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Research Article

Co-administration of N-Acetylcysteine and Acetaminophen Efficiently Blocks Acetaminophen Toxicity

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ABSTRACT Although acetaminophen (APAP) is an effective analgesic and anti-pyretic, APAP overdose is the most frequent cause of serious, often lethal, drug-induced hepatotoxicity. Administration of *N*-acetyl cysteine (NAC) within 8 hours of APAP overdose effectively mitigates APAP-induced hepatotoxicity. Thus, preventing APAP toxicity before it occurs by formulating APAP with NAC is logical and, as we show here in a mouse model, is effective in preventing APAP toxicity. Thus, toxic oral APAP doses sufficient to cause severe widespread liver damage do not cause significant damage when administered concurrently with equal amounts of NAC, that is, in the NAC-APAP treated animals, hepatic transaminases increase only marginally and liver architecture remains fully intact. Thus, we conclude that concomitant oral dosing with APAP and NAC can provide a convenient and effective way of preventing toxicity associated with large dosage of APAP. From a public health perspective, these findings support the concept that a co-formulation of APAP plus NAC is a viable over-the-counter (OTC) alternative to the current practice of providing APAP OTC and treating APAP toxicity if/when it occurs. In essence, our findings indicate that replacing the current OTC APAP with a safe and functional APAP/NAC formulation could prevent the accidental and intentional APAP toxicity that occurs today. Drug Dev Res 76 : 251–258, 2015.

Key words: acetaminophen (APAP); N-acetylcysteine (NAC); hepatoxicity; drug-induced liver damage; acetaminophen toxicity

INTRODUCTION

Acetaminophen-*N-acetyl-p-amino-phenol-* (APAP) is commonly used for analgesic, antipyretic, and peripheral antiinflammatory purposes. [Vad et al., 2009] The major problem with APAP treatment, including self-dosing with over-the-counter (OTC) APAP, is its hepatoxicity, [Priyadarsiny et al., 2008] including acute liver failure [Lee, 2003] at doses that can be readily consumed by children and adults. APAP overdose is estimated to be responsible for over 56,000 emergency department visits and 26,000 hospitalizations annually [Nourjah et al., 2006].

N-acetyl cysteine (NAC) can be administered either orally (p.o.) or intravenously (i.v.) to counteract APAP overdose. Available data suggest that either route is equally effective [Green et al., 2013; Schwarz

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and Cohn, 2014], although some reports suggest that NAC administration p.o. can result in better outcomes [Smilkstein et al., 1988, 1991]. NAC administration i.v. is simpler and easier to administer in hospital settings but is accompanied by anaphylactic reactions that require treatment in less than 10% of patients [Dawson et al., 1989; Kerr et al., 2005; Prescott, 2005]. As these adverse reactions are readily treated in hospital settings (usually by administration of epinephrine), they do not pose a serious problem when NAC is administered i.v., in hospital or other settings prepared to deal with the issue.

Overall, NAC p.o. would be preferable, particularly in settings, for example, during pregnancy, where NAC i.v. administration is difficult or medically counter-indicated. However, until recently, approved NAC p.o. preparations have a disagreeable taste (some patients to liken it to rotten eggs) reserving p.o. treatment to situations in which NAC i.v. may be expected to induce adverse reactions or where APAP antidotes must be administered without adequate medical backup to deal with anaphylactiod or other adverse reactions [Kanter, 2006; Bronstein et al., 2010].

Fortunately, the taste perversion associated with older NAC formulations is not present in modern formulations, making these preferable *and readily available* for oral dosing. A single study has shown that at least one of these preparations is well tolerated [De Rosa et al., 2000] even when used repeatedly for long periods at the very high doses (e.g., 1,800 mg bid for 6 months) [Tirouvanziam et al., 2006]. This ready toleration of long-term oral NAC administration at high doses suggests a simple solution [Andrus et al., 2001; Mehrpour and Ballali-Mood, 2011] to the persistent problem of APAP overdosing with OTC APAP preparations: why not simply co-formulate or co-package APAP with sufficient NAC to avoid potential APAP toxicity? We discuss these findings in terms of the public health gains that could be accomplished by (i) counseling (prescribing) the use of NAC together with APAP; (ii) making co-formulated NAC and APAP available OTC; or better yet, and (iii) by requiring that all OTC APAP be formulated with a toxicity-reducing dose of NAC.

