Paracetamol metabolite concentrations following low risk overdose treated with an abbreviated 12-h versus 20-h acetylcysteine infusion

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ABSTRACT
Context: To compare degree of liver injury and paracetamol metabolite concentrations after treatment with standard of care (20-h) vs. abbreviated (12-h) acetylcysteine regimens used in paracetamol overdose (NACSTOP trial).

Methods: Timed blood samples from a cohort of subjects enrolled in the cluster-controlled NACSTOP trial evaluating a 12-h acetylcysteine regimen (200 mg/kg over 4 h, 50 mg/kg over 8 h) were assayed for paracetamol metabolites as a pilot study, using liquid chromatography/mass spectrometry. Control group subjects received a 20-h course of acetylcysteine (200 mg/kg over 4 h, 100 mg/kg over 16 h). The intervention group received a 12-h acetylcysteine regimen (stopped after at least 12 h of treatment). Please note that this is not the control group. Positive control groups not in the trial with acute liver injury (ALI) or hepatotoxicity were also studied.

Results: One hundred and forty-four blood samples were collected from 40 patients receiving acetylcysteine after paracetamol overdose. Median ALT after 20 h of acetylcysteine was 12 U/L (IQR 8.14) in the abbreviated regimen group, compared to the control group 16 U/L (IQR 11.21) (p = .46). There was no significant difference in median metabolite concentrations on presentation and after 20 h of acetylcysteine between these two groups (p > .05). Presentation median sum CYP-metabolite/total metabolite percentages were 2.5 and 3.0 in the abbreviated and control NACSTOP groups, respectively.

Conclusions: An abbreviated 12-h acetylcysteine regimen for paracetamol overdose used in the NACSTOP trial had similar circulating metabolite concentrations compared to a 20-h regimen in selected subjects with low risk of hepatotoxicity. This suggests that further acetylcysteine may not be needed in the abbreviated group at time of cessation.

Introduction
Paracetamol is one of the commonest medications taken in overdose in the world. Annually, the UK sees approximately 100,000 presentations per annum and is the number-one most frequent call to poisons information centres in Australia [1,2]. Paracetamol overdose is the most common cause of acute liver failure in developed countries, and in the worst circumstances can lead to death [3].

Paracetamol primarily undergoes glucuronidation (APAP-gluc) (60%) and sulfation (APAP-Sul) (35%) with less than 5% metabolized via the cytochrome P450 system to N-acetyl-para-benzoquinamine (NAPQI) [4]. NAPQI is conjugated with glutathione (APAP-GSH), which is further metabolized to APAP-cysteine (APAP-Cys) and APAP-mercapturate (APAP-Mer) [5]. After a paracetamol overdose, glucuronidation and sulfation pathways become saturated. This leads to more NAPQI formation. Hepatic glutathione stores deplete rapidly and NAPQI binds to sulfhydryl groups on cellular proteins. Binding of NAPQI to cellular proteins results in the formation of APAP protein adducts which can be detected in the protein fraction of serum. NAPQI concentration is difficult to measure in blood or serum because of its volatile nature. Previous studies have examined the non-protein fraction of plasma for APAP-Cys in the setting of paracetamol overdose, which is a surrogate marker for NAPQI production [6].

Acetylcysteine is the antidote used to prevent hepatotoxicity following paracetamol overdose. The initial intravenous acetylcysteine regimen was first proposed in the 1970s [7]. The 20.25 h duration of infusion was based upon an assumed paracetamol half-life of 4 h, with five elimination half-lives used for infusion duration. Serum paracetamol concentration assays were not routinely available in hospitals in this era. Serial paracetamol and liver function testing are now routine, and can provide reassurance that hepatic function is preserved as paracetamol concentration falls.

A two-bag acetylcysteine regimen consisting of 200 mg/kg infused over 4 h followed by a further 100 mg/kg infused over 16 h has recently been reported to decrease adverse...
reactions and simplifies the standard three bag regimen [8–10]. Subsequent to this the NACSTOP trial compared early cessation of acetylcysteine after 12 h of treatment to a standard 20-h infusion, using this two-bag regimen [11]. Subjects were included in the study if, after 12 h of acetylcysteine, serum paracetamol concentration was low (<132 μmol/L or 20 mg/L) and the alanine transaminase (ALT) was normal (<40 U/L) regardless of the time delay to treatment following overdose. Other studies have also evaluated reduced duration acetylcysteine regimens [12,13].

