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Temporal Study of Acetaminophen (APAP) and S-Adenosyl-L-methionine (S-AdoMet) Effects on Subcellular Hepatic S-AdoMet Levels and Methionine Adenosyltransferase (MAT) Expression and Activity

J. Michael Brown*, John G. Ball*, Amy Hogsett*, Tierra Williams*, and Monica Valentovic*,¹

*Department of Pharmacology, Physiology, and Toxicology, Joan C. Edwards School of Medicine, Marshall University Huntington, WV 25755

Abstract

Acetaminophen (APAP) is the leading cause of drug induced liver failure in the United States. Previous studies in our laboratory have shown that S-adenosyl methionine (S-AdoMet) is protective for APAP hepatic toxicity. S-AdoMet is critical for glutathione synthesis and transmethylation of nucleic acids, proteins and phospholipids which would facilitate recovery from APAP toxicity. S-AdoMet is synthesized in cells through the action of methionine adenosyltransferase (MAT). This study tested the hypothesis that total hepatic and subcellular S-AdoMet levels are decreased by APAP toxicity. Studies further examined MAT expression and activity in response to APAP toxicity. Male C57BL/6 mice (16–22 grams) were treated with vehicle (Veh; water 15ml/kg ip injections). 250 mg/kg APAP (15 ml/kg, ip), S-AdoMet (1.25 mmol/kg) or S-AdoMet administered one h after APAP injection (S-AdoMet and S-AdoMet+APAP). Hepatic tissue was collected 2, 4, and 6 h after APAP administration. Levels of S-AdoMet and its metabolite S-adenosylhomocysteine (SAH) were determined by HPLC analysis. MAT expression was examined by Western blot. MAT activity was determined by fluorescence assay. Total liver S-AdoMet levels were depressed at 4 h by APAP overdose, but not at 2 or 6 h. APAP depressed mitochondrial S-AdoMet levels at 4 and 6 h relative to the Veh group. In the nucleus, levels of S-AdoMet were depressed below detectable limits 4 h following APAP administration. S-AdoMet administration following APAP (S-AdoMet+APAP) prevented APAP associated decline in mitochondrial and nuclear S-AdoMet levels. In conclusion, the maintenance of S-AdoMet may provide benefit in preventing damage associated with APAP toxicity.

Keywords

S-adenosyl-L-methionine; Acetaminophen; Hepatotoxicity; S-adenosylhomocysteine; Methionine Adenosyltransferase

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¹Correspondence should be addressed to: Department of Pharmacology, Physiology, and Toxicology; 1 John Marshall Drive, Joan C. Edwards School of Medicine, Marshall University, Huntington, WV 25755. Phone (304-696-7332); Fax (304-696-7391); Valentov@marshall.edu.

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INTRODUCTION

Acetaminophen (APAP) is the leading cause of drug induced liver disease in the United States resulting in over 56,000 emergency room visits and approximately 500 deaths each year (Nourjah et al., 2006). One of the problems associated with APAP toxicity is the wide availability of the drug. APAP is present in more than 180 over the counter (OTC) products, which increases the probability of accidental overdose. Acute overdose of APAP leads to severe hepatic centrilobular necrosis (Boyd and Berezky, 1966; Golden et al., 1981). Rapid treatment with *N*-acetylcysteine (NAC) is currently the clinical treatment for APAP overdose.

The toxicity of APAP is mediated through its biotransformation into *N*-acetyl-p-benzoquinoneimine (NAPQI) by cytochrome P450 2E1, 3A4, and 1A2 (Corcoran et al., 1980; Dahlin et al., 1984; Patten et al., 1993). NAPQI is a strong electrophile that rapidly adducts sulfhydryl groups like those found on reduced glutathione (GSH) (Streeter et al., 1984). GSH depletion by NAPQI precedes APAP toxicity (Larrauri et al., 1987). In addition to adducting proteins, NAPQI also induces mitochondrial dysfunction leading to a severe energy debt and the formation of reactive oxygen species (ROS) that induce further damage in the hepatocytes (Andersson et al., 1990).

The current treatment for APAP overdose is *N*-acetylcysteine (NAC). NAC functions by replenishing cellular stores of cysteine which is involved in the rate-limiting step in the formation of GSH. NAC has been demonstrated to reduce protein adduction in response to APAP overdose (Corcoran et al., 1985). Also, NAC reduces mitochondrial dysfunction and reactive oxygen generation in hepatocytes following APAP overdose (Reid et al., 2005). In order to be effective as a treatment however, NAC must be administered within 8-10 h following APAP overdose, making the study of alternative therapies attractive (Smilkstein et al., 1988).

Currently, *S*-adenosyl-L-methionine (SAdMe) is available over the counter and has gained acceptance as beneficial for depression and alcoholic liver disease (Purohit et al., 2007; Williams et al., 2005). SAdMe is a ubiquitous cofactor in a variety of biological reactions. SAdMe is found in most tissues and is produced at a rate of 6-8 g per day in the liver. The production of SAdMe is catalyzed by methionine adenosyltransferase (MAT) (Lu, 2000). *MAT1A* is expressed constitutively in the adult liver and encodes the $\alpha 1$ subunit which composes MAT I (tetramer) and MAT III (dimer). The gene coding for MAT II is *MAT2*, which is widely distributed throughout the body with the exception of the adult liver (Kotb et al., 1997). However, MAT II is expressed in the adult liver during liver regeneration and hepatic cancer (Martinez-Chantar et al., 2003; Paneda et al., 2002). Furthermore, *MAT1A* expression is increased during liver regeneration following partial hepatectomy (Chen et al., 2004).

