß reaction in the presence of iron [9, 20]. The highly damaging hydroxyl radical reacts with unsaturated membrane-bound lipids, causing severe endothelial cell damage by lipid peroxidation [10, 35].

Nonparenchymal cells of the hepatic sinusoids, especially resident macrophages (Kupffer cells) are known to be another important source of OFR [13]. Gut-derived endotoxin leads to the calcium-dependent activation of Kupffer cells [33, 34], releasing interleukin-1 (Il-1), tumor necrosis factor (TNF) and OFR [37]. We demonstrated previously that OFR may cause the adhesion of leukocytes to the endothelium [23]. The expression of adhesion molecules, mainly selectins and integrins, is mediated in part by OFR [27], leading to the accumulation of leukocytes in reperfused livers [19, 23]. Furthermore, it is known that increased leukocyte adhesion is associated with microcirculation disturbance and graft dysfunction.

On the one hand, a variety of radical scavengers have been introduced in experimental liver transplantation in the last decade [6, 23, 32], demonstrating that new strategies of organ procurement and postoperative management have succeeded in reducing reperfusion injury of transplanted liver grafts. The influence of preservation solutions containing different radical scavengers on microcirculation during reperfusion has recently been investigated by our group [21, 38]. On the other hand, different protective mechanisms, e.g., superoxide dismutase, catalase, and glutathione peroxidase, are naturally available in each cell to minimize the harmful effects of OFR. Glutathione (GSH) is one important component of the cells that is responsible for the structural and functional integrity of cell membranes. The major intracellular thiol [25] is synthesized from glycin, glutamate, and the thiol providing aminoacid cystein. During reperfusion, reduced GSH could act as a relevant radical scavenger [25], released from parenchymal liver cells [11]. It has been reported that an intact GSH peroxidase system during reperfusion can attenuate oxidant stress without further cell damage [15]. Otherwise, depletion of liver GSH by GSH-reducing agents is directly related to increased lipid peroxidation in the liver [5].

Since GSH was found to be reduced after cold ischemia [36], GSH is added to University of Wisconsin (UW) solution to maintain the endogenous defense mechanisms [3]. While cell membranes, in general, are impermeable to GSH, it is said that GSH is metabolized, providing amino acid cysteine, which is the rate-limiting substance for the intracellular synthesis of GSH [1]. Yu et al. have demonstrated that GSH, apart from other ingredients, is an essential component of UW solution, necessary for improved survival after rat liver transplantation [40].

Apart from the improvement in cellular integrity during cold ischemia, there is evidence that the GSH level in the liver could be maintained or even increased to some extent by the application of precursors of GSH [1, 14, 30]. In this respect, it has been shown that lipid peroxidation and the release of liver enzymes are mitigated by pretreatment of rats prior to and following liver transplantation with γ-glutamylcysteine ethyl ester [18]. The application of GSH precursors prior to ischemia maintained the integrity of liver function following reperfusion [8, 12, 18].

The purpose of this study was, therefore, to determine the effect of *N*-acetylcysteine (NAC) pretreatment of the donor on liver GSH and on microcirculation after orthotopic liver transplantation in the rat. Apart from the possible improvement in the endogenous GSH peroxidase mechanism, substances related to GSH, such as NAC, possibly act as powerful scavengers of hydroxyl radicals [2]. Therefore, in additional experiments NAC was given to recipients to investigate the reducing capacity of NAC during early reperfusion.

Materials and methods

Animals

Female Sprague-Dawley rats weighing 220–260 g (Hannoversche Tierversuchsanstalt, Hannover, Germany) were used as donors and recipients. The animals had free access to water and standard rat chow until the beginning of the experiments. Harvesting was performed between 3 and 4 p.m., followed by transplantation at 11 a.m. and 12 noon of the following day. General anesthesia with ether was given during all surgical procedures. Ninety minutes after transplantation, the rats were reanesthetized with pentobarbital i.v. (20–50 mg/kg body weight, Narkoren, Rhone Merieu, Laubheim, Gemany). At the end of the experiments the rats were sacrificed via an overdose of pentobarbital, and the livers were removed to determine the GSH. The experiments were conducted after permission had been given by the local ethics committee and were done in accordance with the NIH guidelines for the use of laboratory animals.