The aim of this study was to compare degree of liver injury and paracetamol metabolite concentrations in NACSTOP trial subjects receiving standard of care 20-h infusion, to those receiving the abbreviated 12-h infusion regimen. In addition, a separate cohort of patients, not enrolled in NACSTOP, who developed acute liver injury (ALI) or hepatotoxicity after paracetamol overdose treated with acetylcysteine, were included as comparator groups.

Methods

Study population

A cohort of subjects treated with acetylcysteine following single or staggered ingestion paracetamol overdose was collected. These subjects were recruited from the NACSTOP trial during the period February 2015 to July 2016, from three emergency departments at presentation from Monash Health, Victoria, Australia and in a cluster-controlled study design [14]. Patients were treated with acetylcysteine if meeting criteria as per the Australian and New Zealand paracetamol treatment guidelines [15], for example, treat above the paracetamol nomogram line for single ingestions (1000 μmol/L [150 mg/L] at 4 h post-overdose). Patients with a normal serum ALT at presentation (ALT <40 U/L) and therapeutic or lower paracetamol (<132 μmol/L [20 mg/L]) and normal ALT after 12 h of acetylcysteine infusion were included in the trial.

Excluded patients included those <16 years old, pregnancy, unknown time of ingestions and modified-release paracetamol overdose. The control group received the full 20-h course of acetylcysteine (200 mg/kg over 4 h, 100 mg/kg over 16 h), whilst the intervention group received an abbreviated 12-h acetylcysteine regimen (i.e., 200 mg/kg over 4 h, followed by at least half of the 100 mg/kg over 16-h infusion). Full informed consent from the participants was obtained to participate in the study [11].

The primary outcome of this study was the development of ALI at 20 h post-acetylcysteine from a normal presentation ALT (<40 U/L). ALI was defined as an ALT >50 U/L in this study. Hepatotoxicity was defined as an ALT >1000 U/L.

In addition, two comparator groups of patients that developed liver injury or hepatotoxicity secondary to paracetamol overdose and not involved in the NACSTOP trial were also included. As a result, there were four groups that were compared: NACSTOP patients (i) abbreviated treatment regimen, (ii) full treatment regimen (control group); and non-trial patients: (iii) those who developed liver injury and (iv) those who developed hepatotoxicity.

Those developing ALI or hepatotoxicity had prolonged acetylcysteine infusions beyond the standard 20 h until serum ALT had peaked and was falling and International Normalised Ratio (INR) was <2.

Blood samples were collected at presentation (pre-treatment), after 12 and 20 h of acetylcysteine in the NACSTOP patients. More blood samples were collected in those with ALI and hepatotoxicity until ALT was peaked and falling. Serum was separated and stored at ~80°C till analysis. Samples were analysed using liquid chromatography–tandem mass spectrometry (LC–MS/MS) (Clinical Research Facility, Edinburgh, UK). Each sample was tested for APAP-Cys (non-protein fraction), APAP-Sul, APAP-Glu, APAP-GSH, APAP-Mer and APAP LC/MS. Presentation, peak and end of acetylcysteine infusion concentrations of these metabolites were examined. Receiver operating characteristic curve–area under the curve (ROC–AUC) calculations to predict the outcome of ALI was also performed.

Laboratory analysis

Briefly, APAP and metabolites were extracted from plasma by liquid–liquid extraction with acidified methanol. Ten microliters of plasma were enriched with 10 ng APAP-d4 (APAP-d4) and 10 ng APAP-SUL-d3 as internal standards and 0.8 mL methanol (w/0.2% acetic acid) was added, vortexed, and incubated for 20 min on ice. After centrifugation (3000g, 10 min, 10°C) the supernatant was decanted and reduced to dryness under nitrogen at 40°C and reconstituted in mobile phase (200 μL water/methanol (65:35, v/v)) and centrifuged for a second time.

Analysis was carried out by LC–MS/MS. Liquid chromatographic separation was achieved using an Aria CTC autosampler and Allegros pump on an ACE Excel 2 Super C18 column (150 × 3 × 3 mm; 2 lm) protected by a Kinetex KrudKatcher (Phenomenex, UK) at 20°C and detected on a TSQ Quantum Discovery triple quadrupole mass spectrometer (Thermo Fisher Scientific, UK) operated by selective reaction monitoring. Full lab methodology has been described previously [6].