The protective action of SAdMe upon the liver is hypothesized to be mediated via the transmethylation and transsulfuration pathways. SAdMe is the principle biological methyl donor in cells. Following methyl group donation, SAdMe becomes *S*-adenosylhomocysteine (SAH) that can enter the transsulfuration pathway leading to replenishment of cellular GSH (Finkelstein, 2000). Alterations in either SAdMe or its ratio with SAH have been associated with toxicant exposure. For example, SAdMe levels were decreased in humans with alcoholic liver disease (Purohit et al., 2007). Furthermore, any decline in SAdMe or decrease in the ratio of SAdMe:SAH has been demonstrated to inhibit cellular transmethylation reactions (Purohit et al., 2007) as SAH is a competitive inhibitor of transmethylation reactions (Kharbanda 2007). Previous research by our lab and others has demonstrated that SAdMe protects against APAP induced hepatotoxicity when administered just prior to APAP

overdose (Bray et al., 1992; Terneus et al., 2007). Recent studies in our laboratory showed that SAME was protective for APAP hepatotoxicity when SAME was administered 1 h after APAP overdose (Terneus et al., 2008). SAME administration after APAP overdose is a more clinically relevant experimental model since antidotes are not normally administered to humans until after a toxic exposure.

In our laboratory, SAME and NAC displayed a comparable level of protection for APAP toxicity in mice, when comparisons were made on the basis of a mmol/kg dosage (Terneus et al., 2008). However, the mechanism for SAME protection of APAP toxicity remains to be elucidated. Diminished hepatic SAME levels have been linked to liver damage mediated by toxicants including ethyl alcohol and acetaminophen. Alcohol exposure was associated with diminished hepatic SAME levels in baboons and mice (Lieber et al., 1990 and Song et al., 2007). SAME hepatic levels were lower in rats fed 600 mg/kg in food for 4 weeks (Varela-Moreiras et al., 1993). Hepatic SAME levels were diminished 24 h after a very high acute dose of 750 mg/kg APAP in fed BALB/c mice (Oz et al., 2004) which is higher than most human overdose ingestions. Very little research has been done to examine first, the effects of lower APAP doses that are similar to human exposure and second the temporal changes in intracellular SAME levels following APAP overdose. A decrease in intracellular SAME levels following APAP overdose would have deleterious effects on DNA methylation, phospholipid formation and GSH synthesis. Additionally, given that expression of MAT appears to be required for liver regeneration following damage, the exploration of APAP overdose effects on MAT levels warrants further study. Therefore, the purpose of the current study was to investigate the effect of APAP overdose on hepatic, $15,000 \times g$ supernatant, nuclear and mitochondrial SAME levels, as well as alterations of hepatic MATI/III and MATII. By examining these components of SAME metabolism, the present study hopes to shed light on the mechanism of SAME protection against APAP toxicity.

METHODS AND MATERIALS

Materials

SAME toluenesulfonate salt as used in all experiments (Sigma Chemical Co., St. Louis, MO). The ALT reagent kit (TR-71021) was purchased from Thermo Electron Corporation (Louisville, CO). All solvents were HPLC grade and other reagents were of comparable quality and purchased from Sigma Chemical Co. (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA).

Animals

Male C57BL/6 mice were obtained from Hilltop Lab Animals Inc. (Scottsdale, PA). Animals included in the study were between 4-8 weeks of age and weighed 16-24 g. Mice were maintained in a facility in compliance with the American Association for Accreditation of Laboratory Animal Care. Mice were maintained at controlled temperature (21-23°C), humidity (40-55%), and 12 h light cycles (lights on 6:00 AM to 6:00PM). An acclimation period of 7 days was observed prior to the beginning of any experiment. The animals received Purina rodent chow and water *ad libitum*. The mice were fasted for 16 h prior to any experiment, but maintained free access to water.

SAME and NAC Treatment Following APAP Overdose

Mice were randomly allocated into the following groups: vehicle (Veh; 15 ml/kg water by intraperitoneal (ip) injection), SAME (1.25 mmol/kg 5ml/kg ip injection), APAP (250 mg/kg 15ml/kg ip injection), and SAME administered 1 h after APAP (SAME + APAP; doses same as previously listed). Mice treated with NAC were randomly divided into the following groups: Veh, NAC (1.25 mmol/kg with 5 ml/kg ip injection), APAP, and NAC + APAP.

Mice were fasted for 16 h prior to the administration of APAP. SAME and NAC were administered 1 h after APAP. Mice were anesthetized with carbon dioxide 2, 4, and 6 h after APAP administration with SAME treatment, and 4 h following APAP treatment when NAC was used as a comparison for SAME. Blood was collected by cardiac puncture in heparin-rinsed 1 ml syringes for determination of serum ALT activity, which serves as an indicator of liver injury. Livers were then isolated and placed in ice cold Krebs buffer (126 mM NaCl, 5 mM KCl, 3 mM MgSO₄, 3 mM Na₂HPO₄, 1 mM CaCl₂; pH 7.4), blotted, and weighed.

Mitochondrial Isolation

Mitochondria were isolated using a modification of a previously published protocol (Gogvadze et al., 2004). Briefly, the liver was isolated, blotted, weighed and placed in Mitochondrial Isolation Buffer A (225 mM sucrose, 3 mM KH₂PO₄, 5 mM MgCl₂, 20 mM KCl, 20 mM triethanolamine, 2 mM EGTA; pH 7.4). The liver was minced and homogenized in a Dounce homogenizer on ice. Following homogenization, the liver was centrifuged at 600 × g for 10 minutes. The resultant pellet was discarded and the supernatant was centrifuged at 15,000 × g for 5 minutes. After the final centrifugation, the supernatant was retained for analysis of SAME levels. The pellet containing the mitochondria was resuspended in Mitochondrial Isolation Buffer B (Same as Buffer A except lacking EGTA) for a final concentration of 1 mg tissue weight/1 ml Buffer B. Samples were stored at -80°C until analysis.

