N-acetylcysteine does not prevent hepatorenal ischaemia–reperfusion injury in patients undergoing orthotopic liver transplantation

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Abstract

Background. Glutathione (GSH) acts as a free radical scavenger that may be helpful in preventing reperfusion injury. N-acetylcysteine (NAC) replenishes GSH stores. The aims of this study were to evaluate the efficacy of NAC in improving liver graft performance and reducing the incidence of post-operative acute kidney injury (AKI).

Methods. Our study was a randomized, double-blind, placebo-controlled trial of 100 patients; 50 received placebo and 50 received a loading dose of 140 mg/kg of intravenous (IV) NAC over 1 h followed by 70 mg/kg IV repeated every 4 h for a total of 12 doses. Both groups were followed up for 1 year post-orthotopic liver transplant (OLT). We recorded liver function tests, renal function tests, graft survival, patient survival, plasma GSH and duration of hospital and ICU stay. In addition to serum creatinine (SCR) levels, we analysed cystatin C and beta-trace as independent measures of glomerular filtration. All clinical data were recorded daily for the first week after the surgery, then on Days 14, 21, 30, 90 and 180 and at the end of the first year.

Results. IV NAC did not affect survival, graft function or risk of AKI. However, GSH levels were highly variable with only 50% of patients receiving NAC exhibiting increased levels and fewer patients developed AKI when GSH levels were increased. Additional risk factors for AKI in the post-transplant period were female gender ($P = 0.05$), increased baseline serum bilirubin ($P = 0.004$) and increased baseline SCR levels ($P = 0.02$).

Conclusions. IV NAC was not effective in reducing renal or hepatic injury in the setting of liver transplantation. The dose and duration of NAC used, though higher than most renal protection studies, may have been ineffective for raising GSH levels in some patients.

Keywords: acute kidney injury; liver transplantation; N-acetylcysteine

Introduction

N-acetylcysteine (NAC) is commonly used to treat acetaminophen toxicity [1] and has been recently studied for the prevention of acute kidney injury (AKI) in the setting of radiocontrast exposure [2] as well as in cardiac surgery and sepsis [3,4]. NAC is a rich source of sulfhydryl (SH) groups which are important for replenishing glutathione (GSH) stores. GSH acts as a free radical scavenger to decrease damage caused by toxic free radicals. Patients who undergo orthotopic liver transplantation (OLT) have an incidence of post-operative AKI approaching 80% in some studies [5]. Although multiple aetiologic factors are involved in post-OLT AKI, high levels of toxic free radicals are believed to play an important role [6]. Free radicals may also contribute to primary liver graft failure or delayed liver graft function [6].

Therefore, the primary objective of this study was to evaluate the efficacy of NAC for improving liver graft performance and lowering the incidence of post-OLT AKI. The secondary objectives were to investigate the effect of NAC on GSH levels and to examine the relationship between GSH and AKI. Since NAC has been shown to decrease serum creatinine (SCR) levels without changing glomerular filtration [7], we also measured cystatin C as an independent marker of kidney function. Furthermore, since cystatin C could be affected by high-dose corticosteroid therapy [8], we also measured beta-trace in a subgroup of patients [9].

Materials and methods

Study design

We conducted a randomized, double-blind, placebo-controlled trial investigating the use of NAC intravenous (IV) infusion during and after OLT. After approval of the Institutional Review Board of the University of Pittsburgh, we obtained informed consent from 100 patients scheduled to undergo OLT. We randomized 50 patients to the placebo group and 50 to receive IV NAC for 3 days.
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Our inclusion criteria were: age > 18, scheduled to undergo cadaveric OLT for the first time and baseline SCr ≤ 2.5 mg/dl. Exclusion criteria included: allergy to NAC, history of asthma, fulminant hepatic failure, simultaneous other organ transplant (i.e., pancreas, heart and small bowel) and pregnancy. NAC (Abbott Laboratories) was prepared at the hospital pharmacy as a 1:5 solution in 0.9% saline for IV infusion. Matching saline placebo was administered at rates and volumes equivalent to those used for NAC infusion. The dosing regimen of NAC is the same as the FDA-approved protocol for IV NAC administration to treat acetaminophen-induced hepatic toxicity. A loading dose of 140 mg/kg of NAC was given after the induction of general anesthesia and before the skin incision was made, followed by a subsequent dose of 70 mg/kg every 4 h for a total of 12 doses. The placebo received an equal volume of 0.9% saline IV at the same rate and volume every 4 h for 12 doses. A total of four blood samples were drawn from all participants at the following time points: Time point 1, before the start of the NAC loading dose; Time point 2, during the anhepatic phase; Time point 3, after the completion of the last maintenance dose and Time point 4, 48 h after the last NAC dose was given. Blood samples were used to measure the plasma levels of GSH, cystatin C and, in a subgroup comprised of the first 20 participants, beta-trace.