Statistical analysis

From the NACSTOP trial, a sample size of 100 patients was selected based on expected recruiting efficiency and on the feasibility of the trial given the number of sites participating. A low primary outcome rate would give confidence to pursue a larger clinical trial. This exploratory study aimed to enrol as many of these patients for separate paracetamol metabolite testing during the trial period. Descriptive data are reported with continuous variables reported as median (IQR) unless otherwise stated and compared using Mann–Whitney U test and Kruskal–Wallis analysis of variance by ranks, as appropriate. For subjects with an acute ingestion at a known time, we calculated the ratio between each subject’s acetaminophen concentration at presentation (APAP) and the threshold concentration for treatment based on the time interval post ingestion using the Rumack–Matthew...
Table 1. Patient characteristics for the abbreviated and control acetylcysteine treatment groups as well as acute liver injury (ALI), hepatotoxicity groups.

|                                      | Abbreviated (n = 8) | Control (n = 21) | p Value | ALI (n = 9) | Hepatotoxicity (n = 2) |
|--------------------------------------|---------------------|------------------|---------|-------------|------------------------|
| Gender, % female (n)                 | 85 (6)              | 86 (18)          | NS      | 33 (3)      | 50 (1)                 |
| Median age, years (IQR)              | 19 (18, 41)         | 21 (16, 32)      | NS      | 22 (20, 33) | 34 (30, 37)            |
| Median reported paracetamol dose single ingestion, g (IQR) | 20 (13, 30)         | 20 (12, 23)      | NS      | 20 (15, 30) | 29.5 (29.5, 30)        |
| Mean reported paracetamol single ingestions by weight, mg/kg (95%CI) | 398 (138, 657)      | 284 (225, 342)   | NS      | 292 (202, 384) | 514 (500, 527)        |
| Median acetylcysteine dose, g (IQR)  | 15 (13, 17)         | 19 (17, 23)      | .02     | 23 (17, 29) | 30 (30, 30)           |
| Median acetylcysteine duration, h (IQR) | 13 (13, 13)        | 20 (20, 20)      | <.0001  | 20 (16, 20) | 64 (48, 80)           |
| Median time to acetylcysteine post-overdose, h (IQR) | 6 (5.5, 12)        | 6.5 (5.6, 11)    | NS      | 9 (5.3, 16) | 21 (12, 31)           |
| Median presentation alanine aminotransferase, IU/L (IQR) | 13 (9, 29)          | 17 (12, 21)      | NS      | 52 (27, 65) | 492 (281, 703)        |
| Median presentation paracetamol concentration, μmol/L (IQR) | 1050 (668, 1105)   | 1122 (542, 1581) | NS      | 863 (260, 1161) | 576 (60, 1091)        |
| Median alanine aminotransferase 20 hours post-initiation acetylcysteine, U/L (IQR) | 12 (18, 14)        | 16 (11, 21)      | NS      | 62 (49, 87) | 1572 (1249, 2947)     |
| Median INR (IQR) 20 h post-initiation acetylcysteine | 1.1 (1.1, 1.3)      | 1.3 (1.1, 1.4)   | NS      | 1.3 (1.1, 1.3) | 3.2 (1.5, 4.8)        |
| Median creatinine 20 h post-initiation acetylcysteine, μmol/L (IQR) | 63 (44, 75)        | 55 (49, 71)      | NS      | 64 (42, 70) | 90 (85, 90)           |
| Number of patients with ingestion to acetylcysteine <8 h, n (%) | 4 (57)             | 14 (66)          | NS      | 5 (56)      | 0                      |
| Ward admission median length of stay, days (IQR) | 1 (1, 1)           | 1 (1, 1)         | NS      | 1 (1, 1)    | 3.5 (3, 4)            |

The Mann–Whitney U test was used to compare the abbreviated and control groups. NS: not statistically significant.

Results

One hundred and forty-one blood samples were collected from 40 patients receiving acetylcysteine after paracetamol overdose (Table 1). There were eight patients (20%) who received the 12-h treatment regimen (abbreviated) and 21 (52.5%) receiving 20-h of acetylcysteine (control group). Nine (22.5%) patients developed ALI and two (5%) had hepatotoxicity and all received acetylcysteine. The overall median age was 22 years (IQR 18, 32). The majority of patients were male (70%). The APAP₂:APAP₃ ratio was also similar between the NACSTOP groups (1.3 [IQR 1.1, 1.7]) in the abbreviated vs. 1.25 [1, 1.6] in the control group, p = .38; all had a ratio between 1 and 2. In the ALI group, the APAP₂:APAP₃ ratio was 1.2 (IQR 1.1, 1.9) for the eight patients who had a paracetamol concentration performed within 24 h of overdose.

