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A Mechanistic Study of the Protective Effects of S-Adenosyl-L-Methionine Against Hepatotoxicity of Acetaminophen.

by

Marcus V. Terneus, Jr.

**Dissertation submitted to
the Graduate College
of
Marshall University
in partial fulfillment of the requirements
for the degree of**

**Doctor of Philosophy
in
Biomedical Sciences**

Approved by

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ABSTRACT

A MECHANISTIC STUDY OF THE PROTECTIVE EFFECTS OF S-ADENOSYL-L-METHIONINE AGAINST HEPATOTOXICITY OF ACETAMINOPHEN.

by Marcus V. Terneus, Jr.

Hepatic toxicity is known to be associated with excessive doses of the over-the-counter analgesic, acetaminophen (APAP). APAP overdose is the leading cause of drug-induced liver failure in the United States. APAP hepatotoxicity is dependent on the biotransformation of APAP by cytochrome P450 to the toxic metabolite, N-acetyl-*p*-benzoquinone imine (NAPQI). APAP, when taken in excessive doses, can lead to severe liver damage with the potential to progress to liver failure. Despite substantial efforts in past studies, the mechanism by which APAP induces such damaging effects is not completely understood. Recent discoveries suggest that glutathione (GSH) depletion, protein alkylation and reactive metabolite formation may all play a part in APAP's hepatotoxic effects. The present study hypothesized that S-Adenosyl-L-methionine (SAmE) diminished APAP hepatic toxicity by maintaining GSH status, reducing oxidative stress and altering formation of the toxic metabolite. The model selected for this study was a mouse model which is representative of the human response. This study was directed at investigating the mechanism by which APAP elicits its

damaging effects on the liver and attenuating that damage with a nutraceutical, SAME. Male C57Bl/6 mice were given an intraperitoneal injection (i.p.) of water, 250-500 mg/kg of APAP and/or 500 mg/kg of SAME. Livers were removed 1-4 hours post-injection and were measured for toxicities using GSH levels, plasma alanine aminotransferase (ALT) levels, liver:body weight ratios, lipid peroxidation, histological examinations, protein levels, protein carbonyl levels and oxidized protein levels. Data showed that APAP had toxic effects at all of the measurable levels. These data reiterated that APAP was able to elicit damaging effects not only by GSH depletion, but also by other mechanisms. Pre- and post- treatment with SAME attenuated the APAP-induced liver damage successfully in all measurable parameters compared to the animals dosed with APAP only. A further look into the mechanistic properties of SAME was established after the initial findings. These studies included: (1) incorporating a known limiting reagent, vitamin B₆, in SAME's pathway to the synthesis of GSH, (2) measuring and comparing metabolites produced when given APAP only compared to a dose of SAME before or after APAP and (4) directly comparing the therapeutic effects of SAME to the current antidote to APAP overdose, N-acetylcysteine (NAC). It was found that not only was SAME able to reduce and/or eliminate the effects of APAP overdose, but when given on the same mmol basis, it was able to provide better protection than NAC. These results strengthen the potential use of SAME as an antidote for APAP overdose cases.

DEDICATION

I would like to dedicate this dissertation to my wife: Heidi Kay Terneus.

Heidi, without your love, patience and understanding, I would not have been able to achieve my life's goals. To you, I owe this compilation of my work and know that I will forever be appreciative of everything you have done for me.

This dissertation would not have happened without you. Please know that this work, and my love, are forever yours.

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To my committee, I offer a depth of gratitude for their guidance and support throughout my graduate career. It is with individuals like these that make graduate school an enjoyable and rewarding experience.

Monica A. Valentovic, PhD (Advisor)

Kelley K. Kinningham, PhD

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Richard M. Niles, PhD

Gary O. Rankin, PhD (Departmental Chairman)

I would also like to thank R. Chris Harmon, MD, PhD and John Ball not only for the help they provided while compiling this work, but also for their friendship. I do appreciate every contribution Dr. Harmon and Mr. Ball provided during my graduate career.

Finally, I also like to thank the faculty, students and staff here at Marshall University School of Medicine. Thank you for providing such a wonderful learning experience and always being there for me. It has been an honor working with and knowing all of you.

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LIST OF SYMBOLS / NOMENCLATURE

APAP - Acetaminophen

ALF – Acute liver failure

ALT – Alanine aminotransferase

aa – Amino acid

COX - Cyclooxygenase

CYP – Cytochrome P450

DEM – Diethyl maleate

GSH – Glutathione

GSSG – Glutathione disulfides

HPLC – High performance liquid chromatography

4HNE – 4-Hydroxy-2-nonenal

i.p. – Intraperitoneal

LDH – Lactate Dehydrogenase

MDA – Malondialdehyde

NAC – N-Acetylcysteine

NAPQI – N-Acetyl-p-benzoquinone imine

NO – Nitric oxide

NSAIDs – Non-steroidal anti-inflammatory drugs

SAH – S-Adenosylhomocysteine

SAMe – S-Adenosyl-L-methionine

TBARS – Thiobarbituric acid reactive substances

TCA – Trichloroacetic acid

VEH - Vehicle

CHAPTER I

Introduction

Acetaminophen (APAP) overdose is a significant medical problem in the United States as well as other parts of the world, such as Great Britain and Canada. Intentional and accidental overdose with APAP-containing products is a primary cause for visits to the emergency room for both children and adults and accounts for nearly 50% of all acute liver failure in the United States. Toxic doses of APAP can produce hepatic centrilobular necrosis in human and animal models.

APAP hepatotoxicity is dependent on biotransformation of APAP by cytochrome P450 to the toxic metabolite, N-acetyl-p-benzoquinone imine (NAPQI). Glutathione (GSH) is the body's natural defense mechanism against NAPQI and protects the liver from hepatotoxicity by conjugation of this toxic metabolite. GSH also detoxifies NAPQI and prevents further oxidative stress.

Toxicity elicited by APAP overdose is manifested when GSH stores become depleted. Despite efforts to investigate APAP-induced liver failure over the past thirty years, the complete mechanism for this damage is still unknown. However, an induction of oxidative stress compounded with GSH depletion has been suggested as part of the cellular mechanism for hepatic damage. Interventions that increase GSH levels and reduce oxidative stress will limit or eliminate the effects of APAP toxicity. In the United States, the current antidote

for APAP toxicity is oral administration of N-acetylcysteine (NAC). NAC is able to provide a source of cysteine for increased hepatic synthesis of GSH. However, NAC does not have a 100% success rate. Time of administration after APAP overdose and the extent of the amount of APAP taken have a vital effect on the ability of NAC to reduce liver damage.

S-Adenosyl-L-methionine (SAME) is a naturally occurring substance that is present in plasma and most tissues. A healthy adult human produces about 6-8 grams of SAME per day. SAME also plays an important part in much of the body's reactions, for it is only second to ATP in the variety of reactions where it serves as a cofactor. SAME is also marketed worldwide as a nutritional supplement. It is available in many vitamin, grocery and health food stores in a variety of different packaging schemes.

Reasons for SAME's ability to reduce or eliminate stress to the liver induced by APAP lie with its ability to function as a major methyl donor in transmethylation reactions. Adequate hepatic levels of SAME are critical since the liver is responsible for 85% of all transmethylation reactions. SAME is a substrate precursor for formation of the tripeptide GSH. It can be converted to S-Adenosylhomocysteine and homocysteine which ultimately is converted to cysteine, the rate limiting factor in the production of GSH. SAME's abilities to attenuate the damaging effects of APAP overdose not only lie with GSH production. In our laboratory, we have also seen reduced lipid peroxidation, plasma ALT and protein carbonyls when animals are dosed with SAME just prior or 1 hour after APAP dosing.

The goal of the study was to investigate the hypothesis that SAmE can protect the liver from APAP-induced effects. The study was also designed to explore the ability of SAmE to attenuate APAP toxicity within 1 - 4 hours after APAP overdose in a mouse model. Studies further examined the effect of SAmE on APAP mediated alterations in lipid peroxidation, GSH concentrations, plasma ALT levels, protein carbonyls and oxidized proteins levels. Investigations also contained direct comparisons of the protective effects of vitamin B₆ + SAmE, NAC, NAC + SAmE and SAmE. Data collected during this study strengthens the evidence for the potential use of SAmE as a new antidote for APAP overdose.

CHAPTER II

Review of Literature

2.1 Acetaminophen

Acetaminophen (APAP) has been available in the United States since 1960 as an analgesic/antipyretic drug. Since then, APAP has become the most widely-used analgesic in the United States (Paulose-Ram et al., 2005) as well as one of the best documented causes of liver damage and/or acute liver failure (ALF) for over-the-counter medicines (Bae et al., 2001; Langford, 2006; Lee, 2003; Manov et al., 2006). Toxicities include, but are not limited to, induced liver cell death (Grattagliano et al., 2002; Hinson et al., 1981; James et al., 2003a; Koo et al., 1987; Goldring, 2004) and nephrotoxicity (Stern et al., 2005). Despite having comparable analgesic and antipyretic properties as non-steroidal anti-inflammatory drugs (NSAIDs), APAP is not classified as an NSAID, because APAP does not possess any anti-inflammatory properties. Not only has APAP usage been on the rise, but APAP toxicity has also been steadily increasing since the 1980s (Björnsson et al., 2006; Russo et al., 2004; Koo et al., 1987; Goldring, 2004; von Mach et al., 2005). To date, APAP usage accounts for approximately 56,000 emergency room visits and 26,000 hospitalizations yearly in the United States (Nourjah et al., 2006). The majority of overdose cases involve patients who unintentionally exceeded the recommended APAP amount (Larson et al., 2006).

Despite the wide usage of APAP, the direct mechanism of the drug is still unknown. Although progress has been made in identifying potential mechanistic properties of APAP, questions still exist about how APAP is metabolized to the toxic metabolite, N-acetyl-p-benzoquinone imine (NAPQI). Some believe that a cyclooxygenase isozyme might be important in APAP metabolism. In 1991, Simmons and coworkers discovered an isozyme of the prostaglandin synthesizing cyclooxygenase (COX) and named it cyclooxygenase-2 (COX-2) (Xie et al., 1991; DeWitt, 1999). This enzyme was a remarkable find, mainly since neither COX-1 nor COX-2 are sensitive to APAP. The COX-2 discovery has since brought a whole new line of drugs able to more directly fight medical situations. There is even question of the ability of COX-2 to be a part of a cancer prevention regimen (Rigas and Kashfi, 2005). Nearly 11 years after their discovery of COX-2, Simmons characterized and cloned another COX enzyme in dog brain which was sensitive to APAP (Chandrasekharan et al., 2002). However, the field is divided on the possibility of a third COX isozyme. While some argue that there quite possibly is a COX-3 isozyme that metabolizes APAP into NAPQI (Botting and Ayoub, 2005), others strongly disagree dismissing Simmons find as an incompletely spliced mRNA (Meade, 2003).

Even though there are several arguments regarding the mechanism of APAP hepatotoxicity, there are also some agreements between researchers. Currently, necrosis is believed to be the initial mode of cell death and apoptosis has been ruled out (Lawson et al., 1999; Gujral et al., 2002; James et al., 2003). The reactive metabolite, NAPQI, is formed by a direct two-electron oxidation and

elicits its damaging effects in the liver (Dahlin et al., 1984). Recently, it has been found that cytochromes (CYP) 2E1, 1A2, 3A4, and 2A6 are all involved in the oxidation of APAP to NAPQI (Patten et al., 1993; Thummel et al., 1993; Chen et al., 1998; Neve and Ingelman-Sundberg, 2001). There has also been recent data suggesting that nitrated tyrosine residues, as well as APAP adducts, can occur in the necrotic cells after receiving toxic doses of APAP (James et al., 2003; Hinson et al., 2000). These new findings have enabled scientists to move closer to a fuller understanding of the mechanism by which APAP elicits its damaging effects.

If taken by a healthy individual under the specified therapeutic guidelines, APAP can be a very beneficial therapeutic drug. Under these normal circumstances, humans can conjugate APAP with sulfate or glucuronide via sulfotransferase (ST) or uridine diphosphoglucuronosyltransferase (UDPGT), respectively (Mitchell et al., 1974; Nelson & Bruschi, 2003) (FIGURE 1) and safely eliminate the conjugated APAP from the body. However, even using the recommended dose, about 1-2% of the parent APAP compound can be metabolized (mainly via CYP2E1) into the toxic metabolite, NAPQI. However, the body has a defense mechanism in the form of glutathione (GSH) that conjugates NAPQI and allows for its safe removal through the kidneys. Consequently, APAP can become a very deadly drug if not taken with care. Intentional and accidental overdose with APAP containing products is a primary cause for visits to the emergency room for both children and adults (Watson et al., 2003). In humans, any dose greater than 4 grams per day of APAP can

cause acute hepatotoxicity, while a dose of 20 to 25 grams is potentially fatal (Larson et al., 2006). APAP poisoning is believed to cause at least 42% of acute liver failure (ALF) cases and 1 in 3 deaths seen in tertiary-care centers across the United States of America (Larson et al., 2006). This problem is also seen in the United Kingdom and Australia, with 100,000 cases of acute liver failure per year (Nelson and Bruschi, 2003). APAP overdose is a significant medical problem in the United States. In fact, APAP is the most frequent cause of ALF in this country, which can have up to a 90% mortality rate (Larson et al., 2006; Oz et al., 2004).

Acetaminophen hepatotoxicity is dependent on biotransformation of APAP by cytochrome P450 (mostly CYP2E1) to the toxic metabolite, NAPQI (Mitchell et al., 1973b) (FIGURE 1), however the complete mechanism of this liver injury is not completely understood (Oz et al., 2004). In the beginning of APAP-induced hepatotoxicity, toxic doses of APAP produce centrilobular necrosis in zone 3 of the liver in humans (Davidson and Eastham, 1966; McJunkin et al., 1976) and animal models (Mitchell et al., 1973a, b; Jollow et al., 1973; Mudge et al., 1978; Walker et al., 1982). This region around the liver's central vein is concentrated with high levels of CYP and low levels of GSH. It is therefore understandable why this region is the first to show the damaging effects of APAP. The ability of APAP to generate reactive oxygen species (ROS), sensitive transcription factor NF- κ B, GSH depletion, nitric oxide and lipid peroxides are believed to play a major role involved with this induced liver injury (Adamson and Harman, 1993; Blazka et al., 1995; Gordon et al., 1986; Kamiyama et al., 1993; Arnaiz et al.,

1995; Chen et al., 2005; El-Hassan et al., 2003; Dambach et al., 2006; Gardner et al., 1998; Ito et al., 2004; Jaeschke et al., 2003; Ozdemirler et al., 1994). Binding of the toxic APAP metabolite, NAPQI, to target proteins and DNA as well as mitochondrial GSH depletion also appears to be necessary for toxicity induced by APAP (Oz et al., 2004; Cohen and Khairallah, 1997; Cover et al., 2005; Hinson et al., 2004; Bajt et al., 2003). More recently, there has also been data suggesting that APAP overdose may also cause peroxynitrite formation along with other forms of oxidative stress (Knight and Jaeschke, 2004; Placke et al., 1987).

GSH is crucial in the reduction of damage produced by APAP overdose. This potent endogenous antioxidant is produced from cysteine (also the rate-limiting agent) and protects the liver from hepatotoxicity by conjugation of the NAPQI's parent compound (Mitchell et al., 1973a, b). GSH is a tripeptide of γ -Glutamate-Cysteine-Glycine and is one of the most important compounds in the defense against chemically reactive compounds and oxidative stress (Sener et al., 2003; Lomaestro and Malone, 1995; Lucas-Slitt et al., 2005). GSH also detoxifies NAPQI (Walker et al., 1982; Potter and Hinson, 1986), adriamycin (Olson et al., 1980) and naphthalene (Phimister et al., 2004). However, certain factors such as fasting and ethanol ingestion (induces CYP2E1) when combined with APAP overdose can deplete hepatic GSH and therefore allow an increase in hepatotoxicity and mortality (Oz et al., 2004). It is also known that factors such as the amount of the rate-limiting amino acid, cysteine, can slow down production of GSH. Studies have shown the effects of GSH deficiency on injury and sepsis,

and it is therefore understandable that treatment strategies that maintain and/or increase GSH stores may decrease the incidence of organ failure and death. Understandably, APAP accounts for many ALF cases, as it is one of the most commonly used analgesics in the USA and worldwide. Individuals often misuse APAP both acutely (suicide attempts) and chronically (long-term exposure in chronic pain patients) (Ellenhorn, 1997). Therefore it is of major scientific and medical interest to investigate the toxicity associated with APAP because in the end, patient education, as well as further research investigating antidotes to this potentially harmful drug, will not only save lives but could also release extra donor livers to patients on the waiting lists who need livers for other reasons.

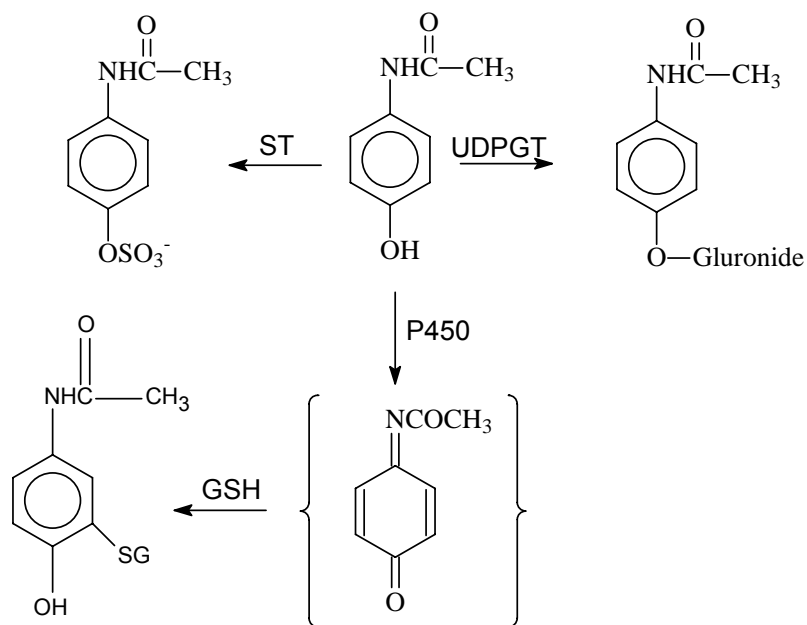


FIGURE 1. Metabolism of APAP. Diagrammatic representation of proposed metabolic pathway of APAP. The APAP structure is shown at the top, in the middle of the reaction. Inside the bracket is a representation of the toxic metabolite, NAPQI and the binding of GSH to the NAPQI (ST = sulfotransferase, UDPGT = uridine diphosphoglucuronosyltransferase).

2.2 N-acetylcysteine

Initial reports of APAP toxicity were described in Dr. Gillette's laboratory in the 1970s. It was found by Dr. Gillette and colleagues that APAP was metabolically activated by cytochrome P450 (CYP) to some reactive metabolite, later identified as N-acetyl-p-benzoquinone imine (NAPQI). NAPQI would deplete glutathione (GSH) up to 90% and covalently bind to protein. It was because of Dr. Gillette's findings that the antidote (that is still used today), N-acetylcysteine (NAC), was discovered. Since its initial discovery, there have been countless studies involving NAC and its protection against APAP-induced toxicity in rodents (Lauterburg et al., 1983) and humans (Marzullo, 2005). The studies involving NAC are not limited to APAP. For example, Raza and colleagues (2003) investigated the possibility of NAC as an antidote to azathioprine overdose. The ability of NAC to create GSH through production of the amino acid (aa) cysteine, makes NAC such an important part of recovery. Since Gillette's initial report of APAP toxicity, a number of other events have been shown to occur during APAP overdose and are likely important in the initiation and repair of toxicity (James et al., 2003). However, despite researchers' best efforts, scientists still do not fully understand and/or agree upon the precise mechanisms of hepatocyte death following APAP overdose (Lawson et al., 1999; Gujral et al., 2002; James et al., 2003).

As mentioned above, NAC is the current antidote to APAP overdose cases in the United States of America, as well as other countries. NAC is the aa L-cysteine plus an acetyl group attached to the amino group (FIGURE 2). Oral

supplementation using NAC can provide a means of boosting intracellular GSH by acting as a sulfhydryl group donor and raising the rate-limiting compound in GSH production, cysteine. NAC is rapidly absorbed after oral administration and reaches a peak plasma level in approximately 2-3 hours, with a half-life of about 6 hours. In the United States, after an individual has overdosed on APAP, patients are generally given oral NAC as a 140 mg/kg loading dose followed by 70 mg/kg every 4 hours for 17 more doses. NAC can be difficult to administer because of its taste and its tendency to cause nausea and vomiting. To maximize tolerance, it can be diluted down to a 5% solution from its commercially available 10% or 20% solutions (Villar et al., 1998). Even though intravenous forms of NAC have been available in other countries for some time (Prescott, 1981), the FDA only recently approved (2004) the administration of NAC through intravenous route in the United States. The justification for this route of administration is to rapidly decrease the damaging effects of APAP quicker.

However, despite the therapeutic effects of NAC in restoring GSH stores, it is not 100% effective. For NAC to be therapeutically effective, most agree that it must be given within 8 hours of APAP overdose (Villar et al., 1998). In fact, in the acute liver failure cases listed above, NAC was administered to all patients but everyone did not fully recover. While a small percentage of some patients did fully recover, others had received irreversible damage to the liver and were placed on the liver transplant list. Some of the patients didn't even survive after the NAC treatment. Questions regarding the potential of NAC as "the best" antidote for APAP toxicity has previously been raised in the past. A study

conducted by Sener and colleagues (2003) compared the protective effects of three antioxidants: melatonin, vitamin E and NAC. Mice were given one of the three antioxidants up to 30 minutes prior to APAP injection. These scientists found that each had therapeutic effects, but it was melatonin that provided better protection and less damage than vitamin E or NAC. This drives the question as to whether there is a better antidote for attenuating the damaging effects of APAP overdose.

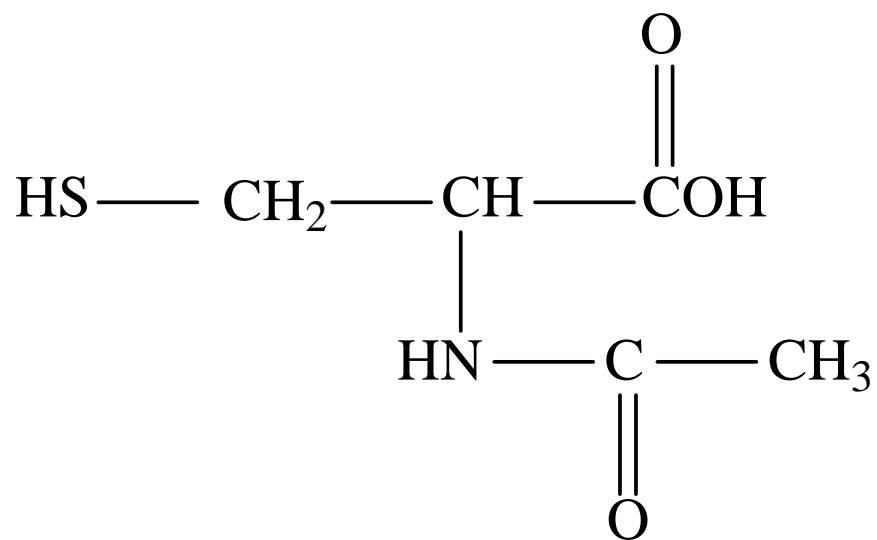


FIGURE 2. Chemical Structure of NAC.

2.3 S-Adenosyl-L-methionine

S-Adenosyl-L-methionine (SAME) (FIGURE 3) is a naturally occurring substance present in plasma and most tissues. SAME levels are critical for normal cellular function (Martinez-Chantar et al., 2002). SAME was first discovered in 1952 and is synthesized from ATP and methionine by the enzyme S-adenosylmethionine synthetase (Lu, 1998). SAME is utilized by three key metabolic pathways (FIGURE 4): (1) transmethylation, (2) trans-sulfuration and (3) polyamine synthesis (Friedel et al, 1989; Mato et al., 1999; Lu, 2000). Transmethylation occurs when a methyl group of SAME is donated to any large variety of acceptor substances such as DNA, phospholipids and proteins. Trans-sulfuration involves the sulfur atom of SAME. The sulfur atom is converted to cysteine, a precursor for taurine and glutathione (GSH). Polyamine synthesis only accounts for about 5% of the SAME metabolic pathways, but this metabolic pathway is required for normal cell growth (Lu, 1998) and it can be induced under conditions of increased polyamine synthesis (Mato et al., 1997). Increased polyamine synthesis can happen during liver regeneration and early hepatocarcinogenesis (Mudd and Poole, 1975).

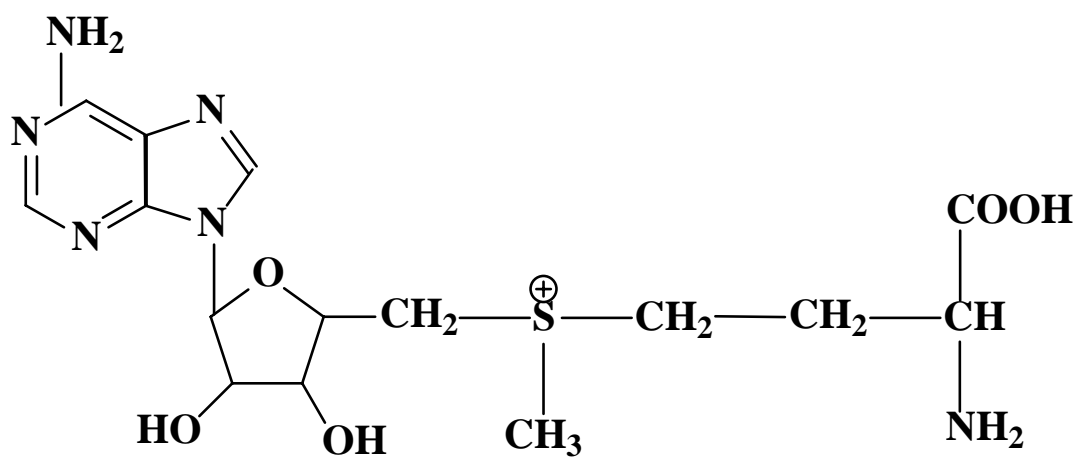


FIGURE 3. Chemical structure of SAMe.

SAMe is probably second only to ATP in the variety of reactions for which it serves as a cofactor (Lu et al., 2005). SAMe is a vital metabolic intermediate in all studied life forms and each cellular organism has enzymes with the ability to utilize SAMe (Kozbial & Mushegian, 2005). SAMe is also the preferred catabolic pathway of methionine, where it can then be converted to S-adenosylhomocysteine (SAH), which is ultimately converted to cysteine, the rate-limiting reagent in GSH synthesis (Lu et al., 1998) (FIGURE 4). SAMe can be synthesized in the cytosol of every cell, but the liver plays host to the central role of SAMe synthesis and degradation. A normal, healthy adult human produces 6-8 grams of SAMe per day (Lu, 2000). The majority of SAMe is used in the transmethylation pathway in the liver mainly due to the fact that the liver is responsible for 85% of all transmethylation reactions (Friedel et al, 1989; Mato et al., 1997; Lu, 2000). An abundance of SAMe is also used for the catabolic trans-sulfuration pathway to generate an important antioxidant, GSH (Lu et al., 1998).

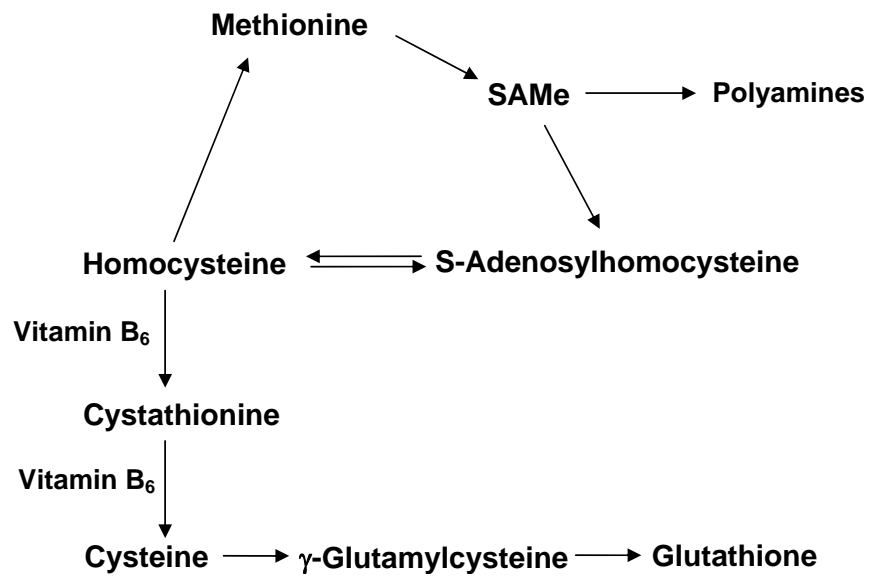


FIGURE 4. SAMe Pathway.

Despite the body's abilities to produce S_{AM}e in such large amounts, there are limiting factors regulating the pathways of S_{AM}e. For instance, SAH is a potent competitive inhibitor of transmethylation reactions. An increase in SAH levels and/or a decrease in the SAM:SAH ratio will result in an inhibition of transmethylation reactions (Finkelstein, 1990; Lu, 2000). For this particular reason, removal of excess SAH is essential for the involvement of S_{AM}e in transmethylation reactions. In vivo, the reaction represented in Figure 4 proceeds in the forward direction only if the products, adenosine and homocysteine, are rapidly removed (Mato et al., 1997; Finkelstein, 1990). The liver plays host to the three pathways that metabolize homocysteine. One of the pathways includes a transformation of homocysteine to cysteine. This particular pathway, the trans-sulfuration pathway, is present largely in the liver and the lens (Lu, 1998). In the trans-sulfuration pathway, serine and homocysteine condense to form cystathionine in a reaction that requires vitamin B₆ as a cofactor (Lu, 2000). Cystathionine is cleaved in a reaction catalyzed by another vitamin B₆-dependent enzyme into free cysteine (Mato et al., 1997; Lu 1998). Cysteine is a precursor for taurine and the rate-limiting precursor for GSH synthesis (Lu 1998). The two other pathways involving the breakdown of homocysteine resynthesize methionine from homocysteine. One is catalyzed by methionine synthase and the other is catalyzed by betaine-homocysteine methyltransferase (Mato et al., 1997; Finkelstein, 1990).

S_{AM}e participates in many major biological functions through its involvement in the three main metabolic pathways listed above: transmethylation,

trans-sulfuration and polyamine synthesis. Given the large spectrum of cellular processes ranging from membrane fluidity (phospholipid methylation) to gene expression (DNA methylation), it is easy to understand the importance of SAMe and the issues that can arise when the availability of SAMe is limited by a disease state or other conditions (Mato et al., 1997; Friedel et al., 1989).

SAMe is commercially available and marketed worldwide as a nutritional supplement that is readily available in vitamin and health food stores. SAMe became available in the United States in 1999 as an over-the-counter nutraceutical (Bottiglieri, 2002). It is currently marketed to provide relief for stiff joints, provide a healthy liver and create emotional well-being. These marketing schemes were not created without some evidence. Regarding past studies, SAMe has been found to reduce toxic effects of ethanol and carbon tetrachloride (Song et al., 2003; Garcia-Ruiz et al., 1995; Lieber et al., 1997), APAP (Valentovic et al., 2004), cytokines, galactosamine, thioacetamide and ischemia-reperfusion (Bray et al., 1992; Lieber et al., 1990; Mato et al., 1997; Wu et al., 1996; Wu and Cederbaum, 2006; Lieber et al., 2002). There was also a study conducted by Pascale et al. (1993) that investigated the ability of SAMe to prevent the development of liver cancer in rodents. Exogenous SAMe has also been shown to decrease the rate of cell growth in a liver cancer cell line (Cai et al., 1998), treat intrahepatic cholestasis (Frezza et al., 1990) and prevent the decrease of liver GSH produced by various hepatotoxins (Wu and Cederbaum, 2005).