Nucleus Isolation

The protocol for isolating the nuclei of liver cells was adapted from Graham (2001). Briefly, liver was homogenized in ice cold Nuclear Isolation Medium (0.25 M sucrose, 25 mM KCl, 5 mM MgCl₂, and 10 mM Tris-Cl; pH 7.4) using a Dounce homogenizer and adjusted to a final 3 mL volume. The homogenate was centrifuged at 800 × g, the supernatant discarded, new buffer was added and centrifuged at 800 × g and the supernatant discarded. The pellet was resuspended in 1 mL Nuclear Isolation Medium followed by 2 mL of Sucrose Density Barrier (1.15 M sucrose, 10 mM KCl, 2.5 mM MgCl₂, and 5 mM Tris-Cl; pH 7.4) and vortexed. Six mL of Sucrose Density Barrier was then layered under the homogenate and centrifuged for 1 h at 100,000 × g. The pellet was resuspended in 1 mL Nuclear Isolation Medium and stored at -80°C until use.

HPLC Analysis of Hepatic SAME Levels

A 200 mg aliquot of liver was homogenized on ice in 0.4 mM HClO₄ and adjusted to a final 1 ml volume. Mitochondrial and 15,000 × g supernatant suspensions were added to an equal volume of 0.4 mM HClO₄ to precipitate protein. Nuclear samples were concentrated by lyophilizing the 1 ml of sample (total liver weight 600-900 mg) and reconstituting the sample in 125 µl 0.4 mM HClO₄. The samples were centrifuged at 10,000 × g for 10 min at 4°C and filtered through 0.45 µm Millex®-HV filters (Millipore; Billerica, MD). A 20 µl sample of the filtrate was analyzed for whole liver, mitochondrial, and 15,000 × g supernatant fractions, while 40 µl of sample was required for detection of nuclear SAME levels. SAME and SAH levels were detected using a Beckman Coulter HPLC system (Fullerton, CA) with a 126 Solvent Module and a 166 Variable Wavelength Detector. The column was a YMC ODS-AQ 3 µm 120 Å 4.6x150 mm column. The mobile phase was a gradient at a flow of 1 ml/min (Waters Corporation; Milford, MA). The mobile phase gradient program was 8 minutes of 90:10 A:B followed by 12 minutes of 60:40 A:B. Mobile phase A consisted of 8 mM 1-heptane sulfonic acid sodium salt and 50 mM sodium phosphate monobasic (pH 3). Mobile phase B was 100% HPLC grade methanol. The wavelength for detection was 254 nm (Wang et al., 2001).

Analysis of MAT I/II/III Expression in Mouse Liver

Western blot analysis was conducted to examine expression of MAT I/III and MAT II. Approximately 200 mg of liver was homogenized in 1 mL ice cold Krebs buffer and protein levels were determined using the Bradford protein assay. A 100 µg protein aliquot was denatured by boiling for 5 minutes. Samples were separated on a 12.5% polyacrylamide gel and transferred to a PVDF membrane (Pall Corporation; Pensacola, FL). Transfer efficiency was verified using MemCode® Reversible Protein Stain Kit (Thermo Scientific; Rockford, IL). The membrane was then blocked using a 5% (w/v) milk/TBST solution (10 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20; pH 8.0) for 1 h. Membranes were next incubated overnight with constant shaking at 4°C in antibody for MAT I/III (sc-28029, Santa Cruz Biotechnology; Santa Cruz, CA) or MAT II (sc-28031, Santa Cruz Biotechnology; Santa Cruz, CA) each at 1:1000 dilution in 5% (w/v) milk/TBST. The membranes were washed four times with TBST. The donkey anti-goat HRP secondary antibody (sc-2020, Santa Cruz Biotechnology; Santa Cruz, CA) was diluted 1:3000 and incubated with the membrane for 1 h. The membrane was again washed with TBST and developed using Amersham™ ECL™ Western Blotting Detection Reagents (GE Healthcare; Buckinghamshire, UK). Blots were then reprobed with GAPDH as a loading control (2275-PC-100, Trevigen; Gaithersburg, MD). Goat anti-rabbit HRP secondary antibody was used to develop the blots (sc-2004, Santa Cruz Biotechnology; Santa Cruz, CA). Densitometry was performed on each gel.

Determination of MAT Activity

An endpoint fluorometric assay for determination of MAT activity developed by Wang, et al. (2003) was modified for a high throughput assay using a fluorescent plate reader. The assay measures the formation of scopoletin through MAT activity. A standard curve was constructed from scopoletin with a lower detection limit of 50 pmol. The protocol of Wang was followed except at the end when the extracted scopoletin was transferred to a 96 well plate and read using a BioTeck Synergy 2 (BioTek Instruments; Winooski, VT) with excitation and emission at 347 and 415 nm, respectively. Activity was expressed as pmol scopoletin/min·mg protein.

Statistical Analysis

Values represent Mean ± S.E.M. with n=3-10 animals/group. Differences in the groups were analyzed using a one-way ANOVA followed by a Tukey's post-hoc test (SigmaStat; SPSS Inc. Chicago, IL). All statistical analyses were conducted using a 95% confidence interval.

RESULTS

The body weights of the animals were not significantly different between groups (Table 1). In addition, liver to body weight ratios were diminished in the SAME+APAP group 4 h after APAP overdose when compared to APAP treated mice (Table 1). However, the same reduction in the liver to body weight ratio was not observed when the livers were collected at 6 h following APAP overdose (Table 1). To provide a comparison for the current standard of treatment for APAP overdose, NAC (1.25 mmol/kg) was administered 1 h following APAP overdose and livers collected 4 h following APAP overdose. When adjusted for body weight, NAC prevented the rise in liver weight that typically serves as an indicator of APAP toxicity (Table 2). These liver weight results indicate that equimolar doses of SAME and NAC (1.25 mmol/kg) are able to reduce the liver to body weight ratio when administered following APAP overdose.