METHODS AND MATERIALS

Animals

The protocol used for the study was approved by Stanford University Administrative Panel of Laboratory Animal Care (APLAC), and all animals were humanely cared for according to the NIH criteria outlined in the "Guide for the Care and Use of Laboratory Animals." Ten week old male (C57BL6 × Balb/c) F1 mice were obtained from Jackson Laboratories (Sacramento, CA) and maintained on standard animal facility chow for 2–8 weeks prior to study. All animals were housed in plastic cages in a room maintained at 22–25°C and 20–50% humidity with a 12-hour/light dark cycle. Standard mouse chow (Harlan & Teklad, Livermore, CA) and water were available ad libitum. Food was removed 16 hours prior to treatment; water was allowed ad libitum.

Treatment Protocols

All animals (23–27 g) were acclimated for 1 week before commencement of the study. NAC and APAP were dissolved in double-distilled water. The mice randomized into 10 groups of 3 mice each and were treated by gavage with a solution containing NAC (300, 400, and 600 mg/kg) alone, APAP (300,400, and 600 mg/kg) alone, and APAP+NAC (300, 400, and 600 mg/kg). Control mice received an equivalent amount of warm double-distilled water; all animals were allowed free access to feed and water ad libitum after dosing and were observed for 24 hours before being sacrificed by carbon dioxide asphyxiation prior to collection of whole blood from the heart via cardiac puncture and excision of liver which were immediately sectioned and preserved in phosphate buffered formalin.

Chemicals

Acetaminophen–*N*-acetyl-p-amino-phenol (APAP) was obtained from Sigma Chemical Co., St. Louis, MO. *N*-acetylcysteine (NAC) was obtained from Bio-Adventex Pharma, Inc., Mississauga, Ontario, Canada, in foil-wrapped packets to prevent oxidation. Each packet contained one tablet contained 900 mg of NAC formulated with inactive ingredients: citric acid, sodium bicarbonate, sodium carbonate, manitol, flavoring, acesulfame potassium, and trisodium citrate with a total weight per tablet of 3 g.

Assay Kits

Alanine, aspartate and γ-glutamyl transferase assay kits and lactate dehydrogenase reagents were purchased from Thermos Scientific (Middletown, VA). Thiobarbituric acid and the GSH-Glo™ Glutathione assay kit were purchased from Oxford Biomedical Research (Oxford, MI) and Promega (Madison, WI) respectively.