The ability of SAME to participate in such a wide spectrum of reactions in the body has made it a valuable tool that has the potential to decrease the effects of different toxicities. One of the main vital reactions for SAME is during situations that cause low GSH levels: ethanol consumption, APAP overdose, fasting, etc. Our laboratory (Valentovic et al., 2004) and others (Stramentinoli et al., 1979; Bray et al., 1992; Carrasco et al., 2000; Song et al., 2004) have further investigated the potential of SAME attenuating chemical-induced forms of liver toxicity.

Due to the major part APAP plays in so many different people's lives every day and the vast increase in APAP toxicity reported around the world, we had decided to study the potential of SAME as an antidote for APAP overdose cases. In vivo experiments were designed using male C57Bl/6 mice. Mice were chosen as an in vivo model based on their ability to metabolize APAP similarly to humans. Significant differences can exist with respect to various species to the hepatotoxic effects of APAP. For instance, rats, rabbits and guinea pigs are somewhat resistant to APAP-induced liver injury, most likely due to their ability to excrete higher amounts of APAP metabolites through the kidney (Bessemers and Vermeulen, 2001). The C57Bl/6 mice were dosed with APAP based on previous mouse studies (Bray et al., 1992; Carrasco et al., 2000; Stramentinoli et al., 1979; Song et al., 2004). SAME doses were given as an intraperitoneal (i.p.) injection just prior or post-APAP treatment and were comparable to doses used in previous studies (Carrasco et al., 2000; Stramentinoli et al., 1979; Song et al., 2004).

One of the protective mechanisms of SAmE is believed to include the production of GSH. After GSH reduces an active metabolite, such as NAPQI, GSH then becomes oxidized. Oxidized GSH is inactive and cannot protect the liver from damage. GSH can be converted back to its reduced form by glutathione reductase. The effects of SAmE on glutathione reductase are not known. If SAmE increases the activity of glutathione reductase, this too could be another protective mechanism of SAmE. GSH in the presence of glutathione peroxidase can donate one electron to NAPQI and convert it back to APAP. Glutathione peroxidase activity is dependent on high GSH levels and its activity is diminished in conditions of GSH depletion. SAmE may provide sufficient GSH to maintain intact glutathione peroxidase activity, which would lessen the levels of NAPQI. To further understand the mechanistic properties of SAmE, our studies give a better description of how and where SAmE initiates GSH synthesis within the cell.

SAmE has been shown to reduce APAP-induced hepatotoxicity by our laboratory (Valentovic et al., 2004) as well as others (Stramentinoli et al., 1979; Bray et al., 1992; Carrasco et al., 2000; Song et al., 2004). SAmE administration just prior to, as well as after, APAP treatment greatly diminished the extent of centrilobular necrosis in C57Bl/6 male mice when compared to animals given only APAP (Valentovic et al., 2004). SAmE has also shown beneficial effects in reducing and reversing hepatic damage induced by alcohol in humans (Lieber, 2002) and in intrahepatic cholestasis (Frezza et al., 1990).

CHAPTER III

Materials

Chemicals/Equipment Including Sources (Company & Catalog Number)

Acetic acid (Fisher, UN-2789)

N-acetylcysteine (NAC) (Sigma, A-8199)

Acrylamide (Fisher, BP170)

Ammonium Persulfate (APS) (Fisher, BP179)

Anti-Nitrotyrosine rabbit immunoaffinity purified IgG (Upstate, 06-284)

Bis-acrylamide (Bio-Rad, 161-0201)

Bromophenol blue (Pharmacia Biotech, 17-1329-01)

N-butanol (Sigma, BT-105)

Coomassie brilliant blue (Calbiochem, 234735)

Disodium EDTA (Fisher, S-311)

5,5'-Dithiobis(2-nitrobenzoic acid) (Aldrich, D21820-0)

ECL Western Blot Kit (Amersham Biosciences, RPN2106)

Ethylenediaminetetraacetic acid disodium salt dehydrate (EDTA) (Sigma, E-5134)

Glutathione disulfide (GSSG) (Sigma, G-6654)

Glutathione reductase (Sigma, G-3664)

Glycerol (Fisher, BP229)

Glycine (Sigma, G7126)

Goat anti-rabbit IgG – HRP (Santa Cruz Biotechnology, SC-2004)

Infinity ALT kit (Thermo, TR71021)

2-Mercaptoethanol (2-ME) (Fisher, BP-176-100)

Methanol (Fisher, AY12-20)

NADPH (Sigma, N-7505)

Nitrotyrosine immunoblotting control (Upstate, 12-354)

Opitran 0.45µm supported nitrocellulose membrane (Whatman, BA-585)
OxyBlot Protein Oxidation Detection Kit (Chemicon International, S7150)
Polyclonal antibody to (e)-4-hydroxynonenal (Alexis Biochemicals, ALX-210-767)
Polyclonal antibody goat anti-rabbit (Santa Cruz Biotechnology)
Ponceau (Sigma, P3504)
Potassium chloride (Fisher, P-217)
Potassium phosphate monobasic (Fisher, P-285)
Reduced glutathione (GSH) (Sigma, G-6529)
S-Adenosyl-L-methionine p-toluenesulfonate (Sigma, A2408)
Sodium chloride (Fisher, S671-3)
Sodium dodecyl sulfate (SDS) (Fisher, L-4390)
Sodium phosphate dibasic anhydrous (Mallinckrodt, 7917)
5-Sulfosalicylic acid (SSA) (EMD, SX 1220-1)
TEMED (Sigma, T9281)
2-Thiobarbituric acid (Sigma, T-5500)
Trichloroacetic acid (Fisher, A322-500)
Triethanolamine (Sigma, T1377)
Trizma Base (Sigma, T6066)
TWEEN-20 (Sigma, P-1379)
2-Vinylpyridine (Aldrich, 13229-2)
0.45µm syringe filter (Durapore; Millex-HV)
Dubnoff metabolic shaker (Precision Scientific; Chicago, IL)
Sorval MC12V centrifuge
Guilford Stasar II Spectrophotometer
Beckman 126 model HPLC
Bio-Rad transblot apparatus
BRL vertical gel V-16 electrophoresis system

Methods

Selection of Animal Species and In Vivo Model

The male C57Bl/6 mouse animal model was selected for this project because it has been shown that these mice have susceptibility to hepatic toxicants similar to that of humans (Bessemers and Vermeulen, 2001). Previous studies have also shown that acetaminophen (APAP) is hepatotoxic in this animal model (Carrasco et al., 2000; Bray et al., 1992; Stramentinoli et al., 1979). Male mice were chosen to minimize hormonal variations in the model.

Male C57Bl/6 mice (16-20 g) were obtained from Hilltop Lab Animals, Inc. (Scottsdale, PA, USA). Animals were maintained under a controlled ambient temperature (21-23°C), humidity (40-55%) and light cycle (lights on 0600-1800 h). Animals were provided free access to tap water and Rodent Chow. All mice were given a minimum 7-day acclimation period prior to initiation of any experiments. This model was the only animal model used throughout the study.

The animals were given an intraperitoneal (i.p.) injection of water, APAP, S-Adenosyl-L-methionine (SAME), N-acetylcysteine (NAC), vitamin B₆, diethyl maleate (DEM) and/or corn oil. These treatments were chosen on the basis that this model provides an overall look at how the body reacts to an overdose of a toxicant such as APAP. It also allows for a realistic view of how we could expect the liver to react/look after a severe overdose of APAP. This model allowed cells to maintain communication and metabolism. Furthermore, the in vivo model avoided confounding factors that may be seen in cell culture models where

results cannot be directly compared to what would be expected in real-life situations.

Treatment of Animals with SAME just prior to APAP

Each experiment consisted of 20 fasted animals randomly separated into 4 different groups with 5 animals per group. Experiments included animals that were fed and those that were fasted overnight (1700 – 0900 h). These experiments are labeled in the results section. The first group, designated as the vehicle (VEH), was injected i.p. with water (15 ml/kg). The second group was injected (i.p.) with 500-1000 mg/kg of SAME (5 ml/kg) only. This allowed a direct comparison to the VEH animal to confirm that the SAME treatment did not have any toxic effects. The third group was given an i.p. dose between 250-500 mg/kg of APAP (15 ml/kg). The fourth and final group was given 500-1000 mg/kg of SAME just prior to 250-500 mg/kg of APAP. After treatment, the mice were anesthetized with CO₂ by inhalation and blood was collected by cardiac puncture. The mice were laparotomized, the abdominal aorta severed and the liver was excised 1-4 hours post-APAP and placed in ice-cold Krebs-Ringer buffer. Blood collected from the animals was spun at 10,000 xg for 10 minutes at room temperature to separate the plasma. ALT assays (Infinity Kit #TR71021) were performed on the plasma as a parameter of hepatic function.

Treatment of Animals with NAC just prior to APAP

Animals were fasted overnight (1700 – 0900 h), prepared and divided into groups as described above. However, in these experiments animals were given

the same 1.25 mmol/kg dose of NAC, i.p., as SAME. The first group was designated as the VEH, the second received (i.p.) with 204 mg/kg of NAC (5 ml/kg at pH 7), the third group received 250-500 mg/kg of APAP and the final group was given 204 mg/kg of NAC just prior to 250-500 mg/kg of APAP. After treatments, livers were excised 1-4 hours post-APAP injection.

Treatment of Animals with DEM after SAME or NAC

Animals were fasted overnight (1700 – 0900 h) and randomly divided into three groups with five animals per group. The first group (VEH) received an i.p. injection of 15 ml/kg corn oil. The second group received 1000 mg/kg DEM. The third group received (i.p.) 1000 mg/kg DEM just after 500 mg/kg SAME or 204 mg/kg NAC. Livers were removed 2 or 4 hours post-DEM treatments.

Treatment of Animals with SAME + NAC just prior to APAP

Animals were fasted overnight (1700 – 0900 h) and randomly divided into four groups, as described previously. The first group contained animals dosed with water and were designated “VEH”. The second group received a total 0.625 mmol/kg SAME and NAC, i.p., injected as 250 mg/kg SAME + 102 mg/kg NAC. The third group received 300 mg/kg APAP and the fourth group received 250 mg/kg SAME + 102 mg/kg NAC just prior to 300 mg/kg APAP. After i.p. injection, the livers were excised 1 or 4 hours post-APAP injection.

Treatment of Animals with Vitamin B₆ + SAmE just prior to APAP

Animals were fasted overnight (1700 – 0900 h) and prepared as described above. The first group was given water and labeled as the VEH, the second group received 3.84 mg/kg vitamin B₆ + 500 mg/kg SAmE, the third group was treated with 300 or 400 mg/kg APAP and the final group was injected i.p. with 3.84 mg/kg vitamin B₆ + 500 mg/kg SAmE just prior to 300 or 400 mg/kg APAP. After injections, livers were excised 1 or 4 hours post-APAP administration.

Experiments Involving SAmE or NAC Dosing Post-APAP Overdose

Animals were fasted overnight (1700 – 0900 h) and randomly divided into four groups with five animals per group. The first group received an i.p. injection of water (VEH). The second group received an i.p. injection of either 500 mg/kg SAmE or 204 mg/kg NAC. The third set of animals received 250 mg/kg of APAP. The final group received 250 mg/kg APAP 1 hour prior to 500 mg/kg SAmE or 204 mg/kg NAC. Blood was collected and livers were excised 4 hours post-APAP.

Isolation of Livers

Food was removed the evening prior to all experiments involving fasting (1700-0900 h). The initial studies included fed animals and are explained in further detail in the results section. During the initial studies, animals were allowed free access to both food and water. All other studies were conducted on animals that had been fasting but were still allowed free access to water. The

afternoon of the experiments, animals were anesthetized within a carbon dioxide (CO₂) chamber. Blood was removed via cardiac puncture using 22-gauge needle on a 1 cc syringe and stored in microcentrifuge tubes. After blood removal, the cervical vertebrae were dislocated to kill the animals. The liver (FIGURE 5) was then removed, trimmed of unwanted tissues (skin, intestines, diaphragm and/or spleen) and immediately placed in 3 ml of ice-cold Krebs-Ringer buffer (see Appendix for preparation of Krebs-Ringer buffer preparation) or Buffer A (see Appendix for preparation of Buffer A) for mitochondrial isolation experiments. The total liver tissue was blotted dry, weighed and separated into specified amounts for each assay. Assay measurements included GSH, protein and lipid peroxidation levels. Each assay had specific ways to store the tissues and is further discussed and described below. Blood collected from the animals was centrifuged at 10,000 *xg* for 10 minutes at room temperature to separate the plasma. Plasma was collected and stored at 4°C in a second set of microcentrifuge tubes.

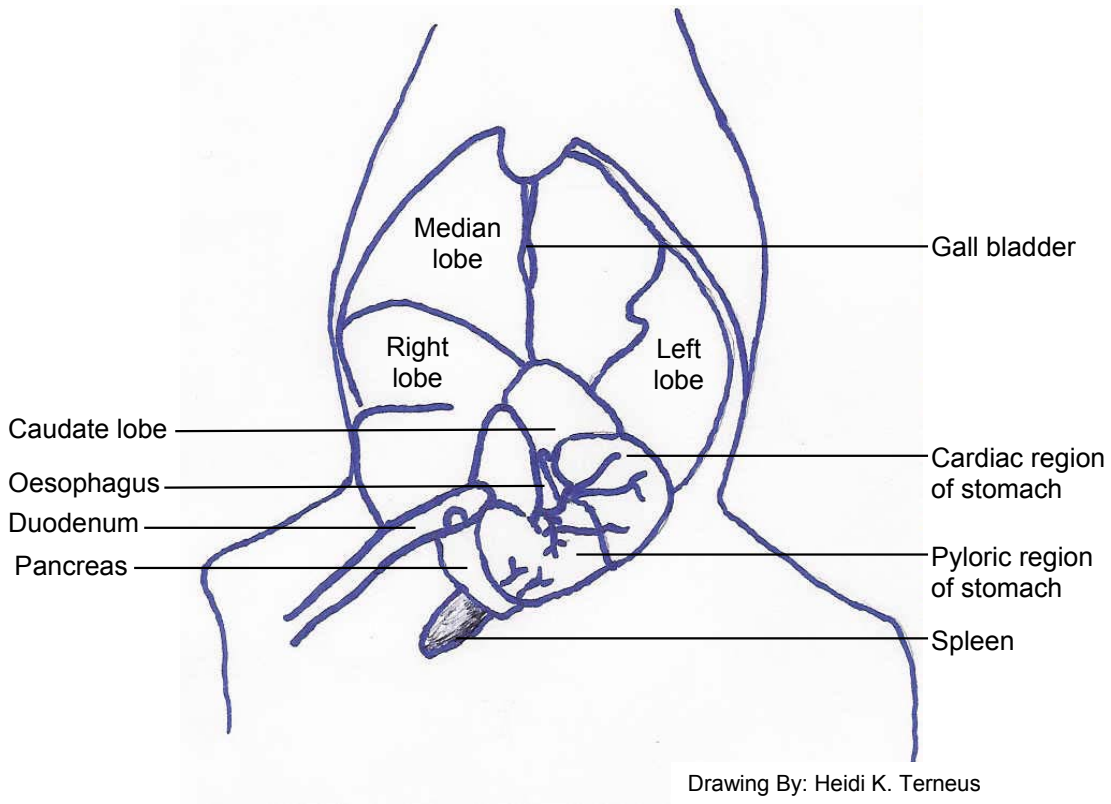


FIGURE 5. Diagram of Mouse Anatomy.

Mitochondrial Isolation

A series of studies examined the subcellular effects of APAP on hepatic mitochondria and whether SAME localized in a specific organelle. Other experiments required mitochondrial isolation to assess the damage of APAP as well as the protection provided by either SAME or NAC in attenuating APAP-induced damage. After collection of blood, liver was excised, blotted, weighed and minced using sharp-pointed scissors. Blood collected from the animals was spun at 10,000 xg for 10 minutes at room temperature to separate the plasma. ALT assays (Infinity Kit #TR71021) were performed on the plasma as a parameter of hepatic function. The liver samples were hand homogenized on ice in 1.5 ml of Buffer A using a dounce tissue grinder. Homogenized tissues were collected and the grinders were rinsed with 1.5 ml of Buffer A and the rinse buffer added to the sample. The 3 ml samples were then centrifuged at 600 xg for 10 minutes at 4°C. Supernatant was then removed and spun at 15,000 xg for 5 minutes at 4°C. After the 15,000 xg spin, the supernatant (cytosolic content) was removed and stored in microcentrifuge tubes for GSH determination, protein concentrations and LDH assays (to confirm mitochondria were isolated). The pellet created after the 15,000 xg spin is mitochondrial based components. The pellet was rinsed with 250 μ l of Buffer B (see Appendix for preparation of Buffer B). The pellet was then resuspended in 1 ml of Buffer B per gram of tissue weight. The resuspension was then divided into tubes to perform GSH determination, protein concentration and LDH levels. See Appendix for further details regarding mitochondrial isolation.

LDH Assay

Upon completion of centrifugation for mitochondrial isolation, cytosolic and mitochondrial lactate dehydrogenase (LDH) levels were determined using a spectrophotometric kinetic assay designed by Sigma (Sigma, Kit #228). The principle for the assay uses LDH to catalyze the oxidation of lactate to pyruvate with the simultaneous reduction of NAD (FIGURE 6). The formation of reduced nicotinamide adenine dinucleotide (NADH) results in an increase of absorbance at 340 nm. The rate of absorbance is directly proportional to LDH activity. LDH release was expressed as percent of total LDH.



FIGURE 6. Reaction involved in LDH assay.

Glutathione Determination

After liver was blotted and weighed, 200 ± 15 mg were collected and homogenized in 5% sulfosalicylic acid (SSA) in a 1 ml total volume. Total GSH was determined by an enzymatic reaction with glutathione reductase using 5,5'-dithiobis(2-nitrobenzoic acid) and NADPH (Anderson, 1985). Glutathione disulfides (GSSG) were measured on samples derivatized with 2-vinylpyridine (Griffith, 1980) prior to enzymatic measurement of GSH (nonspecific). Values were expressed as nmol/g tissue or $\mu\text{mol/g}$ tissue. See Appendix for detailed methods.

Lipid Peroxidation

Thiobarbituric acid reactive substances (TBARS) were measured in the homogenized liver samples as a measure of lipid peroxidation. Livers were collected from each group as described previously. Liver samples (300 ± 15 mg) were homogenized in 1.5 ml of Krebs-Ringer buffer. The homogenizer probe was rinsed with an additional 1.5 ml of Krebs-Ringer buffer and the sample volume was adjusted to 3 ml. A 1.5 ml aliquot was divided evenly between two microcentrifuge tubes and used for protein concentration determination. The other 1.5 ml homogenate volume was added to an equivalent volume of 15% trichloroacetic acid (TCA) dissolved in 0.25 N HCl. Protein was precipitated for 15 min on ice, and the supernatant was collected following centrifugation ($2000 \times g$ at 4°C , 10 minutes). The supernatant was heated for 15 minutes at 90°C with an equal volume of 0.67% thiobarbituric acid (Udea and Shah, 1996). Following

a 10 minute cooling period, the absorbance was measured at 535 nm. The amount of TBARS was calculated based on a standard curve using malondialdehyde (MDA) and expressed as $\mu\text{mol MDA/mg protein}$.

Protein Determination

Livers were collected and prepared as described in the lipid peroxidation section. The 1.5 ml aliquot collected before lipid peroxidation determination was stored at -80°C until analysis. Protein concentrations were analyzed as described by Bradford (1976).

4-Hydroxy-2-nonenal-Adducted Protein

Animals were treated and livers collected as described above. Liver tissues were weighed and then homogenized in Krebs-Ringer buffer in a 1 ml/100 mg of tissue total volume. Western blots were used to look for the presence of 4-hydroxy-2-nonenal (4HNE)-adducted proteins. See Appendix for detailed methods.

Protein Carbonyl Determination

Animals were treated and livers collected as described previously. Livers were weighed and then homogenized in Krebs-Ringer buffer in a total volume of 1 ml of buffer per 100 mg tissue. An OxyBlot kit (Chemicon International, S7150) was used to calculate the total concentration of protein carbonyls present. An antibody specific to regions on protein carbonyls derivatized with 2,4-

Dinitrophenylhydrazine (DNPH) included in the OxyBlot kit was used (FIGURE 7).

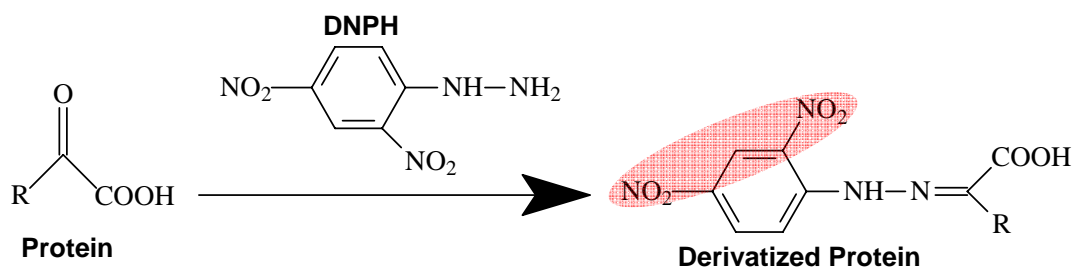


FIGURE 7. Reaction involving derivatization of protein with DNP.

Region highlighted represents the area recognized by the primary antibody included with the OxyBlot kit.

Oxidative Stress

The role of oxidative stress in APAP toxicity was evaluated using three approaches: GSH status, 4HNE-adducted protein determinations and production of protein carbonyls through measurement using the OxyBlot kit. These methods are described above.

Proteomics

Proteomic data represented in the results section were generated in collaboration with Serrine Lau, PhD at the University of Arizona. Protein samples were prepared as explained above in the protein determination section and protein concentrations were calculated using the Bradford Assay. Samples were analyzed by Dr. Lau's laboratory to complete the identification of proteins in question. Dr. Lau is Director of the proteomics center at the University of Arizona.

The following methods were written in collaboration with Dr. Lau's laboratory. It illustrates the steps performed after receiving samples from our laboratory:

Initial sample preparation: Samples were received from Marshall University frozen on dry ice and promptly placed in a -80°C freezer. The samples were thawed on ice and approximately 100 µL of whole tissue homogenate was spun at 14,000 rpm for 5 minutes at 4°C. Then the supernatant (soluble protein fraction) was transferred to a clean tube on ice.

Oxyblot: The exact protocol from Chemicon International was followed using 18 µg of protein/lane (OxyBlot Protein Oxidation Detection Kit S7150).

Gel Electrophoresis: Approximately 40 µg of protein was added to each lane of a 12% gel (Invitrogen) and separated for 1.5 hours at a constant 150 volts. The gel was then stained overnight with Coomassie blue.

LC/MS-MS: Analyses of in-gel trypsin digested H3.3 protein bands were carried out using a quadruple ion trap ThermoFinnigan LCQ DECA XP PLUS (San Jose, CA) equipped with a Michrom Paradigm MS4 HPLC, and a nanospray source based on the design of the University of Washington. Peptides were eluted from a 15 cm pulled tip capillary column (100 µm I.D. x 360 µm O.D; 3-5 µm tip opening) packed with 8-9 cm Vydac C18 (Hesperia, CA) material (5 µm, 300Å pore size), using a gradient of 0-65% solvent B (98% methanol/ 2% water/ 0.5% formic acid/ 0.01% trifluoroacetic acid) over a 60 minute period at a flow rate of 200-300 nl/min. The LCQ ESI positive mode spray voltage was set at 1.6 kV, and the capillary temperature at 200°C. Dependent data scanning was performed by the Xcalibur v 1.3 software, with a default charge of 2, an isolation width of 1.5 amu, an activation amplitude of 35%, activation time of 30 msec, and a minimal signal of 10,000 ion counts. Global dependent data settings were as follows: reject mass width of 1.5 amu, dynamic exclusion enabled, exclusion mass width of 1.5 amu, repeat count of 1, repeat duration of 1 minute, and exclusion duration

of 5 minutes. Scan event series included one full scan with mass range 350 – 2000 Da, followed by 3 dependent MS/MS scan of the most intense ion.

Data Analysis: The criteria that were used for preliminary positive peptide identification are the same as previously described. All matched peptides were confirmed by visual examination of the spectra. All spectra were searched against the latest version of the non-redundant protein database downloaded from the National Center for Biotechnology Information (NCBI).

Computer Program Descriptions for Data Analysis:

Turbo SEQUEST: Tandem MS spectra of peptides were analyzed with Turbo SEQUEST™, a program that allows the correlation of experimental tandem MS data with theoretical spectra generated from known protein sequences.

X!Tandem: Is an open source software program that matches tandem mass spectra with peptide sequences. It identifies and scores possible peptides and their corresponding proteins from the MS/MS data, and is capable of searching the input data for specified amino acid additions.

SALSA: (scoring algorithm for spectral analysis) Is a program that is capable of identifying and scoring spectra that contain a specified neutral loss of a user specified modification, such as a phosphate or small molecule. Spectra showing

promising scores are then compiled into DTA files and processed using X!Tandem.

PMOD: Algorithm screens data files for MS-MS spectra corresponding to peptide sequences in a search list. Modification of the primary peptide sequence will result in a shift in the peptide mass. This shift in the peptide mass may be experimentally observed as a difference between the measured mass of the modified peptide precursor ion (adjusted for charge state) and the predicted mass of the unmodified peptide. If a modification is located at a particular amino acid residue in the sequence, the mass shift also will be observed in the m/z values of some of the fragment ions.

SpecPlot: Is a novel program that generates theoretical b and y ions from a user specified peptide containing an adduct. It then compares the theoretical ions to a spectrum from raw data specified by the user and shows which ions correlate between the two. Essentially, it increases throughput of manual validation of adducted peptides, which is a requirement for acceptance.

High Performance Liquid Chromatography (HPLC)

HPLC methods used were similar to Moldéus (1978) and Vertzoni and colleagues (2003). Metabolites were quantified and normalized to a standard curve (concentration of 0-125 $\mu\text{g/ml}$). Standard curves were established using

APAP, acetaminophen glucuronide and acetaminophen sulfate. Plasma and liver samples were prepared as described above. As described in the methods published by Vertzoni (2003), 50 μ l and 30 μ l of an internal standard are combined in a microcentrifuge tube. A volume of 30 μ l of HClO₄ and 190 μ l of water are also added to the mixture (total volume of 300 μ l). The sample is then vortexed for 1 minute and spun at 11,000 rpm for 10 minutes at room temperature. The supernatant can then be stored at -20°C or filtered with a 0.45 μ m syringe filter. After filtration, 20 μ l is injected and ran on the Beckman 126 model HPLC using a mobile phase similar to that published in Moldéus (1978).

Statistical Analyses

All statistical calculations were performed using SigmaStat software package (version 2.03). Values were reported as a mean \pm SEM. Differences between groups were analyzed using a repeated-measures analysis of variance (RM-ANOVA) followed by a Newman-Keuls or Tukey test at a 95% confidence interval (Sokal and Rohlf, 1969). All groups consisted of 4-10 animals.

CHAPTER IV

Results

4.1 General Toxicity

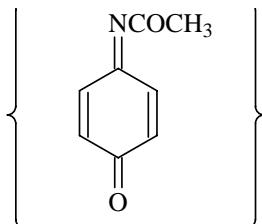
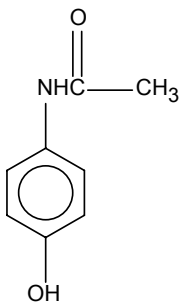
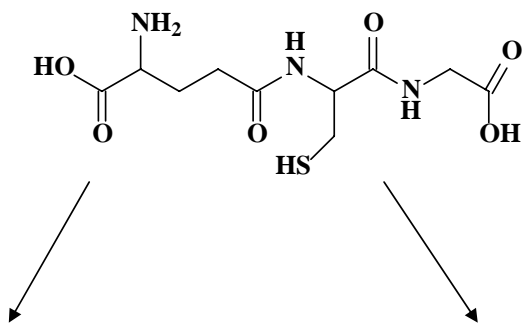
Since acetaminophen (APAP) biotransformation is similar between humans and mice, the APAP dosages were selected to closely mimic a human overdose. The doses of APAP selected were 250-500 mg/kg. In this study, APAP was directly toxic to the liver as noted by glutathione (GSH) depletion, elevated plasma ALT levels, increased liver: body weight ratio and abnormal morphology viewed using light microscopy. APAP induced both concentration- and time-dependent hepatotoxicity as indicated by the following studies. The toxic effects were observed with concentrations as low as 250 mg/kg APAP. These studies reiterate the harmful effects of APAP to the body, especially the liver, and how it alters tissue viability as measured by GSH levels, plasma ALT levels, liver:body weight ratio, protein levels, lipid peroxidation and histology studies.

4.2 Treatments Just Prior to APAP

4.2.1 Time- and Dose-Dependent APAP Toxicity in Fed Mice

Effect of 1000 mg/kg or 500 mg/kg SAmE just prior to 500 mg/kg APAP dose

Initial studies examined the protective effect of SAmE on APAP hepatic toxicity in fed mice. The concentration of SAmE was chosen based on results published in past studies (Stramentinoli et al., 1979; Bray et al., 1992), as well as what we had seen in our laboratory (Valentovic et al., 2004). It has been postulated (Lu, 1998) that one of the protective mechanisms of SAmE is to induce production of GSH (FIGURE 4). GSH is then able to bind to either N-acetyl-p-benzoquinone imine (NAPQI) or the parent compound, APAP, and the conjugated pair is then eliminated from the body (FIGURE 8). Therefore, we examined the effects on GSH production when SAmE was administered just prior to APAP.



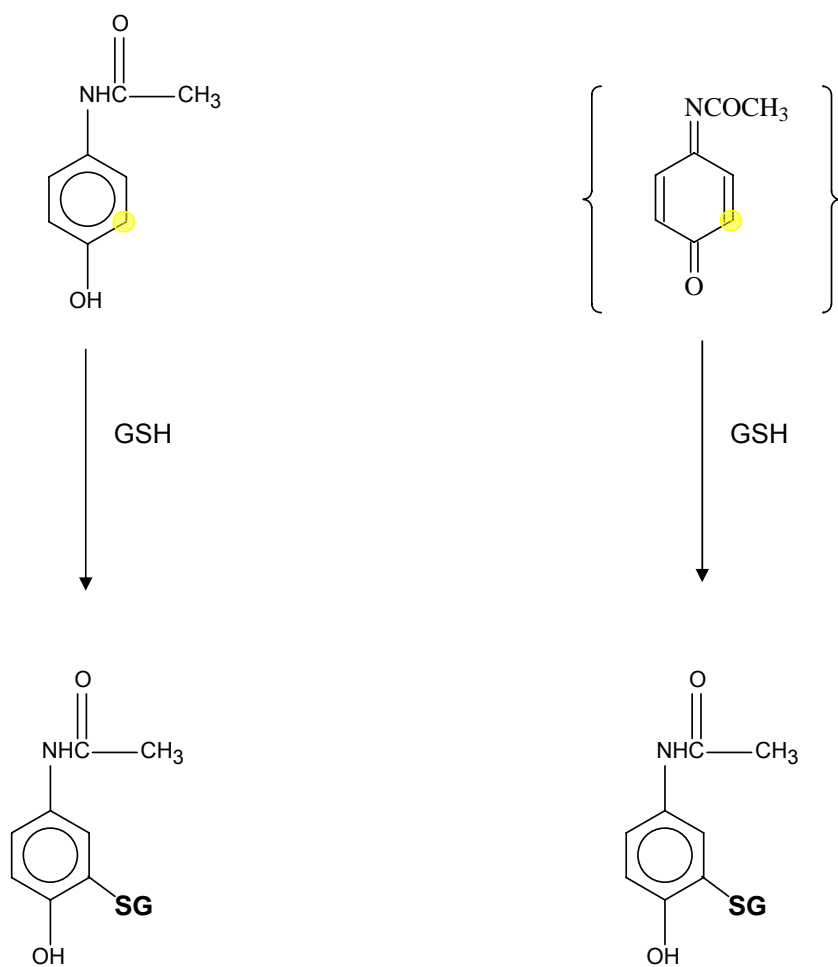


FIGURE 8. GSH conjugation of APAP and NAPQI.