Plasma ALT is a common measure of liver toxicity as ALT is released into the serum following damage to the liver. In humans, ALT values may be in excess of 1000 U/L in APAP overdose. Prior experiments on APAP toxicity have indicated that toxicity induces a

steady increase in plasma ALT between 2 and 6 h. Plasma ALT levels typically range between 2000 and 6000 IU/L for the time periods studied in mice (Andringa et al., 2008; Hinson et al., 2002; Knight et al., 2003). Toxicity from APAP overdose was demonstrated 2 h following APAP administration (Figure 1). At 4 h following APAP administration, equivalent doses of SAME and NAC protect the liver as indicated by a significant decrease in serum ALT when compared to the APAP treatment group (Figure 1). NAC, when administered 1h following APAP, significantly reduced ALT levels at 4 h when compared to the APAP group (Figure 1). NAC did not totally correct hepatic damage as ALT levels were higher than the Veh group in the NAC+APAP treated animals. ALT levels in the SAME+APAP group were comparable to the Veh group at 4 h. SAME partially corrected APAP hepatic toxicity as ALT levels at 6 h following APAP treatment were higher than Veh but less than the APAP treatment group (Figure 1).

Hepatic SAME levels were measured following APAP overdose because perturbations in SAME can inhibit transmethylation and transsulfuration reactions (Lu, 2000). At 4 and 6 h following APAP administration SAME levels were significantly ($p<0.05$) decreased compared to the Veh groups (Figure 2). Administration of SAME 1 h following APAP prevented the decrease in whole cell lysate SAME levels (Figure 2). Peak SAME levels were observed in the SAME and SAME + APAP groups 1 h following SAME administration (Figure 2). Mice were collected at 1 h after SAME administration but 2 h following APAP administration (Figure 2). In Table 3 it can be observed that levels of SAH significantly decreased 2 h following APAP administration when compared to the Veh group. In addition, the ratio of SAME:SAH increases in the APAP treatment group at 2 h (Figure 2). Also, by 6 h the ratio of SAME:SAH in the SAME group decreases because of the drop in SAME levels as the levels of SAH remain constant (Figure 2; Table 3).

As would be expected, administration of NAC did not increase SAME levels in the NAC group (Figure 3). Perhaps the most interesting finding in the NAC study was that NAC administration following APAP prevented a significant decrease in the levels of SAME in the NAC + APAP group (Figure 3). No changes were observed in SAH levels following APAP administration (Table 4). The ratio of SAME:SAH in the APAP treatment groups is decreased when compared to the Veh group (Figure 3). Administration of NAC following APAP was not able to significantly increase the ratio of SAME:SAH when compared with the APAP at 4 h (Figure 3). SAME was also unable to increase the ratio of SAME:SAH at the same time period (Figure 2). Based on these findings, the decline in hepatic SAME:SAH ratios is due to a decline in SAME and not an increase in SAH levels.

After analyzing whole liver levels of SAME, the experiment next proceeded to measuring subcellular levels of SAME. First, the effect of APAP overdose on hepatic mitochondrial SAME levels were analyzed. Mitochondria are known to have SAME transporters (Aqrini et al., 2004). Levels of SAME were decreased at both 4 and 6 h following APAP overdose in the APAP treatment groups but not at 2 h following APAP administration (Figure 4). At 2, 4, and 6 h following APAP overdose, administration of SAME after APAP causes a significant increase in levels of mitochondrial SAME when compared to the Veh group (Figure 4). Mitochondrial SAH levels are decreased significantly 4 h following APAP overdose, but remain unchanged for all other groups and time periods analyzed (Table 5). Additionally, the ratio of SAME:SAH is increased in the SAME + APAP group for the mitochondria at all time periods (Figure 4). One other aspect of note, is that the ratio of SAME:SAH in the mitochondria is not decreased in the APAP treatment group (Figure 4).

Levels of $15,000 \times g$ supernatant SAME were determined in addition to the levels of mitochondrial SAME. Hepatic $15,000 \times g$ supernatant SAME levels were significantly decreased at 4 and 6 h following APAP overdose (Figure 5). The drop in SAME observed in

the APAP treatment group at 4 and 6 h was prevented by SAME administration following APAP overdose (Figure 5). $15,000 \times g$ supernatant SAH levels were decreased in the SAME and SAME + APAP groups at two h (Table 5). SAH levels were also significantly depressed at 4 and 6 h in the $15,000 \times g$ supernatant APAP treatment groups and this was reversed by administration of SAME following APAP overdose (Table 5). In the SAME + APAP group at both 2 and 4 h the ratio of SAME:SAH was significantly increased (Figure 5). In the APAP treatment group at 6 h, there was no observed alteration of the ratio of SAME:SAH (Figure 5).

APAP treatment altered subcellular hepatic nuclear levels of SAME. No change was observed in nuclear SAME levels 4 h following APAP administration between Veh, SAME and SAME+APAP groups. The detection limit was 12.5 pmol and the APAP treated group all had nuclear SAME levels below the detection limit of 12.5 pmol (Figure 6). Levels of SAH could be determined 4 h following APAP overdose, but there was no significant change observed in any of the groups (Table 6). The ratios of SAME:SAH in the nuclei of the liver cells was also unchanged when observed at 4 h (Figure 6). The ratio for the APAP group could not be determined because of the lack of SAME data.

Analysis of MAT I/III expression in the liver indicates that there is a significant decrease in the levels of MAT I/III in the APAP group when compared to the Veh group 2 and 4 h following APAP administration (Figure 7). The administration of SAME 1 h after APAP increased levels of MAT I/III back to Veh level at 2 and 4 h following APAP overdose (Figure 7). The presence of MAT II was not detected in hepatic tissue 2 and 4 h following APAP overdose (Figure 7). Protein transfer was confirmed by staining as described in *Methods and Materials* and recombinant MAT II protein was used to confirm the viability of the MAT II antibody (Figure 7).