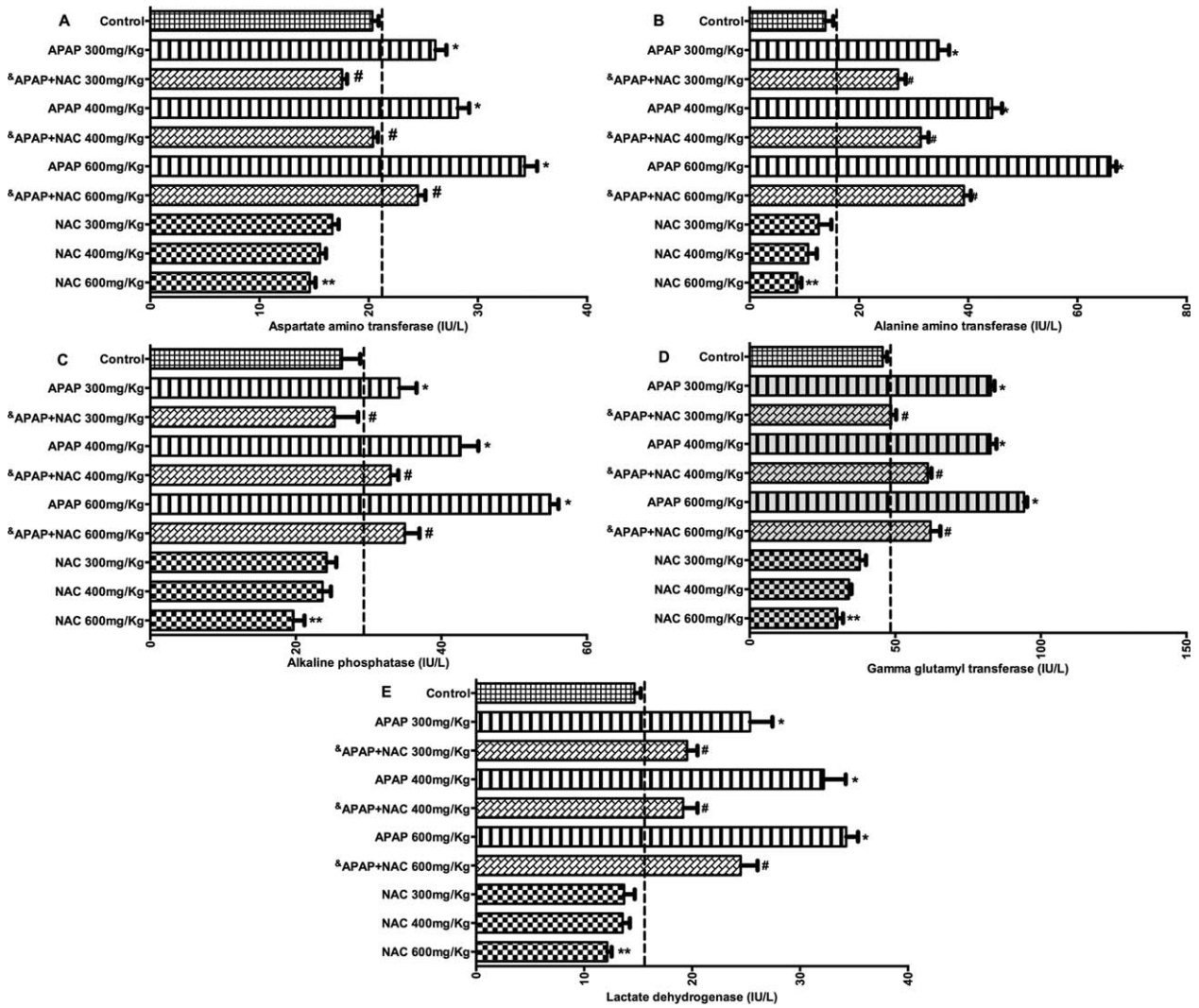

Fig. 1. (**A–D**) Effect of acetaminophen (APAP; A) and N-acetyl cysteine (NAC; N) co-administration on hepatic transaminases and Lactate dehydrogenase (LDH) in C57/BalbC mice. Mice were treated with APAP and NAC as shown, after 24 hours serum activity of hepatic transaminases and LDH were assessed. The results are expressed as mean ± S.D (IU/L) $P < 0.05$. AST, aspartate amino transferase; ALT, alanine amino transferase; ALP, alkaline phosphatase; GGT, γ-glutamyl transferase; AST, aspartate transferase. Results are expressed as means ± S.D ($n = 3$). *($P < 0.01$) from control, #($P < 0.01$) APAP group, **($P < 0.01$) from A+N group. A, acetaminophen; N, *N*-acetylcysteine; and (A+N), acetaminophen + *N*-acetylcysteine. AST, aspartate amino transferase; ALT, alanine amino transferase; ALP, alkaline phosphatase; γ-GT, gamma glutamyl transferase, and LDH, lactate dehydrogenase.