The following data were abstracted from the medical records: haemodynamic parameters (BP, HR and cardiac output), blood gas, liver function tests, kidney function tests, blood products transfused, adverse drug reactions and medications. These data were collected during the intra-operative period, daily for the first week post-OLT, at the end of Months 1, 3 and 6 post-OLT and, finally, at the end of the first year.

Definitions

Post-reperfusion syndrome (PRS) was evaluated and classified as ‘mild’ when the decrease in blood pressure and/or heart rate was <30% of the pre-perfusion levels and short-lived (not more than 5 min) which responds to calcium chloride and/or epinephrine IV boluses without the need for continuous infusion. PRS was considered to be ‘severe’ when patients continued to have a systemic blood pressure decrease >30% from the pre-perfusion level after IV calcium chloride and epinephrine boluses and required vasopressor therapy during the intra-operative course. In addition, PRS was considered to be severe when it was accompanied by prolonged and recurrent fibrinolysis that showed up on the TEG tracing 30 min after reperfusion and required therapeutic intervention.

Extended donor criteria grafts (EDC) was described as the presence of any of the following in the donor or graft: age > 60 year, non-heart-beating status, serum sodium > 150 mmol/L, cold ischaemia time > 12 h, heart ischaemia time > 90 min or the presence of >30% macrovesicular steatosis.

Post-operative AKI was defined according to the RIFLE criteria: abrupt (1–7 days) and sustained (>24 h), changes from baseline of SCr and urine output [10,11].

Post-hepatic evaluation. The progress of graft recovery from the ischaemia–reperfusion injury was evaluated using liver function tests (bilirubin total/direct, ALT/AST, lactate, PT, INR, APTT, platelet count, quality and volume of bile production) on a daily basis during the first week post-transplant. The allograft outcome was evaluated at 1, 3 and 6 months and at completion of the first year post-transplant.

Assays

Plasma cystatin C was analysed by commercial ELISA (BioVendor, LLC, USA). Cystatin C is a protein that is produced by the majority of nucleated cells. Cystatin C occurs in the blood in high concentration and is considered as an important extracellular inhibitor of cysteine proteases [8,9]. Cystatin C concentration is considered to be an excellent correlate of the levels of GF that is not affected by diet, infection, malignancy or liver diseases. The production of cystatin C in the body is constant and it is freely filtrated in the glomerulus and completely reabsorbed in the proximal renal tubule cells. Accordingly, cystatin C is considered almost 100% specific and sensitive for decreased GF [8].

Beta-trace was analysed using particle-enhanced immunonephelometry on a Behring Nephelometer II (Dade Behring, Germany). The nephelometric test kits were a gift from Dade Behring and the serum analysis was performed at the Mayo Clinic Lab. The measurement is based on the principle of latex particle-enhanced immunonephelometry using rabbit polyclonal antibodies against beta-trace protein. Beta-trace is a GSH-independent prostaglandin D (PGD) synthase enzyme which is responsible for the biosynthesis of PGD2 in the CNS, in the male reproductive organs of various mammals and in the human heart. Beta-trace acts as a neuromodulator in the CNS and as a mediator of allergy and inflammatory response in the peripheral tissue [25,26]. In the serum, the levels of beta-trace reportedly increase in renal failure and it was found to be a sensitive marker of renal injury.