Transplantation procedure

Twenty-four livers were transplanted orthotopically after cold storage (0°–4°C) for 20 h in UW solution (Via Span, DuPont Pharma, Germany/NBPI, The Netherlands) using the cuff technique developed by Kamada and Calne and by Zimmermann and colleagues [17, 41]. Before harvesting the organ, an intraluminal stent was in-

Table 1 Permanent leukocyte adhesion was determined 90 min after surgical procedure. Data of permanent adherent leukocytes are expressed as a percentage of all observed, free-passing and adherent leukocytes for 30 s and are given in sublobular distribution (mean ± SEM). In all groups, leukocyte adhesion was highest in the periportal area, followed by the midzonal and the pericentral areas. Leukocyte adhesion was significantly increased after transplantation compared to sham-operated animals, while no differences were observed between the transplanted groups

	Sham	Donor		Recipient	
Pretreatment		Placebo	NAC	–	–
Treatment		–	–	Placebo	NAC
n	8	6	6	6	6
Periportal	14.2 ± 1.0	23.5 ± 4.6*	23.9 ± 2.3**	22.2 ± 3.7*	20.5 ± 1.7*
Midzonal	9.6 ± 1.0	16.0 ± 2.3*	17.6 ± 2.4**	18.8 ± 3.6**	16.3 ± 0.7**
Pericentral	6.4 ± 0.6	11.8 ± 2.0*	12.3 ± 1.3**	12.9 ± 2.4**	12.0 ± 0.5**

* $P < 0.05$; ** $P < 0.01$

serted into the bile duct. In the back-table bath, the infrahepatic vena cava and the portal vein were prepared with polyethylene cuffs (Venflon, 2.0 mm outside diameter, Pfrimmer-Viggo, Erlangen, Germany). The suprahepatic vena cava was anastomosed with a running suture (Prolene 7–0, Ethicon, Norderstedt, Germany). Prior to the insertion of the portal vein cuff into the appropriate recipient vessels, the livers were rinsed with 5 ml of Ringer's solution. During the first minutes after declamping, the stent of the bile duct was led into the recipient's bile duct. Volume replacement after transplantation consisted of 2.5 ml Ringer's solution and 2.5 ml human albumin (5 %) i. v. Postoperatively, the rats were allowed to take water ad libitum until the time came for microscopic investigation of the liver.

Experimental setting

Five experimental groups with six experiments per group were investigated (Table 1). Donors and recipients were given NAC (Fluimucil, Inpharz, Gräfelfing, Germany) or an identical volume of placebo (NaCl) in a blind, random fashion. Six hours prior to harvesting, the donors were pretreated with 150 mg/kg body weight NAC or placebo i. v. via a tail catheter, followed by two injections of 50 mg/kg body weight each, 4 and 2 h before explantation, respectively. In additional groups, the recipients were given a bolus of 83 mg/kg body weight NAC three times: at the beginning of the recipient operation, 1 min prior to reperfusion, and after 60 min of reperfusion. Additional experiments with time and procedure-matched controls were investigated. They underwent the entire surgical procedure of the donor operation with ligation of the hepatic artery, except that transplantation was not performed.

Intravital microscopy

The technique of intravital microscopy has been described recently [23, 24]. Ninety minutes after transplantation, the rats were anesthetized with pentobarbital (20–50 mg/kg body weight, Narcoren, Rhone Merieux, Laupheim, Germany). The abdomen was opened again and the rats were positioned on their left sides on a specially designed plexiglas stage. The lower surface of the left liver lobule was exteriorized for in vivo investigation of liver microcirculation using a epifluorescence microscope (Nikon MM 11, 100 W mercury lamp, with a filter set consisting of an excitation filter bandpass 470–490 nm, a dichroic mirror 510 nm, and a barrier filter 520 nm, optical magnification of × 330). Circulating leukocytes were labelled with 0.1 mg/kg body weight acridine orange (Sigma, Deisenhofen, Germany) [16] via the tail vein catheter. Five to six liver lobules were recorded for a period of 30 s with a charge-coupled device (CCD) low-light video camera (FK 6990, Pieper, Schwerte, Germany) connected to a time-date generator (VTG 33, FOR-A company, Tokyo, Japan) and a SVHS video recorder (Panasonic, NV-FS1 HQ, Japan). The following parameters describing the microcirculatory situation were investigated by a blinded observer:

1. Leukocytes adhering temporarily (> 200 ms and < 20 s) or permanently (> 20 s) to the sinusoidal wall were determined using off-line, frame-by-frame analysis. Following Rappaport's example [28], periportal, midzonal, and pericentral sublobular regions were defined at one-third of the distance between the edge of the central vein and the periportal area [24]. Data of temporarily adherent leukocytes were given as a percentage of all observed circulating leukocytes; data of permanently adherent leukocytes were expressed as the percentage of all observed, unrestrained, flowing, and adherent leukocytes.
2. Perfused sinusoids were given as the percentage of all sinusoids of the sublobular region.
3. Sinusoidal width in the middle of the sublobular regions was measured with a computer-supported analyzing system (Lobulus, Medvis, Homburg/Saar, Germany) [22].