The left side of the figure represents GSH conjugation of APAP. The right side shows the ability of GSH to attach itself and inactivate NAPQI. The highlighted region represents the area upon which GSH conjugates the compound.

During our initial studies, we used an APAP dose of 500 mg/kg. The male C57Bl/6 mice (16 – 22 g) were divided into four treatment groups (TABLE 1): control treated (VEH), APAP treated (APAP), SAME treated (SAME) and SAME pretreatment just prior to APAP (SAME + APAP). SAME was administered intraperitoneal (i.p.) at a dose of 500 or 1000 mg/kg (5 ml/kg in water) and given just prior to APAP. APAP was administered i.p. at a dose of 500 mg/kg (15 ml/kg). The VEH group was injected with water (15 ml/kg). These first studies included animals that were not fasted. The mice had free access to both food and water.

APAP hepatotoxicity was evaluated 2 and 4 hours post-APAP treatment. The following results have been published (Valentovic et al., 2004). APAP hepatic toxicity was confirmed by increased liver weight, elevated plasma ALT values and histological alterations. APAP increased liver weight within 4 hours when compared to the VEH group (TABLE 2). Liver weight was not altered 2 hours after APAP injection in these fed animals. APAP increased ($p < 0.05$) plasma ALT levels (TABLE 3) above VEH values 2 and 4 hours post-APAP injection. APAP-induced hepatotoxicity was reduced by the SAME pretreatment. Both SAME pretreatment doses prevented the increase in liver weight attributed to APAP treatment at all time periods. Both doses of SAME injected prior to APAP prevented the APAP induced rise in plasma ALT levels. These findings support the hypothesis that SAME attenuates APAP toxicity.

Examination by light microscopy showed normal morphology for the VEH treated group at 2 hours (FIGURE 9a) and 4 hours (FIGURE 10a). Within 2

hours of APAP treatment, moderate focal centrilobular changes were observed (FIGURE 9c). SAME (1000 mg/kg) pretreatment showed slight changes in the centrilobular region at 2 hours (FIGURE 9b) and 4 hours (FIGURE 10b). The SAME + APAP group showed less damage within the centrilobular region at both time points (FIGURES 9d and 10d). High power magnification of 4 hour tissue samples showed extensive centrilobular necrosis in the APAP group and almost complete protection in the SAME + APAP group (FIGURES 10e and 10f).

Lipid peroxidation was increased within 2 hours after APAP injection when compared to all other groups (FIGURE 11). Lipid peroxidation continued to be increased at 4 hours after treatment in the APAP group. SAME (1000 mg/kg) treatment just prior to APAP prevented an increase in lipid peroxidation in the SAME + APAP group when compared to the SAME or VEH groups (FIGURE 11). Lipid peroxidation levels were comparable between VEH and SAME groups. SAME pretreatment just prior to APAP prevented a rise in lipid peroxidation 2 and 4 hours after APAP injection.

Pretreatment with a lower dose of SAME (500 mg/kg) just prior to APAP also prevented a rise in lipid peroxidation (FIGURE 12). These results indicate that pretreatment with 500 mg/kg rather than 1000 mg/kg SAME prevented the rise in lipid peroxidation induced by APAP. The prevention of APAP mediated rise in lipid peroxidation by the administration of 500 mg/kg SAME (not 1000 mg/kg SAME) just prior to APAP may have allowed the liver to utilize the SAME more efficiently instead of overloading the liver with too much SAME. It also reiterated the conclusion first published by Lu (1998) that if the ratio of

SAH:SAMe favors SAH, the reaction to produce GSH is reversed and no GSH is made.

GSH levels were markedly diminished within 2 hours after APAP injection when compared to the VEH group (FIGURE 13). GSH levels continued to be severely diminished in the APAP group 4 hours after treatment when compared to the VEH group (FIGURES 13 and 14). SAMe administration (1000 mg/kg) did not alter baseline GSH levels (FIGURE 13). However, the 500 mg/kg dose of SAMe resulted in a small increase ($p < 0.05$) in baseline GSH levels when compared to the vehicle (FIGURE 14). The increase in baseline GSH levels may be attributed to rapid tissue handling and immediate treatment with sulfosalicylic acid (SSA) to prevent degradation of GSH.

Group	APAP	SAMe
VEH	No	No
APAP	Yes	No
SAMe	No	Yes
SAMe + APAP	Yes	Yes

TABLE 1. SAMe and APAP treatment regimens.

Group	SAMe dose (mg/kg)	Time post-APAP treatment (h)	Body weight (g)	Liver weight (g/10g body wt.)
VEH	0	2	20.4 ± 0.4	0.60 ± 0.01 ^a
APAP	0	2	18.0 ± 0.6	0.59 ± 0.04 ^a
SAMe	1000	2	19.6 ± 0.7	0.58 ± 0.02 ^a
SAMe + APAP	1000	2	18.0 ± 0.6	0.59 ± 0.04 ^a
VEH	0	4	25.2 ± 0.5	0.48 ± 0.01 ^a
APAP	0	4	25.0 ± 0.4	0.55 ± 0.01 ^b
SAMe	1000	4	25.6 ± 0.8	0.45 ± 0.02 ^a
SAMe + APAP	1000	4	25.4 ± 0.7	0.46 ± 0.02 ^a
VEH	0	4	19.6 ± 0.50	0.42 ± 0.02 ^a
APAP	0	4	19.2 ± 0.33	0.56 ± 0.03 ^b
SAMe	500	4	19.5 ± 0.40	0.42 ± 0.02 ^a
SAMe + APAP	500	4	19.0 ± 0.45	0.49 ± 0.02 ^a

TABLE 2. Body and liver weight following APAP administration.

APAP was administered as 500 mg/kg (15 ml/kg), i.p. SAMe was administered as 500 or 1000 mg/kg (5 ml/kg), i.p. Values are reported at mean ± SEM with n = 4-10 animals/group. Groups with different superscripts denote statistical difference ($p < 0.05$) within each treatment experiment.

Group	SAMe dose (mg/kg)	Time post-APAP treatment (h)	ALT (U/l)
VEH	0	2	34 ± 12
APAP	0	2	185 ± 50 ^a
SAMe	1000	2	63 ± 19
SAMe + APAP	1000	2	73 ± 9
VEH	0	4	25 ± 1
APAP	0	4	376 ± 160 ^a
SAMe	1000	4	76 ± 34
SAMe + APAP	1000	4	54 ± 24
VEH	0	4	23 ± 2
APAP	0	4	315 ± 62 ^a
SAMe	500	4	55 ± 14
SAMe + APAP	500	4	104 ± 63

Comment [V1]: Need to change the alignment for the ALT values so that all the ± are in the same column, it looks better. Check any publication and you will see the ± are all aligned

TABLE 3. Plasma ALT levels following APAP administration.

Groups represent vehicle (VEH); acetaminophen (APAP) injected i.p. 500 mg/kg; S-Adenosyl-L-methionine (SAMe) i.p. injected 1000 mg/kg; SAMe and acetaminophen (SAMe + APAP) at doses listed for individual agents. Unlike letters (a) indicate groups are different ($p < 0.05$) from each other using a one way ANOVA and Tukey's post hoc test. Values represent mean ± S.E.M. with $n = 5$ per group.

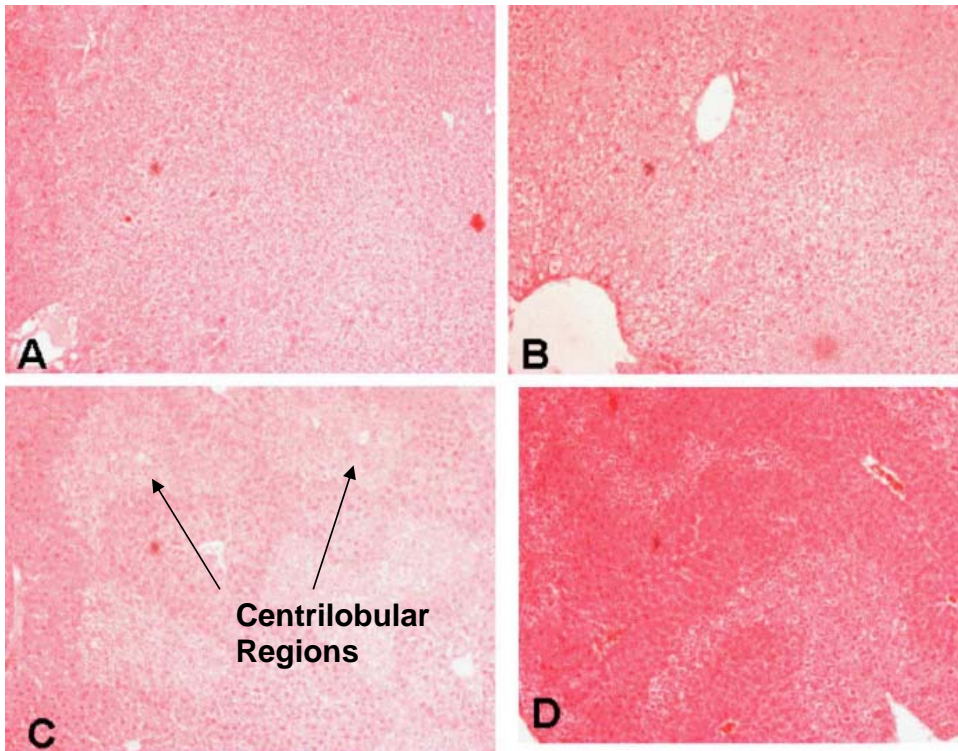


FIGURE 9. Histological examination 2 hours after APAP injection.

Hepatic tissues were prepared and stained with H&E. Representative slides of (a) vehicle (VEH); (b) SAME (1000 mg/kg) pretreated; (c) 2 hours post-acetaminophen (APAP) injection and (d) mice treated with SAME and APAP. Normal morphology was noted in the VEH and SAME animals. Moderate damage was noted in the APAP group and is labeled with arrows. Magnification is 200 \times .

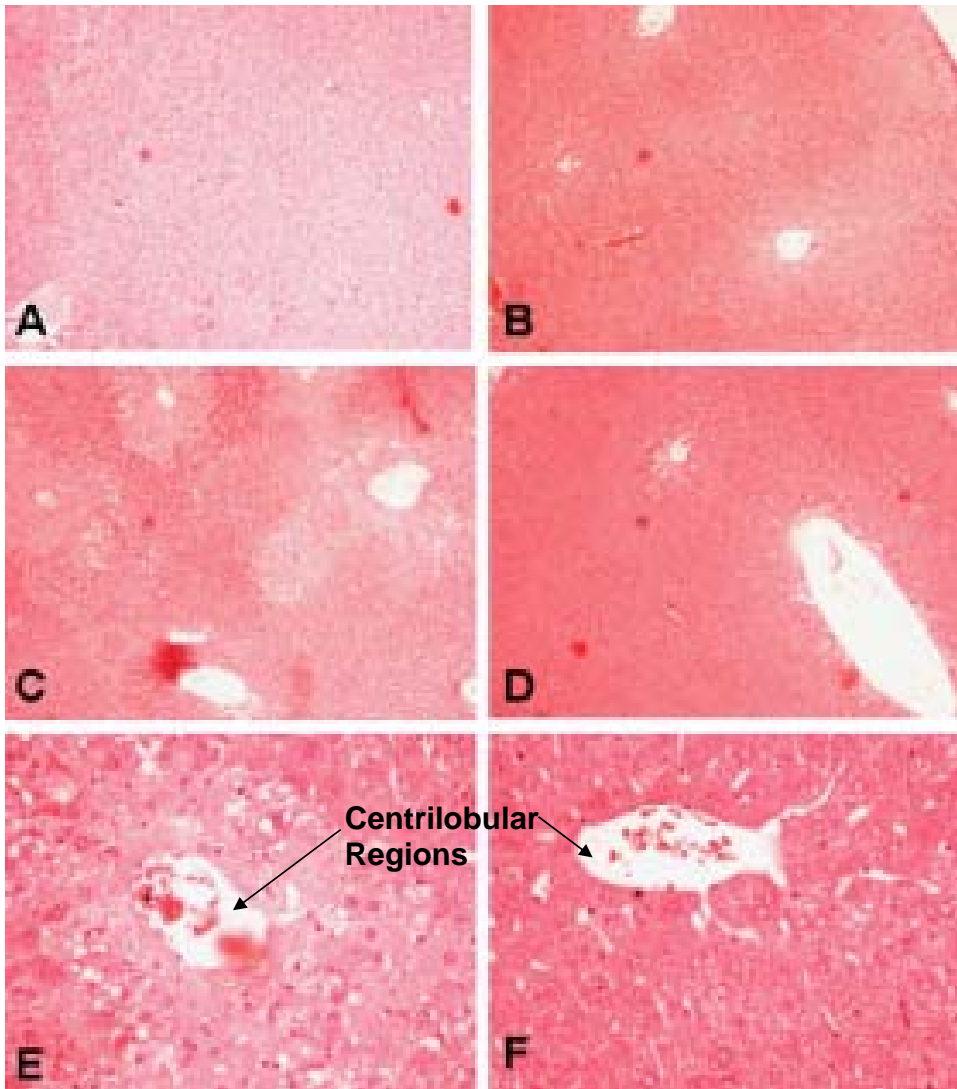


FIGURE 10. Histological examination 4 hours after APAP injection.

Hepatic tissues were collected 4 hours after APAP injection and stained with H&E. Representative slides are of (a) vehicle (VEH); (b) SAME only; (c) 4 hours post-acetaminophen (APAP) injection; (d) mice treated with SAME and APAP; (e) 4 hours post APAP injection; (f) mice treated with SAME and APAP. Normal

morphology was noted in the VEH and SAME animals. Centrilobular necrosis was apparent in the APAP group around the central vein. The SAME + APAP group had moderate changes in the centrilobular region. Magnification is 200x for (a)–(d) and 400x for (e)–(f).

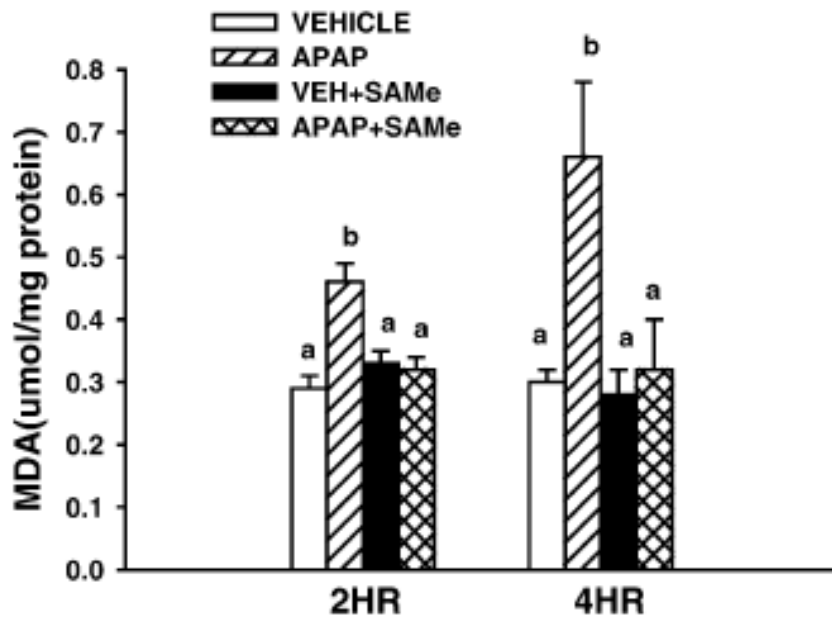


FIGURE 11. Lipid Peroxidation following APAP injection in C57Bl/6 mice.

Animals were randomly divided into vehicle (VEH), acetaminophen 500 mg/kg treated (APAP), SAMe 1000 mg/kg treated (SAMe) and SAMe and APAP treated (SAMe + APAP). Values represent mean S.E.M., n = 4–6 mice per group. Lipid peroxidation was measured 2 and 4 hours after injection (i.p.) of 500 mg/kg APAP. Lipid peroxidation was measured as μmol malondialdehyde (MDA)/mg protein. Groups with unlike superscripts are statistically different.

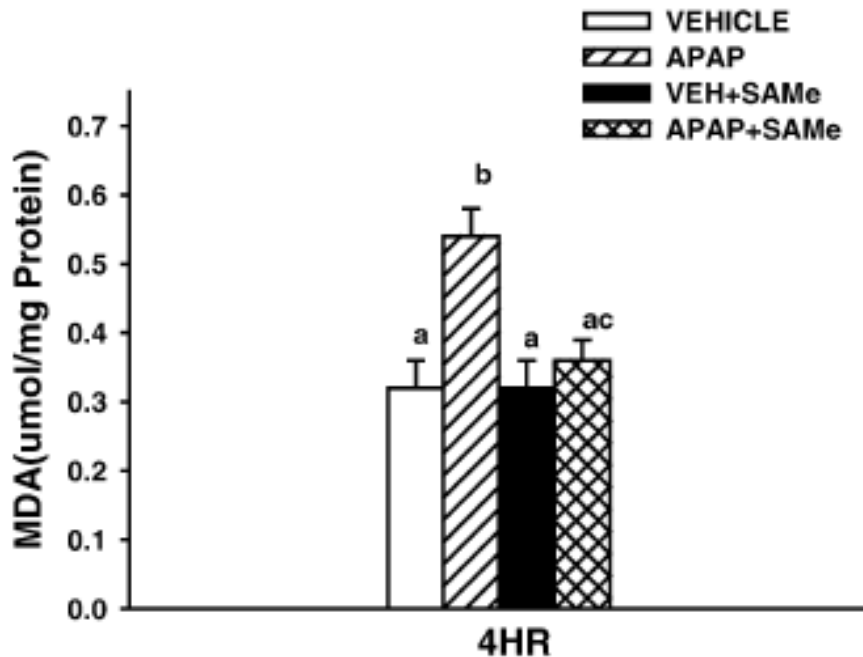


FIGURE 12. Lipid Peroxidation 4 hours post-APAP injection in C57Bl/6 mice.

Animals were randomly divided into vehicle (VEH), acetaminophen 500 mg/kg treated (APAP), SAMe 500 mg/kg treated (SAMe) and SAMe and APAP treated (SAMe + APAP). SAMe was administered just prior to APAP injection. Values represent mean S.E.M., $n = 4-6$ mice per group. Lipid peroxidation was measured 2 and 4 hours after injection (i.p.) of 500 mg/kg APAP. Lipid peroxidation was measured as μmol malondialdehyde (MDA)/mg protein. Groups with unlike superscripts are statistically different. SAMe + APAP group is statistically different ($p < 0.05$) from the APAP group as designated by "c".

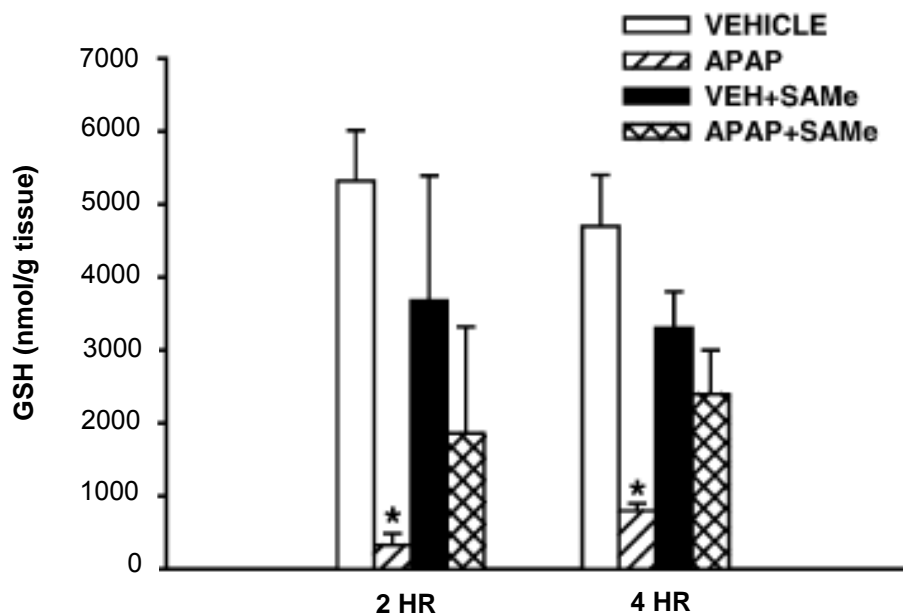


FIGURE 13. Total hepatic glutathione levels following APAP injection in C57Bl/6 mice.

Animals were randomly divided into vehicle (VEH), acetaminophen 500 mg/kg treated (APAP), SAMe 1000 mg/kg treated (SAMe) and SAMe and APAP treated (SAMe + APAP). Values represent mean \pm S.E.M., $n = 4-6$ mice per group. Total hepatic GSH levels were measured 2 and 4 hours after injection (i.p.) of 500 mg/kg APAP. Groups with an asterisk (*) are statistically different ($p < 0.05$).

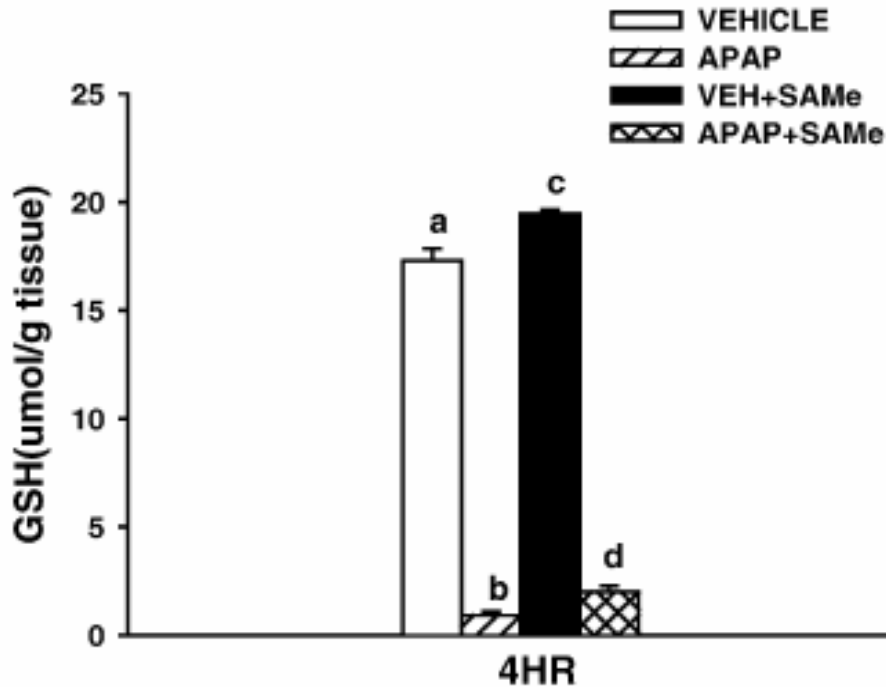


FIGURE 14. Total hepatic glutathione levels following APAP injection in C57Bl/6 mice.

Animals were randomly divided into vehicle (VEH), acetaminophen 500 mg/kg treated (APAP), SAMe 500 mg/kg treated (SAMe) and SAMe and APAP treated (SAMe + APAP). Values represent mean \pm S.E.M., n = 4–6 mice per group. Total hepatic glutathione levels were measured 2 and 4 hours after injection (i.p.) of 500 mg/kg APAP. Groups with unlike superscripts are statistically different.

4.2.2. APAP Toxicity in Fasted Mice

Effect of 500 mg/kg SAME just prior to 400 mg/kg APAP dose

For these studies, the animals were randomly divided into four treatment groups, as shown in Table 1. The animals were fasted overnight (1700 – 0900 h), but had free access to water. The “VEH” group was dosed with water, the “SAME” group was administered with 500 mg/kg SAME, the third group, “APAP”, was injected with 400 mg/kg APAP and the final group (SAME + APAP) received 500 mg/kg SAME just prior to 400 mg/kg APAP. Mice were anesthetized and livers were removed 1 hour post-APAP.

APAP-induced hepatotoxicity was reduced by the SAME pretreatment. APAP induced hepatic toxicity was confirmed by increased liver weight and elevated plasma ALT levels when compared to VEH. Liver weight was increased in animals dosed with APAP within 1 hour when compared to the VEH group (TABLE 4). SAME pretreatment doses prevented the increase in liver weight. APAP increased plasma ALT levels (TABLE 5) above VEH values. SAME prevented the APAP induced rise in plasma ALT levels.

There is no published data containing information about early effects of APAP-induced hepatic toxicity. We were able to investigate some of the initial effects of APAP-induced hepatotoxicity. Lipid peroxidation levels were unaltered 1 hour post-APAP (data not shown). This was most likely due to the short time frame. GSH levels were severely depleted by APAP (FIGURE 15), but SAME was unable to significantly increase the GSH values. Once again, this was most

likely due to the short time frame. Early on, damage is occurring at a rapid rate and it is most likely that SAME had not yet been able to relinquish all damaging effects after 1 hour. These results also showed that fasting reduced total hepatic GSH when compared to fed mice. We knew from our previous study that damage is seen as early as two hours. During the first hour of APAP toxicity, APAP lyses cells causing the cell's contents to flush into the liver resulting in a rise in liver weight. We also showed GSH levels are depleted by almost 90%, the toxicity initiates a rise in plasma ALT levels that continually increased for at least 4 hours (Valentovic et al., 2004) and damage begins to become evident around the centrilobular region of the liver.

Group	SAMe dose (mg/kg)	Time post-APAP treatment (h)	Body weight (g)	Liver weight (g/10g body wt.)
VEH	0	1	17.6 ± 0.3 ^a	0.46 ± 0.01 ^a
APAP	0	1	18.6 ± 0.4	0.50 ± 0.01 ^b
SAMe	500	1	17.8 ± 0.5	0.45 ± 0.01 ^a
SAMe + APAP	500	1	17.4 ± 0.3	0.45 ± 0.01 ^a

TABLE 4. Body and liver weight following APAP administration.

APAP was administered as 400 mg/kg (15 ml/kg), i.p. SAMe was administered as 500 mg/kg (5 ml/kg), i.p. Values are reported at mean ± SEM with n = 4-10 animals/group. Groups with different superscripts denote statistical difference (p < 0.05) within each treatment experiment.

Group	SAMe dose (mg/kg)	Time post-APAP treatment (h)	ALT (U/l)
VEH	0	1	61 ± 9 ^a
APAP	0	1	293 ± 86 ^b
SAMe	500	1	69 ± 20 ^a
SAMe + APAP	500	1	104 ± 15 ^a

TABLE 5. Plasma ALT levels following APAP administration.

Groups represent vehicle (VEH); acetaminophen (APAP) injected i.p. 400 mg/kg; S-Adenosyl-L-methionine (SAMe) i.p. injected 500 mg/kg; SAMe and acetaminophen (SAMe + APAP). Unlike letters (a, b) indicate groups are different ($p < 0.05$) from each other using a one way ANOVA and Tukey's post hoc test. Values represent mean ± S.E.M. with $n = 4-10$ per group.

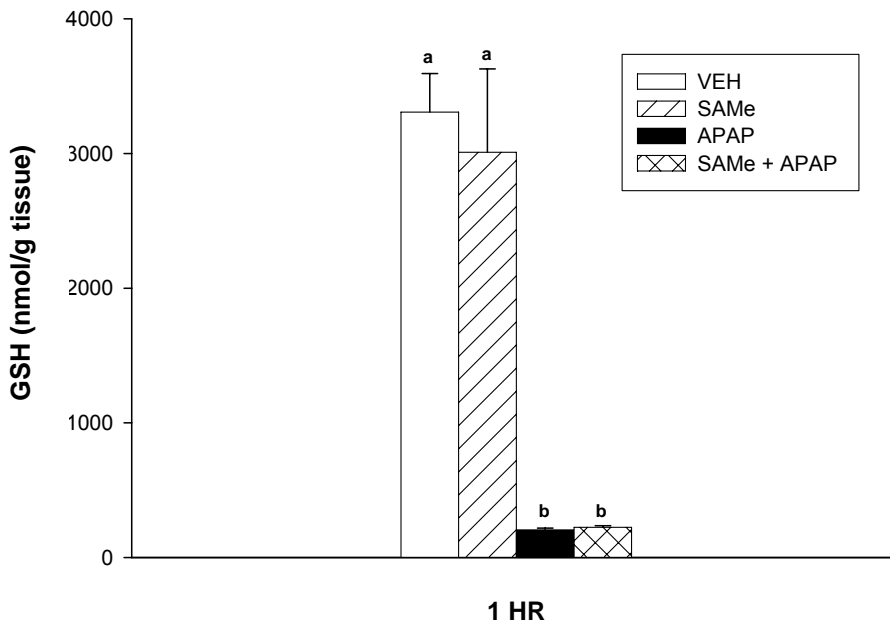


FIGURE 15. Total hepatic glutathione levels 1 hour post-APAP injection in C57Bl/6 mice.

Animals were randomly divided into vehicle (VEH), acetaminophen 400 mg/kg treated (APAP), SAMe treated (SAMe) and SAMe + APAP treated (SAMe + APAP). Values represent mean \pm S.E.M., $n = 4-10$ mice per group. Total hepatic GSH levels were measured 1 hour after injection (i.p.) of 400 mg/kg APAP. Groups with unlike superscripts are statistically different ($p < 0.05$).

Effect of 500 mg/kg SAME just prior to 300 mg/kg APAP dose

After receiving more consistent results for GSH levels and other parameters of hepatotoxicity, we decided to continue fasting all mice prior to treatment with APAP or VEH. We had seen APAP induced hepatic toxicity with doses of 500 mg/kg and 400 mg/kg. To establish a threshold of overdose in C57Bl/6 mice, we decided to lower the injection to 300 mg/kg APAP. While we did see favorable results with SAME attenuating APAP at 400 and 500 mg/kg APAP, it was decided to attempt a determination of the range of possibilities with SAME to attenuate less “extreme” damage. Stramentinoli et al. (1979) had used 710 mg/kg APAP but the parameter of measurement was death vs. survival. Valentovic et al. (2004), Bray et al. (1992) and Carrasco et al. (2000) had used 250-500 mg/kg APAP, 500 mg/kg APAP and 400 mg/kg APAP respectively. Valentovic et al. (2004) was the only study to not use death vs. survival as the main measure of protection by SAME.

Animals were randomly divided as described previously (TABLE 1). The groups remained the same: VEH, SAME, APAP and SAME + APAP. Animals were fasted overnight (1700 – 0900 h), but allowed free access to water. The mice were anesthetized (CO₂) and livers were excised 1, 2 and 4 hours post-APAP.