Serum Samples

Blood was drawn by cardiac puncture from the heart into centrifuge tubes and was allowed to clot at room temperature. The clotted blood was centrifuged (3000 *g* at 4°C, for 10 min) in a TOMY MX-300 centrifuge and the serum collected for the estimation of hepatic enzymes and lipid peroxidation.

Enzyme Assays

Hepatic transaminases (alanine aminotransferase [ALT], aspartate amino transferase (AST), γ-glutamyl transferase, alkaline phosphatase [ALP]) and lactate dehydrogenase (LDH) activities were assessed biochemically in serum. In addition, the 2-thiobarbituric acid reacting species (TBARS) formation was also measured an index of lipid peroxidation and oxidative stress in serum.

Histology

Excised livers were rinsed in phosphate buffered saline, blotted and weighed. Sections from each liver lobe were processed for histopathology.

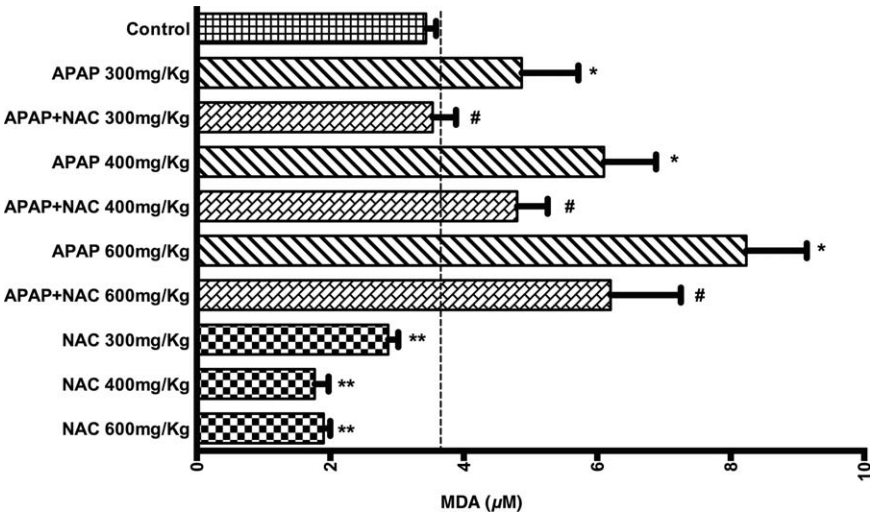

Fig. 2. Effect of acetaminophen and N-acetylcysteine co-treatment on hepatic lipid peroxidation in C57/Balb C mice. Data represents means ± S.D ($n = 3$), *significantly different ($P < 0.05$) from control, **significantly different ($P < 0.05$) from control group, # significantly different ($P < 0.05$) from APAP only group. A, acetaminophen; *N*, *N*-acetylcysteine; (A+N), acetaminophen+*N*-acetylcysteine; and MDA, malondialdehyde.

Formalin fixed liver section embedded in paraffin was layered on glass slides and stained with hematoxylin and eosin (H&E) following standard protocol prior to examination.

Lipid Peroxidation Assay

Lipid peroxidation was assessed by quantifying thiobarbituric acid reacting substances (TBARS) malondialdehyde (MDA) levels in freshly prepared and deproteinized serum using an assay kit (Oxford Biomedicals) as described by the manufacturer. The absorbance of the chromogenic product formed was obtained at 532 nm using a SpectraMax Plus^{384} Microplate Reader (Molecular Devices, CA) and the results obtained from the Standard curve generated.

