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Original Article

A prospective randomized trial of N-acetyl cysteine administration during cold preservation of the donor liver for transplantation

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Abstract

Aims: **N-acetyl cysteine (NAC), an anti oxidant and a glutathione precursor, is effective in ameliorating liver injury of Tylenol overdose. There is experimental evidence that it also reduces ischemia reperfusion (I/R) injury. This clinical study was undertaken to study the effect of NAC administered in the donor operation.**

Methods: **22 patients were randomized to receive NAC (IV & Portal flush) or no NAC (Control Group) during donor operation. Peak AST levels and 1-hour post-reperfusion biopsies were used to assess I/R injury. Episodes of acute rejection were recorded together with immunosuppressive drug levels.**

Results: **There were 4 exclusions (re-exploration for post-operative hemorrhage x3, OLT for acute liver failure x1). The two groups (n = 9 each) were matched for recipient and donor ages and sex. Viral hepatitis accounted for cirrhosis in 3 patients in NAC Group and 6 patients in Control Group. Statistically, Cold and warm ischemia times were not significantly different as was the use of blood and blood products in both groups. Serum peak AST levels were similar and post-reperfusion biopsy showed moderate to severe reperfusion injury in 3 recipients in the NAC Group and 4 in the Control Group. Excluding ones associated with low Tacrolimus levels (n = 4), there were 6 episodes of acute rejection (2- mild, 4- moderate) in the NAC Group and 5 in the Control Group (3- mild,1- moderate, 1- severe).**

Conclusion: **In this pilot study, NAC administered during donor operation did not show a protective effect on I/R injury or on acute cellular rejection.**

Key words: Ischemia reperfusion injury, N-acetyl cysteine, Orthotopic liver transplantation.

Introduction

It has been postulated that oxidative stress and leucocyte-endothelial interations are critical events in the development of ischemia reperfusion (I/R) injury[1] or allograft rejection[2] during liver transplantation. There is currently no effective treatment to prevent I/R injury in solid organ transplantation.[3] Antioxidants such as allopurinol, S-adenosylmethionine, superoxide dismutase and N-acetyl cysteine (NAC) have shown promising results in reducing reperfusion injury in animal models.[4,5] Anti-oxidants warrant further study of their role in human liver transplantation. In order to develop a cost effective anti-oxidant therapy without serious adverse effects, recent studies have investigated the therapeutic potential of the endogenous anti-antioxidant Glutathione (GSH).[6] NAC, a GSH precursor, has been in use for the several years as an effective antidote for fulminant hepatic failure.[7,8] Maintenance of the hepatic intracellular GSH pool (especially in mitochondria) is essential for a variety of physiological functions, and the Thiol status is central to the signalling pathways dictating, on the one hand, normal cell homeostasis, or on the other hand, inflammatory response, necrosis or programmed cell death.[9,10] Apart from having a direct effect in suppressing oxygen free radicals, NAC enhances the vasodilator properties of nitric oxide. It also inhibits the activation of thromobocytes, neutrophils and monocytes, which play a dominant role in I/R injury. In a rat model of hepatic cold ischemia, NAC administered pre-ischemic phase and in the post-ischemic and reperfusion phases has shown attenuation of I/R injury and production of better quality and quantity of bile as compared to controls.[11] In a previous clinical study in human liver transplantation by Thies et al.[12] NAC was administered in the post-ischemic and reperfusion phases of orthotopic liver transplantation (OLT) and demonstrated a trend towards reduction of I/R injury in the NAC treated group. It

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was our contention that if NAC was administered prior to hypoxia, it might be possible to maximize its benefits. The present pilot study was undertaken to study the effect of NAC administered to the organ donor prior to cold preservation. We chose to study this approach to seek a simple protocol for liver protection against I/R injury. In this setting, it was decided to administer the NAC in such a way that protection was in place in the donor organ prior to reperfusion, since it has been shown in experimental studies that during cold liver preservation, GSH becomes depleted.[13] Glutathione is present in the University of Wisconsin solution, but oxidizes to varying degrees during manufacture and its permeation is reduced by cold temperatures. Hence, NAC administration both centrally before cooling and via the flush could maximize potential protection of the intracellular Thiol status.