Pretreatment with SAME just prior to APAP reduced APAP-induced hepatotoxicity. APAP hepatic toxicity was confirmed at 1, 2, and 4 hours post-APAP by increased liver weight (TABLE 6), elevated plasma ALT levels (TABLE

7) and histological alterations (at 1 and 2 hours post-APAP injection) (FIGURE 16 and FIGURE 17 respectively). At 1 and 2 hours post-APAP, plasma ALT values were not statistically higher than the control animals, but the trend was leading toward increases in plasma ALT levels. This finding was confirmed at 4 hours post-APAP when animals dosed with APAP produced a statistically significant higher plasma ALT level than the other treatment groups.

As expected, 1 hour post-APAP showed the least amount of damage. At 4 hours post-APAP, plasma ALT levels were highly elevated (over 8,000 Units/liter) and the liver weight showed a statistically significant raise. SAME was able to prevent a rise in liver weight and decreased plasma ALT values at all time periods.

GSH levels were markedly diminished within 1 hour after APAP injection when compared to VEH group (FIGURE 18). GSH levels continued to be severely diminished in the APAP group at 2 and 4 hours post-APAP (FIGURE 19 and FIGURE 20 respectively). SAME administration did not alter baseline GSH levels at 1, 2, or 4 hours post-liver excision. However, SAME was able to increase GSH levels at all time frames measured when given just prior to APAP as compared to groups given only APAP. The largest gain in GSH stores was seen 4 hours post-APAP (FIGURE 21).

Oxidative stress measurements were only conducted at 4 hours post-APAP due to the lack of serious damage seen at 1 and 2 hours post-APAP. Measurements of oxidative stress caused by APAP overdose were shown by

increased protein carbonyl levels at 4 hours post-APAP of proteins between 37.1 – 67.2 kDa (FIGURE 22). There was no significant oxidative stress illustrated by these procedures at 1 or 2 hours post-APAP (data not shown).

Group	SAMe dose (mg/kg)	Time post-APAP treatment (h)	Body weight (g)	Liver weight (g/10g body wt.)
VEH	0	1	18.2 ± 0.5	0.47 ± 0.01 ^a
APAP	0	1	18.2 ± 0.5	0.51 ± 0.01 ^b
SAMe	500	1	19.0 ± 0.5	0.50 ± 0.01 ^a
SAMe + APAP	500	1	19.6 ± 0.6	0.48 ± 0.01 ^a
VEH	0	2	17.6 ± 0.4	0.49 ± 0.01 ^a
APAP	0	2	17.8 ± 0.5	0.57 ± 0.02 ^b
SAMe	500	2	18.0 ± 0.6	0.49 ± 0.01 ^a
SAMe + APAP	500	2	18.4 ± 0.4	0.52 ± 0.02 ^a
VEH	0	4	18.0 ± 0.30	0.51 ± 0.01 ^a
APAP	0	4	17.8 ± 0.36	0.59 ± 0.01 ^b
SAMe	500	4	18.0 ± 0.30	0.51 ± 0.01 ^a
SAMe + APAP	500	4	17.8 ± 0.36	0.52 ± 0.02 ^a

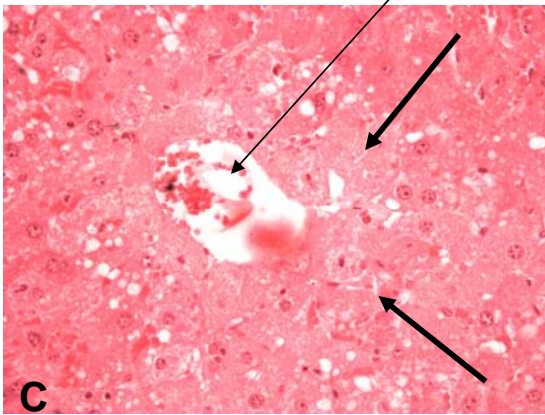
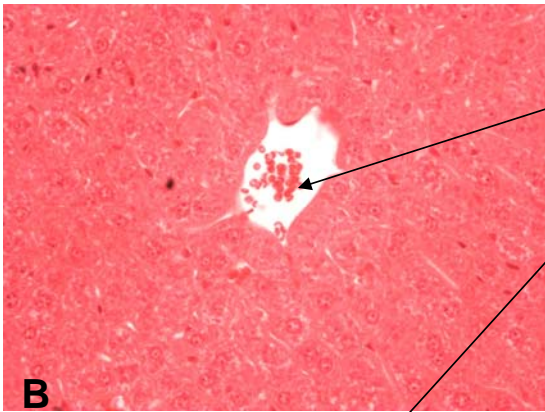
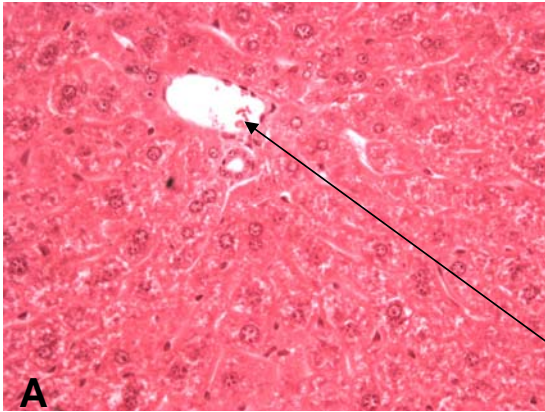
TABLE 6. Body and liver weight following APAP administration.

APAP was administered as 300 mg/kg (15 ml/kg), i.p. SAMe was administered as 500 mg/kg (5 ml/kg), i.p. Values are reported at mean ± SEM with n = 4-10 animals/group. Groups with different superscripts denote statistical difference (p < 0.05) within each treatment experiment.

Group	SAMe dose (mg/kg)	Time post-APAP treatment (h)	ALT (U/l)
VEH	0	1	63 ± 18
APAP	0	1	243 ± 76
SAMe	500	1	143 ± 41
SAMe + APAP	500	1	143 ± 46
VEH	0	2	290 ± 79
APAP	0	2	647 ± 367
SAMe	500	2	235 ± 57
SAMe + APAP	500	2	369 ± 115
VEH	0	4	81 ± 19 ^a
APAP	0	4	8533 ± 623 ^b
SAMe	500	4	373 ± 70 ^a
SAMe + APAP	500	4	2811 ± 346 ^c

TABLE 7. Plasma ALT levels following APAP administration.

Groups represent vehicle (VEH); acetaminophen (APAP) injected i.p. 300 mg/kg; S-Adenosyl-L-methionine (SAMe) i.p. injected 300 mg/kg; SAMe and acetaminophen (SAMe + APAP) at doses listed for individual agents. Unlike letters (a, b) indicate groups are different ($p < 0.05$) from each other using a one way ANOVA and Tukey's post hoc test. There was no statistical difference seen at 1 or 2 hours post APAP administration. Values represent mean ± S.E.M. with $n = 4-10$ per group.



Central Vein

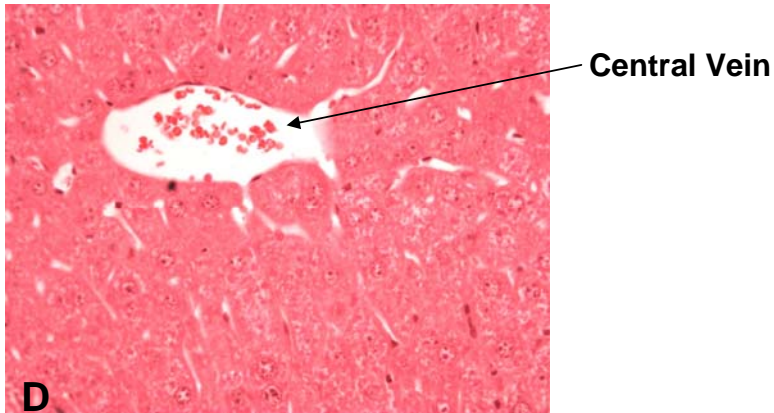
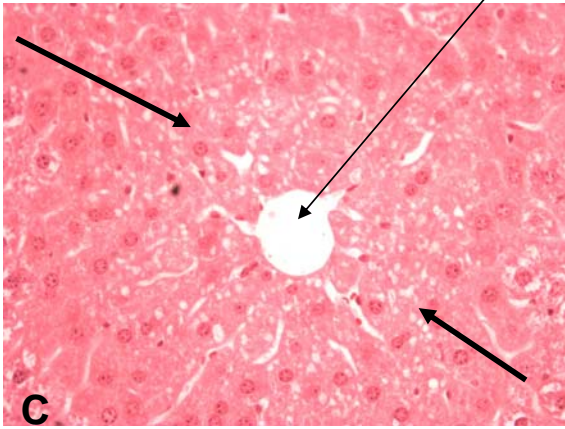
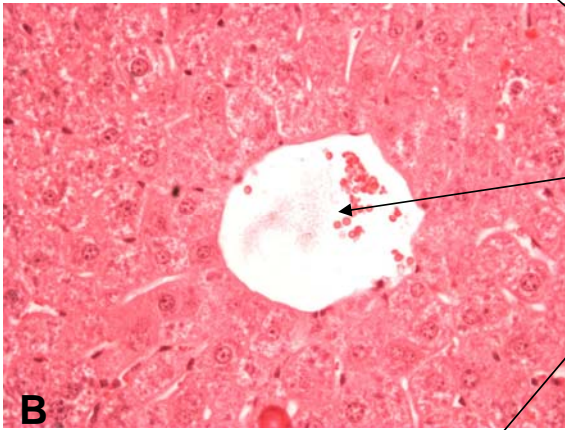
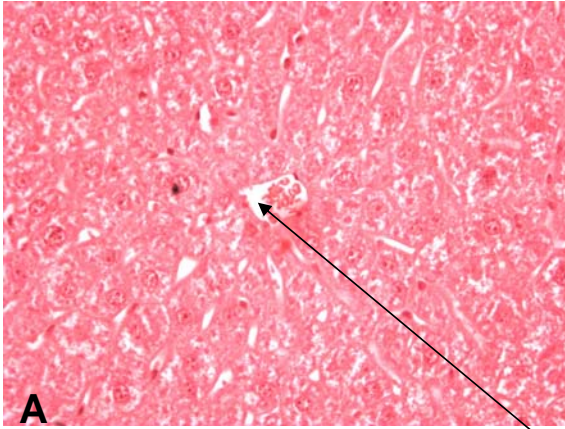


FIGURE 16. Histological examination 1 hour after APAP injection.

Hepatic tissues were prepared and stained with H&E. Representative slides of (A)VEH; (B) SAMe pretreated; (C) 1 hour post-APAP injection and (D) mice treated with SAMe just prior to APAP. Normal morphology was noted in the VEH and SAMe animals. Damage was noted around the centrilobular region (pointed out in slide C) in the APAP group. Magnification is 200 \times .



Central Vein

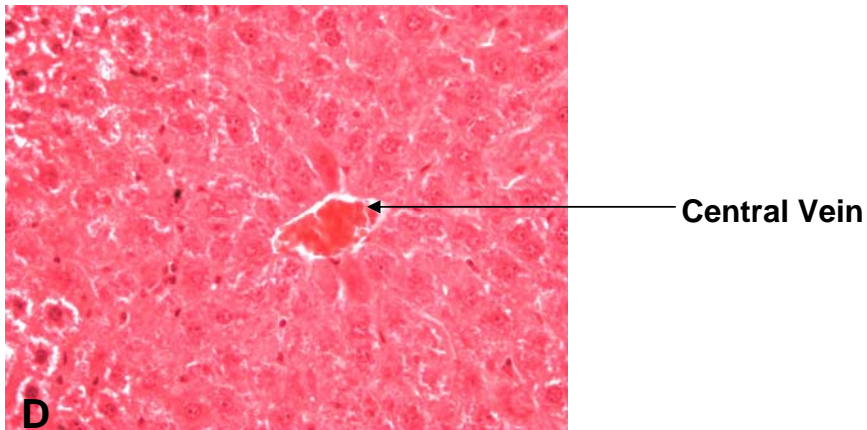


FIGURE 17. Histological examination 2 hours after APAP injection.

Hepatic tissues were prepared and stained with H&E. Representative slides of (A) VEH; (B) SAmE pretreated; (C) 2 hours post-APAP injection and (D) mice treated with SAmE just prior to APAP. Normal morphology was noted in the VEH and SAmE animals. Moderate damage was noted in the APAP group around the central vein and is denoted with black arrows. Magnification is 200 \times .

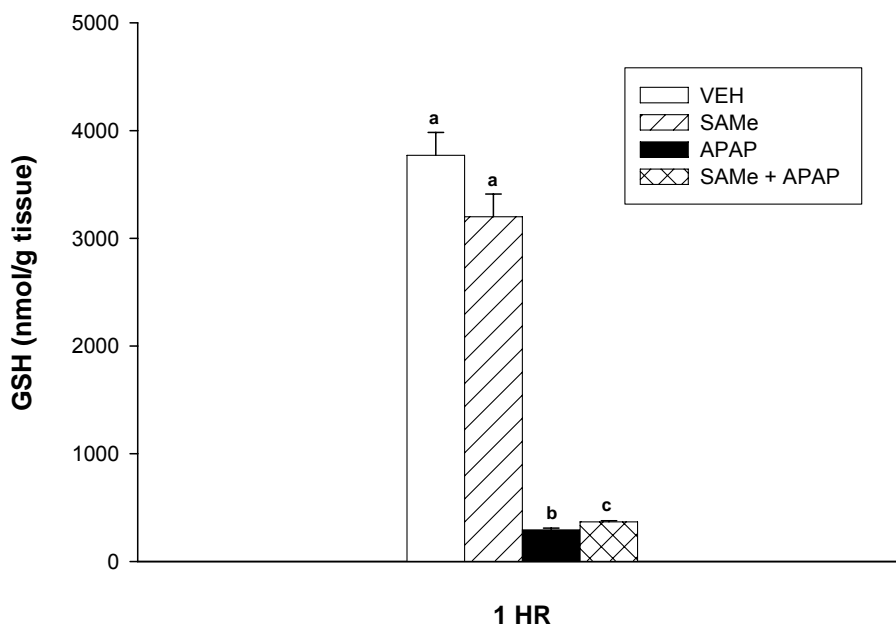


FIGURE 18. Total hepatic glutathione levels 1 hour post-APAP injection in C57Bl/6 mice.

Animals were randomly divided into vehicle (VEH), acetaminophen 300 mg/kg treated (APAP), SAmE 500 mg/kg treated (SAmE) and SAmE + APAP treated (SAmE + APAP). Values represent mean \pm S.E.M., $n = 4-6$ mice per group. Total hepatic GSH levels were measured 1 hour after injection (i.p.) of 500 mg/kg APAP. Groups with unlike superscripts are statistically different.

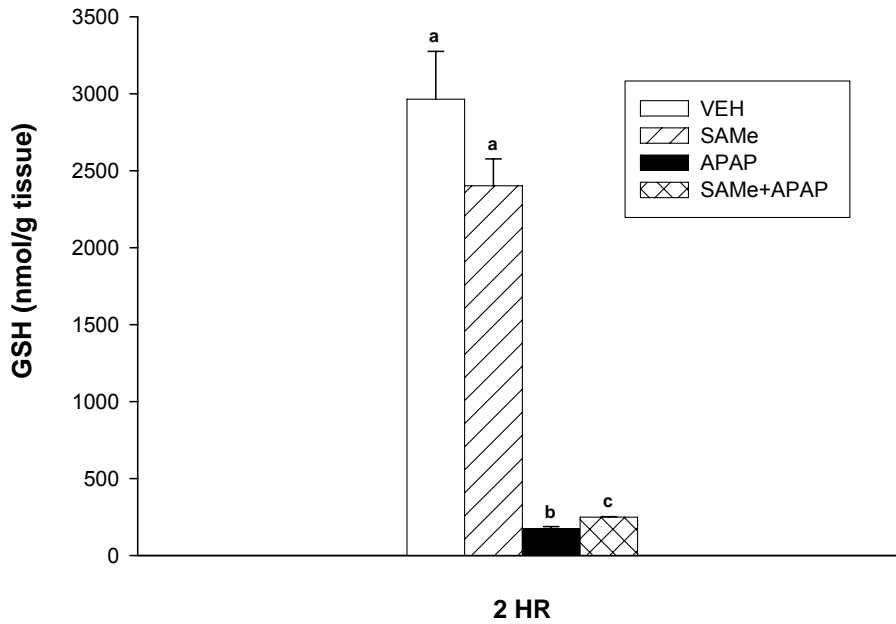


FIGURE 19. Total hepatic glutathione levels 2 hours post-APAP injection in C57Bl/6 mice.

Animals were randomly divided into vehicle (VEH), acetaminophen 300 mg/kg treated (APAP), SAmE 500 mg/kg treated (SAmE) and SAmE + APAP treated (SAmE + APAP). Values represent mean \pm S.E.M., $n = 4-6$ mice per group. Total hepatic GSH levels were measured 2 hours after injection (i.p.) of 500 mg/kg APAP. Groups with unlike superscripts are statistically different.

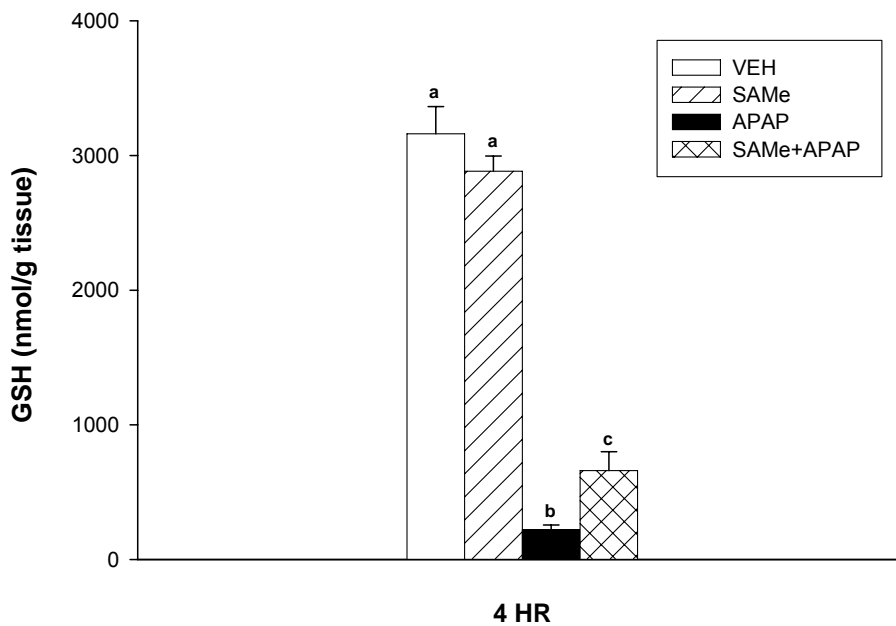


FIGURE 20. Total hepatic glutathione levels 4 hours post-APAP injection in C57Bl/6 mice.

Animals were randomly divided into vehicle (VEH), acetaminophen 300 mg/kg treated (APAP), SAMe 500 mg/kg treated (SAMe) and SAMe + APAP treated (SAMe + APAP). Values represent mean \pm S.E.M., $n = 4-6$ mice per group. Total hepatic GSH levels were measured 4 hours after injection (i.p.) of 500 mg/kg APAP. Groups with unlike superscripts are statistically different.

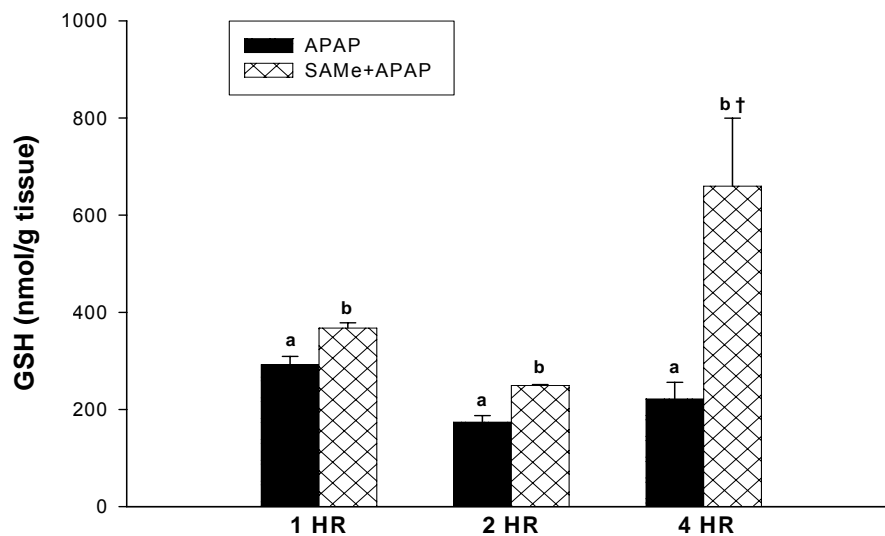


FIGURE 21. Total hepatic glutathione in APAP or SAMe + APAP dosed groups 1, 2 or 4 hours post-APAP injection in C57BI/6 mice.

Animals were randomly divided into vehicle (VEH), acetaminophen 300 mg/kg treated (APAP), SAMe 500 mg/kg treated (SAMe) and SAMe + APAP treated (SAMe + APAP). Values represent mean \pm S.E.M., $n = 4-6$ mice per group. Total hepatic GSH levels were measured 1, 2 or 4 hours after injection (i.p.) of 500 mg/kg APAP. Groups with unlike superscripts are statistically different. The “†” symbol depicts a statistically significant value ($p < 0.05$) when directly compared to all other groups shown in the figure.

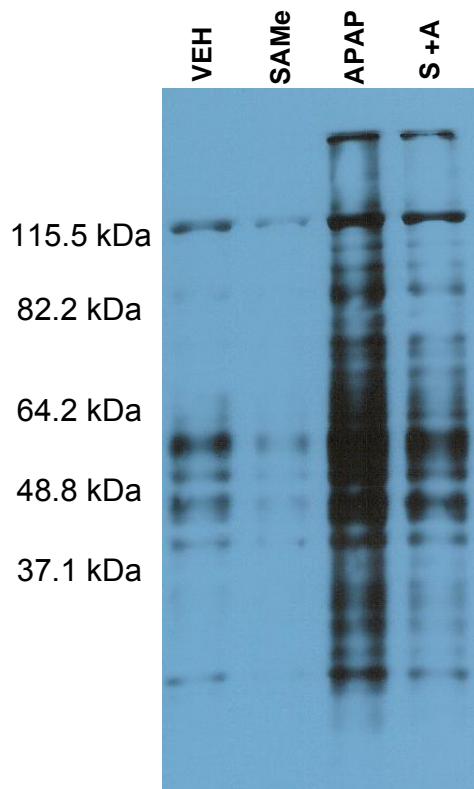


FIGURE 22. OxyBlot of samples 4 hours post-APAP injection.

Lanes are denoted as: Lane 1 VEH, Lane 2 SAMe, Lane 3 APAP and Lane 4 SAMe + APAP (S + A). SAMe was administered as 1.25 mmol/kg, i.p. (5 ml/kg). APAP was injected i.p. at a dose of 300 mg/kg (15 ml/kg). VEH animals were injected i.p. with water.

Effect of 500 mg/kg SAME just prior to 250 mg/kg APAP dose

For the final set of experiments using SAME as a pretreatment, 250 mg/kg APAP was used. We had already established APAP toxicity at 500, 400 and 300 mg/kg APAP in our laboratory as well as the successes of SAME attenuating this induced hepatic toxicity at 1, 2 and 4 hours post-APAP. At a dose of 250 mg/kg APAP, the effect of SAME during a minor overdose (as compared to 300 – 500 mg/kg) could be established.

The animals were randomly divided into four groups as previously described and shown in Table 1. The animals were fasted overnight (1700-0900 h) with free access to water. SAME was administered as a 500 mg/kg dose just prior to 250 mg/kg APAP. Animals were anesthetized in a CO₂ chamber and livers were excised 2 and 4 hours post-APAP injection.

APAP-induced hepatotoxicity was reduced by the SAME pretreatment at both 2 and 4 hours post-injection. APAP hepatic toxicity was confirmed by increased liver weight and elevated plasma ALT values 2 and 4 hours post APAP injection. APAP increased liver weight within 2 hours when compared to the VEH group (TABLE 8). At 4 hours post-APAP, liver values were statistically different when compared to VEH. SAME administration just prior to APAP prevented the rise in liver weight at both 2 and 4 hours post-APAP injection. Plasma ALT levels were increased post-APAP at 2 and 4 hours ($p < 0.05$) (TABLE 9). When administered alone, SAME had no effect on liver weight or plasma ALT values at 2 or 4 hours. SAME pretreatment did prevent a rise in liver

weight and plasma ALT values were lowered relative to animals only receiving APAP.

Glutathione levels were markedly diminished at both 2 and 4 hours after APAP injection when compared to the VEH group (FIGURES 23-25). When SAME was administered prior to APAP, GSH levels were higher when directly compared to the animals dosed with only APAP. Pretreatment with SAME provided the best protection, with regards to APAP-induced GSH depletion.

Oxidative effects of APAP on the liver were confirmed with Western blots at 2 and 4 hours post-APAP injection (FIGURES 26 and FIGURE 27 respectively). Protein carbonyl levels (FIGURE 27) as well as 4HNE values (FIGURE 28) were higher ($p < 0.05$) in animals dosed with 250 mg/kg APAP at 4 hours post injection relative to the control animals. At 2 hours post-APAP, protein carbonyl levels were higher in animals given only APAP. Given as a predose, SAME was able to prevent the rise ($p < 0.05$) in oxidized proteins as well as protein carbonyls. SAME, when given alone, had no significant effect on oxidation when directly compared to VEH.

Group	SAMe dose (mg/kg)	Time post-APAP treatment (h)	Body weight (g)	Liver weight (g/10g body wt.)
VEH	0	2	19.5 ± 0.6	0.47 ± 0.02 ^a
APAP	0	2	18.2 ± 0.1	0.57 ± 0.01 ^b
SAMe	500	2	18.9 ± 0.5	0.49 ± 0.01 ^a
SAMe + APAP	500	2	18.3 ± 0.4	0.49 ± 0.02 ^a
VEH	0	4	20.0 ± 0.5	0.47 ± 0.01 ^a
APAP	0	4	19.8 ± 0.7	0.57 ± 0.01 ^b
SAMe	500	4	19.4 ± 0.4	0.49 ± 0.01 ^a
SAMe + APAP	500	4	20.8 ± 0.4	0.51 ± 0.01 ^a

TABLE 8. Body and liver weight following APAP administration.

APAP was administered as 250 mg/kg (15 ml/kg), i.p. SAMe was administered as 500 mg/kg (5 ml/kg), i.p. Values are reported at mean ± SEM with n = 4-10 animals/group. Groups with different superscripts denote statistical difference (p < 0.05) within each treatment experiment.

Group	SAMe dose (mg/kg)	Time post-APAP treatment (h)	ALT (U/l)
VEH	0	2	34 ± 10 ^a
APAP	0	2	982 ± 120 ^b
SAMe	500	2	223 ± 81 ^a
SAMe + APAP	500	2	348 ± 66 ^a
VEH	0	4	96 ± 25 ^a
APAP	0	4	10630 ± 1057 ^b
SAMe	500	4	125 ± 20 ^a
SAMe + APAP	500	4	4675 ± 1223 ^c

TABLE 9. Plasma ALT levels following APAP administration.

Groups represent vehicle (VEH); acetaminophen (APAP) injected i.p. 250 mg/kg; S-Adenosyl-L-methionine (SAMe) i.p. injected 500 mg/kg; SAMe and acetaminophen (SAMe + APAP) at doses listed for individual agents. Unlike letters (a, b) indicate groups are different ($p < 0.05$) from each other using a one way ANOVA and Tukey's post hoc test. Values represent mean ± S.E.M. with $n = 4-10$ per group.

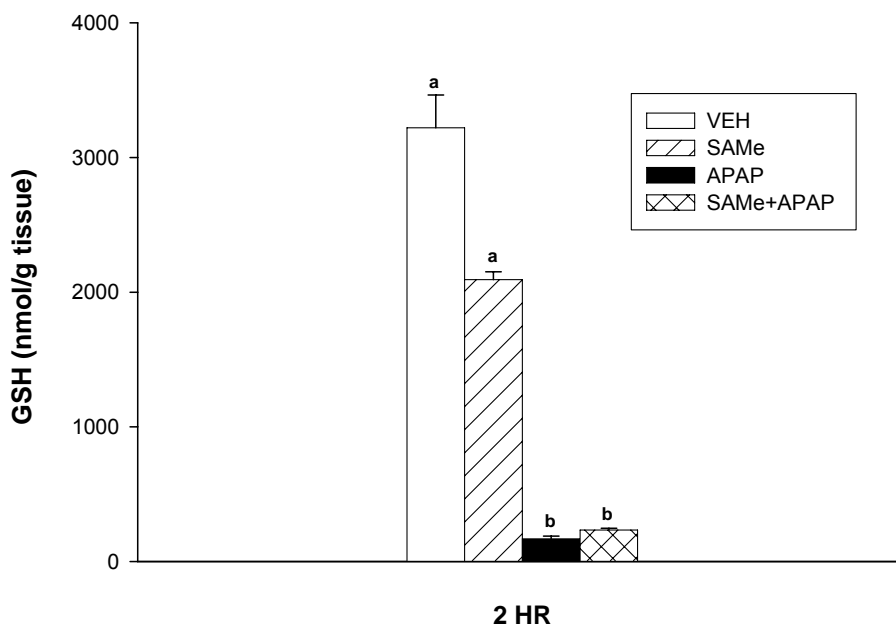


FIGURE 23. Total hepatic glutathione levels 2 hours post-APAP injection in C57Bl/6 mice.

Animals were randomly divided into vehicle (VEH), acetaminophen 250 mg/kg treated (APAP), SAMe 500 mg/kg treated (SAMe) and SAMe + APAP treated (SAMe + APAP). Values represent mean \pm S.E.M., $n = 4-10$ mice per group. Total hepatic GSH levels were measured 2 hours after injection (i.p.) of 250 mg/kg APAP. Groups with unlike superscripts are statistically different.

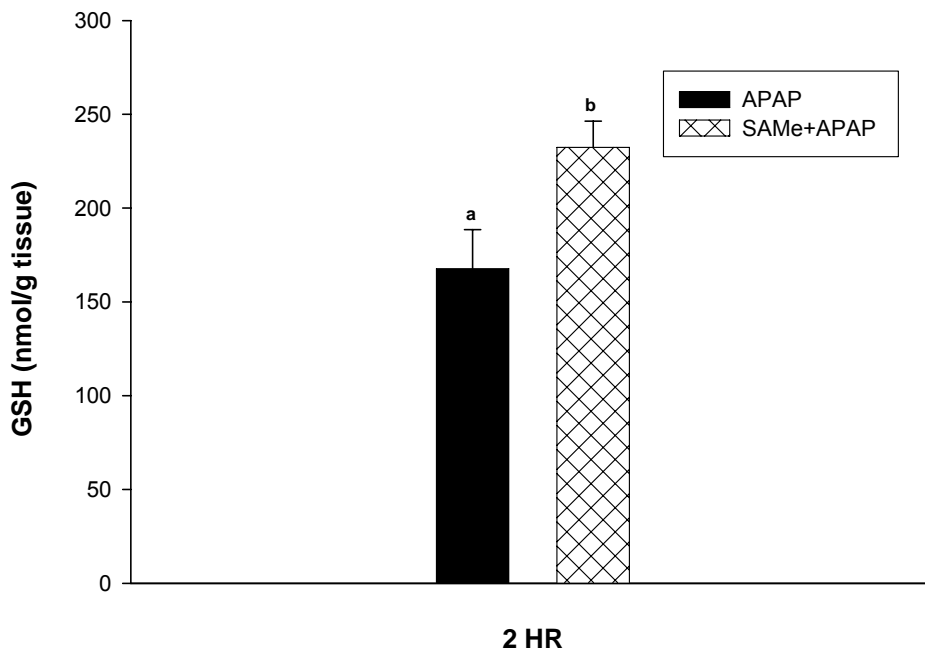


FIGURE 24. Total hepatic glutathione levels 2 hours post-APAP injection in C57Bl/6 mice injected with APAP or SAmE just prior to APAP.