GSH measurements were performed at the Department of Environment and Occupational Health, University of Pittsburgh. To estimate GSH content in human plasma, ThioGlo™-1 was added to samples pre-treated with GSH peroxidase (1 U/50 μl plasma) (Sigma) in the presence of cumene hydroperoxides (1 mM) (Sigma) for 30 min at 25 °C. Differences in the fluorescence responses between LMTW and the samples treated with GSH peroxidase corresponded to the fluorescence of labelled GSH adduct with ThioGlo™-1. A standard curve was established by addition of GSH (0.5–20 μM) to phosphate buffer saline, pH 7.4, containing 10 μM ThioGlo™-1. A Fusion plate reader (Packard) was employed for the assay of fluorescence using excitation at 388 nm and emission at 500 nm [12].

Statistical analysis

The primary analysis was performed as intent-to-treat. Survival analysis was used to analyse time-to-event variables, including survival time, time to primary non-functional or delayed liver function. Comparisons between treatment and placebo groups were made using logrank tests. The Kolmogorov–Smirnov test was used to assess the variable normality within both groups. Student's t-test was also used to compare post-reperfusion syndrome and post-operative renal and liver function between the two groups. A Pearson correlation test was used to assess the correlation between cystatin C and beta-trace level within the treatment and placebo groups. Multi-variable modelling was used to evaluate the effect of risk factors (age, gender, pre-transplant liver function, pre-transplant kidney function, body mass, MELD score, cold and warm ischaemia time), treatment assignment and GSH. Variables were retained in the model if the P-value was <0.05. Sample size was determined assuming an incidence of post-OLT AKI of ~60% and an effect size of 30%. A sample size of 100 subjects achieves nearly 90% power to detect this difference or 80% power to detect a difference of 27%. Data are expressed as mean ± standard deviation and P < 0.05 was considered to be statistically significant.

Results

A total of 93 patients completed the study; 46 in the NAC group and 47 in the placebo group (see flowchart in Figure 1). The participants’ demographic and baseline clinical data are presented in Table 1, which showed that the two groups were similar as far as age, gender, weight, MELD score and the aetiology of the liver failure. Intra-operative data of both groups are also provided in Table 1, which showed that there were no significant differences between the groups as far as warm and cold ischaemia time or blood product transfusion requirements. However, the duration of surgery was longer in the NAC group (P = 0.016). Utilization of EDC (NAC/placebo, 10/9) and utilization of veno-venous bypass (NAC/placebo, 45/47) were not significantly different between groups.

The post-operative data showed that there was no significant difference between the groups as far as the days spent on the ventilator; in the NAC group (5.58 ± 9.79) and the placebo group (4.30 ± 9.37); P = 0.51. The duration of ICU stay in days was similar for both groups; in the NAC group (10.33 ± 11.60) and in the placebo group (8.84 ± 13.29); P = 0.55. The duration of hospitalization was not different in the NAC group (18.23 ± 15.49) compared to the placebo group (19.00 ± 21.07); P = 0.84.

One-year patient survival rate was 78.4% in the NAC group compared to 80.9% in the placebo group (P = 0.87). Figure 2 shows the survival time as a function of treatment group (Kaplan–Meier). Figure 3 shows that the 1-year organ survival rate is 74% in the NAC group and
80% in the placebo group ($P$-value is 0.54). Similarly, the frequency of severe PRS (NAC/placebo, 26/25) was similar and no significant effects of NAC on post-operative recovery of liver functions were detected. The post-operative serum levels of ALT, AST, INR and total bilirubin were similar in both groups during the first 3 months of the study period. Finally, AKI occurred over the first 14 days in 18 (36%) patients in the NAC group and 16 (32%) in the placebo group ($P = 0.83$). Table 2 shows the proportion of patients developing AKI by Day 7, by Day 14, limited to survivors and by various severities cutoff points. Table 2 also shows the proportion of patients receiving renal replacement therapy by 14 days and 1 year and shows the mean RIFLE scores (maximum for each patient) over the first 7 and 14 days. There are no differences between the groups for any of these comparisons.