GSH of liver tissue

In additional groups with the identical procedure except for microscopy, tissue samples of the livers weighing approximately 1 g were taken before pretreatment, after pretreatment just before the donor operation, after 20 h of cold storage, and 90 min after transplantation with a freeze clamp. Samples were pulverized in liquid nitrogen prior to homogenization in perchloric acid (1.0 M). After centrifugation (2800 *g*), the supernatant was separated from the perchlorate and adjusted to pH 7.0. After the second centrifugation, reduced glutathione (GSH) and oxidized glutathione (GSSG) were determined enzymatically in the supernatant according to the method of Bernt and Bergmeyer [4]. All chemicals used in the study were obtained from Boehringer Chemicals (Mannheim, Germany).

Statistical analysis

Data are given as means ± –SEM. Statistical significance concerning sinusoidal perfusion and sinusoidal width was determined by

applying an analysis of variance with a post-hoc *t*-test and Bonferroni correction. Comparisons between glutathione values in livers and permanent leukoyte adhesion were made with the Kruskal-Wallis test for non-normally distributed data. A P level below 0.05 was considered significant.

Results

Microcirculation

The sinusoidal perfusion in sublobular distribution after transplantation (Fig. 1 A) was significantly lower than in sham-operated animals, whereas no differences were observed between the transplantation groups. In all groups, the integrity of microcirculation was maintained in the pericentral region better than in the midzonal and the periportal areas, as described recently [24].

Sinusoidal width in all sublobular regions (Fig. 1 B) was decreased after transplantation with placebo (e.g., periportal area 8.0 ± 0.1 μm) and NAC-pretreated donors (8.1 ± 0.1 μm), as well as in recipients treated with placebo (7.9 ± 0.3 μm) and in recipients treated with NAC (7.5 ± 0.2 μm), in contrast to sham controls (10.1 ± 0.2 μm).

Leukocyte adhesion

Ninety minutes after transplantation, permanent leukocyte adhesion (Table 1) was significantly greater in all treated groups than in sham-operated animals (e.g., periportal region 14.2 % ± 1.0 %; $P < 0.05$). Neither donor pretreatment (23.9 % ± 2.3 %) nor treatment of recipients (20.5 % ± 1.7 %) with NAC was able to reduce leukocyte adhesion, any more than was placebo-pretreatment of donors (23.5 % ± 4.6 %) or recipients (22.2 % ± 3.7 %). With respect to temporary leukocyte adhesion, no differences were observed between the application of placebo and NAC (data not shown).

GSH and GSSG content of tissue samples

GSH in liver (Fig. 2 A) was reduced after 6 h of pretreatment, followed by a marked decrease after 20 h of cold ischemia with slight differences between NAC and placebo-pretreated donors. After 90 min of reperfusion, GSH was significantly reduced in the group of NAC-pretreated donors ($P < 0.01$), whereas GSH remained unchanged in the group of placebo-pretreated donors. Liver GSH of recipients treated with NAC or placebo was in the same range as liver GSH of donors pretreated with the placebo.

GSSG in livers (Fig. 2 B) obtained from NAC or placebo-pretreated donors was equal 6 h preoperatively