Patients and methods

Twenty-two donors were randomized to receive NAC (NAC Group) or no NAC (Control Group) during donor operation performed by the RFH team. All organs were procured using in-situ flushing with citrate solution and University of Wisconsin storage solution by the established Royal Free Hospital methods.[14] This was administered as follows:

a) IV infusion of NAC (20% solution dissolved in 0.9% saline, 150 mg/kg, ~ 10 grams) was started about 15 minutes before cardiac arrest and
b) via the portal vein (75 mg/kg, ~ 5 grams) in the perfusion solution during the cold phase dissection (1 gram/liter for 3 liters of citrate solution) and bench perfusion (1 gram/liter for 2 liters of University of Wisconsin solution) before bagging.

The liver was preserved in a bag containing the cold UW solution with or without NAC and retained in an ice box. The liver was subjected to bench dissection just before implantation for 15-30 minutes retained in a bowl containing cold UW solution. Reperfusion was started after completion of inferior vena cava (top and bottom) and portal vein anastomoses and after flushing with albumin solution (500 mL). Trucut needle liver biopsies were obtained approximately 60 minutes after graft revascularization, inserted in 10% buffered formalin, conventionally processed, embedded in paraffin, and stained with hematoxylin-eosin. Specimens were analyzed by light microscopy with respect to steatosis and preservation injury.

All patients received an ABO-compatible allograft. Liver function tests were recorded daily post-operatively. The analysis of liver function tests was restricted to first 7 days because the enzymes were found to start rising again around day 7 concomitant with occurrence of acute cellular rejection. I/R injury was assessed by recording peak aspartate aminotransferase (AST) levels during the first 72-hours and by histomorphologic analysis of 1-hour post-reperfusion graft biopsies. Biopsy was not performed in one patient in each group. According to the extent and location of hepatocellular necrosis, I/R injury was classified into four categories by an independent pathologist, and in a blinded fashion (0 = absent, 1-3 foci of necrosis/lobule = mild, 4-5 foci of necrosis/lobule = moderate, > 5 foci of necrosis/lobule = severe injury). I/R injury was statistically evaluated using the above categories (categorical variable) and also as a continuous variable (number of necrotic foci/lobule). The immunosuppressive protocols included Tacrolimus monotherapy for all patients except those transplanted for PBC, PSC and AIH who received triple therapy with Prednisolone 16 mg daily and Tacrolimus (via NG tube/ orally) and Azathioprine 1.0 mg/kg daily (IV). A protocol liver biopsy was performed around day 7 and then at one year. Otherwise, a liver biopsy was performed whenever clinically indicated. Incidence and severity of acute rejection (Royal Free Hospital grading) was recorded during the post-operative period for a period of one year. This was assessed according the Royal Free grading system,[15] which in addition to the BANFF grading system[16] (mononuclear portal infiltration, bile duct damage, and arterial endothelial wall damage) also separately grades eosinophils in the mononuclear portal infiltrate. The serum levels of Tacrolimus were also recorded.

The donor and recipient ages, the sex and diagnosis of the recipient, cold and warm ischemia times, peri-operative blood and blood product transfusions and histology of the donor livers as judged by the post-reperfusion biopsy were also recorded. Livers from donors older than 50 years or those having macrovesicular steatosis were considered to be marginal livers. Statistical comparison between groups was performed using mainly the t-test for parametric data and Mann-Whitney test for non-parametric data. The level of significance was set at $P< 0.05$. All data were expressed as mean (SD).