GSH levels are shown in Figure 4. At baseline, there were no significant differences between the groups: NAC $3.04 \pm 4.59 \mu M$, placebo $2.52 \pm 1.88 \mu M$; $P = 0.49$. The GSH levels showed an increased in the NAC group at Time points 2 and 3 ($18.43 \pm 26.56$ and $10.83 \pm 23.25 \mu M$, respectively), while in the placebo group, levels remained close to the baseline level ($2.33 \pm 1.62$ and $2.67 \pm 3.27 \mu M$, respectively), $P = 0.0001$ and 0.024, respectively. At Time point 4,

### Table 1. Pre-operative and intra-operative variables

<table>
<thead>
<tr>
<th>Variable</th>
<th>Placebo group ($N = 50$)</th>
<th>NAC group ($N = 50$)</th>
<th>$P$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>$59.82 \pm 9.37$</td>
<td>$59.16 \pm 8.67$</td>
<td>0.715</td>
</tr>
<tr>
<td>Male/female</td>
<td>68/32%</td>
<td>70/30%</td>
<td>0.829</td>
</tr>
<tr>
<td>Weight</td>
<td>$83.66 \pm 15.55$</td>
<td>$88.22 \pm 18.35$</td>
<td>0.185</td>
</tr>
<tr>
<td>MELD score</td>
<td>$14.1 \pm 4.33$</td>
<td>$14.38 \pm 4.66$</td>
<td>0.756</td>
</tr>
<tr>
<td>Baseline SCr</td>
<td>$1.16 \pm 0.42$</td>
<td>$1.15 \pm 0.48$</td>
<td>0.895</td>
</tr>
<tr>
<td>PNC HBV/HCV</td>
<td>20%</td>
<td>18%</td>
<td>0.799</td>
</tr>
<tr>
<td>PNC HBV/HCV + ethanol</td>
<td>18%</td>
<td>22%</td>
<td>0.617</td>
</tr>
<tr>
<td>PNC + ethanol</td>
<td>22%</td>
<td>22%</td>
<td>1.00</td>
</tr>
<tr>
<td>Autoimmune and metabolic</td>
<td>26%</td>
<td>22%</td>
<td>0.640</td>
</tr>
<tr>
<td>HCC</td>
<td>6%</td>
<td>6%</td>
<td>1</td>
</tr>
<tr>
<td>Other aetiologies</td>
<td>8%</td>
<td>10%</td>
<td>0.727</td>
</tr>
<tr>
<td>RBC units</td>
<td>$7.52 \pm 8.54$</td>
<td>$9.02 \pm 7.28$</td>
<td>0.351</td>
</tr>
<tr>
<td>Fresh frozen plasma</td>
<td>$5.20 \pm 7.00$</td>
<td>$7.13 \pm 7.00$</td>
<td>0.177</td>
</tr>
<tr>
<td>Cryoprecipitate</td>
<td>$1.74 \pm 3.47$</td>
<td>$2.52 \pm 3.69$</td>
<td>0.284</td>
</tr>
<tr>
<td>Platelet</td>
<td>$6.28 \pm 6.63$</td>
<td>$8.21 \pm 6.90$</td>
<td>0.162</td>
</tr>
<tr>
<td>Cold ischaemia time in minutes</td>
<td>$671.5 \pm 193.08$</td>
<td>$644.56 \pm 133.88$</td>
<td>0.426</td>
</tr>
<tr>
<td>Warm ischaemia time in minutes</td>
<td>$27.1 \pm 5.85$</td>
<td>$29.56 \pm 8.64$</td>
<td>0.376</td>
</tr>
<tr>
<td>Duration of surgery in minutes</td>
<td>$409.44 \pm 81.45$</td>
<td>$455.12 \pm 102.49$</td>
<td>0.016</td>
</tr>
</tbody>
</table>
however, the GSH had returned to baseline for the NAC group (3.0 ± 1.65 μM) and was not different compared to the placebo group (3.27 ± 2.35 μM); P = 0.56.