Median ALT after 20 h of acetylcysteine was 12 U/L (IQR 8.14) in the abbreviated NACSTOP regimen group, compared to 16 U/L (IQR 11.21) in the NACSTOP control group (p = .46) (Table 1). No patients in the NACSTOP trial developed ALI. In the ALI and hepatotoxicity groups, median ALT after 20 h of initiation of acetylcysteine was 62 U/L (IQR 49.87) and 1572 U/L (IQR 1249, 2947), respectively.

Median time to starting acetylcysteine post-overdose was 6-h (IQR: 5.5, 12), 6.5 (5.6, 11), 9 (5.5, 16), 21 (12, 31) in the abbreviated, control, ALI and hepatotoxicity groups, respectively. Median acetylcysteine duration was 13 h (IQR 13, 13) for the abbreviated group, significantly shorter than the controls (20 h [20, 20]), ALI (20 h [16, 20]) and hepatotoxicity groups (64 h [48, 80]) (p < .0001).

Paracetamol metabolite concentrations were examined and compared between the study groups (Figure 1). There was no significant difference in median presentation metabolite concentrations in the abbreviated and control groups (p > .9) (Table 2). Median metabolite concentrations after 20 h of acetylcysteine were also similar between the two groups (p > .05). Specifically, there was no significant difference comparing APAP-Cys in the abbreviated (0.95 μmol/L [IQR 0.60, 1.8]) versus control groups (2.2 μmol/L [1.29, 5.51]) (p = .07). All median paracetamol metabolite concentrations were decreased on presentation in the hepatotoxicity group compared to the abbreviated group, except APAP-GSH which was mildly increased (0.03 μmol/L [IQR 0.027, 0.036] vs. 0.016 μmol/L [0.004, 0.024]), but this was not statistically significant (p = .8).

Median presentation metabolite concentrations were examined across the study groups (Table 2). There was no significant difference in all median presentation metabolite concentrations when comparing the abbreviated and control groups (p > .05). When comparing both the abbreviated group with those who developed hepatotoxicity, there was a significant decrease in median presentation APAP-Glu (765 μmol/L [IQR 572, 972] vs. 61 μmol/L [33, 91]) and APAP-Sul (108 μmol/L [82, 202] vs. 21 μmol/L [10, 32]), respectively (p < .04).

To compare the relative amounts of metabolites formed by CYP activity and non-CYP conjugation, sum CYP metabolites (APAP-Cys, APAP-Mer, APAP-GSH) was expressed as fraction of total metabolites on presentation and a cumulative value after 20 h post-initiation of acetylcysteine (AUC₀–₂₀h). Presentation median sum CYP-metabolite/total metabolite percentages were 2.5, 3.0, 2.8 and 14.9 in the abbreviated, NACSTOP-control, ALI and hepatotoxicity groups, respectively (p < .0001). There was no significant difference in CYP-metabolite percentage at presentation or with AUC₀–₂₀h in those not developing any liver injury (p > .6).

The ROC–AUC on presentation using the definition of ALI as an end point of each paracetamol metabolite is present in Table 3. Of the paracetamol metabolite concentrations at presentation, APAP-GSH had the highest predictive value for ALI with a ROC AUC 0.68 (95% CI 0.5, 0.85).
Paracetamol metabolites can be useful to compare the effectiveness of different acetylcysteine treatment regimens in preventing liver injury. In our study, similar metabolite concentrations were evident when comparing an abbreviated 12-h to a 20-h acetylcysteine treatment regimen for treating low risk patients taking paracetamol overdoses. Identification of patients with low risk of developing hepatotoxicity (i.e., ALT < 40 U/L and low paracetamol concentrations) after at least 12 h of acetylcysteine were key inclusion criteria in selecting subjects suitable for an abbreviated regimen.

Presentation and 20-h ALT concentrations were similar between the two NACSTOP trial treatment groups enrolling patients at low risk of liver injury. Paracetamol metabolite concentrations were also similar on presentation and after 20 h of acetylcysteine in both the groups. If there was a significant increase in NAPQI production, one might expect higher concentrations of CYP metabolites. Therefore, there is unlikely have been a significant difference in NAPQI production between the two groups when acetylcysteine was ceased early in the NACSTOP-abbreviated group. This suggests that further acetylcysteine may not be needed in the abbreviated group at time of cessation.

Similarly, the ALI group also followed similar metabolite concentrations over the 20-h treatment course of acetylcysteine. This is likely due to the minimal degree of liver injury in this group. When analysing the CYP metabolites, those who developed hepatotoxicity had higher concentrations of APAP-GSH throughout their admission, which correlates with results of a similar previous study. In addition, APAP-Mer was not significantly different between those who developed ALI and those that did not. Of the CYP metabolites, APAP-GSH concentration is most likely to be continually elevated in patients that develop liver injury.