Glutathione Assay

Total hepatic glutathione was assessed using an assay kit (Promega, WI) as described by the manufacturer. Liver extract (50 μL) was dispensed in triplicate into a 96-well plate in addition to 50 μL of 2X GSH-Glo reagent and incubated for 30 min at room temperature. The reaction was terminated by the addition of previously reconstituted Luciferin detection reagent (100 μL), the resulting solution was mixed briefly on a plate shaker and allowed to incubate for another 15 min at room temperature. Luminescence generated from each sampled well was estimated using a TECAN Infinite M-1000 PRO 96-well plate reader. The net GSH-dependent relative luminescence units (net-RLU) were estimated by subtracting the mean luminescence of the negative control from that of the GSH-containing reaction. This represents the GSH activity expressed as RLU.

Statistical Analysis

Data are expressed as mean ± sd, and analyzed with one-way analysis of variance (ANOVA) using JMP version 10. Statistical significance was set at $P < 0.05$.

RESULTS

Co-administered NAC Decreases APAP Toxicity: Hepatic Enzyme Levels Increase in Sera

Oral administration of APAP to adult mice induces liver damage in a dose-dependent manner. Liver enzyme levels (AST, ALT, GGT, ALP, and LDH) in serum increase in a dose-dependent manner when mice are treated with increasing doses of APAP ($P < 0.05$) (Fig. 1). Liver enzymes do not rise when NAC, an antidote to APAP toxicity, is co-administered with APAP.

Co-administered NAC Decreases APAP-Toxicity: Lipid Peroxidation (LP) Decreases in the Liver

Hepatic lipid peroxidation (LP) in mice treated with NAC alone were reduced ($P < 0.05$) below levels obserserved in control in a dose-dependent manner. APAP treatment alone dose-dependently increased hepatic LP. In the presence of NAC, APAP-induced LP was reduced. These decreases were observed in the 300 and 400 mg/Kg A-N treated groups.

TABLE 1. Effect of Acetaminophen and N-acetylcysteine Treatment on Body Weight, Liver and Relative Liver Weight of C57/Balb C Mice

Groups	Body weight		Liver		Kidney Appearance
	Initial (g)	Final (g)	Wt. (g)	RLW (%)	
Control	24.90 ± 1.24	25.10 ± 1.21	1.10 ± 0.10	4.37 ± 0.19	Normal
APAP 300 mg/Kg	25.00 ± 1.73	25.26 ± 1.58	1.33 ± 0.05*^{S}	5.29 ± 0.48	Normal
APAP 400 mg/Kg	24.40 ± 1.87	24.00 ± 1.83	1.26 ± 0.05	5.30 ± 0.53	Normal
APAP 600 mg/Kg	24.63 ± 1.30	24.60 ± 1.15	1.36 ± 0.05*^{S}	5.57 ± 0.47*	Normal
NAC 300 mg/Kg	25.50 ± 0.87	25.80 ± 0.96	1.13 ± 0.05$^{\#}$	4.40 ± 0.35	Normal
NAC 400 mg/Kg	24.56 ± 1.43	24.73 ± 1.40	1.10 ± 0.10	4.45 ± 0.37	Normal
NAC 600 mg/Kg	24.86 ± 0.23	25.06 ± 0.49	1.16 ± 0.05	4.65 ± 0.14	Normal
A/N 300 mg/Kg	24.63 ± 1.02	24.60 ± 1.31	1.20 ± 0.00	4.88 ± 0.26	Normal
A/N 400 mg/Kg	25.33 ± 1.30	25.23 ± 0.75	1.20 ± 0.00	4.75 ± 0.13	Normal
A/N 600 mg/Kg	24.23 ± 2.15	24.33 ± 2.14	1.13 ± 0.05	4.66 ± 0.16	Normal

Results are expressed as means ± S.D ($n = 3$).
*Significantly different ($P < 0.05$) from control.
$^{\#}$Significantly different ($P < 0.05$) from APAP-NAC-paired group.
^{S}Significantly different ($P < 0.05$) from NAC only group.
A, acetaminophen; *N*, *N*-acetylcysteine; and (A+N), acetaminophen +*N*-acetylcysteine.