In multivariable analysis, risk factors for post-OLT AKI were: pre-operative total bilirubin (odds ratio 1.53; 95% confidence limits 1.14 to 2.05; P = 0.004), pre-operative SCr (cutoff 2.5 mg/dl) (odds ratio 1.02; 95% confidence limits 1.00 to 1.03; P = 0.025) and female gender (odds ratio 2.88; 95% confidence limits 0.979 to 8.49; P = 0.05). Group assignment (NAC or placebo) was not a significant determinant of AKI by the RIFLE criteria using SCr or cystatin C. However, increased GSH (above 5 μM) was associated with fewer cases of AKI either by change in SCr (19% vs 30%) or cystatin C (15% vs 30%) although these differences were not statistically significant (P = 0.18). In 50% of the NAC patients, GSH increased above 5 μM, and in this subgroup, risk of AKI was substantially reduced (odds ratio 0.176; 95% confidence limits 0.036 to 0.87; P = 0.033).

Comparison of post-treatment (Time point 4) cystatin C and beta-trace for 20 patients are shown in Figure 5; the two measures correlated significantly (R² = 0.79, P <

<table>
<thead>
<tr>
<th>Table 2. Development of AKI by treatment group</th>
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<tr>
<td></td>
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<tr>
<td>14-day AKI (any)*</td>
</tr>
<tr>
<td>14-day AKI (I or F)</td>
</tr>
<tr>
<td>14-day AKI (F)</td>
</tr>
<tr>
<td>14-day RRT</td>
</tr>
<tr>
<td>1-year RRT</td>
</tr>
<tr>
<td>14-day AKI (survivors)</td>
</tr>
<tr>
<td>14-day AKI (cystatin C)</td>
</tr>
<tr>
<td>Max RIFLE to Day 7 (mean)</td>
</tr>
<tr>
<td>Max RIFLE to Day 14 (mean)</td>
</tr>
</tbody>
</table>

*aPrimary endpoint for cystatin C, a 50% increase from baseline was used as the minimum criterion for AKI.

AKI, acute kidney injury; RRT, renal replacement therapy.
micromolars) at the four sampling time points.

Also, we could not demonstrate any beneficial effects of NAC (a thiol-containing antioxidant) is the acetylated version of the amino acid L-cysteine and is a rich source of GSH synthesis. GSH promotes detoxification and acts as a free radical scavenger. NAC is currently the mainstay of treatment for acetaminophen-induced hepatotoxicity [1]. Because of its hepatoprotective effect, NAC has been used in some clinical trials in patients receiving OLT to improve the function of the liver allograft [13,14]. More recently, NAC has been used with mixed results in patients undergoing radiocontrast to protect the kidneys from the toxic effect of IV contrast agents [7].

The SH group is responsible for the metabolic activity of NAC, while the acetyl-substituted amino group makes the molecule more stable against oxidation. Following IV administration, the terminal half-life is 5.58 h. The renal clearance may account for 30% of the total body clearance [16,17]. The total clearance of NAC in cirrhotic patients is markedly impaired and could be less than half of that of healthy individuals [17]. NAC appears to support the synthesis of GSH under conditions where the demand for GSH is high; however, NAC may not increase the plasma GSH levels under normal conditions [18].

Discussion

NAC (a thiol-containing antioxidant) is the acetylated version of the amino acid L-cysteine and is a rich source of GSH groups. When administered to patients, NAC increases GSH synthesis. GSH promotes detoxification and acts as a free radical scavenger. NAC is currently the mainstay of treatment for acetaminophen-induced hepatotoxicity [1]. Because of its hepatoprotective effect, NAC has been used in some clinical trials in patients receiving OLT to improve the function of the liver allograft [13,14]. More recently, NAC has been used with mixed results in patients undergoing radiocontrast to protect the kidneys from the toxic effect of IV contrast agents [7].