Represents direct comparison of GSH levels in animals dosed with 250 mg/kg APAP or 500 mg/kg SAmE just prior to 250 mg/kg APAP. Values represent mean \pm S.E.M., $n = 4-10$ mice per group. Total hepatic GSH levels were measured 2 hours after injection (i.p.) of 250 mg/kg APAP. Groups with unlike superscripts are statistically different.

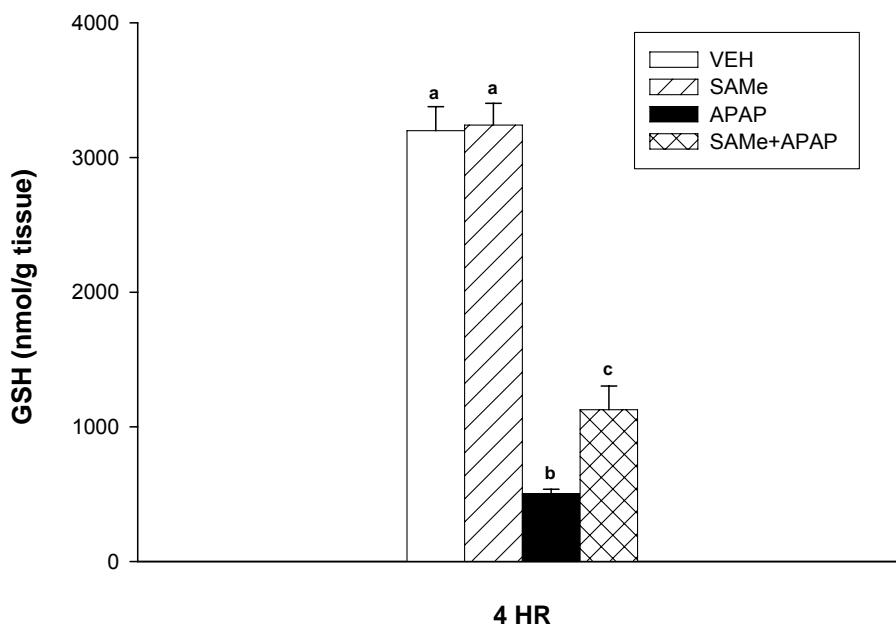


FIGURE 25. Total hepatic glutathione levels 4 hours post-APAP injection in C57Bl/6 mice.

Animals were randomly divided into vehicle (VEH), acetaminophen 250 mg/kg treated (APAP), SAmE 500 mg/kg treated (SAmE) and SAmE + APAP treated (SAmE + APAP). Values represent mean \pm S.E.M., $n = 4-10$ mice per group. Total hepatic GSH levels were measured 4 hours after injection (i.p.) of 250 mg/kg APAP. Groups with unlike superscripts are statistically different.

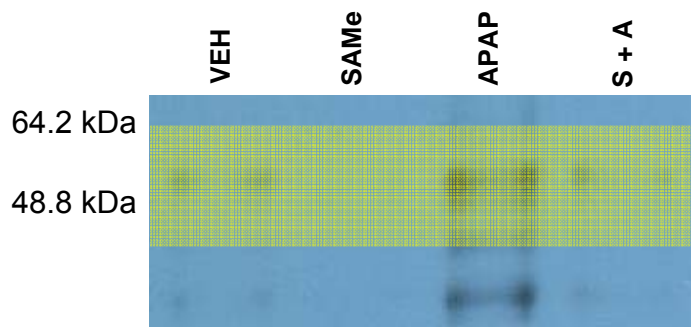


FIGURE 26. OxyBlot of samples 2 hours post-APAP injection.

Lanes are denoted as: Lane 1 vehicle, Lane 2 SAmE, Lane 3 acetaminophen, Lane 4 SAmE prior to acetaminophen (S + A). SAmE was administered as 500 mg/kg, i.p. (5 ml/kg). APAP was injected i.p. at a dose of 250 mg/kg (15 ml/kg). VEH animals were injected i.p. with water (15 ml/kg). Highlighted region represents area of greatest differences.

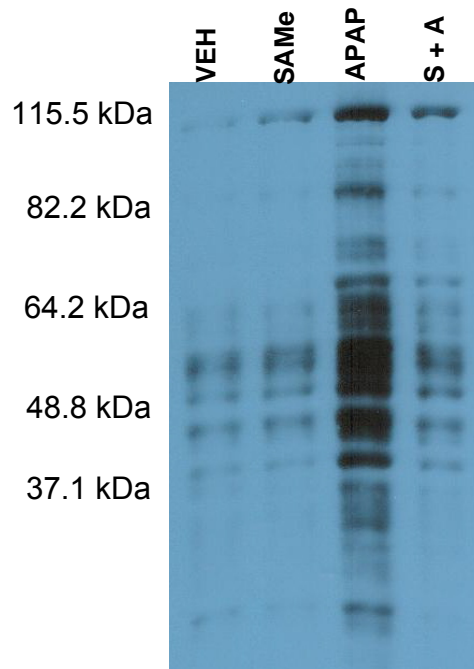


FIGURE 27. OxyBlot of samples 4 hours post-APAP injection.

Lanes are denoted as: Lane 1 vehicle, Lane 2 SAMe, Lane 3 acetaminophen, Lane 4 SAMe prior to acetaminophen (S + A). SAMe was administered as 500 mg/kg, i.p. (5 ml/kg). APAP was injected i.p. at a dose of 250 mg/kg (15 ml/kg). VEH animals were injected i.p. with water (15 ml/kg).

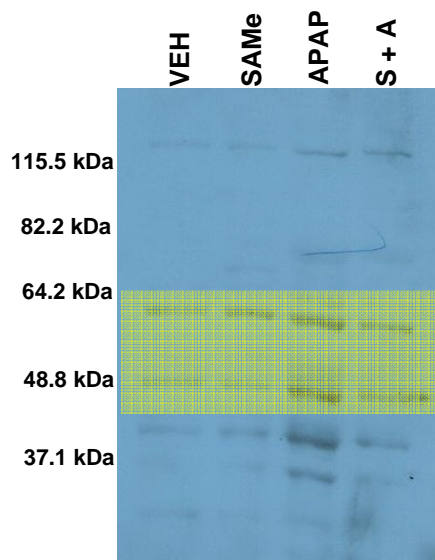


FIGURE 28. 4HNE Blot of samples 4 hours post-APAP injection.

Lanes are denoted as: Lane 1 vehicle, Lane 2 SAmE, Lane 3 acetaminophen, Lane 4 SAmE prior to acetaminophen (S + A). SAmE was administered as 500 mg/kg, i.p. (5 ml/kg). APAP was injected i.p. at a dose of 250 mg/kg (15 ml/kg). VEH animals were injected i.p. with water (15 ml/kg). Highlighted region represents area of greatest differences.

Effect of 204 mg/kg NAC just prior to 300 mg/kg APAP dose

Studies were conducted using the current clinical antidote, NAC, for APAP overdose in order to directly compare the benefits of NAC vs. SAME. Animals were randomly divided into four treatment groups (TABLE 10): control treated (VEH), APAP treated (APAP), NAC treated (NAC) and NAC pretreatment just prior to APAP (NAC + APAP). NAC was administered at a dose of 204 mg/kg (the exact same mmol concentration [1.25 mmol/kg] of SAME that was given in previous studies). Animals were fasted overnight (1700 – 0900 h) with free access to water, and livers were excised 1 or 4 hours post-APAP injection.

When given alone, NAC had no effect on liver weight, plasma ALT levels or histological alterations when directly compared to the VEH. APAP hepatic toxicity was confirmed by increased liver weight (TABLE 11), and elevated plasma ALT levels (TABLE 12). APAP-induced hepatotoxicity was not prevented when NAC was administered just prior to APAP when toxicity was elevated 1 or 4 hours after APAP injection. NAC did not prevent increases in liver weight (TABLE 11), damage to the centrilobular region 4 hours post-APAP (FIGURE 29) nor did it prevent rises in plasma ALT values (TABLE 12).

GSH levels were diminished in groups dosed with APAP only at 1 hour post-APAP injection (FIGURE 30). There was a gradual trend for increase in total GSH in groups given NAC just prior to APAP at both 1 (FIGURE 30) and 4 (FIGURE 31) hours, but unlike animals dosed with SAME just prior to APAP, these values failed to produce statistically significant results.

Oxidative stress in animals dosed with APAP was confirmed with an increase in protein carbonyls near 48.8 kDa (FIGURE 32) and 4HNE values mainly between 65 – 115 kDa (FIGURE 33) 4 hours post-APAP injection. There is no measurable oxidative damage in our animal model 1 hour post-APAP (data not shown). NAC, when given just prior to APAP, was unable to prevent a rise in protein carbonyls or 4HNE values as illustrated by the Western blots. When directly compared to the VEH, NAC had no significant effect on the production of oxidized protein or protein carbonyls.

Group	APAP	NAC
VEH	No	No
APAP	Yes	No
NAC	No	Yes
NAC + APAP	Yes	Yes

TABLE 10. NAC and APAP treatment regimens.

Group	NAC dose (mg/kg)	Time post-APAP treatment (h)	Body weight (g)	Liver weight (g/10g body wt.)
VEH	0	1	16.8 ± 0.5	0.50 ± 0.02
APAP	0	1	16.8 ± 0.5	0.53 ± 0.02
NAC	204	1	17.6 ± 0.7	0.50 ± 0.01
NAC + APAP	204	1	17.2 ± 0.5	0.50 ± 0.02
VEH	0	4	17.6 ± 0.4	0.46 ± 0.02 ^a
APAP	0	4	18.4 ± 0.8	0.58 ± 0.01 ^b
NAC	204	4	18.0 ± 0.6	0.50 ± 0.01 ^a
NAC + APAP	204	4	18.2 ± 0.4	0.58 ± 0.01 ^b

TABLE 11. Body and liver weight following APAP administration.

APAP was administered as 300 mg/kg (15 ml/kg), i.p. NAC was administered as 204 mg/kg (5 ml/kg), i.p. Values are reported at mean ± SEM with n = 4-5 animals/group. Groups with different superscripts denote statistical difference (p < 0.05) within each treatment experiment.

Group	NAC dose (mg/kg)	Time post-APAP treatment (h)	ALT (U/l)
VEH	0	1	35 ± 13
APAP	0	1	266 ± 92
NAC	204	1	124 ± 43
NAC + APAP	204	1	116 ± 30
VEH	0	4	84 ± 19 ^a
APAP	0	4	9147 ± 468 ^b
NAC	204	4	108 ± 30 ^a
NAC + APAP	204	4	10421 ± 1125 ^b

TABLE 12. Plasma ALT levels following APAP administration.

Groups represent vehicle (VEH); acetaminophen (APAP) injected i.p. 300 mg/kg; N-acetylcysteine (NAC) i.p. injected 204 mg/kg; NAC and acetaminophen (NAC + APAP) at doses listed for individual agents. Unlike letters (a, b) indicate groups are different ($p < 0.05$) from each other using a one way ANOVA and Tukey's post hoc test. There was no statistical difference seen regarding plasma ALT values 1 hour post-injection. Values represent mean ± S.E.M. with $n = 4-5$ per group.

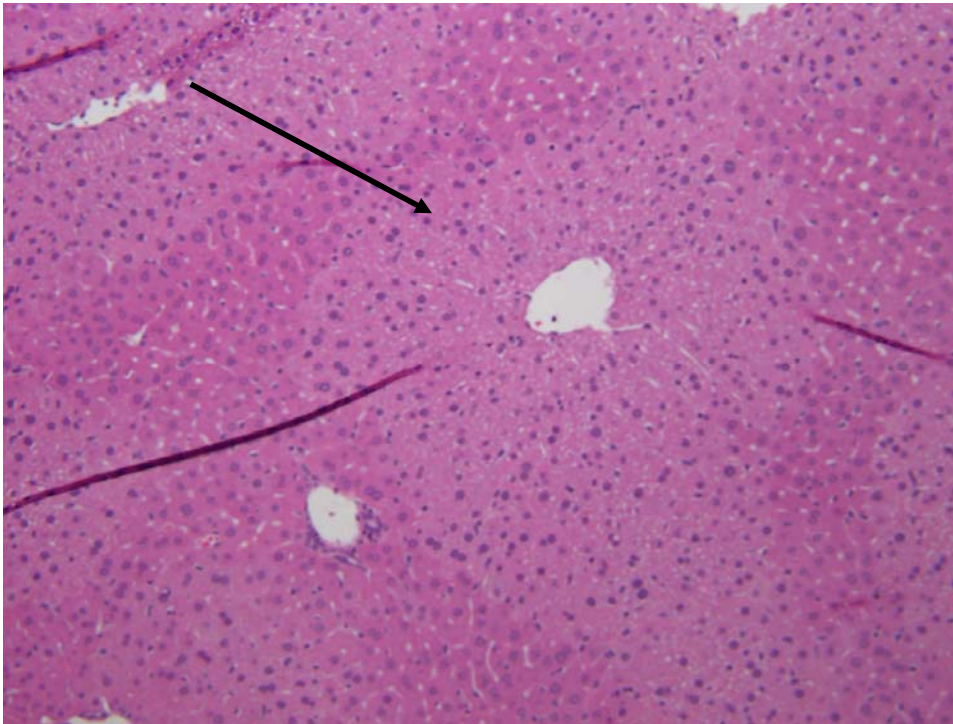


FIGURE 29. Histological examination of NAC given just prior to APAP 2 hours post-APAP injection.

Necrosis still evident around central vein area. Some protection seen, but areas of damage are more evident than when compared to animals given SAME just prior to APAP. Areas of damage around centrilobular region shown by black arrow.

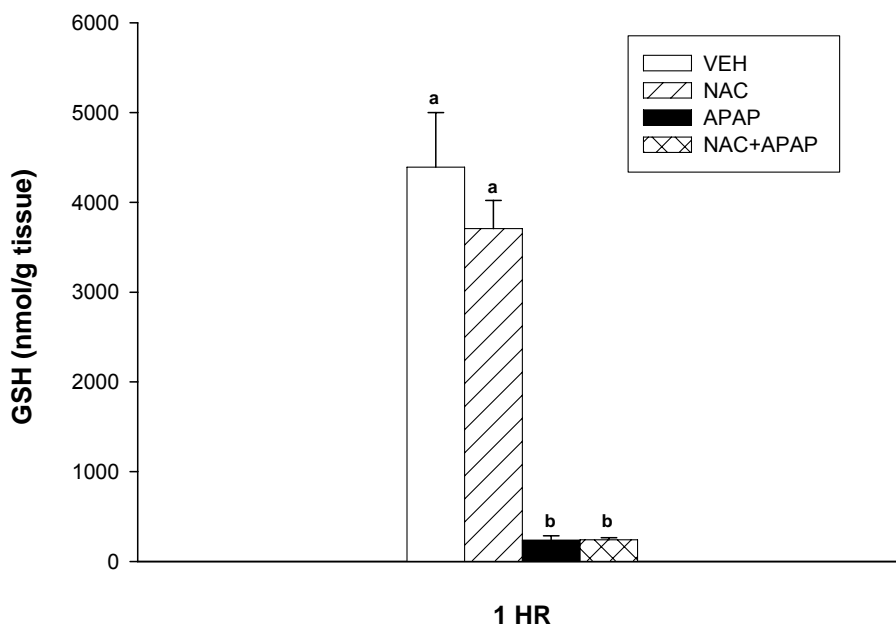


FIGURE 30. Total hepatic glutathione levels 1 hour post-APAP injection in C57Bl/6 mice.

Animals were randomly divided into vehicle (VEH), acetaminophen 300 mg/kg treated (APAP), NAC 204 mg/kg treated (NAC) and NAC + APAP treated (NAC + APAP). Values represent mean \pm S.E.M., n = 4–10 mice per group. Total hepatic GSH levels were measured 1 hour after injection (i.p.) of 300 mg/kg APAP. Groups with unlike superscripts are statistically different.

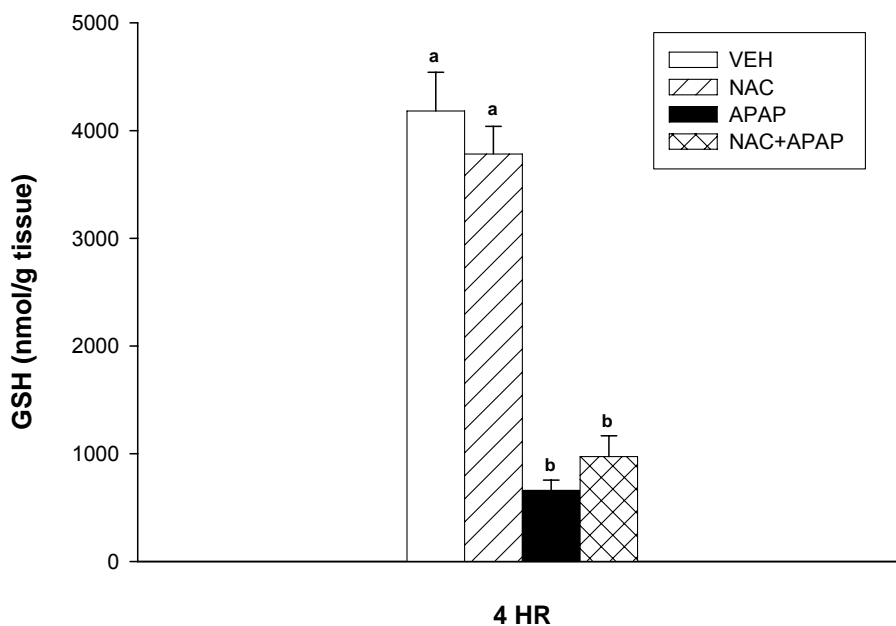


FIGURE 31. Total hepatic glutathione levels 4 hours post-APAP injection in C57Bl/6 mice.

Animals were randomly divided into vehicle (VEH), acetaminophen 300 mg/kg treated (APAP), NAC 204 mg/kg treated (NAC) and NAC + APAP treated (NAC + APAP). Values represent mean \pm S.E.M., $n = 4-10$ mice per group. Total hepatic GSH levels were measured 4 hours after injection (i.p.) of 300 mg/kg APAP. Groups with unlike superscripts are statistically different.

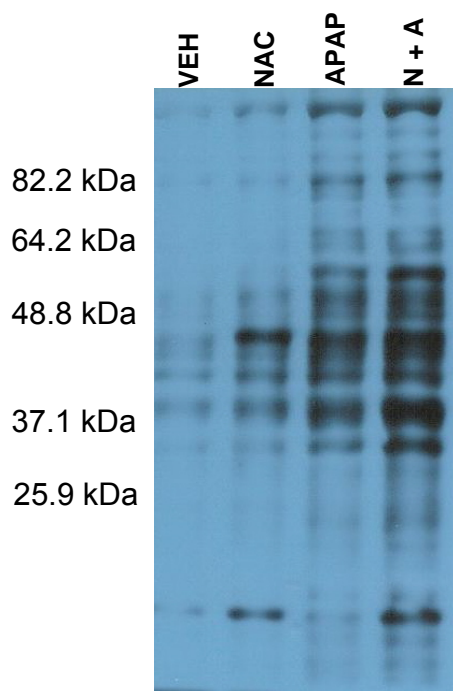


FIGURE 32. OxyBlot of samples 4 hours post-APAP injection.

Lanes are denoted as: Lane 1 VEH, Lane 2 NAC, Lane 3 APAP, Lane 4 NAC prior to APAP. NAC was administered as 204 mg/kg, i.p. (5 ml/kg). APAP was injected i.p. at a dose of 300 mg/kg (15 ml/kg). VEH animals were injected i.p. with water (15 ml/kg).

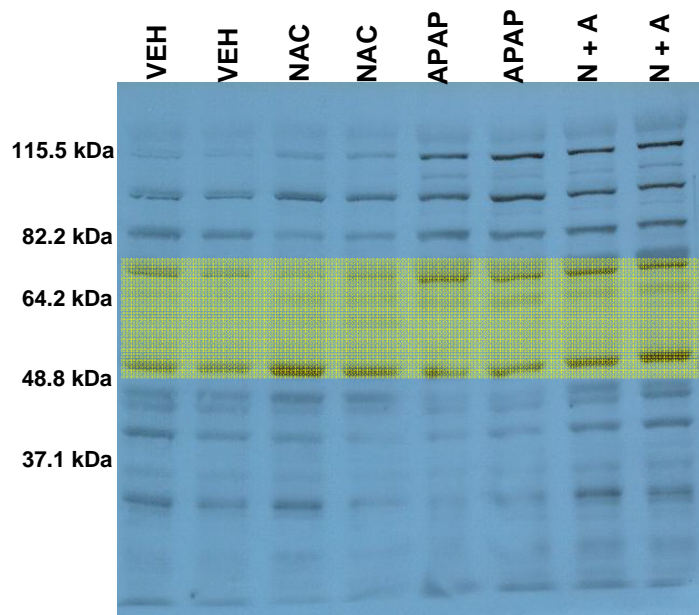


FIGURE 33. 4HNE Blot of samples 4 hours post-APAP injection.

Lanes are denoted as: Lanes 1-2 VEH, Lanes 3-4 NAC, Lane 5-6 APAP, Lane 7-8 NAC prior to APAP (N + A). NAC was administered as 204 mg/kg, i.p. (5 ml/kg). APAP was injected i.p. at a dose of 300 mg/kg (15 ml/kg). VEH animals were injected i.p. with water (15 ml/kg). Highlighted region represents area of greatest differences.

Effect of 500 mg/kg SAME just prior to 1000 mg/kg DEM

Diethyl maleate (DEM) and buthionine sulfoximine are GSH depleting agents. Both inhibit GSH already in the body. Once inhibited, the GSH is incapable of providing any type of antioxidant support (Casey et al., 2002; Hosoya et al., 2002). However new GSH can be produced in the presence of DEM since DEM binds directly to the GSH compound. Buthionine sulfoximine, on the other hand, will prohibit production of new GSH by selectively inhibiting γ -glutamylcysteine synthetase, the rate-limiting enzyme in the synthesis of new GSH (Anderson et al., 1999; Griffith and Meister, 1979; Griffith, 1982). This experiment was designed to measure the efficiency of SAME to produce GSH using DEM to block the activity of pre-existing GSH. This study also evaluated whether NAC and SAME would have comparable effects on GSH.

Animals were divided into three treatment groups (TABLE 13): control treated with corn oil (VEH), DEM treated (DEM) and SAME treated just prior to DEM (SAME + DEM). The animals were fasted overnight (1700 – 0900 h) but were allowed free access to water. The following day, the animals were injected (i.p.) with SAME and/or DEM. SAME was administered in a dose of 500 mg/kg and DEM was given at 1000 mg/kg. The mice were anesthetized in a CO₂ chamber 2 and 4 hours post-DEM and livers were excised.

As expected, DEM had no effect on liver weight (TABLE 14). However, GSH levels were severely depleted ($p < 0.05$) in animals dosed with only DEM at both 2 and 4 hours (FIGURE 34 & FIGURE 35 respectively). When pretreated with SAME, GSH stores increased almost 50% (FIGURE 35) at 4 hours post-

DEM. There was no noticeable difference in GSH levels between DEM and SAME + DEM 2 hours post-DEM injection (FIGURE 34).

Group	DEM	SAMe
VEH	No	No
DEM	Yes	No
SAMe + DEM	Yes	Yes

TABLE 13. SAMe and DEM treatment regimens.

Group	SAMe dose (mg/kg)	Time post-DEM treatment (h)	Body weight (g)	Liver weight (g/10g body wt.)
VEH	0	2	21.4 ± 0.5	0.46 ± 0.01
DEM	500	2	21.6 ± 1.0	0.45 ± 0.02
SAMe + DEM	500	2	22.0 ± 0.7	0.45 ± 0.01
VEH	0	4	23.2 ± 0.8	0.42 ± 0.01
DEM	500	4	24.4 ± 0.7	0.41 ± 0.01
SAMe + DEM	500	4	24.0 ± 0.0	0.44 ± 0.01

TABLE 14. Body and liver weight following DEM administration.

DEM was administered as 1000 mg/kg, i.p. SAMe was administered as 500 mg/kg (5 ml/kg), i.p. Values are reported as mean ± SEM with n = 4-10 animals/group. Groups with different superscripts denote statistical difference ($p < 0.05$) within each treatment experiment.

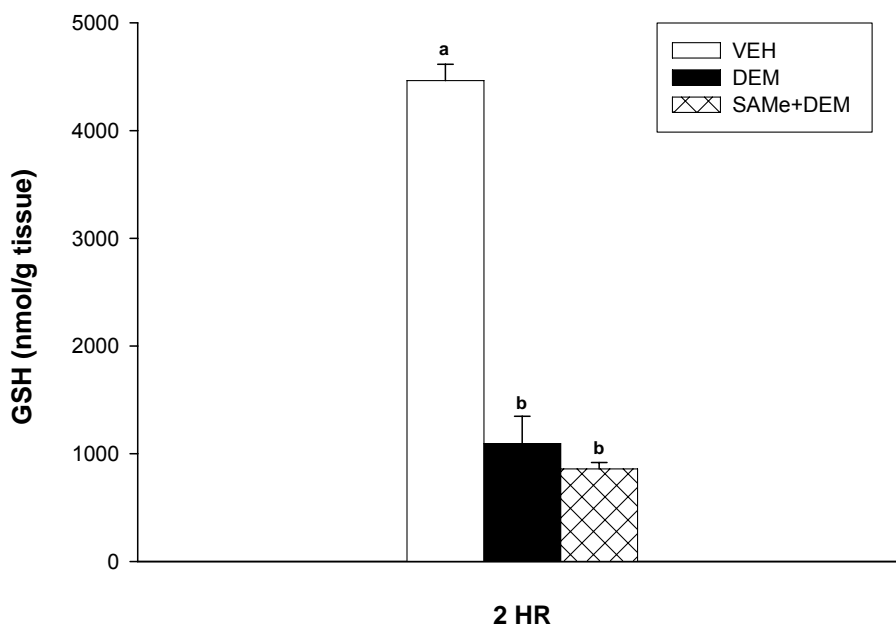


FIGURE 34. Total hepatic glutathione levels 2 hours post-DEM injection in C57Bl/6 mice.

Animals were randomly divided into vehicle (VEH), DEM treated (DEM) and SAMe + DEM treated (SAMe + DEM). Values represent mean \pm S.E.M., $n = 4-10$ mice per group. Total hepatic GSH levels were measured 2 hours after injection (i.p.) of 1000 mg/kg DEM. Groups with unlike superscripts are statistically different.

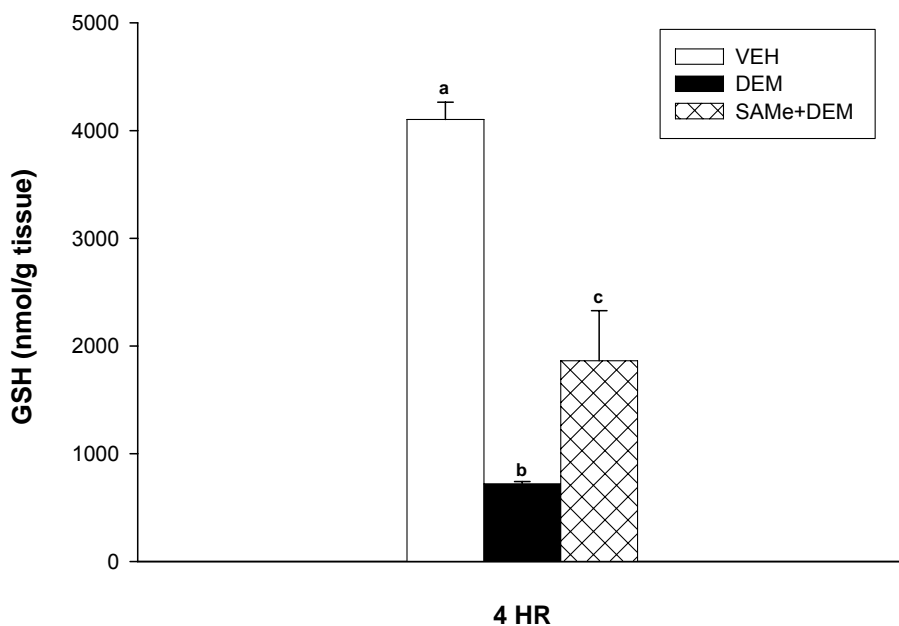


FIGURE 35. Total hepatic glutathione levels 4 hours post-DEM injection in C57Bl/6 mice.

Animals were randomly divided into vehicle (VEH), DEM treated (DEM) and SAMe + DEM treated (SAMe + DEM). Values represent mean \pm S.E.M., $n = 4-10$ mice per group. Total hepatic GSH levels were measured 4 hours after injection (i.p.) of 1000 mg/kg DEM. Groups with unlike superscripts are statistically different.

Effect of 204 mg/kg NAC just prior to 1000 mg/kg DEM

Using the same mmol dose as SAME (1.25 mmol/kg), NAC was administered the same way as previously described. Animals were divided into three treatment groups (TABLE 15): control treated with corn oil (VEH), DEM treated (DEM) and NAC treated just prior to DEM (NAC + DEM). NAC was administered in a dose of 204 mg/kg and DEM was given at 1000 mg/kg. The animals were fasted overnight (1700 – 0900 h) but were allowed free access to water. The following day, the animals were injected (i.p.) with NAC and/or DEM. The mice were anesthetized in a CO₂ chamber 2 and 4 hours post-DEM and livers were excised.

Similar to the SAME studies, DEM had no effect on liver weight at 2 or 4 hours post-injection (TABLE 16). However, GSH levels were severely depleted ($p < 0.05$) at both 2 (FIGURE 36) and 4 (FIGURE 37) hours. When pretreated with NAC, GSH stores were similar between DEM and DEM + NAC 2 hours post-DEM (FIGURE 36). However at 4 hours, the increase of GSH stores when NAC was administered just prior to DEM was less noticeable when directly compared to SAME + DEM GSH levels (FIGURE 37). DEM inhibited GSH levels, but neither NAC nor SAME altered GSH levels when measured 2 hours after DEM treatment. However, GSH levels with SAME provided better protection when monitored 4 hours after DEM injection. These results suggest that SAME is more potent than NAC at producing new GSH.

Group	DEM	NAC
VEH	No	No
DEM	Yes	No
NAC + DEM	Yes	Yes

TABLE 15. NAC and DEM treatment regimens.

Group	NAC dose (mg/kg)	Time post-DEM treatment (h)	Body weight (g)	Liver weight (g/10g body wt.)
VEH	0	2	23.6 ± 0.7	0.42 ± 0.01
DEM	204	2	24.4 ± 0.7	0.40 ± 0.0
NAC + DEM	204	2	22.4 ± 0.7	0.42 ± 0.01
VEH	0	4	22.8 ± 0.8	0.42 ± 0.01
DEM	204	4	23.6 ± 0.4	0.41 ± 0.01
NAC + DEM	204	4	22.8 ± 0.5	0.43 ± 0.01

TABLE 16. Body and liver weight following DEM administration.

DEM was administered as 1000 mg/kg, i.p. NAC was administered as 204 mg/kg (5 ml/kg), i.p. Values are reported as mean ± SEM with n = 4-10 animals/group. Groups with different superscripts denote statistical difference ($p < 0.05$) within each treatment experiment.