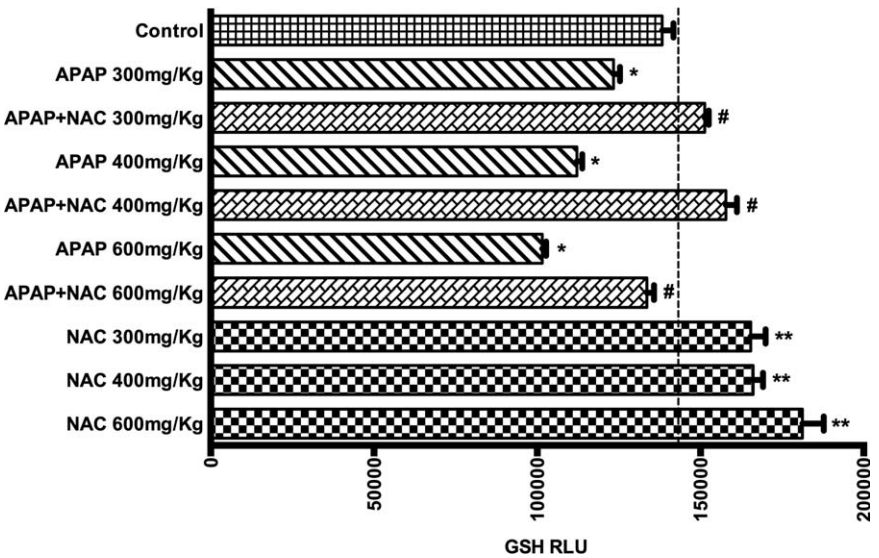

Fig. 3. Effect of acetaminophen and N-acetylcysteine co-treatment on hepatic glutathione in C57/Balb C mice. Data represents means ± S.D ($n = 3$), *significantly different ($P < 0.05$) from control, # significantly different ($P < 0.05$) from APAP only group **significantly different ($P < 0.05$) from control group. A, acetaminophen; *N*, *N*-acetylcysteine; A+N, (acetaminophen +*N*-acetylcysteine; and RLU, relative lumenescence unit.

In animals treated with A-N (400 and 600 mg/Kg) LP was reduced but was higher than control (Fig. 2).

Co-administered NAC Decreases APAP-Toxicity: Liver Weight Does Not Decrease

The body weight of mice in treated groups at the termination of treatment was not significantly different (Table 1) compared to control mice. However, the weight of the liver was significantly increased in all APAP-treated groups ($P < -0.03$ to 0.05).

Co-administered NAC Decreases APAP-Toxicity: Increases Baseline Hepatic Glutathione

Treatment with NAC alone increased hepatic GSH levels in mice (Fig. 3) to levels higher than control. However GSH was depleted ($P < 0.05$) in mice treated with APAP ($600 > 400 > 300$ mg/Kg) compared to controls. A-N treatment abrogated APAP-induced hepatic GSH depletion; with an overall increase ($P < 0.05$) in GSH levels with co-administration compared to APAP alone and control groups.