Results

Of the 22 patients randomized, 4 patients were later excluded from the final analysis. One was excluded as the recipient was in fulminant liver failure. Three patients had re-exploration for post-operative hemorrhage within 48 hours after OLT and were excluded as they suffered another ischemic insult. All had more than 30 units of blood transfused during the first 72 hours of OLT. Two of the three belonged to the NAC group.

Of the 18 remaining patients, 9 belonged to each of the two groups. The mean ages of donors and recipients were comparable in the two groups *(Table I)*. Seven donors were aged more than 60 years in the NAC Group and five in the Control Group while two recipients in the NAC Group and 4 in the Control Group was older than

Table I. Comparison of donor and recipient variables in NAC and control groups.

Variable	NAC group (n = 9)	Control group (n = 9)	Significance (P)
Donor age	48.9 years (SD 13.7)	52.0 years (SD12.2)	0.62
Recipient age	48.3 years (SD13.6)	56.3 years (SD 9.7)	0.17
Fatty donor liver	Mild = 3, moderate = 1	Mild = 2	
Cold ischemia time	660 min (SD 132)	800 min (SD 144)	0.06
Warm ischemia time	44.7 min (SD 18.8)	48.2 min (SD 11.5)	0.65
Blood (48 hours)	6.6 units (SD 3.9)	10.2 units (SD 7.8)	0.23
FFP* (48 hours)	5.2 units (SD 2.8)	9.2 units (SD 7.0)	0.14
AST** (Peak)	990 IU (SD 974)	1,163 IU (SD 530)	0.65
ALT*** (Peak)	685 IU (SD 421)	682 IU (406)	0.99
AST (Peak, marginal donors)	1,350 IU (SD 983)	1,116 IU (SD 513)	0.88

(* = Fresh frozen plasma, ** = Aspartate aminotransaminase, *** = Alanine aminotransferase)

60 years. There were 6 males in the NAC group and 4 in the Control group. The etiology of the recipient was viral hepatitis in 2 patients in the NAC group and 5 patients in the Control group *(Table II)*. Two patients died in the postoperative period (day 12 and day 21), one of sepsis and the other of stroke. Both belonged to the control group.

The donor liver was assessed, on 1-hour post-reperfusion biopsy, to be mildly fatty in 2 patients in the Control Group while in the NAC Group, 3 livers were mildly fatty and one was moderately fatty with evidence of steatohepatitis and macrovesicular steatosis. The mean Cold Ischaemia Time (CIT) was longer in the control group but was not statistically significant (P=0.06) *(Table I)*. CIT of 12 or more hours occurred in 5 patients in the NAC Group and 6 patients in the control group. None of the livers had a CIT greater than 18 hours. The mean peri-operative (48 hours) blood transfusion was less in the NAC Group (P= 0.17). Two patients had 10 or more units transfused in the NAC Group while 4 patients had more than 10 units transfused in the Control Group, one of whom received 27 units *(Table I)*.

I/R injury: (Table III) Peak serum transaminase levels as well as levels during the first 7 days were quite similar in the two groups. Patients who were considered to receive marginal livers (donor age > 50 years or macrovesicular steatosis [n= 1]) comprised 7 in the NAC group and 5 in the Control group. They generally had higher peak transaminase levels but differences in the peak values did not achieve statistical significance in the two groups. The 1-hour post- reperfusion biopsy did not show any statistical difference on the basis of number of foci of hepatocyte necrosis/lobule when assessed as a categorical variable (P= 0.65) nor when analyzed as a continuous variable (P= 0.25).

Acute Cellular Rejection: There were nine episodes of acute rejection (8 patients) in the NAC group and 7 episodes (6 patients) in the control group analyzed for one year post-transplant. The severity of rejection is shown in *table IV*. Serum Tacrolimus levels at the time of rejection were noted to be < 5.0 ng/mL (normal 5 -15 ng/mL) in 3 episodes in the NAC group and 2 in the Control group *(Table IV)*. After excluding the rejections associated with low Tacrolimus levels, the mean Tacrolimus levels at the time of rejection were 10.0 SD, 4.1 for NAC and 9.8 SD, 2.1 for the Control groups. All the rejections were noted within the first 3 months in both groups. One-year protocol biopsies in the 16 patients surviving the immediate post-transplant period showed no evidence of rejection.