MAT activity was significantly increased in the APAP treatment group when compared to the SAME and SAME + APAP groups 2 h following administration of APAP (Figure 8). Although the trend is there, the Veh group was not significantly elevated compared to the SAME and SAME + APAP groups at 2 h (Figure 8). No differences were observed in MAT activity 4 h following APAP overdose (Figure 8). When taken together with the 2 h MAT expression data, it is observed that MAT activity in the SAME and SAME + APAP groups decrease compared to the APAP group, while expression of MAT in the APAP group is decreased 2 h following APAP administration.

DISCUSSION

SAME has been demonstrated to be an effective treatment for APAP overdose in mouse animal models (Stramentinoli et al., 1979; Valentovic, et al., 2004). Although it is known that SAME can participate in the replenishment of GSH through the transsulfuration pathway (Lu, 1998), other mechanisms of protection remain to be explored. In order to gain an understanding of SAME metabolism in the liver following APAP overdose, the purpose of the current study was to analyze hepatic SAME and SAH levels. The observed decrease in SAME levels following APAP overdose could prevent any protection that SAME could afford, and also could act as a precursor to APAP toxicity, much as the depletion of GSH precedes APAP toxicity (Moore et al., 1985). In addition, the observed decrease in MAT I/III expression in the liver at 4 h would lead to SAME depletion following APAP overdose.

SAME is the primary methyl donor in cells and is capable of methylating phospholipids, proteins, and nucleic acids. Polyamine synthesis is dependent on adequate cellular SAME levels (Lieber and Packer, 2002). The study of effects of toxicity on SAME levels in the cell are lacking, especially in the area of subcellular analysis of SAME levels. Perturbations in

the ratio of SAME:SAH can inhibit transmethylation reactions and conceivably the ability of SAME to protect against APAP toxicity (Finkelstein et al., 1974). In the current study of total liver SAME, the ratio of SAME:SAH was increased in the SAME + APAP group at 2 h following APAP administration (Figure 2). The levels of SAME were decreased in whole liver 4 and 6 h after APAP treatment, which would inhibit crucial transmethylation reactions. When the ratios of SAME:SAH in whole cell lysate was calculated there was an observed increase in the ratio at 2 h when comparing the Veh and APAP groups, no change at 4 h, and a decrease in the APAP treatment group when compared to Veh at 6 h. The trend observed in the ratios was due to the SAH levels decreasing 2 h following APAP administration and steadily increasing thereafter. Decrease of the ratio of SAME:SAH could inhibit crucial transmethylation reactions. Of interest for clinical situations, was that the administration of SAME following APAP overdose lead to an increase in hepatic SAME back to control levels.

Mitochondria are dependent on the presence of SAME for proper function. Inhibition of SAME transport into hepatic mitochondria has been demonstrated to sensitize the cells to tumor necrosis factor (TNF) hepatotoxicity (Song et al., 2007). TNF has been shown to be unregulated in response to APAP overdose (Dambach et al., 2006). Also, SAME has been demonstrated to aid in the maintenance of mitochondrial membrane potential in a cell culture model (Lotkova et al., 2005). One of the hallmarks of APAP toxicity is mitochondrial dysfunction (Bajt et al., 2008). We present here, for the first time, analysis of mitochondrial and $15,000 \times g$ supernatant levels of SAME following APAP overdose.

The observed decrease in mitochondrial SAME levels could make mitochondria more susceptible to APAP toxicity. We were also able to establish a time course for the depression of mitochondrial SAME levels occurring between 2 and 4 h following APAP administration. A spike in cellular SAME levels was observed in whole cell lysate, mitochondria, and $15,000 \times g$ supernatant at 2 h following SAME administration. When SAME was administered following APAP, it was able to dramatically increase levels of mitochondrial SAME at 2, 4, and 6 h. SAME increased to levels surpassing the control mice indicating that SAME transport into mitochondria is not inhibited by APAP toxicity. Further studies may provide better insight into mitochondrial SAME transport regulation in response to APAP toxicity.

The purposes of SAME in the cytosol is to function as a methylator and in the synthesis of polyamines as well as participating in the transsulfuration pathway to replenish cellular reduced glutathione. At 4 and 6 h following APAP overdose, the levels of SAME in the $15,000 \times g$ supernatant are depressed when compared to the control mice. When SAME was administered following APAP it was able to aid in the recovery of SAME levels to amounts surpassing controls at 2 and 4 h.

Given the role of SAME in methylation of DNA, we examined the amount of SAME present in the nuclei of cells treated with APAP. Caudill et al., (2001) noted that in cases of SAH elevation in the liver the DNA tended toward hypomethylation regardless of SAME levels. We found that levels of SAH were not elevated in the APAP treatment group compared to any other group indicating that it would be unlikely for the hepatocytes to experience DNA hypomethylation as a result of APAP toxicity even though SAME levels dropped below the detectable limit of 12.5 pmol in the APAP treatment groups.

Expression of MAT I/III is important for the protection of the liver, and its absence can lead to unwanted proliferation of hepatocytes (Lu et al., 2001). These findings have significance because the presence of MAT I/III appears to be necessary for the normal function of hepatocytes (Lu and Mato, 2008). Furthermore, SAME has been found to prevent

dysfunction of MAT I/III following administration of carbon tetrachloride, a prototypical liver toxicant (Corrales et al., 1992). MAT expression levels were significantly depressed at 2 and 4 h following APAP administration when compared with the Veh groups. SAME levels and have been found to fall initially following partial hepatectomy; however, expression of *MAT1A* increases as soon as 3 h following partial hepatectomy (Huang et al., 1998). Our data did not demonstrate the increase in expression of MAT I/III proteins, but we did not look at mRNA expression as did Huang and associates.