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GSH is mainly an intracellular molecule; its active form is chemically reduced and is referred to as GSH. GSH serves as a substrate for glutathione peroxidase and glutathione S-transferase enzymes. GSH is synthesized mainly in the liver cells where the availability of cysteine is the rate-limiting substrate when GSH is depleted [19]. GSH depletion may occur during ischemia or during the presence of oxidative stress [20–22]. However, as shown in Figure 4, in the placebo group, there were no changes in the levels of GSH, probably due to the fact that ESLD is itself associated with GSH depletion. Nevertheless, baseline GSH in these patients was close to that published in normal individuals, suggesting that, while intracellular levels may be reduced, plasma levels do not represent GSH stores or the ability to increase GSH production during exposure to stress.

In an animal study, the use of NAC was found to prevent hepatic damage during ischemia–reperfusion [23]. In our study, we used IV NAC as approved by the FDA for the treatment of acetaminophen poisoning. The baseline (Time point 1) GSH levels in cirrhotic patients were (2.76 ± 3.40). There are no documented serum GSH levels for cirrhotic patients in the literature; however, the published levels for GSH in various organs and cell types range from 0.5 to 10 mM [19]. Previous studies have shown gender differences in the activities of GSH-related enzymes, with greater activity in females [24]. In our study, levels in both sexes were not significantly different, 3.27 ± 5.18 (females) vs 2.50 ± 1.87, P = 0.433.

In the group receiving NAC, GSH levels showed a large increase at Time points 2 and 3 while NAC was being administered, but at Time point 4, levels had already fallen to baseline, suggesting that the affect of NAC on GSH levels is not sustained. However, in spite of the increase in GSH levels which lasted for about 48 h, there was no significant change in the recovery of the graft function as measured by liver function tests.

Both groups were similar as far as demographic characteristics, MELD score, aetiology of the liver failure, the type of the allograft (EDC vs conventional), warm and cold ischaemia time, intra-operative transfusion requirement, severity of PRS and the utilization of piggyback technique with or without venous bypass. The only significant difference between the groups was the length of the surgical time where it was longer in the NAC group (P = 0.016) for undetermined reasons.

We used the RIFLE criteria [10,11] to assess for AKI in the post-operative period. Furthermore, we used two additional markers to characterize renal functions: cystatin C and beta-
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trace. Cystatin C and beta-trace were strongly correlated (Figure 3) and the rates of AKI classified using RIFLE were similar to those using cystatin C in place of creatinine.

We used modelling to assess the effect of various factors on the development of AKI: age, gender, baseline serum bilirubin, baseline SCr, change in GSH levels (delta GSH), body weight and the use of NAC. Female gender (P = 0.04), baseline serum bilirubin (P = 0.007) and baseline SCr (P = 0.01) were significant risk factors for post-OLT AKI. Female gender as a risk factor in AKI has been reported in previous studies [24]. Serum bilirubin level is a well-known risk factor in the aetiology of post-operative AKI as is SCr [27,28].

When GSH levels exceeded 5 μM, AKI defined by cystatin C was less frequent (14.8%) compared to 30% when GSH ≤ 5 μM. While this difference fails to achieve statistical significance (P = 0.21; 0.18 Fisher's exact), it would represent a >50% risk reduction (Table 2). However, our primary endpoint was RIFLE max over the first 2 weeks and this outcome was not different between the NAC and placebo groups, with the occurrence of AKI exactly the same (32%) in both groups.

Conclusion

In summary, NAC was not effective in reducing the risk of AKI after OLT, nor was it beneficial in terms of liver function or survival. Risk factors for AKI included female gender, baseline serum bilirubin and SCr. We speculate that one possible reason for the lack of effect with NAC may have been that only 50% of patients achieved a significant increase in GSH despite receiving a high-dose IV protocol. The reason that some patients did not respond to NAC with increased GSH is unclear. Poor early allograft function might have limited GSH synthesis, but the highest levels of GSH were seen during the anhepatic phase. The effect of NAC on GSH was also fairly short-lived. For future studies in patients undergoing OLT, it may be better to give even higher doses of NAC and to continue the drug for a longer duration. The use of NAC in the donor as well as the recipient might also be helpful.

Conflict of interest statement. None declared.

References


Received for publication: 21.11.09. Accepted in revised form: 29.1.10