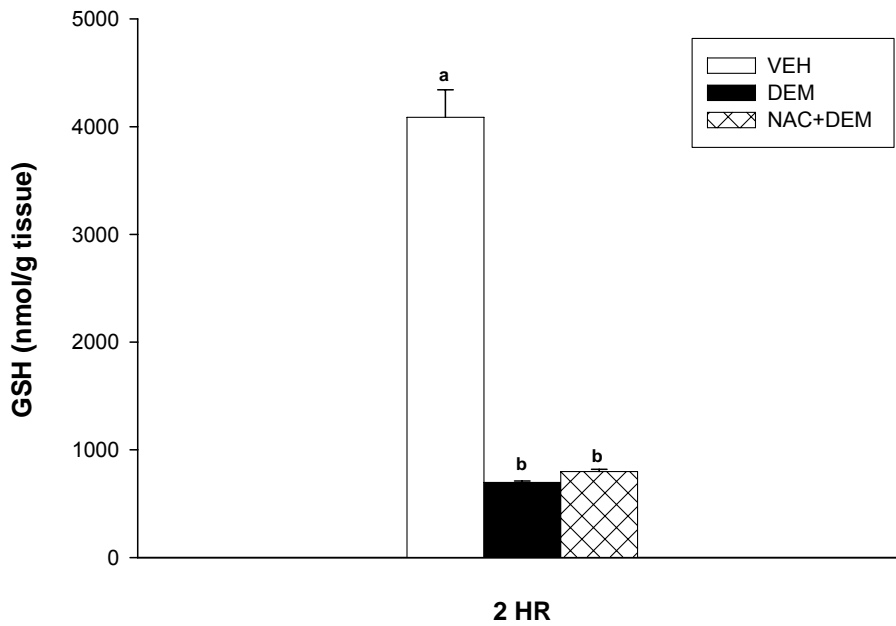


FIGURE 36. Total hepatic glutathione levels 2 hours post-DEM injection in C57Bl/6 mice.

Animals were randomly divided into vehicle (VEH), DEM treated (DEM) and NAC + DEM treated (NAC + DEM). Values represent mean \pm S.E.M., n = 4–10 mice per group. Total hepatic GSH levels were measured 2 hours after injection (i.p.) of 1000 mg/kg DEM. Groups with unlike superscripts are statistically different.

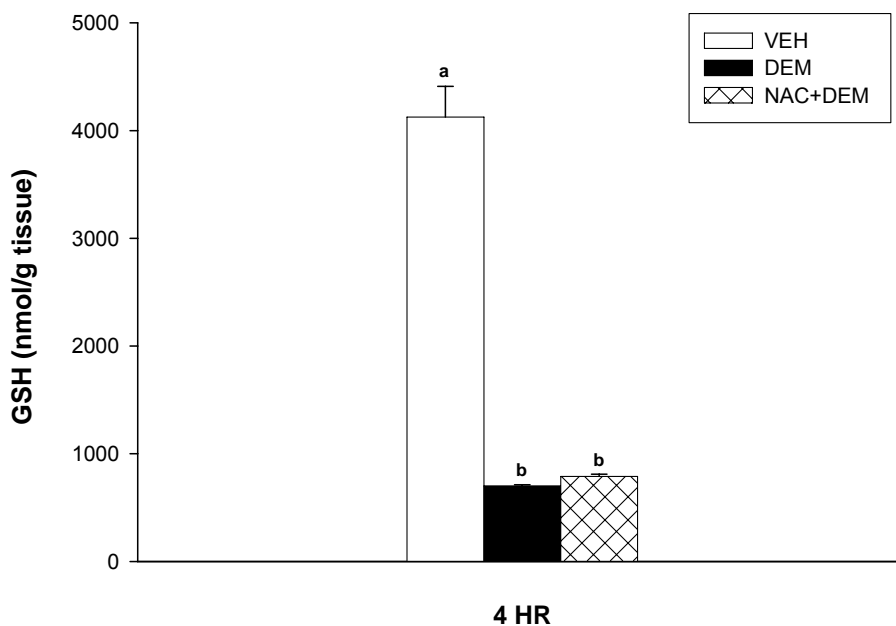


FIGURE 37. Total hepatic glutathione levels 4 hours post-DEM injection in C57Bl/6 mice.

Animals were randomly divided into vehicle (VEH), DEM treated (DEM) and NAC + DEM treated (NAC + DEM). Values represent mean \pm S.E.M., n = 4–10 mice per group. Total hepatic GSH levels were measured 4 hours after injection (i.p.) of 1000 mg/kg DEM. Groups with unlike superscripts are statistically different.

Effect of 250 mg/kg SAME + 102 mg/kg NAC just prior to 300 mg/kg APAP dose

To investigate the possibility of synergistic effects when combining SAME + NAC and to study the possibility of the two drugs working via different mechanisms, a combination therapy cocktail was developed. We wanted to directly compare the therapeutic effects of the cocktail to the prior experiments where SAME or NAC were given individually. Therefore, the total mmol concentration had to equal that of prior experiments involving SAME and NAC. We used ½ of what we had normally been using for both SAME and NAC: 250 mg/kg SAME and 102 mg/kg NAC.

It is believed that the mechanism by which SAME and NAC produce GSH is very similar (FIGURE 38), but there is not agreement that this is the only mechanism by which the two drugs elicit the protective effects seen. Both, SAME and NAC, can produce the rate limiting compound, cysteine, that is needed for GSH production.

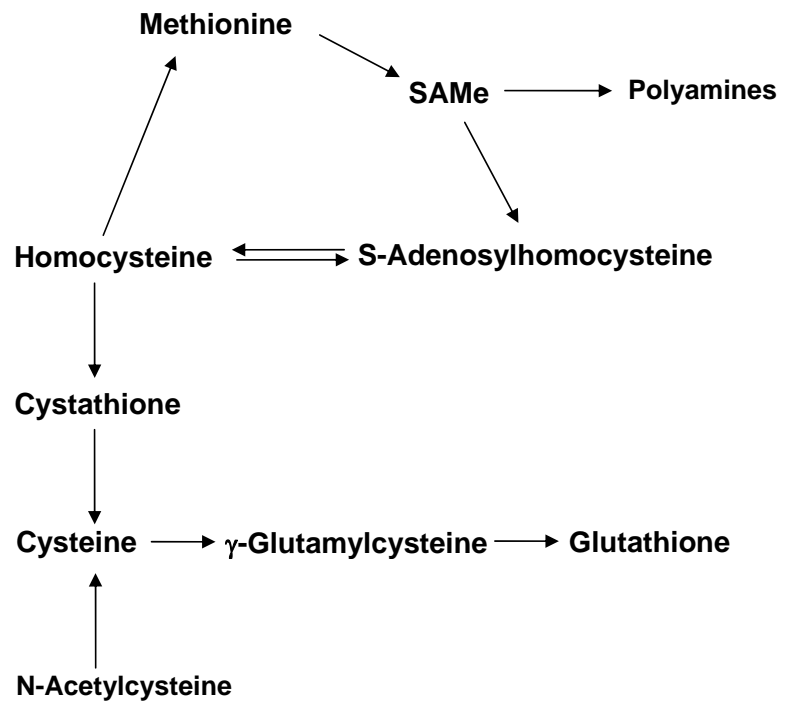


FIGURE 38. Diagrammatic representation of GSH production with both SAMe and NAC.

Animals were divided, just as before, into four treatment groups (TABLE 17): control treated (VEH), APAP treated with 1.98 mmol/kg (APAP), SAME + NAC treated with 0.625 mmol/kg of SAME and NAC (SAME + NAC) and SAME + NAC pretreatment just prior to APAP (SAME + NAC+ APAP). Animals were fasted overnight from 1700 – 0900 h, but allowed free access to water. At 0900 h the morning following fasting, the mice were injected i.p. with the specified amount of water, SAME + NAC, APAP or SAME + NAC + APAP. At 1 and 4 hours post-APAP injection, the mice were anesthetized with CO₂, blood was drawn via cardiac puncture and the livers were removed.

APAP-induced hepatotoxicity was seen at 4 hours post-APAP injection. Liver weight (TABLE 18) and plasma ALT values (TABLE 19) were significantly ($p < 0.05$) raised 4 hours after APAP had been injected. No severe toxicity was seen 1 hour post-APAP. The SAME + NAC pretreatment at 4 hours post-injection showed limited protection. Liver weight was decreased (TABLE 18) when the SAME + NAC cocktail was given just prior to APAP, but the protection seen was not as great as when SAME alone was given just prior to APAP. There was no protection seen at 1 or 4 hours post-APAP with regards to the plasma ALT values (TABLE 19).

GSH levels were markedly diminished at both 1 (FIGURE 39) and 4 (FIGURE 40) hours after APAP injection when compared to the VEH group. When SAME + NAC were administered prior to APAP, GSH levels were slightly higher than the group dosed with APAP at both 1 and 4 hours post-APAP. There

was no statistically significant difference in the groups given SAME + NAC vs. SAME + NAC just prior to APAP at either time periods.

Oxidative effects of APAP on the liver were confirmed with lipid peroxidation, protein carbonyl values and 4HNE adducted proteins 4 hours post-APAP (FIGURES 41-43 respectively). Oxidative stress was not seen 1 hour post-APAP based on indicators such as protein carbonyls, etc. (data not shown). While we did not see any significant protective effects with SAME + NAC attenuating damage done by APAP regarding decreased GSH levels, increases in ALT levels and high liver weights, we did see strong effects of decreasing lipid peroxidation levels when SAME + NAC was given just prior to APAP and livers were excised 4 hours post-injection (FIGURE 41). However, the SAME + NAC cocktail did not protect against the production of APAP-induced protein carbonyls or 4HNE adducted proteins (FIGURE 42 and FIGURE 43 respectively).

In summary, we did not see any protective effects of the pretreatment with the ALT and GSH assays nor the liver weight values at 1 or 4 hours post-APAP injection. SAME + NAC pretreatment was also unable to prevent the amount of protein carbonyls produced when given just prior to APAP. The only protective effect that was seen with the cocktail was that SAME + NAC did provide positive results regarding prevention of TBAR production 4 hours post-APAP. The combination of SAME + NAC at combined equimolar dose of 1.24 mmol/kg did not provide better protection than SAME.

Group	APAP	SAMe + NAC
VEH	No	No
APAP	Yes	No
SAMe + NAC	No	Yes
SAMe +NAC + APAP	Yes	Yes

TABLE 17. SAMe + NAC and APAP treatment regimens.

Group	SAMe/NAC dose (mg/kg)	Time post-APAP treatment (h)	Body weight (g)	Liver weight (g/10g body wt.)
VEH	0	1	17.6 ± 0.4	0.49 ± 0.02
APAP	0	1	16.0 ± 0.6	0.51 ± 0.01
SAMe + NAC	250/102	1	16.8 ± 0.5	0.47 ± 0.02
SAMe + NAC + APAP	250/102	1	16.8 ± 0.5	0.50 ± 0.02
VEH	0	4	17.8 ± 0.4	0.47 ± 0.01 ^a
APAP	0	4	17.6 ± 0.3	0.59 ± 0.01 ^b
SAMe + NAC	250/102	4	17.8 ± 0.4	0.48 ± 0.01 ^a
SAMe + NAC + APAP	250/102	4	18.0 ± 0.4	0.53 ± 0.01 ^c

TABLE 18. Body and liver weight following APAP administration.

APAP was administered as 300 mg/kg (15 ml/kg), i.p. SAMe was administered as 250 mg/kg, i.p. and NAC was administered as 102 mg/kg, i.p. Values are reported as mean ± SEM with n = 4-10 animals/group. Groups with different superscripts denote statistical difference (p < 0.05) within each treatment experiment.

Group	SAMe/NAC dose (mg/kg)	Time post-APAP treatment (h)	ALT (U/l)
VEH	0	1	58 ± 31
APAP	0	1	62 ± 24
SAMe + NAC	204	1	54 ± 22
SAMe + NAC + APAP	204	1	125 ± 54
VEH	0	4	78 ± 26 ^a
APAP	0	4	10671 ± 584 ^b
SAMe + NAC	204	4	80 ± 36 ^a
SAMe + NAC + APAP	204	4	10566 ± 1102 ^b

TABLE 19. Plasma ALT levels following APAP administration.

Groups represent vehicle (VEH); acetaminophen (APAP) injected i.p. 300 mg/kg; SAMe i.p. injected 250 mg/kg + NAC injected i.p. 102 mg/kg (SAMe + NAC); SAMe + NAC and acetaminophen (SAMe + NAC + APAP) at doses listed for individual agents. Unlike letters (a, b) indicate groups are different ($p < 0.05$) from each other using a one way ANOVA and Tukey's post hoc test. Values represent mean ± S.E.M. with $n = 4-10$ per group.

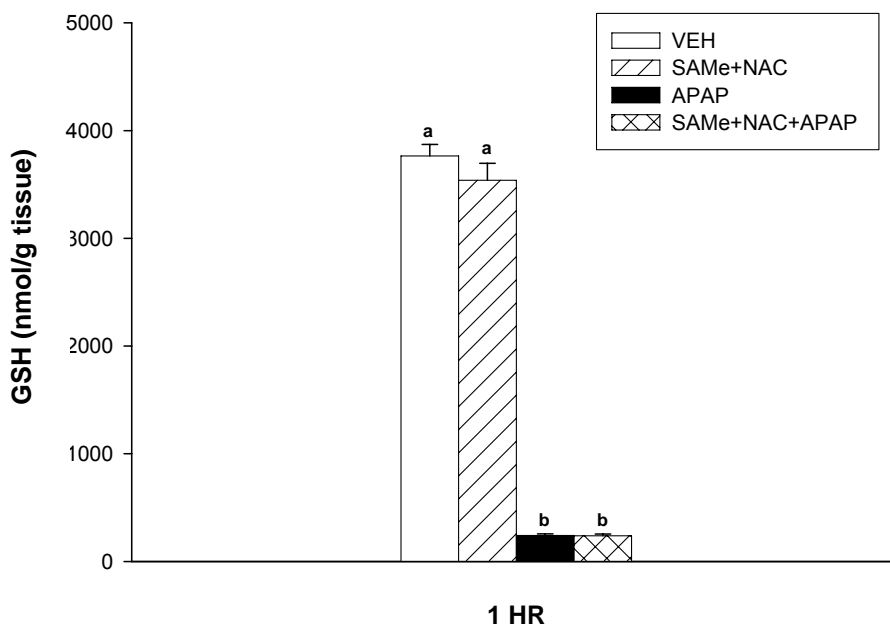


FIGURE 39. Total hepatic glutathione levels 1 hour post-APAP injection in C57Bl/6 mice.

Animals were randomly divided into vehicle (VEH), acetaminophen treated (APAP), SAMe 250 mg/kg + NAC 102 mg/kg treated (SAMe + NAC) and SAMe + NAC + APAP treated (SAMe + NAC + APAP). Values represent mean \pm S.E.M., $n = 4-10$ mice per group. Total hepatic GSH levels were measured 1 hour after injection (i.p.) of 300 mg/kg APAP. Groups with unlike superscripts are statistically different.

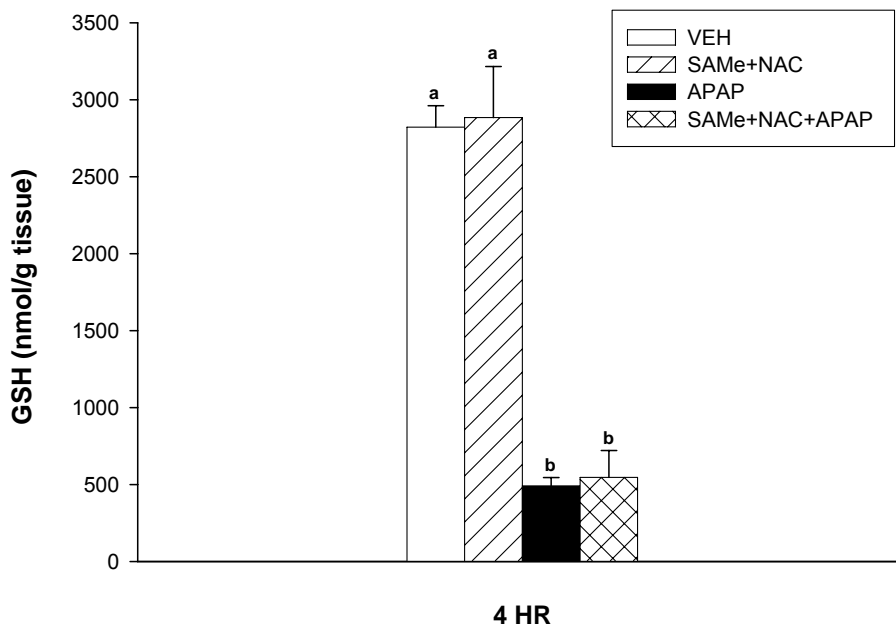


FIGURE 40. Total hepatic glutathione levels 4 hours post-APAP injection in C57Bl/6 mice.

Animals were randomly divided into vehicle (VEH), acetaminophen treated (APAP), SAMe 250 mg/kg + NAC 102 mg/kg treated (SAMe + NAC) and SAMe + NAC + APAP treated (SAMe + NAC + APAP). Values represent mean \pm S.E.M., $n = 4-10$ mice per group. Total hepatic GSH levels were measured 4 hours after injection (i.p.) of 300 mg/kg APAP. Groups with unlike superscripts are statistically different.

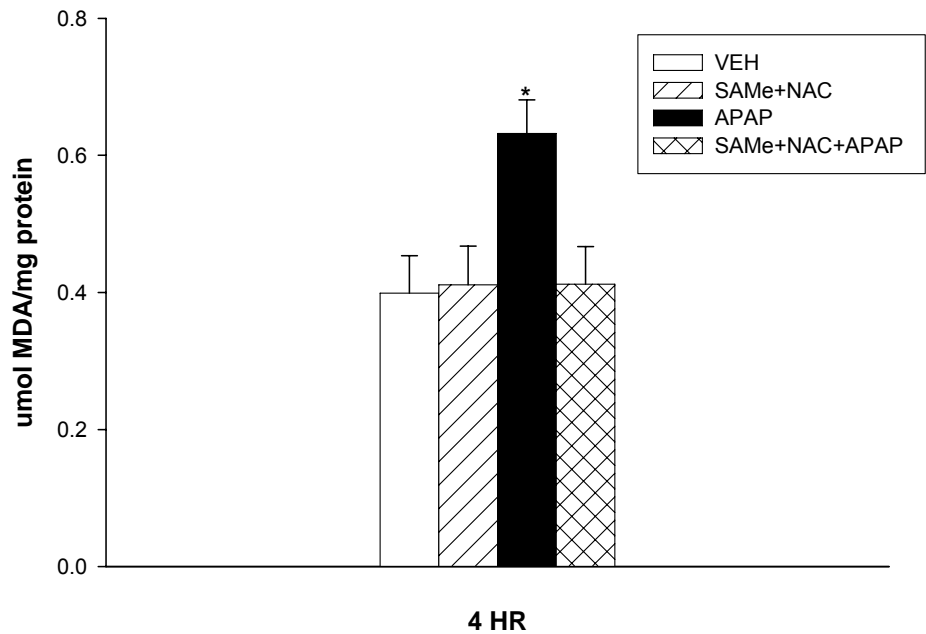


FIGURE 41. Lipid peroxidation levels 4 hours post-APAP injection in C57Bl/6 mice.

Animals were randomly divided into vehicle (VEH), acetaminophen treated (APAP), SAMe 250 mg/kg + NAC 102 mg/kg treated (SAMe + NAC) and SAMe + NAC + APAP treated (SAMe + NAC + APAP). Values represent mean S.E.M., *n* = 4–10 mice per group. Lipid peroxidation was measured 4 hours after injection (i.p.) of 300 mg/kg APAP. Lipid peroxidation was measured as μmol malondialdehyde (MDA)/mg protein. Group denoted with an asterisk (*) are statistically different.

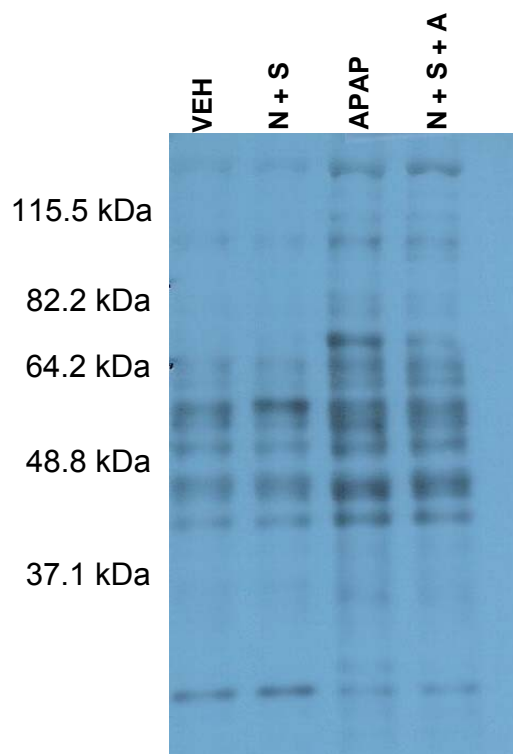


FIGURE 42. OxyBlot of samples 4 hours post-APAP injection.

Lanes are denoted as: Lane 1 vehicle, Lane 2 SAME + NAC (N + S), Lane 3 acetaminophen, Lane 4 SAME + NAC prior to acetaminophen (N + S + A). SAME was administered as 250 mg/kg and NAC was given as 102 mg/kg, i.p. APAP was injected i.p. at a dose of 300 mg/kg (15 ml/kg). VEH animals were injected i.p. with water (15 ml/kg).

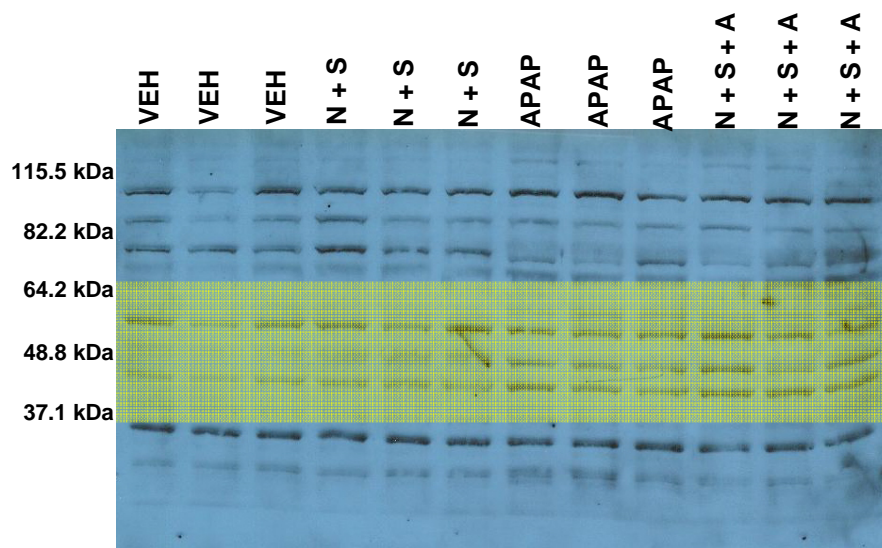


FIGURE 43. 4HNE Blot of samples 4 hours post-APAP injection.

Lanes are denoted as: Lane 1-3 VEH, Lane 4-6 SAME + NAC (N +S), Lane 7-9 APAP, Lane 10-12 SAME + NAC prior to APAP (N + S + A). SAME was administered as 250 mg/kg and NAC was given as 102 mg/kg, i.p. APAP was injected i.p. at a dose of 300 mg/kg (15 ml/kg). VEH animals were injected i.p. with water (15 ml/kg). Highlighted region represents area with greatest differences.

Positive Control: High dose of NAC

It is known that NAC can attenuate APAP-induced hepatic toxicity. However in emergency situations, the doses of NAC that are given are quite large. We have shown that on a similar mmol basis, NAC is unable to protect the liver as effectively as SAME. To disprove any hesitations of the ability of NAC to attenuate APAP-induced toxicity in our laboratory, we used a high amount of NAC (1200 mg/kg) to show its protective effects. This dose is roughly 10 times larger than our experiments directly comparing NAC vs. SAME.

Animals were prepared as previously described. Twenty animals were randomly divided into 4 groups consisting of a vehicle (VEH), NAC treated (NAC), APAP treated (APAP) and NAC given just prior to APAP (NAC + APAP). The animals were fasted from 1700-0900 h but allowed free access to water. The following morning, animals (NAC and NAC + APAP) were injected at 0900 h with NAC at a dose of 1200 mg/kg (5 ml/kg adjusted to pH 7). The APAP and NAC + APAP groups were injected i.p. with 300 mg/kg APAP (15 ml/kg). The VEH group was injected i.p. with water (15 ml/kg). The animals were anesthetized with CO₂ 4 hours after APAP injection (1300 h), blood was collected via cardiac puncture and the livers were removed and placed in ice-cold Krebs buffer.

As expected, the high dose of NAC provided protection in all of the measurable parameters. APAP caused increased liver weight (TABLE 20), large increases in plasma ALT values (TABLE 21) and histological alterations

(FIGURE 44). NAC, when given at the 1200 mg/kg dose, was able to prevent the rise in liver weights and plasma ALT values, plus reverse the damage around the centrilobular region of the liver.

GSH levels were markedly diminished at 4 hours after APAP injection when compared to the VEH group (FIGURE 45). When the high dose of NAC was administered prior to APAP, GSH levels were markedly higher than the group dosed with APAP 4 hours post-injection. There was statistically significant difference in the groups given NAC vs. NAC just prior to APAP.

Further protection was illustrated by the ability of the high dose of NAC to attenuate oxidative stress measured by the production of 4HNE adducted proteins (FIGURE 46). The 4HNE gel showed a lower concentration of the amount of oxidized proteins in the NAC + APAP animals compared to the APAP dosed animals. The high dose of NAC showed no effect on the production of 4HNE adducted proteins when directly compared to the VEH group.

Group	NAC dose (mg/kg)	Time post-APAP treatment (h)	Body weight (g)	Liver weight (g/10g body wt.)
VEH	0	4	19.6 ± 0.4	0.46 ± 0.01 ^a
APAP	0	4	17.6 ± 0.4	0.56 ± 0.01 ^b
NAC	1200	4	18.4 ± 0.7	0.49 ± 0.01 ^a
NAC + APAP	1200	4	19.5 ± 0.5	0.50 ± 0.01 ^a

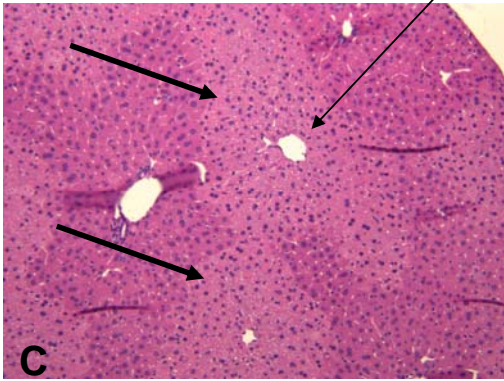
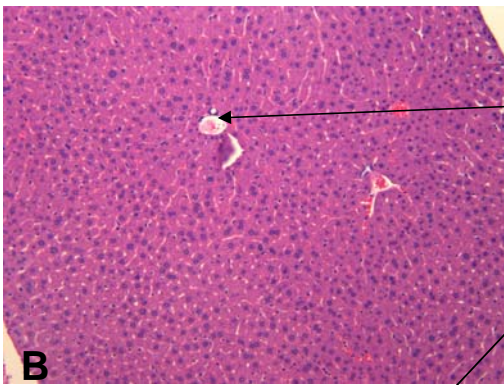
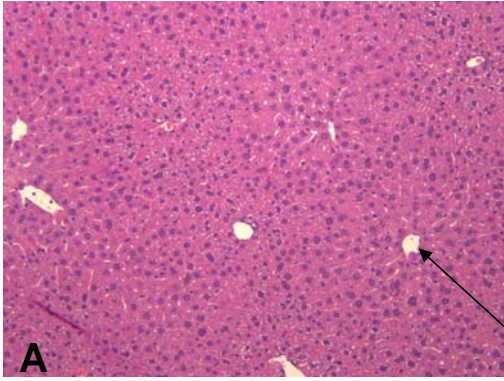
TABLE 20. Body and liver weight following APAP administration.

APAP was administered as 300 mg/kg (15 ml/kg), i.p. NAC was administered as 1200 mg/kg (5 ml/kg), i.p. Values are reported at mean ± SEM with n = 4-10 animals/group. Groups with different superscripts denote statistical difference (p < 0.05) within each treatment experiment.

Group	NAC dose (mg/kg)	Time post-APAP treatment (h)	ALT (U/l)
VEH	0	4	34 ± 13 ^a
APAP	0	4	6674 ± 1377 ^b
NAC	1200	4	108 ± 52 ^a
NAC + APAP	1200	4	606 ± 215 ^a

TABLE 21. Plasma ALT levels following APAP administration.

Groups represent vehicle (VEH); acetaminophen (APAP) injected i.p. 300 mg/kg; NAC i.p. injected 1200 mg/kg (NAC); NAC and acetaminophen (NAC + APAP) at doses listed for individual agents. Unlike letters (a, b) indicate groups are different (p < 0.05) from each other using a one way ANOVA and Tukey's post hoc test. Values represent mean ± S.E.M. with n = 4-5 per group.



**Centrilobular
Regions**

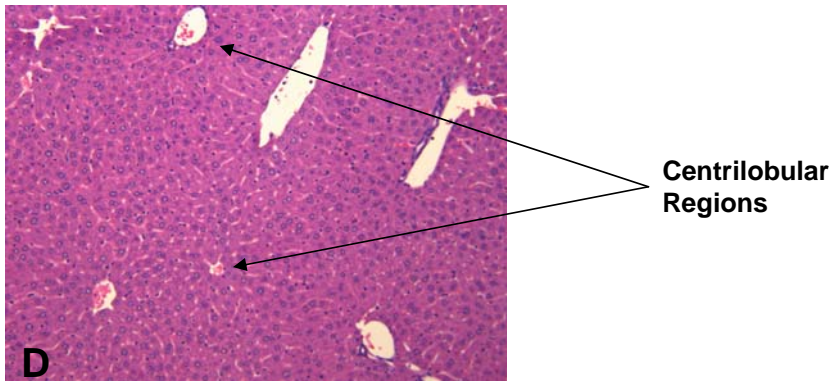


FIGURE 44. Histological examination 4 hours after APAP injection.

Hepatic tissues were prepared and stained with H&E. Representative slides of (A) vehicle (VEH); (B) NAC (1200 mg/kg) treated; (C) 4 hours post-acetaminophen (APAP) injection and (D) mice treated with NAC just prior to APAP. Normal morphology was noted in the VEH, NAC and NAC + APAP animals. Centrilobular damage was noted in the APAP group as shown by the arrows in slide C. Magnification is 200 \times .

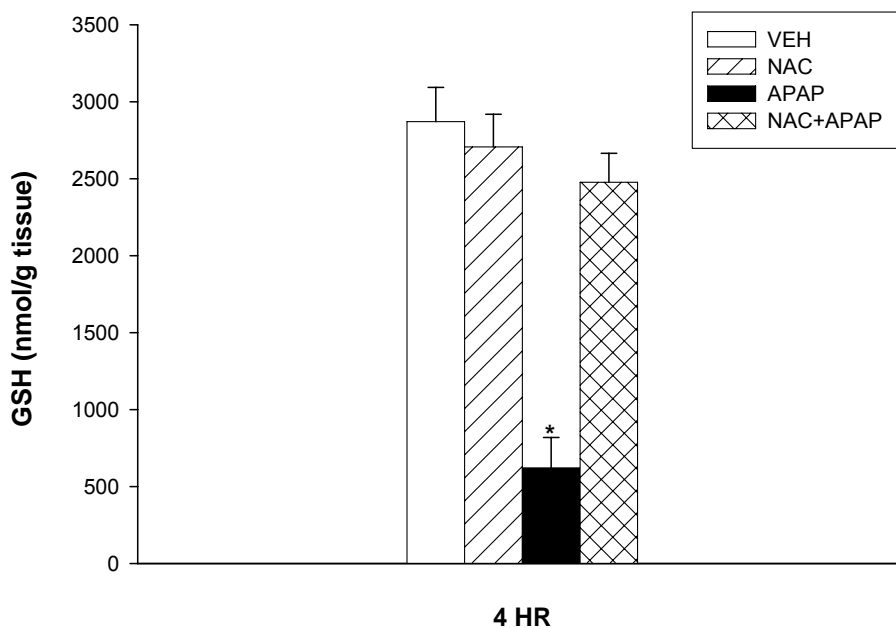


FIGURE 45. Total hepatic glutathione levels following APAP injection in C57Bl/6 mice.