Unfortunately, the rise in MAT II expression that typically accompanies liver regeneration (Latasa et al., 2001) was not observed by our lab at 2 or 4 h following APAP overdose. Perhaps future studies monitoring time periods beyond 4 h following APAP administration will yield an appreciable expression of MAT II in APAP treated mice. In a prior study looking at MAT II following partial hepatectomy, a rise in the mRNA of *MAT2A* was observed as soon as 6 h following the surgery (Horikawa et al., 1996). In our study, APAP toxicity was quite pronounced as ALT levels at 6 h were approximately 10,000 U/L in the APAP group. Hepatic SAME levels at 6 h in these same APAP treated animals was beginning to recover relative to 4 h treatment groups suggesting MAT activity was functional for formation of SAME. Hepatic SAME levels were maintained in the SAME + APAP group suggesting MAT expression and function may be regenerating in the SAME + APAP group. Future studies would be needed to examine longer time periods to characterize MAT II expression and examine mRNA levels for MAT II which could yield earlier indications that MAT II was being upregulated.

Studies by other investigators on MAT activity concluded that MAT I and MAT II activity are inhibited by SAME, while MAT III is actually activated by SAME (Sullivan and Hoffman, 1983). Given that there was no expression of MAT II observed at the time periods studied, the activity present comes from MAT I/III. When SAME was administered either alone or after APAP overdose, activity of MAT was significantly decreased when compared with the APAP group at 2 h. MAT activity was not different between groups 4 h post APAP treatment. Given the propensity of SAME to inhibit MAT, the data at 2 h following APAP administration is easily understood. A spike in SAME levels occurs 2 h following APAP overdose in whole cell lysate which could lead to the observed MAT inhibition without a significant change in expression for the SAME or the SAME administered following APAP group. By 4 h the increase in SAME brought about by SAME administration in whole cell samples has largely dissipated leading to the return of MAT activity to Veh levels in the SAME and SAME + APAP groups. Previous research found a link between reduced glutathione depletion and decreased MAT activity (Corrales et al., 1991). The same group found that cysteine modification was most likely responsible for the observed decrease in MAT activity (Pajares et al., 1991). Cellular reduced glutathione depletion is a hallmark of APAP toxicity making the current findings interesting in the sense that APAP administration did not reduce activity of MAT at 2 or 4 h (Mitchell et al., 1973). The observed decrease in MAT I/III expression could be because of covalent modifications at the antibody binding site and, if so, does not translate to a decrease in MAT activity for the time periods studied.

In conclusion, this is the first study to report a time analysis of SAME levels in mitochondria, $15,000 \times g$ supernatant, and the nucleus following APAP overdose. APAP overdose decreased SAME in whole liver, mitochondria, and $15,000 \times g$ supernatant at 4 and 6 h, while there is no depression of SAME levels observed at 2 h. When SAME was administered following APAP, the levels of SAME did not decline. SAME was not detectable in the nucleus at 2 h following APAP treatment even though our level of sensitivity was 12.5 pmol of SAME. We have also demonstrated here for the first time that administration of NAC following APAP aided in the maintenance of whole cell SAME levels 4 h after APAP overdose. At both 2 and 4 h following APAP administration, levels of

MAT were significantly decreased with respect to the Veh group in the APAP treatment group. MAT activity tended to decrease when SAME was administered and livers collected at 2 h following APAP overdose, but no changes were observed at 4 h. These results provide important insight into the effects of APAP overdose on SAME metabolism in the livers of C57BL/6 mice that can be used in future studies to evaluate alterations in MAT enzymes.

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Figure 1. The effect of SAmE and NAC on serum ALT levels following APAP overdose in C57BL/6 mice

In panel A of the figure, mice were randomly allocated into the following groups: Veh (water), SAmE (1.25 mmol/kg), APAP (250 mg/kg), SAmE 1 h after APAP (S + A). Plasma was collected 2, 4 and 6 h following the initial APAP overdose. Panel B of the figure consisted of mice allocated into groups of Veh (water), NAC (1.25 mmol/kg), APAP (250 mg/kg), and NAC administered 1 h following APAP (NAC + APAP). Serum was collected 4 h following APAP administration. Values represent mean $\pm$ S.E.M. with n=5-10 mice/group. Superscripts denote statistical significance ($p < 0.05$).

Figure 2. Alterations of SAmE levels following APAP overdose

C57BL/6 mice were randomly allocated into Veh (water), SAmE (1.25 mmol/kg), APAP (250 mg/kg), and SAmE administered 1 h after APAP (S + A). Livers were collected at 2, 4 and 6 h following APAP overdose and processed for analysis of SAmE levels by HPLC. Values represent mean $\pm$ S.E.M. Panel A represents SAmE levels (nmol/g tissue) following APAP administration, while panel B represents the ratio of SAmE:SAH. Superscripts denote statistically significant differences ($p < 0.05$) with $n = 5-10$ mice/ group.

Figure 3. The observed effects of NAC on SAME levels in the liver after APAP overdose
C57BL/6 mice were randomly allocated into Veh (water), NAC (1.25 mmol/kg), APAP (250 mg/kg), and NAC administered 1 h following APAP (N + A). Livers were collected 4 h following administration of APAP and processed for determination of SAME levels via HPLC. Panel A represents SAME levels when NAC was given following APAP overdose, and panel B represents the ratio of SAME:SAH under the same experimental conditions. All values represent mean $\pm$ S.E.M. with $n=5$ mice/group, and superscripts denote statistical differences ($p<0.05$).