There was no difference in the non-CYP metabolite concentrations (APAP-Glu and APAP-Sul) in abbreviated, control and ALI groups. Similar to a previous study, these were less useful at distinguishing between patients who did and did not develop hepatotoxicity.
not develop ALI [18]. The decreased concentration of APAP-Glu and APAP-Sul in patients who developed hepatotoxicity in our study were related to the delay in presentation to hospital. Thus paracetamol metabolism, most likely, had peaked prior to presentation.

Presentation and AUC0–20 sum CYP metabolite percentages were increased in patients developing hepatotoxicity compared to those who did not. These findings are similar to a previous study where CYP metabolites percentages were higher in those who developed ALI (>50% ALT rise) compared to those that did not [18]. In our study, the difference in CYP metabolite percentages was less pronounced between the NACSTOP and ALI groups. This is the result of a smaller range in ALT rise in our ALI group. However, our study demonstrated much higher peak ALTs in the hepatotoxicity group in comparison.

APAP-GSH outperformed the other metabolites in the prediction of development of ALI in our study. Sum CYP metabolites/total % and APAP Cys/Sul ratio also performed well. In the study by Vliegenthart et al. [18], APAP Cys/Sul ratio and sum CYP metabolites performed better compared with other paracetamol metabolites as predictors of ALI on presentation. However, ALT on presentation performed better than any of the paracetamol metabolites at predicting ALI in our study. This discordance may relate to a smaller sample size and higher proportion of patients with ALI used as positive controls in our study. Other risk prediction tools such as the paracetamol-multiplication product [19,20] or miR-122 assays [21] may be more sensitive or available methods to determine risk of liver injury upon presentation.

There are several limitations of this study. The stated ingestion times of paracetamol were based on patient recollection, but were asked on multiple occasions to minimise any inaccuracies. The study was not randomized at the individual subject level, and a cluster design for the study was chosen for both simplicity and to minimize protocol violations and crossover. The primary endpoint was based on biochemical test results and hence unlikely to be biased. The sample size in this study was small, but appropriate for an initial feasibility study. We believe that future studies can now test our abbreviated acetylcysteine regimen and utilise paracetamol metabolites as comparative biomarkers to demonstrate minimal differences in CYP metabolism with low risk patients.

Table 3. Predictive value of paracetamol metabolites on presentation for development of acute liver injury prior to initiation of acetylcysteine.

| ROC–AUC (95% CI) | Sensitivity % (95% CI) | Likelihood ratio |
|------------------|------------------------|-----------------|
| ALT              | 0.94 (0.87, 1.0)       | 75 (20, 99)     | 7.3 |
| APAP-GSH         | 0.68 (0.5, 0.85)       | 27 (6, 61)      | 2.6 |
| APAP lab concentration | 0.66 (0.47, 0.84) | 27 (6, 61) | 2.6 |
| APAP-Sul         | 0.66 (0.44, 0.89)      | 47 (16, 77)     | 4.4 |
| Sum CYP/total metabolites | 0.65 (0.47, 0.84) | 27 (6, 61) | 2.6 |
| APAP Cys/Sul     | 0.63 (0.42, 0.83)      | 36 (11, 69)     | 3.5 |
| APAP-Glu         | 0.59 (0.38, 0.81)      | 36 (11, 69)     | 3.5 |
| APAP-Mer         | 0.57 (0.35, 0.79)      | 27 (6, 61)      | 2.6 |
| Sum CYP metabolites | 0.52 (0.30, 0.74) | 27 (6, 61) | 2.6 |
| APAP-Cys         | 0.51 (0.29, 0.73)      | 18 (2, 52)      | 1.7 |

Sensitivity (at 90% specificity), ALT: alanine transaminase; APAP: paracetamol, APAP-Glutathione (GSH), APAP-Sulfate (sul), CYP: cytochrome P450, APAP-Cysteine (cys), APAP-Glucuronide (glu), APAP-Mercapturate (mer).

Conclusions

An abbreviated NACSTOP 12-h acetylcysteine regimen for paracetamol overdose showed no difference in circulating metabolites compared to a 20-h regimen in subjects at low risk of hepatotoxicity. This suggests that further acetylcysteine may not be needed in the abbreviated group when paracetamol concentration is undetectable and liver transaminases are normal at the time of treatment cessation.

Disclosure statement

No potential conflict of interest was reported by the authors.

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