Animals were randomly divided into vehicle (VEH), acetaminophen treated (APAP), NAC 1200 mg/kg treated (NAC) and NAC + APAP treated (NAC + APAP). Values represent mean \pm S.E.M., $n = 4-5$ mice per group. Total hepatic GSH levels were measured 4 hours after injection (i.p.) of 300 mg/kg APAP. An asterisk (*) marks statistically significant difference ($p < 0.05$) from the rest of the treatment groups.

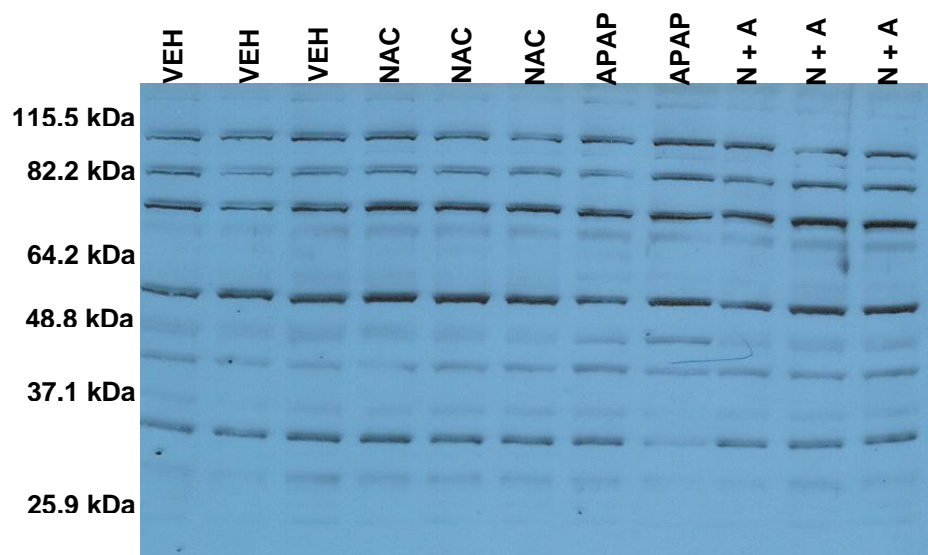


FIGURE 46. 4HNE Blot with high dose of NAC.

Lanes are denoted as: Lane 1-3 vehicle, Lane 4-6 NAC, Lane 7-8 acetaminophen, Lane 9-11 NAC + acetaminophen. NAC was administered as 1200 mg/kg. APAP was injected i.p. at a dose of 300 mg/kg (15 ml/kg). VEH animals were injected i.p. with water (15 ml/kg).

Effect of 3.84 mg/kg vitamin B₆ + 500 mg/kg SAmE just prior to 300 mg/kg APAP

According to Lu (1998), vitamin B₆ is a rate limiting agent in the production of SAmE → GSH (FIGURE 4). If vitamin B₆ were in short supply during APAP overdose, it is possible that the mechanism would be halted at the production of homocysteine. As described earlier, if the ratio of SAH:SAmE favors SAH, the reaction will be reversed and no GSH will be produced. To further investigate the mechanistic properties of SAmE and to discover if vitamin B₆ could provide additional therapeutic effects as compared to SAmE alone, we injected (i.p.) the animals with a dose of vitamin B₆ with SAmE just prior to APAP. The dose of vitamin B₆ that was used was comparable to a human's recommended daily allowance.

Animals were divided, just as before, into four treatment groups (TABLE 22): control treated (VEH), APAP treated (APAP), SAmE + vitamin B₆ treated (SAmE + vitamin B₆) and SAmE + vitamin B₆ pretreatment just prior to APAP (SAmE + vitamin B₆ + APAP). Vitamin B₆ was administered in a dose of 3.84 mg/kg, SAmE was dosed at 500 mg/kg and APAP was injected (i.p.) 300 mg/kg. The animals were fasted overnight (1700 – 0900 h) but were allowed free access to water. The following day, the animals were anesthetized in a CO₂ chamber 1 and 4 hours post-APAP and livers were excised.

APAP hepatic toxicity was confirmed by increased liver weight (only at 4 hours post-APAP injection) (TABLE 23) and elevated plasma ALT levels (TABLE

24) at 4 hours post-APAP. One hour post-APAP showed change in plasma ALT levels but not in the liver weight.

APAP-induced hepatotoxicity was prevented, to an extent, when vitamin B₆ was administered with SAME just prior to APAP 4 hours post-injection. The treatment prevented increases in liver weight and it prevented extreme rises in plasma ALT values. However, 1 hour post-APAP the treatment showed virtually no protection. When directly comparing the benefits seen with vitamin B₆ + SAME to the benefits of SAME when given alone, there is either no difference or SAME works better by itself.

GSH levels were diminished in groups dosed with APAP at 1 and 4 hours post-APAP injection (FIGURE 47 and FIGURE 48 respectively). There was a gradual trend for increase in total GSH in groups given vitamin B₆ + SAME just prior to APAP at both 1 and 4 hours, but only the values at 4 hours post-APAP produced statistically significant results (FIGURES 48 and 49). There was a drop in GSH levels in the group given vitamin B₆ + SAME only when directly compared to the VEH group 4 hours post-injection.

Oxidative stress in animals dosed with APAP only was confirmed with an increase in lipid peroxidation (FIGURE 50). The vitamin B₆ + SAME predose was able to prevent a rise in lipid peroxidation at 4 hours post-APAP. There was no notable difference in lipid peroxidation levels 1 hour post-APAP injection (data not shown).

Group	APAP	SAMe + Vitamin B ₆
VEH	No	No
APAP	Yes	No
SAMe + Vitamin B ₆	No	Yes
SAMe +Vitamin B ₆ + APAP	Yes	Yes

TABLE 22. SAMe + Vitamin B₆ and APAP treatment regimens.

Group	SAMe/Vitamin B ₆ dose (mg/kg)	Time post-APAP treatment (h)	Body weight (g)	Liver weight (g/10g body wt.)
VEH	0	1	18.0 ± 0.0	0.48 ± 0.01
APAP	0	1	17.6 ± 0.4	0.49 ± 0.02
SAMe + Vitamin B ₆	500/3.84	1	18.8 ± 0.5	0.49 ± 0.01
SAMe + Vitamin B ₆ + APAP	500/3.84	1	17.2 ± 0.5	0.49 ± 0.01
VEH	0	4	19.2 ± 0.5	0.48 ± 0.01 ^a
APAP	0	4	19.0 ± 0.5	0.57 ± 0.01 ^b
SAMe + Vitamin B ₆	500/3.84	4	18.6 ± 0.5	0.49 ± 0.01 ^a
SAMe + Vitamin B ₆ + APAP	500/3.84	4	19.6 ± 0.5	0.50 ± 0.01 ^a

TABLE 23. Body and liver weight following APAP administration.

APAP was administered as 300 mg/kg (15 ml/kg), i.p. SAMe was administered as 500 mg/kg (5 ml/kg) with 3.84 mg/kg vitamin B₆, i.p. Values are reported as mean ± SEM with n = 4-10 animals/group. Groups with different superscripts denote statistical difference (p < 0.05) within each treatment experiment.

Group	SAMe/Vitamin B ₆ dose (mg/kg)	Time post-APAP treatment (h)	ALT (U/l)
VEH	0	1	179 ± 80 ^a
APAP	0	1	237 ± 80 ^a
SAMe + Vitamin B ₆	500/3.84	1	131 ± 39 ^a
SAMe + Vitamin B ₆ + APAP	500/3.84	1	503 ± 110 ^b
VEH	0	4	96 ± 39 ^a
APAP	0	4	7905 ± 148 ^b
SAMe + Vitamin B ₆	500/3.84	4	233 ± 148 ^a
SAMe + Vitamin B ₆ + APAP	500/3.84	4	4311 ± 874 ^c

TABLE 24. Plasma ALT levels following APAP administration.

Groups represent vehicle (VEH); acetaminophen (APAP) injected i.p. 300 mg/kg; SAMe i.p. injected 500 mg/kg + vitamin B₆ injected i.p. 3.84 mg/kg (SAMe + Vitamin B₆); SAMe + vitamin B₆ and acetaminophen (SAMe + vitamin B₆ + APAP) at doses listed for individual agents. Unlike letters (a, b) indicate groups are different ($p < 0.05$) from each other using a one way ANOVA and Tukey's post hoc test. Values represent mean ± S.E.M. with $n = 4-10$ per group.

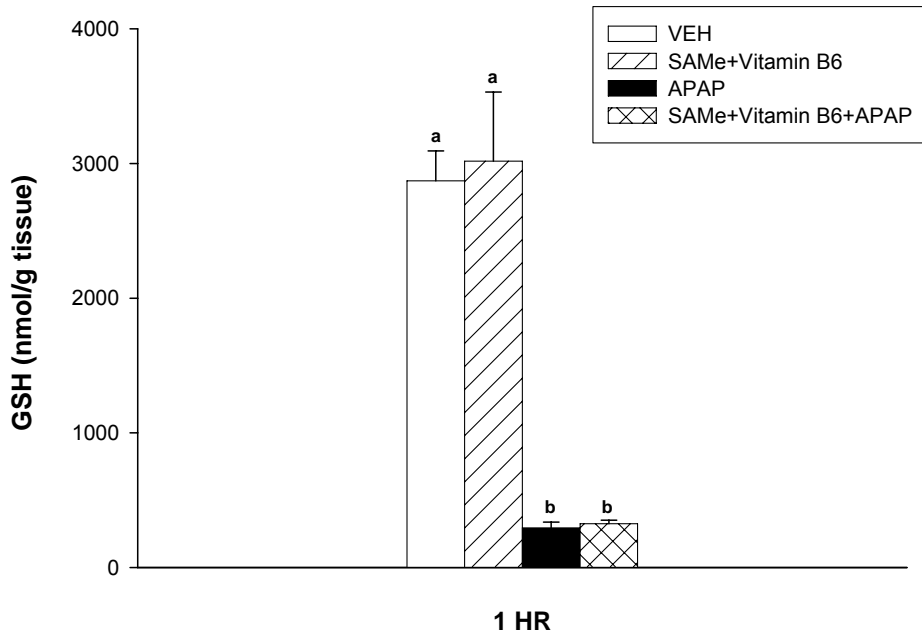


FIGURE 47. Total hepatic glutathione levels following APAP injection in C57Bl/6 mice.

Animals were randomly divided into vehicle (VEH), acetaminophen treated (APAP), SAmE 500 mg/kg + vitamin B₆ 3.84 mg/kg treated (SAmE + Vitamin B₆) and SAmE + Vitamin B₆ and APAP treated (SAmE + Vitamin B₆ + APAP). Values represent mean ± S.E.M., *n* = 4–10 mice per group. Total hepatic GSH levels were measured 1 hour after injection (i.p.) of 300 mg/kg APAP. Groups with unlike superscripts are statistically different.

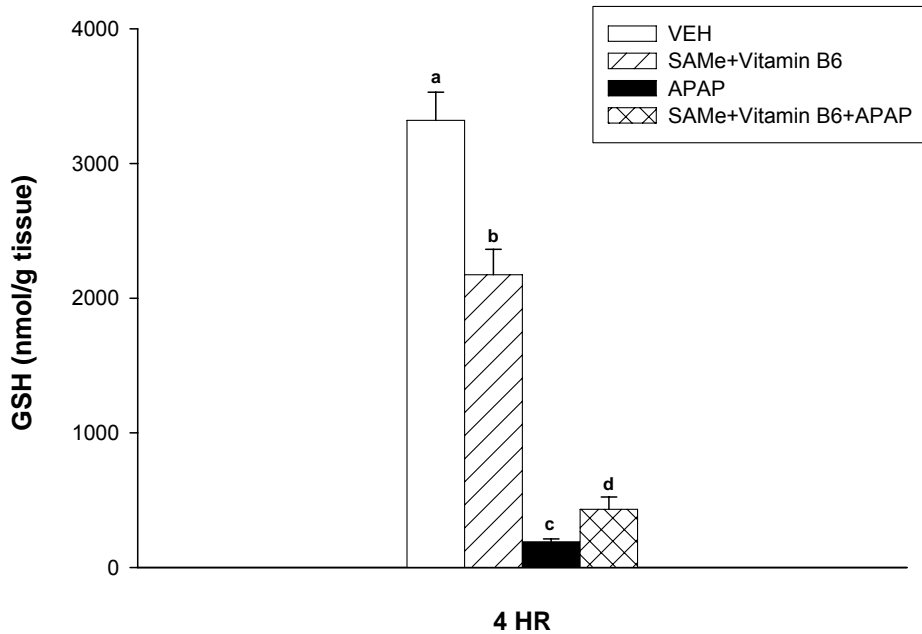


FIGURE 48. Total hepatic glutathione levels following APAP injection in C57Bl/6 mice.

Animals were randomly divided into vehicle (VEH), acetaminophen treated (APAP), SAmE 500 mg/kg + vitamin B₆ 3.84 mg/kg treated (SAmE + Vitamin B₆) and SAmE + Vitamin B₆ and APAP treated (SAmE + Vitamin B₆ + APAP). Values represent mean ± S.E.M., *n* = 4–10 mice per group. Total hepatic GSH levels were measured 4 hours after injection (i.p.) of 300 mg/kg APAP. Groups with unlike superscripts are statistically different.

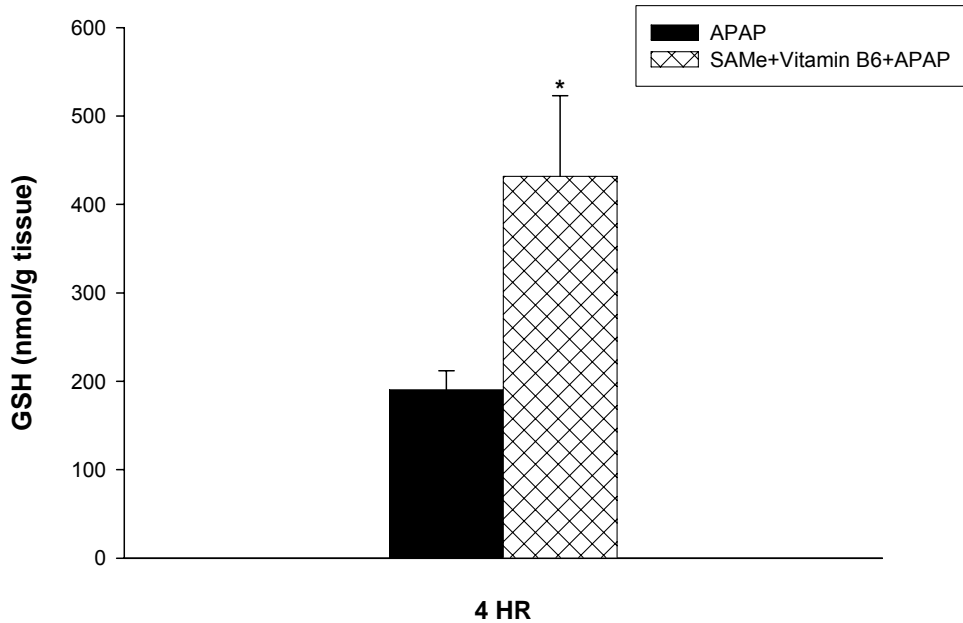


FIGURE 49. Total hepatic glutathione levels following APAP injection in C57Bl/6 mice dosed with APAP or SAMe + Vitamin B6 just prior to APAP. Animals were randomly divided into acetaminophen treated (APAP) and SAMe + Vitamin B6 and APAP treated (SAMe + Vitamin B6 + APAP). Values represent mean \pm S.E.M., n = 4–10 mice per group. Total hepatic GSH levels were measured 4 hours after injection (i.p.) of 300 mg/kg APAP. An asterisk (*) marks a statistically significant difference ($p < 0.05$).

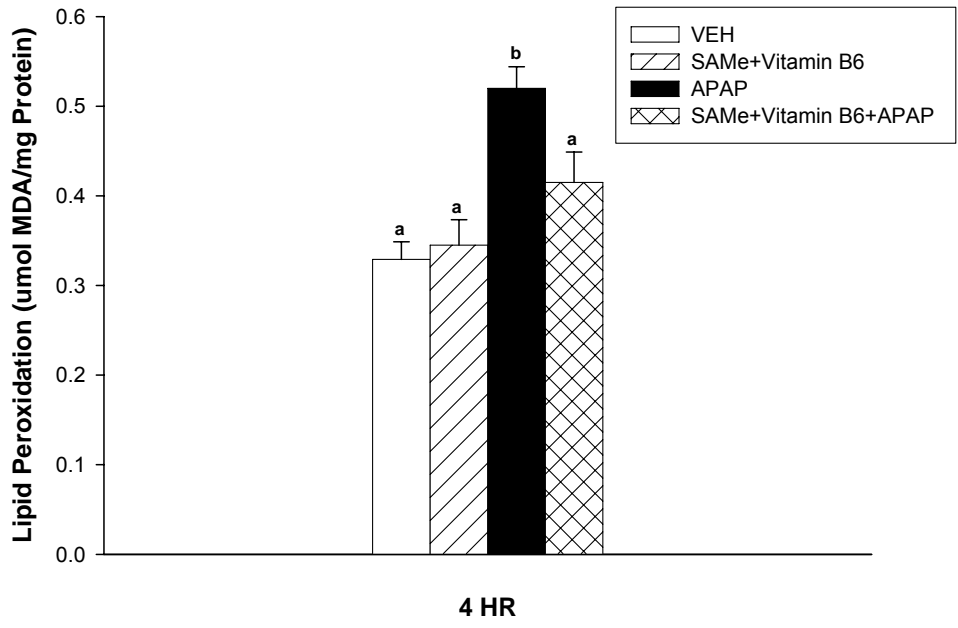


FIGURE 50. Lipid Peroxidation following APAP injection in C57Bl/6 mice.

Animals were randomly divided into vehicle (VEH), acetaminophen treated (APAP), SAME 500 mg/kg + Vitamin B₆ treated (SAME + Vitamin B₆) and SAME + Vitamin B₆ and APAP treated (SAME + Vitamin B₆ + APAP). Values represent mean S.E.M., *n* = 4–10 mice per group. Lipid peroxidation was measured 4 hours after injection (i.p.) of 300 mg/kg APAP. Lipid peroxidation was measured as μmol malondialdehyde (MDA)/mg protein. Groups with unlike superscripts are statistically different.

4.3 Treatments Post-APAP

Effect of 250 mg/kg APAP 1 hour prior to 500 mg/kg SAME dose

After reviewing the initial results of the abilities of SAME to attenuate APAP-induced hepatotoxicity, the next step was to investigate the possibilities of SAME as an antidote to APAP overdose. This experiment enabled us to mimic what occurs in the clinical setting since treatment is administered after overdosing on APAP. This experiment provided some of the most valuable information regarding the possibility of SAME as a true antidote to APAP-induced hepatic toxicity.

Experiments were set up as before. Animals were randomly divided into 4 treatment groups with 5 animals per group (TABLE 25): control (VEH), APAP (APAP), SAME (SAME) and APAP injected 1 hour prior to the administration of SAME (SAME + APAP). Animals were fasted overnight from 1700 – 0900 h, but had free access to water. The following morning at 0900 h, animals were injected with water or APAP. At 1000 h, the SAME treatment was administered i.p. to the SAME + APAP group. Three hours later (4 hours post-APAP), the mice were anesthetized with CO₂, blood was collected via cardiac puncture and livers were excised and placed on ice-cold Krebs buffer.

APAP-induced hepatotoxicity was reduced by the SAME treatment. APAP hepatic toxicity was confirmed by increased liver weight (TABLE 26), elevated plasma ALT values (TABLE 27) and histological alterations of visible centrilobular necrosis (FIGURE 51). APAP increased liver weight when directly compared to

the VEH group. SAME treatment doses prevented the increase in liver weight attributed to APAP treatment. APAP increased ($p < 0.05$) plasma ALT levels above VEH values 4 hours post-APAP injection. SAME injection post-APAP prevented the dramatic APAP-induced rise in plasma ALT levels. These findings support the possibility of SAME as an antidote to APAP toxicity.

Examination by light microscopy showed normal morphology for the VEH treated group. In the groups treated only with APAP, focal centrilobular changes were observed (FIGURE 51). The SAME and SAME + APAP groups showed no differences when directly compared to the VEH group. The SAME + APAP group showed no damage within the centrilobular region (FIGURES 51).

GSH levels were markedly diminished 4 hours after APAP injection when compared to the VEH group (FIGURE 52). SAME administration (500 mg/kg) did not alter baseline GSH levels probably due to the ratio of SAH:SAME levels. Amazingly, the group that had been administered SAME 1 hour after APAP completely recovered GSH levels when compared to the VEH group (FIGURE 52). SAME was able to replenish GSH lost within 3 hours of administration. Results seen were better than when SAME was given as a pretreatment to APAP. Once again, this was most likely due to the mechanistic properties of SAME in the production of GSH. If SAH levels are favored (no GSH is needed), the reaction is reversed and no GSH is produced. However if GSH levels are low (as depicted in this experiment), then the reaction proceeds forward and GSH is produced.

Group	APAP	SAMe
VEH	No	No
APAP	Yes	No
SAMe	No	Yes
SAMe + APAP	Yes	Yes

TABLE 25. SAMe and APAP treatment regimens.

All injections were i.p. APAP and VEH were administered 0900 h. SAMe was administered at 1000 h at a dose of 500 mg/kg.

Group	SAMe dose (mg/kg)	Time post-APAP treatment (h)	Body weight (g)	Liver weight (g/10g body wt.)
VEH	0	4	16.4 ± 0.4	0.47 ± 0.01 ^a
APAP	0	4	16.8 ± 0.4	0.55 ± 0.01 ^b
SAMe	500	4	17.0 ± 0.3	0.47 ± 0.01 ^a
SAMe + APAP	500	4	16.4 ± 0.3	0.50 ± 0.01 ^a

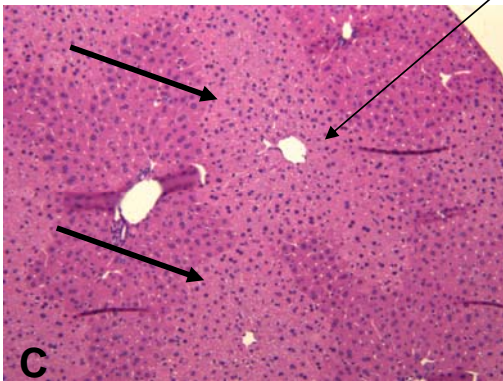
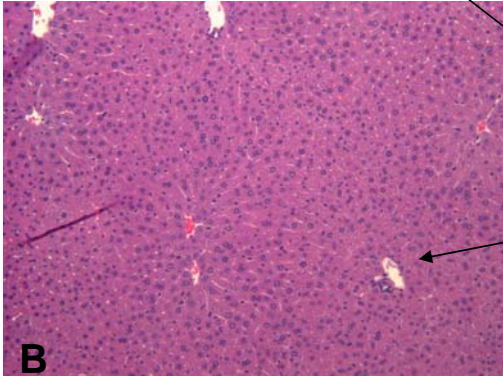
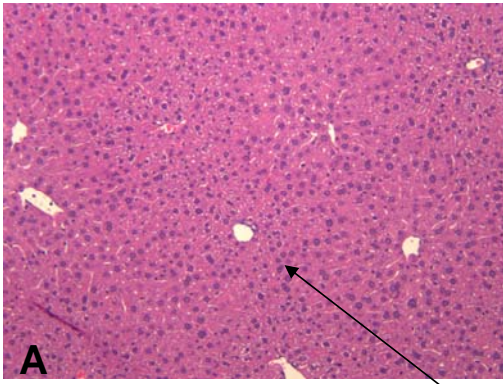
TABLE 26. Body and liver weight following APAP administration.

APAP was administered as 250 mg/kg (15 ml/kg), i.p. 1 hour prior to SAMe (500 mg/kg (5 ml/kg), i.p.). Statistical differences ($p < 0.05$) are denoted by superscripts. Values are reported as mean ± SEM with $n = 4-10$ animals/group. Groups with different superscripts denote statistical difference ($p < 0.05$) within each treatment experiment.

Group	SAMe dose (mg/kg)	Time post-APAP treatment (h)	ALT (U/l)
VEH	0	4	96 ± 25 ^a
APAP	0	4	12476 ± 407 ^b
SAMe	500	4	84 ± 16 ^a
SAMe + APAP	500	4	403 ± 107 ^c

TABLE 27. Plasma ALT levels following APAP administration.

Groups represent vehicle (VEH); acetaminophen (APAP) injected i.p. 250 mg/kg; S-Adenosyl-L-methionine (SAMe) i.p. injected 500 mg/kg; SAMe and acetaminophen (SAMe + APAP) at doses listed for individual agents. Unlike letters (a, b) indicate groups are different ($p < 0.05$) from each other using a one way ANOVA and Tukey's post hoc test. Values represent mean ± S.E.M. with $n = 4-10$ per group.



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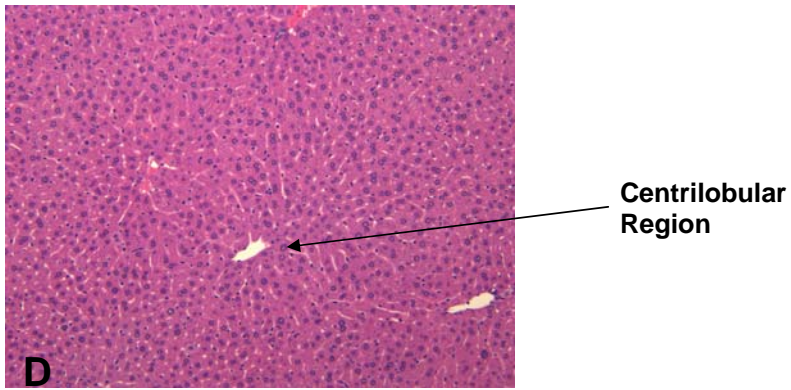


FIGURE 51. Histological examination 4 hours after APAP injection.

Hepatic tissues were prepared and stained with H&E. Representative slides of (A) vehicle (VEH); (B) SAME (500 mg/kg) treated; (C) 4 hours post-acetaminophen (APAP) injection and (D) mice treated with SAME 1 hour after APAP. Normal morphology was noted in the VEH, SAME and SAME + APAP animals. Moderate damage was noted in the APAP group around the centrilobular region as shown by the arrows in slide C. Magnification is 200 \times .

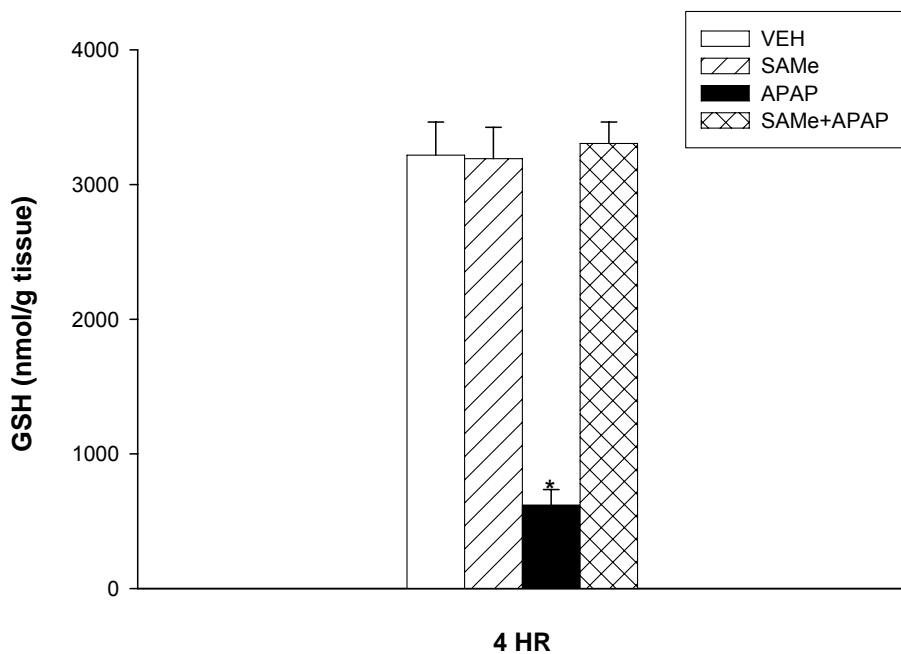


FIGURE 52. SAmE antidote studies: Total hepatic glutathione levels 4 hours post-APAP injection in C57Bl/6 mice.

Animals were randomly divided into vehicle (VEH), acetaminophen treated (APAP), SAmE 500 mg/kg treated (SAmE) and SAmE + APAP treated (SAmE + APAP). Values represent mean \pm S.E.M., $n = 4-10$ mice per group. Total hepatic GSH levels were measured 4 hours after injection (i.p.) of 250 mg/kg APAP. An asterisk (*) marks statistically significant difference ($p < 0.05$) from the rest of the treatment groups.

Effect of 250 mg/kg APAP 1 hour prior to 204 mg/kg NAC dose

After reviewing the initial results of the abilities of SAME to attenuate APAP-induced hepatotoxicity and comparing that to results seen with NAC, we wanted to test the abilities of SAME vs. NAC in attenuating APAP-induced hepatic toxicity. Since NAC is the main antidote of choice for APAP related overdoses, we wanted to investigate the protective effects of NAC in mice and directly compare the effects to SAME. This experiment provided valuable data regarding the possibility of SAME vs. NAC as an effective antidote for hepatic toxicity resulting from APAP overdose.

Animals were randomly divided into 4 treatment groups with 5 animals per group (TABLE 28): control (VEH), APAP (APAP), NAC (NAC) and APAP treated 1 hour prior to the administration of NAC (NAC + APAP). Animals were fasted overnight (1700 – 0900 h) and had free access to water. The following morning at 0900 h, animals were injected with either water or APAP. At 1000 h, the NAC treatment was administered i.p. to the NAC + APAP group. Three hours later (4 hours post-APAP), the mice were anesthetized with CO₂, blood was collected via cardiac puncture and livers were excised and stored on ice-cold Krebs buffer for analysis.

APAP-induced hepatotoxicity was reduced by the NAC treatment, but not to the same extent as the animals given SAME. Just as before, APAP hepatic toxicity was confirmed by increased liver weight, elevated plasma ALT values and histological alterations. APAP increased liver weight when directly

compared to the VEH group (TABLE 29), but NAC treatment doses were not able to prevent the increase in liver weight attributed to APAP. APAP increased ($p < 0.05$) plasma ALT levels (TABLE 30) above VEH 4 hours post-APAP injection. NAC injection post-APAP minimally prevented APAP-induced rise in plasma ALT levels.

Examination by light microscopy showed normal morphology for the VEH treated group. In the groups treated only with APAP, focal centrilobular changes were observed (FIGURE 53). The NAC group showed no differences when directly compared to the VEH group. The NAC + APAP group showed less damage within the centrilobular region when compared to the APAP group. However, there was less protection seen in the NAC + APAP group vs. the SAME + APAP animal set.