Figure 4. Examination of the effect of exogenous S-AdoMet on levels of S-AdoMet in liver mitochondria of C57BL/6 mice

Mice were randomly divided into Veh (water), S-AdoMet (1.25 mmol/kg), APAP (250 mg/kg), and S-AdoMet administered 1 h following APAP (S + A). Livers were collected and mitochondria isolated at 2, 4 and 6 h following APAP overdose. Levels of S-AdoMet and SAH were determined by HPLC. Panel A represents levels of mitochondrial S-AdoMet when S-AdoMet was administered following APAP overdose. Panel B is the ratio of S-AdoMet:SAH in the same treatment groups. All values represent mean $\pm$ S.E.M. with $n=5$ mice/group. Superscripts denote statistical significance ($p<0.05$).

Figure 5. Alterations of 15,000 × g supernatant fraction SAME levels following APAP overdose in C57BL/6 mice

The mice were randomly allocated into Veh (water), SAME (1.25 mmol/kg), APAP (250 mg/kg), and SAME administered one h following APAP (S + A). Livers were collected at 2, 4 and 6 h following APAP administration and processed for collection of the 15,000 × g supernatant. Levels of SAME were determined by HPLC. Panel A represents SAME levels in the 15,000 × g supernatant fraction, while panel B is the ratio of SAME:SAH in the same samples. All values represent mean ± S.E.M. with n=5 mice/group. Superscripts denote statistically significant differences (p<0.05).

Figure 6. Alterations of nucleus SAd levels following APAP overdose in C57BL/6 mice
Mice were randomly put into Veh (water), SAd (1.25 mmol/kg), APAP (250 mg/kg), or SAd administered 1 h following APAP groups. Livers were collected at 4 h and nuclei isolated. SAd levels were determined by HPLC analysis. All groups had n=3-5 and values are listed as mean $\pm$ S.E.M. Panel A represents levels of SAd in the nucleus of cells following APAP overdose, and panel B represents the ratio of SAd:SAH in the same groups. SAd levels in the APAP treatment group were below the current detection limit of 12.5 pmol (ND group). Superscripts denote statistically significant differences ($p < 0.05$).

Figure 7. MAT I/II/III expression in hepatic tissue 2 h and 4 h following APAP overdose in C57BL/6 mice

Hepatic tissue was collected after administration of Veh (water), 1.25 mmol/kg SAMe, 250 mg/kg APAP, and SAMe one h following APAP. Each lane was loaded with 100 μ g protein and detection was accomplished with chemiluminescence. Panel A of the figure represents the density of the individual MAT I/III bands expressed as % Veh (n=3-5 animals/group). Values represent mean $\pm$ S.E.M. with superscripts denoting statistical significance. Panel B is a representative blot 2 h following APAP administration. Lanes 1, 2, and 3 represent Veh samples. Lanes 4, 5, and 6 represent SAMe samples. Lanes 7 and 8 represent APAP samples, while 9 and 10 represent S + A treatment groups. Panel C is a representative blot 4 h following APAP administration. Lanes depicted are as follows: Veh (1 and 2), SAMe (3 and 4), APAP (5 and 6), and S + A (7 and 8). Panel D is a representative example of MAT II expression 4 h following APAP administration. Lane assignments include: recombinant MAT II (1), Veh (2 and 3), SAMe (4 and 5), APAP (6 and 7), and S + A (8 and 9). Similar blots were conducted for MAT II expression 2 h following APAP administration with lack of expression also observed (data not shown).

Figure 8. MAT activity alterations following APAP overdose

Mice were allocated at random into Veh (water), S+Me (1.25 mmol/kg), APAP (250 mg/kg), or S+Me administered one h following APAP groups. Livers were collected 2 and 4 h following APAP injection and MAT activity was determined by an endpoint fluorescence assay measuring pmol of scopoletin formed per minute per mg protein. Values represent mean $\pm$ S.E.M. (n=5 mice per group). Superscripts denote statistical significance ($p < 0.05$).

TABLE 1

Body weight and liver weight per 10 g body weight changes following APAP and SAME.

	Group	Body Weight (g)	Liver weight /10 g body weight
SAME 2 h	Veh	24.00 ± 0.00 ^a	0.461 ± 0.00552 ^a
	SAMe	23.60 ± 0.40 ^a	0.400 ± 0.0256 ^a
	APAP	24.40 ± 0.40 ^a	0.456 ± 0.0231 ^a
	SAMe + APAP	22.80 ± 0.80 ^a	0.467 ± 0.0108 ^a
SAME 4 h	Veh	20.80 ± 0.49 ^a	0.395 ± 0.0164 ^a
	SAMe	20.40 ± 0.75 ^a	0.415 ± 0.00650 ^{ac}
	APAP	20.80 ± 0.49 ^a	0.477 ± 0.0102 ^b
	SAMe + APAP	20.80 ± 0.49 ^a	0.464 ± 0.0177 ^c
SAME 6 h	Veh	18.40 ± 0.40 ^a	0.363 ± 0.00475 ^a
	SAMe	19.20 ± 0.49 ^a	0.385 ± 0.00858 ^a
	APAP	18.40 ± 0.40 ^a	0.486 ± 0.0192 ^b
	SAMe + APAP	19.00 ± 0.58 ^a	0.470 ± 0.0187 ^b

Mice were randomly allocated into the following groups: Veh (water), SAME (1.25 mmol/kg), APAP (250 mg/kg), SAME 1 h after APAP (SAME + APAP). SAME and APAP were administered by ip injection. Values represent Mean ± S.E.M. (n=5-10). Values were measured 2, 4 or 6 h post APAP or Veh injection.

^aDenote statistically significant differences (p<0.05) between groups.

^bDenote statistically significant differences (p<0.05) between groups.

^cDenote statistically significant differences (p<0.05) between groups.