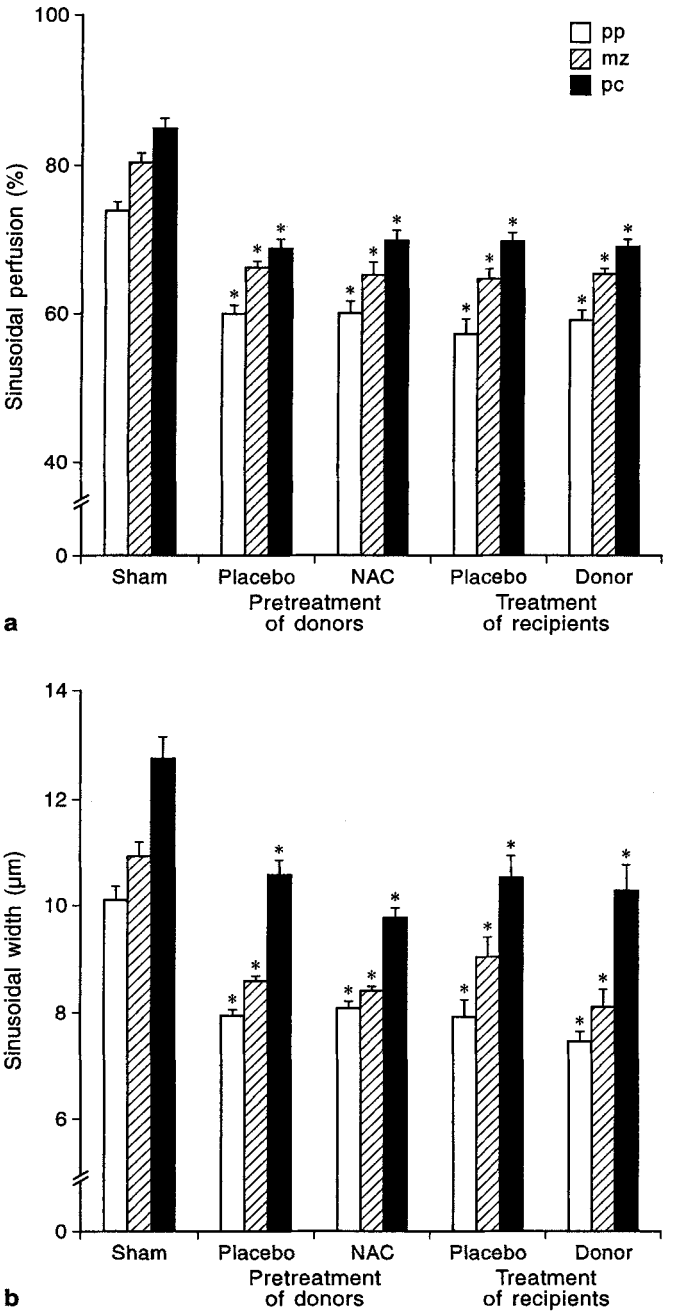

Fig. 1 **a** Sinusoidal perfusion and **b** sinusoidal width of the liver lobule 90 min after the surgical procedure. Sinusoidal perfusion is expressed as a percentage of all sinusoids in the liver lobule observed. Data are given in sublobular distribution. After transplantation both sinusoidal perfusion and sinusoidal width was significantly reduced in all groups compared to sham controls ($P < 0.001$). No differences were observed between donors pretreated with placebo or NAC and recipients treated with placebo or NAC, respectively (*pp* periportal, *mz* midzonal, *pc* pericentral)