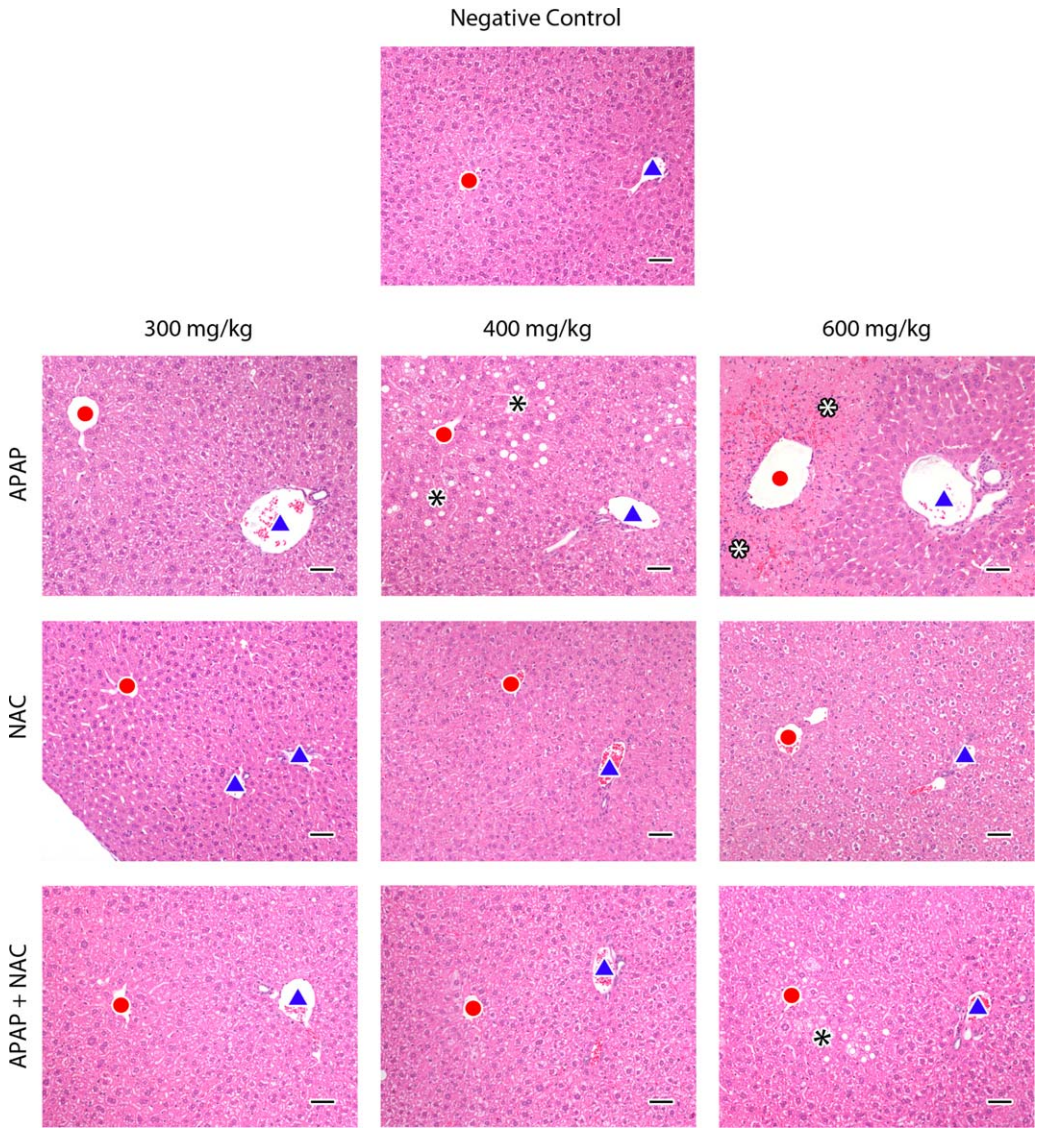

Fig. 4. Photomicrographs of mouse livers treated with acetaminophen (APAP) and *N*-acetylcysteine (NAC) for 24 hours. Histologic architecture/cellular structure is normal in the negative control. There is evidence of coagulation necrosis (white asterisks), and macrovesicular and microvesicular lipidosis (black asterisks) of hepatocytes in mice receiving 600 mg/kg and 400 mg/kg APAP, respectively, compared to the histologically normal liver of the mouse receiving 300 mg/kg APAP (similar to the negative control mouse). In NAC treated mice, the liver remains essentially normal (similar to the negative control mouse) regardless of dose. In mice administered both APAP and NAC, macrovesicular and microvesicular lipidosis (black asterisk) of hepatocytes is only noted in the mouse receiving the 600 mg/kg dose, compared to the essentially normal livers (similar to the negative control mouse) of mice receiving the lower 300 and 400 mg/kg doses. H&E stain; 200 × magnification; bar = 50 microns; blue dots = portal triads; red circles = central veins.