Discussion

There is no standardized methodology for assessing I/R injury which

is characterized by high levels of transaminases in the early transplant period. The primary effect of I/R is damage of hepatic sinusoidal endothelium and subsequent microcirculatory impairment.[1] Quantification of hepatic microcirculation has also been used to categorize preservation injury and graft quality.[17] Various inflammatory mediators such as adhesion molecules and leukotrienes have been implicated in the pathogenesis of reperfusion

Table II. Aetiology of cirrhosis.

Aetiology	NAC (n = 9)	Control (n = 9)
Hepatitis B/C	2	5
ALD*	4	0
AIH**	1	1
Cryptogenic	0	2
PBC***	2	0
Chronic Budd Chiari	0	1

* = Alcoholic liver disease, ** = Autoimmune hepatitis, *** = Primary biliary cirrhosis

Table III. Pathological severity of I/R injury.

Group	None/Mild* I/R-injury	Moderate** I/R-injury	Severe*** I/R- injury
NAC	5	1	2
Control	4	0	4

(*: 1-3 foci of necrosis/lobule, **: 4-5 foci/lobule, ***: > 5 foci/lobule)

injury as also vasomotor mediators such as NO and endothelins.[3] Serum and tissue levels of these mediators have been used to assess extent of I/R injury.

A number of histological features have been attributed to IR injury. These include hepatic microvesicular steatosis, foci of parenchymal neutrophilic infiltration, cholestasis, hepatocyte ballooning, necrosis and apoptosis.[18] These changes are commonly seen to a minor degree in baseline reperfusion biopsies and are generally more prominent in the centrilobular regions. A number of studies have shown that severity of changes seen in time-zero (1-hour post-reperfusion) biopsies may predict subsequent graft function.[19,20] Hepatocyte necrosis and /or apoptosis have been found in time-zero biopsies in the absence of preceding changes in pre-harvest biopsies indicating that they may be a feature of early IR-injury.[21,22] In the present study, there was no significant difference in the severity of I/R injury between the two groups. Although some histological changes may be present in the donor liver before harvesting, clinical trials suggest hepatocellular necrosis before graft removal from the donor is only 6% and unrelated to the clinical course of recipients.[22]

There is evidence that increasing CIT beyond 12 hours may predispose to greater reperfusion injury.[2] Five patients in the NAC group and 6 in the Control group had CIT greater than 12 hours. However, no discernable differences on I/R injury were noted with increasing CIT in either of the groups. None of the patients had CIT of more than 18 hours, which may have implications for graft survival.[23] Donor characteristics, which favor severe I/R injury, include donor age greater than 50 years and fatty infiltration.[22] Such marginal livers were transplanted in 12 recipients, and there was evidence of moderate to severe I/R injury in 6 (50%).