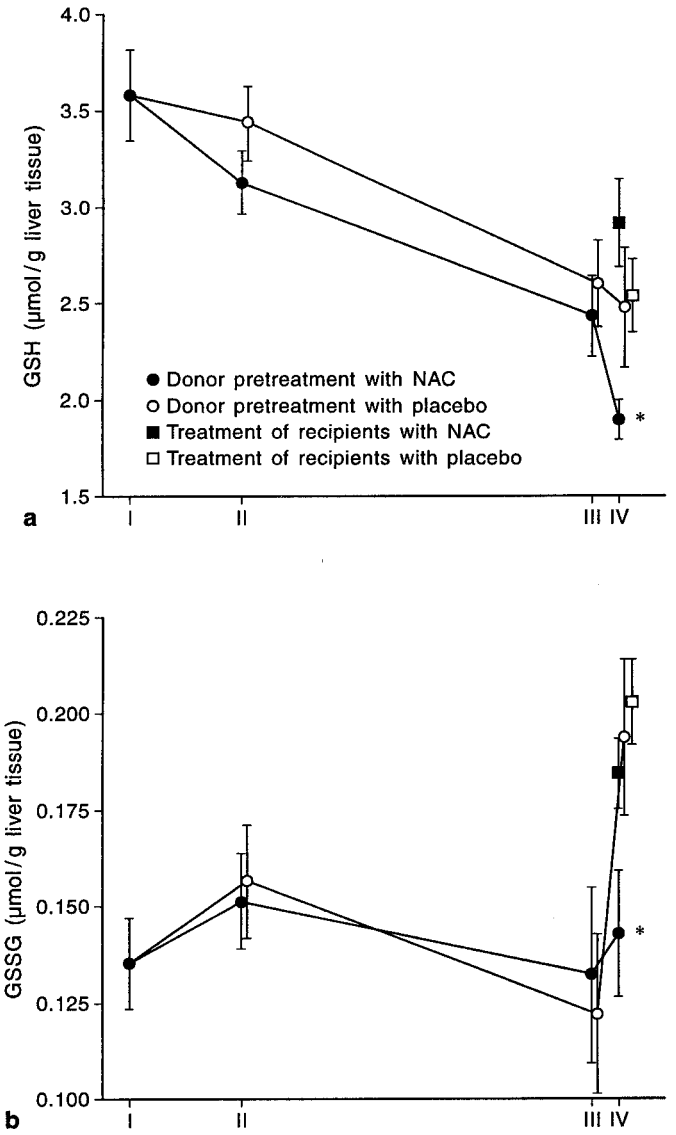

Fig. 2 **a** Reduced and **b** oxidized glutathione was determined enzymatically. Tissue samples of the livers were taken before pretreatment (*I*), after pretreatment (*II*), after 20 h of cold storage (*III*), and 90 min after transplantation (*IV*). Data are given as means ± SEM. Values of liver GSH and GSSG in NAC-pretreated donors were significantly decreased during reperfusion. * $P < 0.05$

and following cold ischemia. During reperfusion, liver GSSG was intensely increased in all groups except in donors pretreated with NAC ($P < 0.05$).

Discussion

The GSH peroxidase system is absolutely necessary for protecting cells from radical injury [11, 25, 39]. GSH predominantly stored intracellularly is partly transported out of cells [11, 12, 25], and it improves the reduction capacity in the space of Dissé and in liver sinusoids. The destruction of the hydrogen peroxide and OFR generated during reperfusion is catalyzed by the GSH peroxidase system [25].

Due to diurnal variation, liver GSH decreases during the daytime and is at a minimum at 6 p.m. [14]. Therefore, we tried to maintain the GSH level in the liver by pretreating donors with NAC. The first bolus of NAC was given at 9 a.m., 6 h before explantation, followed by two injections at an interval of 2 h each. Surprisingly, we observed no increase in liver GSH after pretreatment with NAC, whereas others have shown that administration of GSH, cysteine, or NAC maintains or even increases the cellular GSH to some extent [1, 18, 25, 30]. This aids in reducing reperfusion injury [12]. It can be argued that GSH regulates its biosynthesis by feedback inhibition of the enzymes involved [29]. This may also explain our observation that GSH in livers from NAC-pretreated donors was slightly decreased during the whole observation period. However, it remains unclear why the GSH of livers obtained from NAC-pretreated donors was significantly decreased during reperfusion.

It has been reported that reperfusion of transplanted liver grafts leads to an increase in GSSG [13, 31], a sensitive parameter of oxidant stress [13]. In our study, we saw an increase in GSSG during reperfusion in all groups except that in which donors were pretreated with NAC, indicating that OFR seems to be involved in the early reperfusion period. However, we have to assume that there is also limited increase in GSSG in livers from NAC-pretreated donors during reperfusion as a result of the overall reduction in GSH in these livers.

The influence of NAC pretreatment on leukocyte adhesion and sinusoidal perfusion was studied during early reperfusion to determine the effect of the glutathione peroxidase system on radical-induced microcirculatory disturbance. However, the application of NAC to donor animals failed to reduce leukocyte adhesion or to restore the integrity of microcirculation after liver transplantation.

Not only did we try to maintain liver GSH by pretreating donors with NAC, we also tried to investigate the effect of NAC given prior to and during reperfusion. Aruoma and colleagues have shown that NAC is a powerful scavenger of hydroxyl radicals [2], which are responsible for severe cell damage during reoxygenation. In a recent study, we demonstrated that superoxide dismutase, likewise known to be an effective radical scavenger, given during transplantation can reduce microcirculatory disturbances as well as leukocyte-endothelial interactions [23]. Therefore, superoxide dismutase maintained the integrity of parenchymal cells expressed by decreased serum levels of liver transaminases and was able to increase the survival rate after li-

ver transplantation. However, in the present study, both microcirculation and adhesion of leukocytes were unaffected by the administration of NAC during the transplantation procedure. Since it is known that OFRs are involved in reperfusion injury, leading in part to the expression of adhesion molecules [27] and, therefore, to increased leukocyte adhesion, it seems obvious that NAC given during reperfusion was not able to reduce radical-induced leukocyte adhesion.

In conclusion, neither pretreatment of donors nor treatment of recipients with NAC in the concentration used could improve liver microcirculation after transplantation. Further studies must clarify whether NAC pretreatment of donors with reduced antioxidative capacity, e.g., by starvation, could attenuate reperfusion injury.

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