Co-administered NAC Decreases APAP-Toxicity: Histologic Damage is Greatly Reduced

Livers architecture and cellular structures appeared normal in control mice on histological examination with small numbers of microvesicular lipid droplets that did not result in hepatocellular swelling (physiological lipidosis). There was evidence of acute coagulation necrosis, macrovesicular and microvesicular lipidosis of centrilobular hepatocytes in mice receiving APAP (600 and 400 mg/kg) respectively, compared to the histologically normal liver of the mice receiving 300 mg/kg APAP that are similar to the negative control mice (Fig. 4). In mice receiving NAC, the livers remain essentially normal and are similar to the controls, regardless of dose. In contrast, in mice that received both A+N, macrovesicular and microvesicular lipidosis

of centrilobular hepatocytes was only noted in the mouse receiving the 600 mg/kg dose, compared with the essentially normal livers (negative control mouse) of mice receiving the lower 300 and 400 mg/kg doses.

Overall Animal Survival/Distress

The studies outlined above show that the APAP doses administered were sufficient to routinely cause serious liver damage and other indications of APAP toxicity (see above). However, despite the clear hepatic damage indicated by the hepatic enzyme changes, there were no readily visible symptoms of distress in any of the mice and only one mouse (in the APAP only group) died. Although the doses of APAP used were lethal [350 mg of APAP administered i.p, is widely reported to be the LD_{50}; Shayiq et al., 1999], and even though the doses used caused significant histologically detectable hepatic damage, they were insufficient to cause death in significant numbers of animals during the course of the study.

The one mouse was humanely euthanized 10-hour post treatment as a result of distress and reduced physical activity with no visible sign of recovery. None of the other animals exhibited distress signs during the 24 hour treatment period.

DISCUSSION

Lethal APAP overdose due to suicide attempts and inadvertent overdoses are a major concern in both adult and pediatric populations [Doyon et al., 2013]. When promptly detected, administering NAC, either orally or I.V. (within a 24 hour window for maximal efficacy), readily reverse the potentially lethal consequences of the APAP overdose [Ferner et al., 2011; Mahmoudi et al., 2015]. However, because APAP is present in many OTC and prescription formulations (e.g., Vicodin, cold and cough remedies, etc.), APAP overdose can often go undetected until substantial hepatic damage has occurred. Nonlethal APAP exposures, or co-exposures to APAP and a wide variety of hepatotoxic substances (including alcohol), also represent a serious and often undetected cause/exacerbation of APAP-induced hepatic disease.

NAC is delivered to patients I.V because of the well-known taste perversion associated with oral NAC preparations. As currently available NAC preparations have overcome this limitation, physicians now have the option of treating acute APAP toxicity with i.v. or oral NAC. More importantly, the introduction of palatable NAC formulations opens the possibility of using orally administered NAC to treat chronic or borderline APAP toxicity. From a public health perspective, this also provides the possibility of preventing toxicity by co-formulating NAC with APAP as NAC can mitigate APAP toxicity [Corcoran et al., 1985; Terneus et al., 2007] or that of other drugs that may require adequate glutathione stores for detoxification. We had proposed some time ago[Andrus et al., 2001] that co-administering NAC with APAP could be useful for decreasing the toxicity of APAP and possibly other drugs that rely on glutathione for detoxification.

The findings in the present mouse studies provide addition evidence that co-formulation (or simply joint administration) of APAP with an amount of NAC necessary to prevent APAP toxicity could be preemptive in preventing this toxicity decreasing the lethal consequences of APAP overdose and decreasing the treatment burden for APAP overdose.

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CONFLICT OF INTEREST

The NAC used for this experiment was donated by BioAdventex Pharma, Inc., Mississauga, Ontario, Canada. Dr. Lee Herzenberg serves in the board of BioAdventex and has shares in the company.

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