Our results are in contrast to several animal studies noting a benefit of NAC for attenuating I/R injury. These studies were done either in an isolated perfused rat liver[9,24], or models of warm ischemia[4] produced by hepatic vascular exclusion. It has been suggested that rat models are not optimal for studying I/R injury in the transplant setting[25]. Also the studies in rats are not from brain dead animals. Brain death is associated with a cytokine 'storm' and may make the I/R injury less easy to treat by simple administration of NAC. The exact role of brain death in initiating I/R injury in the clinical setting is unclear[26], but there do seem to be donor-associated agonal factors, which have a negative impact on early liver graft function in comparison to that seen from living donors[27]. In a pig liver transplant model, non-heart beating donors have been used to produce significant I/R injury[23]. However, in this study pre-ischemic and post-ischemic administration of NAC failed to show a benefit in attenuating I/R injury. Also in the two randomized human studies comprising 60 OLTs each (heart beating donors)[12,29], no statistically significant benefit of intraoperative (recipient) NAC administration was demonstrated. However, a trend was noted in one[12], with regard to peak transaminases and primary non-function in favor of the NAC group. In another study, NAC administration to the recipients in a group of 10 transplants resulted in amelioration of some markers of I/R injury[30], but no immediate clinical benefit of this was clear from the report. Our study, as far as we are aware, is the only study to have used NAC solely in the donor operation and added also to the preservation solution. We have preliminary experimental evidence that NAC can permeate liver tissue during cold flushing under conditions, which simulated the clinical procedure[31]. Our rationale was that by exposure to NAC during storage, the liver would be fully primed for reducing I/R injury. This was not shown by our results which suggests that the pharmacokinetics of NAC uptake at low temperature is not sufficiently speedy to provide this priming protection and /or the more significant part of I/R injury, amenable to NAC treatment, occurs during reperfusion. Perhaps NAC is needed both in the storage solution and given to the recipient for several hours after the operation.

Another explanation for the lack of an effect in the present study could be that the dose of administered intravenous NAC (half-life: 2-hours)[32] may have been insufficient to replenish the stores of intracellular GSH. Nankano et al.[24] have suggested a dose of 150 mg/kg to possess an anti-oxidant effect. In the present study, this dose was administered IV, 15 minutes before cardiac arrest and a further 75 mg/kg was administered via the portal vein (perfusion during cold phase dissection and bench perfusion) suggesting that dosage was satisfactory. An alternative explanation is that hepatocytes have been found to be remarkably resistant to intracellular oxidative stress by virtue of large amount of intracellular stores of GSH as compared to the extracellular space. It has been shown in rats that even depletion of hepatic glutathione by 90%, before inducing an intracellular oxidant stress, does not significantly enhance liver injury.[33] However, there are other cells as sinusoidal endothelial cells and Kupfer cells which are primarily involved in I/R injury and they may not be as resistant. Another potentially confounding factor in this study is that UW solution already contains oxidized GSH (3 mMol/L) which may have had a masking effect on any protective response provided by more NAC in the extracellular environment. Finally, the lack of effect from donor treatment with NAC may point to the rel-

Table IV. Severity of acute rejection episodes.

Group	Mild	Moderate	Severe
NAC	2	4 + {1}	0 + {2}
Control	3	1 + {2}	1

Parenthesis represents episodes of rejection when Serum Tacrolimus < 5.0 ng/mL (Normal 5-15 ng/mL)

atively greater influence of the recipient I/R response in contributing to the overall post-reperfusion injury; i.e. the administration of NAC should be focused in the recipient rather than in the donor, or in both phases of the procedure. However, as discussed above, to date, there is little evidence of any strong clinical benefit from giving NAC in the recipient phase.[12,29]

There is experimental and clinical evidence of a link between IR injury and episodes of acute rejection in kidney transplantation.[34] One explanation is that I/R injury makes the allograft more immunogenic by upregulating the molecules involved in the immune response (HLA Class I/II). In liver transplantation there have been conflicting reports as to whether a correlation exists between I/R injury and episodes of acute rejection. A weak correlation was demonstrated in some[35,36] and none in others.[37-39] The current study did not show any trends towards an effect of NAC on the number or severity of acute rejection episodes during the one year follow-up.

We are left to conclude that in this small series, no major benefit could be identified from donor administration of NAC. Given that I/R effects were still seen in 33%-44% of the patients in either group, this suggests that other changes resulting from preservation injury must make a greater impact than Thiol protection during liver cold storage. Although much effort has been placed on studying cold preservation injury, there still remain many areas, which are as yet only poorly understood, and the results again highlight the differences between laboratory studies and clinical application which need more investigation.

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