TABLE 2

The effect of NAC and APAP on body and liver weight per 10 g body weight

	Group	Body Weight (g)	Liver weight/10g body weight
	Veh	20.00 ± 0.63 ^a	0.416 ± 0.0296 ^a
NAC 4 h	NAC	19.60 ± 0.75 ^a	0.443 ± 0.00492 ^a
	APAP	18.00 ± 0.00 ^a	0.547 ± 0.0150 ^b
	NAC + APAP	20.00 ± 0.89 ^a	0.466 ± 0.0101 ^a

Mice were randomly allocated into the following groups: Veh (water), NAC (1.25 mmol/kg), APAP (250 mg/kg), NAC 1 h after APAP (NAC + APAP). NAC and APAP were administered by ip injection. Values represent Mean ± S.E.M. (n=5-10). Values were measured 4 h post APAP or Veh.

^aDenote statistically significant differences (p<0.05) between groups.

^bDenote statistically significant differences (p<0.05) between groups.

TABLE 3

The Effect of SAmE on Total Hepatic SAH levels following APAP overdose.

	Group	Liver SAH Levels (nmol/g tissue)
SAmE 2 h	Veh	67.176 ± 1.540 ^a
	SAmE	75.404 ± 9.352 ^a
	APAP	34.630 ± 9.046 ^b
	SAmE + APAP	49.118 ± 2.549 ^{ab}
SAmE 4 h	Veh	44.130 ± 2.110 ^a
	SAmE	61.770 ± 4.310 ^b
	APAP	42.310 ± 4.410 ^a
	SAmE + APAP	40.580 ± 2.100 ^a
SAmE 6 h	Veh	51.290 ± 5.470 ^{ab}
	SAmE	57.810 ± 4.400 ^{ab}
	APAP	54.630 ± 2.940 ^a
	SAmE + APAP	64.250 ± 0.840 ^b

Mice were randomly allocated into the following groups: Veh (water), SAmE (1.25 mmol/kg), APAP (250 mg/kg), SAmE 1 h after APAP (SAmE + APAP). SAmE and APAP were administered by ip injection. Values represent Mean ± S.E.M. (n=5-10). SAH levels were measured in liver homogenate 2, 4 or 6 h post APAP or Veh.

^a Denote statistical significance (p<0.05) between groups.

^b Denote statistical significance (p<0.05) between groups.

TABLE 4

The Effect of NAC and APAP on Hepatic SAH levels.

	Groups	Whole Cell SAH Levels (nmol/g tissue)
NAC 4 h	Veh	59.230 ± 4.070 ^a
	NAC	60.090 ± 8.500 ^a
	APAP	39.420 ± 11.020 ^a
	NAC + APAP	59.710 ± 3.140 ^a

Mice were randomly allocated into the following groups: Veh (water), NAC (1.25 mmol/kg), APAP (250 mg/kg), NAC 1 h after APAP (NAC + APAP). NAC and APAP were administered by ip injection. Values represent Mean ± S.E.M. (n=5-10). SAH levels were measured in liver homogenate 4 h post APAP or Veh.

^aDenote no statistical difference between groups (p>0.05).

TABLE 5

Mitochondrial and 15,000 × g supernatant SAH levels in animals treated with APAP followed 1 h later by SAmE

	Group	Mitochondria SAH Levels (nmol/g tissue)	15,000 × g SAH Levels (nmol/g tissue)
SAmE 2 h	Veh	8.329 ± 0.250 ^a	20.603 ± 1.146 ^a
	SAmE	9.237 ± 0.810 ^a	7.446 ± 0.212 ^b
	APAP	7.488 ± 1.642 ^a	16.856 ± 3.736 ^{ac}
	SAmE + APAP	9.667 ± 0.855 ^a	5.315 ± 0.656 ^d
SAmE 4 h	Veh	11.359 ± 0.747 ^a	23.871 ± 1.804 ^a
	SAmE	11.609 ± 1.342 ^a	22.147 ± 2.184 ^a
	APAP	3.633 ± 0.389 ^b	3.911 ± 0.334 ^b
	SAmE + APAP	8.544 ± 0.361 ^a	8.991 ± 0.481 ^c
SAmE 6 h	Veh	7.427 ± 0.743 ^a	26.267 ± 1.717 ^a
	SAmE	6.013 ± 0.618 ^a	29.343 ± 2.493 ^a
	APAP	3.006 ± 0.311 ^a	8.218 ± 3.484 ^b
	SAmE + APAP	6.377 ± 1.379 ^a	13.700 ± 3.719 ^c

Mice were randomly allocated into the following groups: Veh (water), SAmE (1.25 mmol/kg), APAP (250 mg/kg), SAmE 1 h after APAP (SAmE + APAP). SAmE and APAP were administered by ip injection. Values represent Mean ± S.E.M. (n=5-10). Mitochondrial and 15,000 × g supernatant SAH levels were measured 2, 4 or 6 h post APAP or Veh.

^aDenote statistically significant differences (p<0.05) between groups within respective sub-cellular region.

^bDenote statistically significant differences (p<0.05) between groups within respective sub-cellular region.

^cDenote statistically significant differences (p<0.05) between groups within respective sub-cellular region.

TABLE 6

Levels of SAH in the nucleus following APAP overdose.

	Group	Nucleus SAH Levels (nmol/g tissue)
SAMe 4 h	Veh	0.109 ± 0.0163^a
	APAP	0.159 ± 0.0122^a
	SAMe	0.143 ± 0.0279^a
	SAMe + APAP	0.167 ± 0.0272^a

Mice were randomly allocated into the following groups: Veh (water), SAMe (1.25 mmol/kg), APAP (250 mg/kg), SAMe 1 h after APAP (SAMe + APAP). SAMe and APAP were administered by ip injection. Values represent Mean $\pm$ S.E.M. (n=5-10). Nuclear SAH levels were measured 4 and 6 h post APAP or Veh treatment.

^aDenotes no statistical differences ($p > 0.05$) between groups.