GSH levels were markedly diminished after APAP injection when compared to the VEH group (FIGURE 54). NAC administration did not alter baseline GSH levels. This may in fact be due to similar reasons of why we don't see an increase in GSH levels when SAME is administered. It is possible that if there is not a necessity to produce more GSH, the reaction does not move forward. Instead, the excess treatment is eliminated from the body.

Similar to the results seen with SAME, the group that had been administered NAC 1 hour after APAP almost completely recovered GSH levels when compared to the VEH group (FIGURE 54). NAC was able to replenish GSH lost within 3 hours of administration. When directly comparing the recovery

GSH results of NAC + APAP vs. SAmE + APAP, we did not see any statistically significant difference. This does however raise some interesting questions about whether the use of NAC or SAmE should be used during emergency room situations involving APAP overdose cases. While both were able to produce a recovery of GSH levels, NAC was not able to prevent rises in liver weights, SAmE produced better recovery of plasma ALT values when given post-APAP and histology favors SAmE as a better antidote in prevention of centrilobular damage caused by high amounts of APAP. All of the GSH experiments thus far suggest that this depletion of the compound may not be the major mediator of APAP-induced liver toxicity.

Group	APAP	NAC
VEH	No	No
APAP	Yes	No
NAC	No	Yes
NAC + APAP	Yes	Yes

TABLE 28. NAC and APAP treatment regimens.

All injections were given i.p. APAP and VEH were administered 0900 h. NAC was administered at 1000 h at a dose of 204 mg/kg.

Group	NAC dose (mg/kg)	Time post-APAP treatment (h)	Body weight (g)	Liver weight (g/10g body wt.)
VEH	0	4	18.0 ± 0.0	0.47 ± 0.01 ^a
APAP	0	4	17.6 ± 0.4	0.54 ± 0.01 ^b
NAC	204	4	17.6 ± 0.4	0.45 ± 0.01 ^a
NAC + APAP	204	4	18.0 ± 0.6	0.51 ± 0.01 ^b

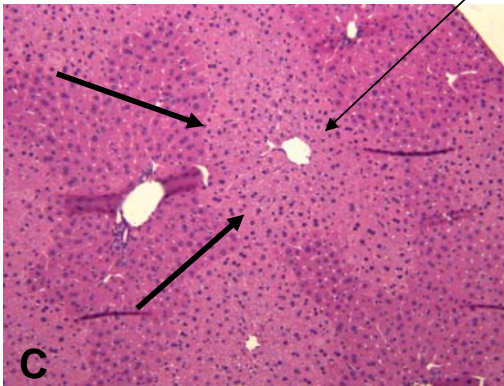
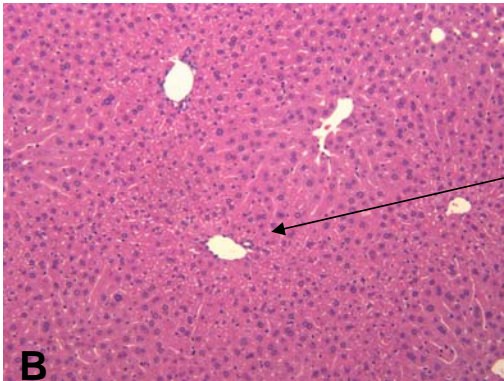
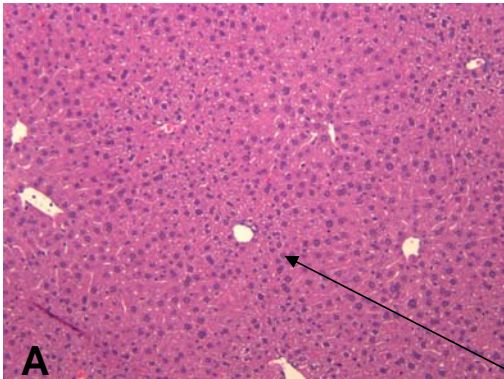
TABLE 29. Body and liver weight following APAP administration.

APAP was administered as 250 mg/kg (15 ml/kg), i.p. 1 hour prior to NAC (204 mg/kg (5 ml/kg), i.p.). Statistical differences ($p < 0.05$) are denoted by superscripts.

Group	NAC dose (mg/kg)	Time post-APAP treatment (h)	ALT (U/l)
VEH	0	4	74 ± 26 ^a
APAP	0	4	12158 ± 1570 ^b
NAC	204	4	47 ± 10 ^a
NAC + APAP	204	4	801 ± 295 ^c

TABLE 30. Plasma ALT levels following APAP administration.

Groups represent vehicle (VEH); acetaminophen (APAP) injected i.p. 250 mg/kg; N-acetylcysteine (NAC) i.p. injected 204 mg/kg; NAC and acetaminophen (NAC + APAP) at doses listed for individual agents. Unlike letters (a, b) indicate groups are different ($p < 0.05$) from each other using a one way ANOVA and Tukey's post hoc test. Values represent mean ± S.E.M. with $n = 4-5$ per group.



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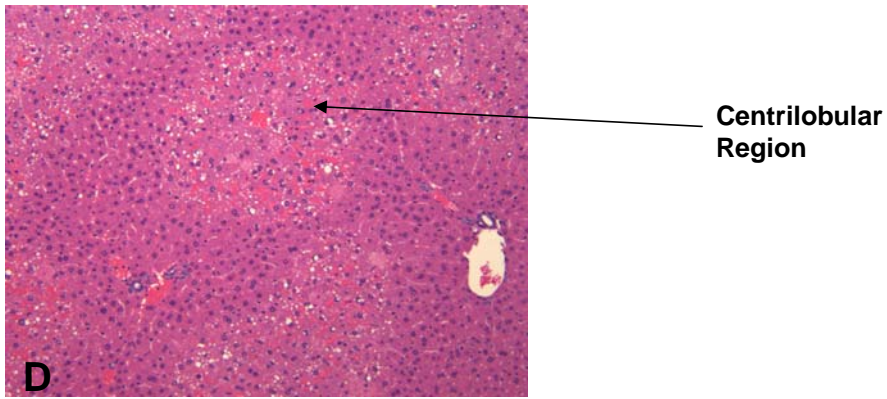


FIGURE 53. Histological examination 4 hours after APAP injection.

Hepatic tissues were prepared and stained with H&E. Representative slides of (A) vehicle (VEH); (B) NAC (204 mg/kg) treated; (C) 4 hours post-acetaminophen (APAP) injection and (D) mice treated with NAC 1 hour after APAP. Normal morphology was noted in the VEH and NAC animals. Moderate damage was noted in the APAP group as shown by the arrows in slide C. There was only limited protection shown in animals dosed with NAC 1 hour post-APAP. Magnification is 200 \times .

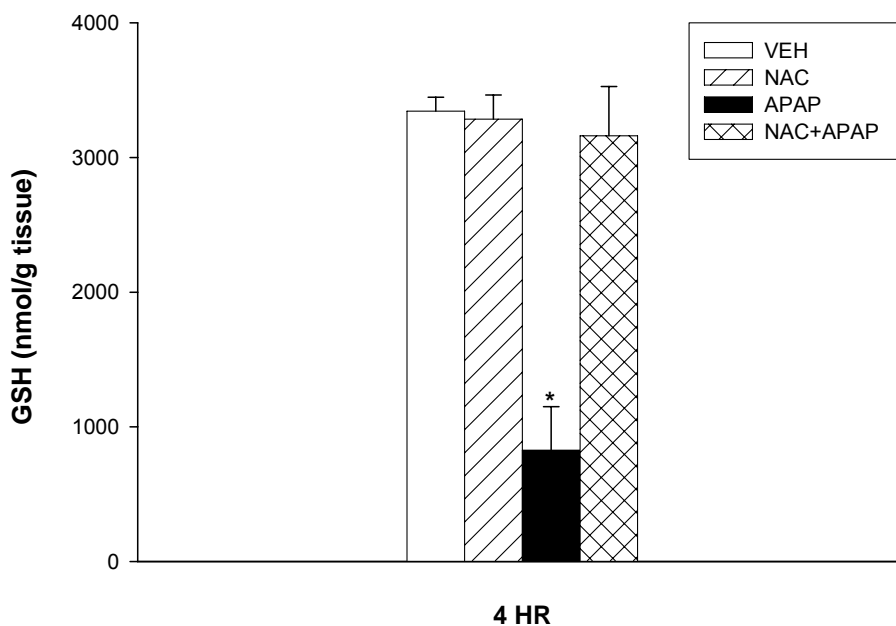


FIGURE 54. NAC antidote studies: Total hepatic glutathione levels 4 hours post-APAP injection in C57Bl/6 mice.

Animals were randomly divided into vehicle (VEH), acetaminophen treated (APAP), NAC 204 mg/kg treated (NAC) and NAC + APAP treated (NAC + APAP). Values represent mean \pm S.E.M., $n = 4-5$ mice per group. Total hepatic GSH levels were measured 4 hours after injection (i.p.) of 250 mg/kg APAP. An asterisk (*) marks statistically significant difference ($p < 0.05$) from the rest of the treatment groups.

4.4 Mitochondrial Isolation: Treatments Post-APAP

Effect of 250 mg/kg APAP 1 hour prior to 500 mg/kg SAME dose

Glutathione can be found in the smooth endoplasmic reticulum, the cytosol and the mitochondria of the cell. Only about 10-15% of the cell's GSH is located in the mitochondria (Fernandez-Checa and Kaplowitz, 2005; Fernandez-Checa et al., 2002). More importantly, GSH cannot be synthesized in the mitochondria. All of the cell's mitochondrial GSH is brought in by carriers. It has been found that the cytosolic GSH is the first line of defense against toxic metabolites such as NAPQI, but under severe circumstances APAP is known to deplete mitochondrial GSH levels (Reid et al., 2005; Masubuchi et al., 2005; Kon et al., 2004; Fernandez-Checa et al., 1998). The effect of SAME on mitochondrial GSH is not known. These studies examined the dose- and time- dependent effects of SAME and APAP on mitochondrial GSH levels. The glutathione reductase and glutathione peroxidase studies helped to identify the effects of SAME and APAP on the enzymes critical in the balance of oxidized and reduced GSH. Information is not available in the literature regarding the effects of SAME and APAP on these enzymes. Consequently, these studies provide new information that may be of importance regarding the mechanism for SAME attenuation of APAP hepatic toxicity.

Body weight and liver weights for all treated animals are shown in Table 31. Liver weight was increased relative to VEH 4 hours after APAP treatment ($p < 0.05$). Liver weight was similar between SAME + APAP, VEH and SAME groups.

APAP hepatic toxicity was confirmed by increased liver weight. APAP increased liver weight when directly compared to the VEH group (TABLE 31), and SAME treatment doses were able to prevent the increase in liver weight attributed to APAP, just as before.

Mitochondria isolation was confirmed by measuring LDH activity. LDH levels were higher in the supernatant relative to mitochondria (FIGURE 55). Mitochondria LDH reflects total amount as mitochondria were suspended in 1 ml of buffer (refer to methods section regarding mitochondrial isolation).

Mitochondrial GSH levels were severely depleted 4 hours post-APAP injection (FIGURE 56). SAME, when given 1 hour after APAP was not able to prevent this decrease in GSH in the mitochondria or the supernatant. This could possibly be due to inadequate time for GSH to be carried into the mitochondria. SAME GSH levels were not statistically different from the VEH in the mitochondria or supernatant. Further investigation regarding mitochondrial GSH will need to be done at later time frames to fully illustrate the protective effects (if any) of SAME on mitochondrial GSH.

Nitrotyrosine levels were investigated using Western blotting. Initial results suggest that SAME reduces the amounts of nitrotyrosine-adducted proteins. This, to our knowledge, is the first data suggesting that SAME might in fact reduce APAP-induced nitrosylated proteins in the mitochondria (FIGURE 57).

Group	SAMe dose (mg/kg)	Time post-APAP treatment (h)	Body weight (g)	Liver weight (g/10g body wt.)
VEH	0	4	26.8 ± 0.5	0.40 ± 0.01 ^a
APAP	0	4	24.8 ± 0.4	0.51 ± 0.02 ^b
SAMe	500	4	24.8 ± 0.8	0.40 ± 0.02 ^a
SAMe + APAP	500	4	26.8 ± 0.5	0.42 ± 0.01 ^a

TABLE 31. Body and liver weight following APAP administration.

APAP was administered as 250 mg/kg (15 ml/kg), i.p. 1 hour prior to SAMe (500 mg/kg (5 ml/kg), i.p.). Statistical differences ($p < 0.05$) are denoted by superscripts.

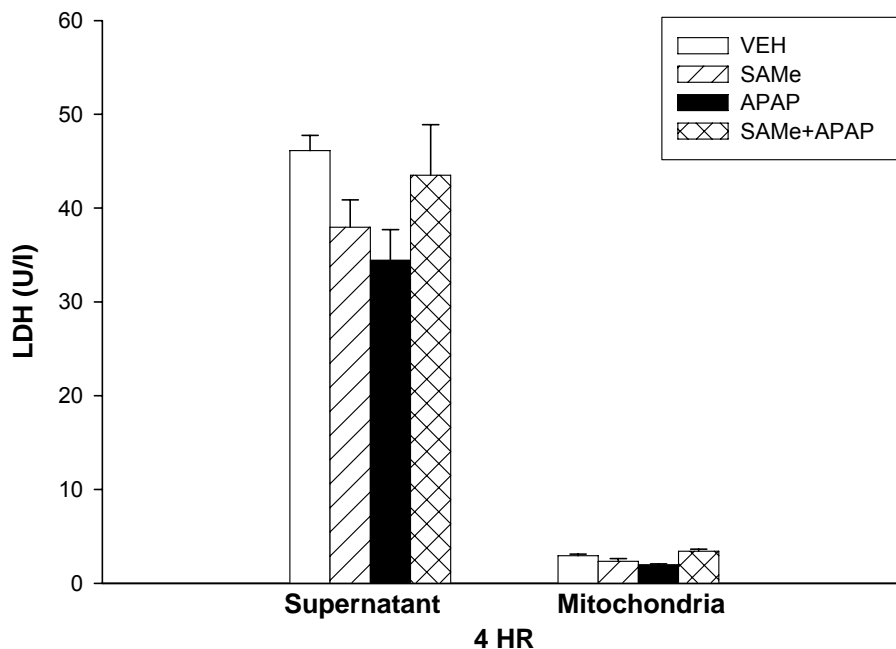


FIGURE 55. LDH activity 4 hours post-APAP treatment.

Mitochondrial and non-mitochondrial LDH activity was measured 4 hours post-APAP injection. Values are expressed as U/l. Groups indicate vehicle (VEH), SAmE treated (SAmE), acetaminophen treated (APAP) and mice treated with SAmE 1 hour after APAP (SAmE + APAP). SAmE was administered as 500 mg/kg, i.p. APAP was injected i.p. at a dose of 250 mg/kg (15 ml/kg). Values represent mean \pm SEM with n=4-5 animals/group. Mitochondrial values were different from respective supernatant (nonmitochondrial) samples.



FIGURE 56. Total glutathione levels post-APAP treatment in supernatant and mitochondria.

Non-mitochondrial GSH levels were measured 4 hours after APAP injection. Values are expressed as $\mu\text{mol/g}$ tissue. Groups are denoted as: vehicle (VEH), SAMe treated (SAMe), acetaminophen treated (APAP) and mice treated with SAMe and APAP (SAMe + APAP). Values represent mean \pm SEM with $n = 4-5$ animals/group. Groups with dissimilar superscripts were statistically different ($p < 0.05$) from each other.

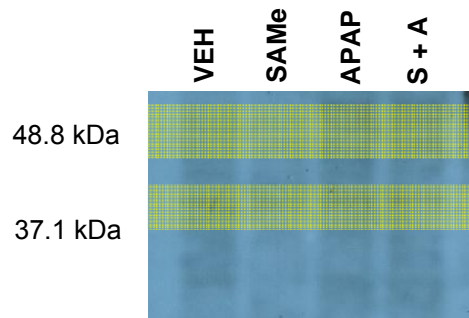


FIGURE 57. A nitrotyrosine Western blot of samples 4 hours post-APAP injection.

Lanes are denoted as: Lane 1 vehicle, Lane 2 SAmE, Lane 3 APAP, Lane 4 SAmE + APAP (S + A). SAmE was administered at 500 mg/kg. APAP was injected i.p. at a dose of 300 mg/kg (15 ml/kg). VEH animals were injected i.p. with water (15 ml/kg). Highlighted regions represent areas of protection vs. damage.

Effect of 250 mg/kg APAP 1 hour prior to 204 mg/kg NAC dose

A direct comparison between the two treatments were established to study the effects on mitochondrial GSH involving NAC or SAME. To our knowledge, this type of study had not yet been conducted. This experiment allowed us to investigate both the ability of SAME as well as NAC to not only synthesize GSH, but to also get it into the mitochondria (or protect the mitochondria from GSH depletion).

Body weight and liver weights for all treated animals are shown in Table 32. Liver weight was increased relative to VEH 4 h after APAP treatment ($p < 0.05$). Liver weight was similar between NAC + APAP, VEH and NAC groups.

APAP hepatic toxicity was confirmed by increased liver weight. APAP increased liver weight when directly compared to the VEH group (TABLE 32), and NAC treatment doses were able to prevent the increase in liver weight attributed to APAP.

Mitochondria isolation was confirmed by measuring LDH, similar to the SAME studies. LDH levels, as expected, were higher in the supernatant relative to mitochondria (Figures 58). Mitochondria LDH reflects total amount as mitochondria were suspended in 1 ml.

Mitochondrial GSH levels were severely depleted 4 hours post-APAP injection. NAC, similar to SAME, when given 1 hour after APAP was not able to prevent this decrease in GSH in the mitochondria or the supernatant (FIGURE 59). This could possibly be due to inadequate time for GSH to be carried into the

mitochondria. NAC GSH levels were not statistically different from the VEH in the mitochondria or supernatant. Further investigation regarding mitochondrial GSH will need to be done.

Nitrotyrosine levels were measured using western blotting. Initial results suggest that, unlike SAME, there is little to no reduction in the amount of nitrotyrosine-adducted proteins. NAC had no effect in producing nitrosylated proteins (FIGURE 60) when compared to VEH, but it also didn't prevent APAP-induced effects on the production of these adducted proteins.

Group	NAC dose (mg/kg)	Time post-APAP treatment (h)	Body weight (g)	Liver weight (g/10g body wt.)
VEH	0	4	25.6 ± 0.4	0.37 ± 0.01 ^a
APAP	0	4	26.4 ± 0.7	0.45 ± 0.02 ^b
NAC	204	4	25.6 ± 0.4	0.37 ± 0.02 ^a
NAC + APAP	204	4	27.0 ± 0.9	0.37 ± 0.01 ^a

TABLE 32. Body and liver weight following APAP administration.

APAP was administered as 250 mg/kg (15 ml/kg), i.p. 1 hour prior to NAC (204 mg/kg (5 ml/kg), i.p.). Statistical differences ($p < 0.05$) are denoted by superscripts.

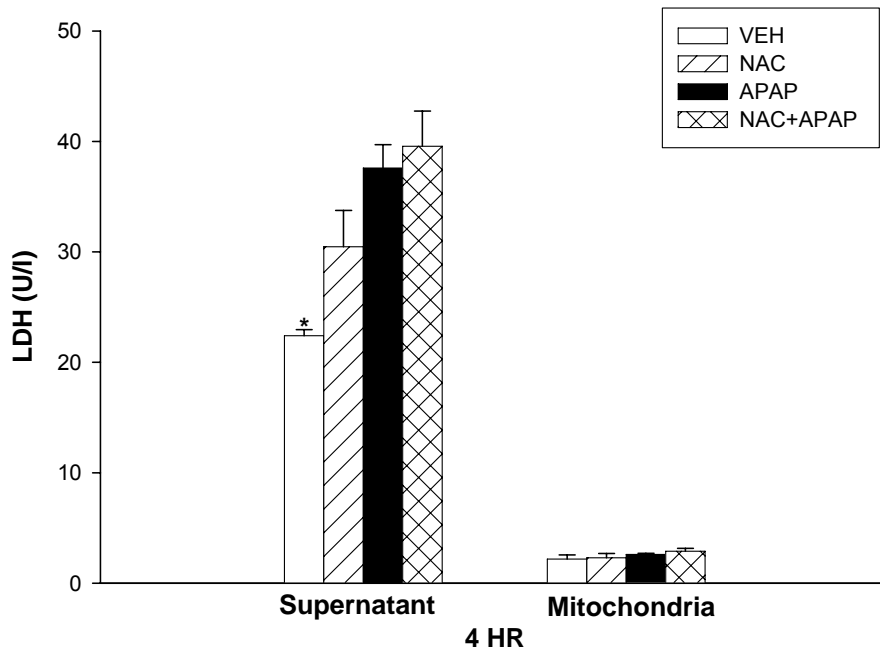


FIGURE 58. LDH activity 4 hours post-APAP treatment.

Mitochondrial and non-mitochondrial LDH activity was measured 4 hours post-APAP injection. Values are expressed as U/l. Groups indicate vehicle (VEH), NAC treated (NAC), acetaminophen treated (APAP) and mice treated with NAC 1 hour after APAP (NAC + APAP). NAC was administered as 204 mg/kg, i.p.. APAP was injected i.p. at a dose of 250 mg/kg (15 ml/kg). Values represent mean \pm SEM with n = 4-5 animals/group. Mitochondrial values were different from respective supernatant (nonmitochondrial) samples. An asterisk (*) denotes statistical difference ($p < 0.05$) within group.

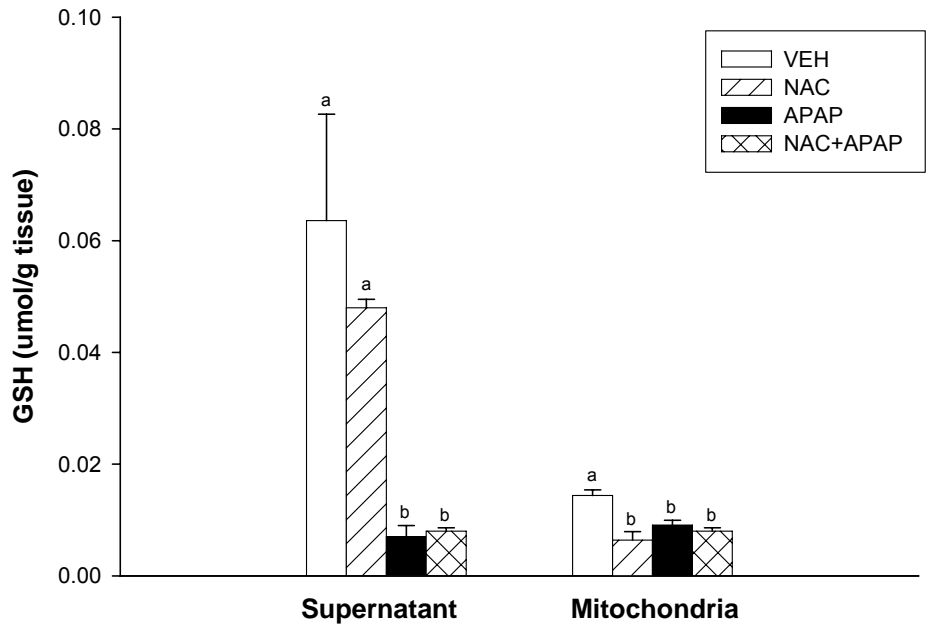


FIGURE 59. Total glutathione levels post-APAP treatment in supernatant and mitochondria.

Non-mitochondrial GSH levels were measured 4 hours after APAP injection and 3 hours post NAC injection. Values are expressed as $\mu\text{mol/g}$ tissue. Groups are denoted as: vehicle (VEH), NAC treated (NAC), acetaminophen treated (APAP) and mice treated with NAC and APAP (NAC + APAP). Values represent mean \pm SEM with $n = 4-5$ animals/group. Groups with dissimilar superscripts were statistically different ($p < 0.05$) from each other.

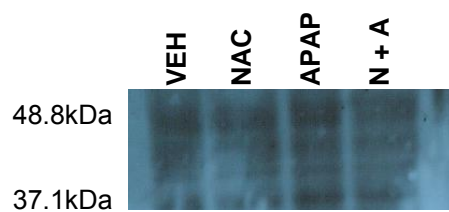


FIGURE 60. Western Blot of samples 4 hours post-APAP injection.

Lanes are denoted as: Lane 1 vehicle, Lane 2 NAC, Lane 3 APAP, Lane 4 NAC + APAP (N + A). NAC was administered as 204 mg/kg. APAP was injected i.p. at a dose of 300 mg/kg (15 ml/kg). VEH animals were injected i.p. with water (15 ml/kg).

4.5 HPLC Analysis

Investigation into the production and quantification of particular APAP metabolites was undertaken to better understand the mechanisms of action for both SAME and APAP. Research techniques for quantification of both drugs have been developed (Aboul-Enein and Abu-Zaid, 2001; Dietze et al., 1997; Esteban et al., 1992; Esteban et al., 1993; Wang, 2001; Fischer et al., 1981; Hamedani et al., 1993; Jensen et al., 2004; Miners et al., 1984; Vertzoni et al., 2003; Wang and Cederbaum, 2006; Moldéus, 1978), but none of the studies examined the effects of SAME on APAP metabolites. Of the procedures listed above, we chose to use the methods of Vertzoni et al. (2003) and Moldéus (1978). These two methods gave detailed instruction on the procedures of isolating the metabolites along with flow rates and column specificities, plus had a fairly short retention time for each sample allowing for an efficient overall procedure (roughly 20 minutes/sample).

We quantified APAP and two of its metabolites, sulfated- and glucuronidated-APAP (Figures 61-64). Data represents APAP and sulfated-APAP concentration differences between animals treated with a dose of 300 mg/kg APAP i.p. (APAP) or animals injected (i.p.) with 500 mg/kg SAME just prior to 300 mg/kg APAP (SAME + APAP). HPLC analysis was done on plasma and hepatic tissue collected 1, 2 or 4 hours post-APAP or VEH injection (FIGURES 61 - 64). The glucuronidated-APAP showed no statistical difference between each of the two treatment groups (data not shown).

In the plasma, animals dosed with APAP had lower concentrations of APAP than animals given SAmE just prior to APAP. At both 1 and 2 hours post-APAP, the SAmE + APAP group showed statistically significant ($p < 0.05$) higher amounts of APAP (FIGURE 61). At 4 hours post-APAP there was still a general trend for higher concentrations of APAP, but it was not statistically significant. Also in the plasma, levels of the sulfated-APAP were higher in the SAmE + APAP group than when directly compared to the APAP dosed animals. For the sulfated-APAP, statistically significant differences between the APAP and SAmE + APAP groups were seen at 2 and 4 hours post-APAP injection (FIGURE 62).

In the liver, no significant difference was seen in APAP concentrations between the groups at 1, 2 or 4 hours post-injection (FIGURE 63). Both groups showed the same general trend of a steady decrease in the amount of APAP in the liver as time passed. However, there were statistically significant differences seen in the amount of sulfated-APAP concentrations between the two groups 1 hour post-APAP. In animals dosed with SAmE just prior to APAP, there was a higher amount of sulfated-APAP present in the liver (FIGURE 64). The other two time studies (2 and 4 hours post-APAP), did not show any significant differences between the two groups.

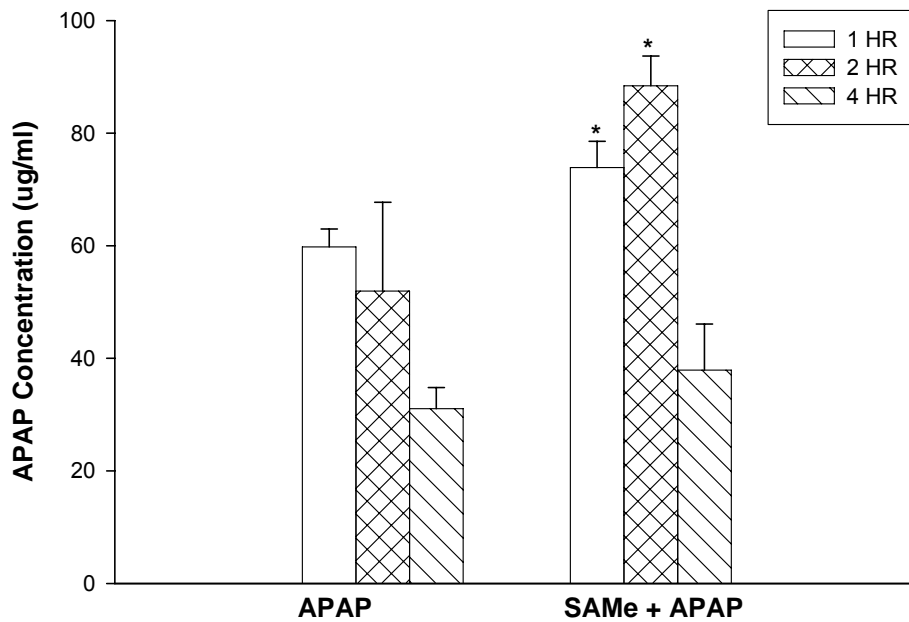


FIGURE 61. HPLC data of APAP concentration in plasma.

Plasma levels were measured 1, 2 and 4 hours after APAP injection. Values are expressed as $\mu\text{g/ml}$. Groups are denoted as: acetaminophen treated (APAP) and mice treated with SAME and APAP (SAME + APAP). Values represent mean \pm SEM with $n = 4-5$ animals/group. Groups with an asterisk (*) were statistically different ($p < 0.05$) from the other group at the same time frame.

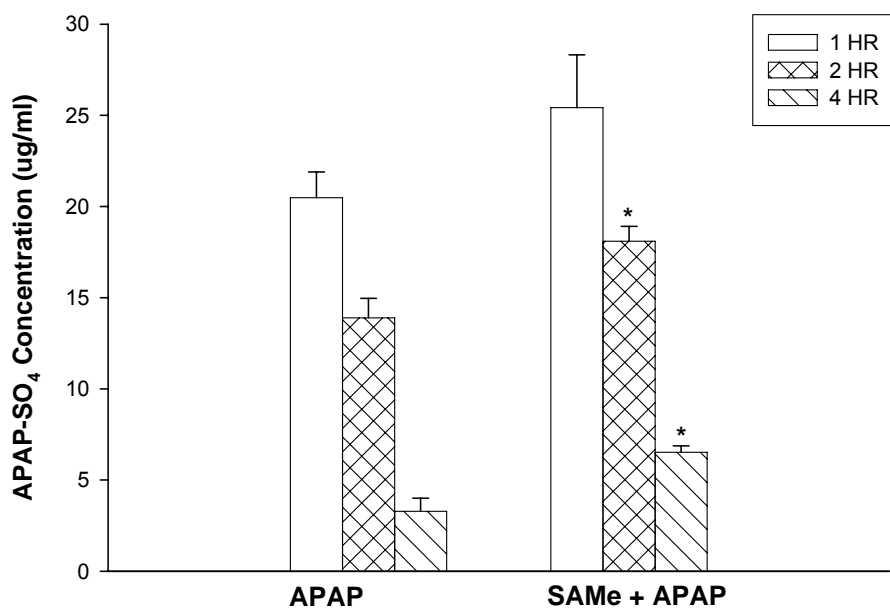


FIGURE 62. HPLC data of APAP-SO₄ concentration in plasma.

Plasma levels were measured 1, 2 and 4 hours after APAP injection. Values are expressed as $\mu\text{g/ml}$. Groups are denoted as: acetaminophen treated (APAP) and mice treated with SAME and APAP (SAME + APAP). Values represent mean \pm SEM with $n = 4-5$ animals/group. Groups with an asterisk (*) were statistically different ($p < 0.05$) from the other group at the same time frame.

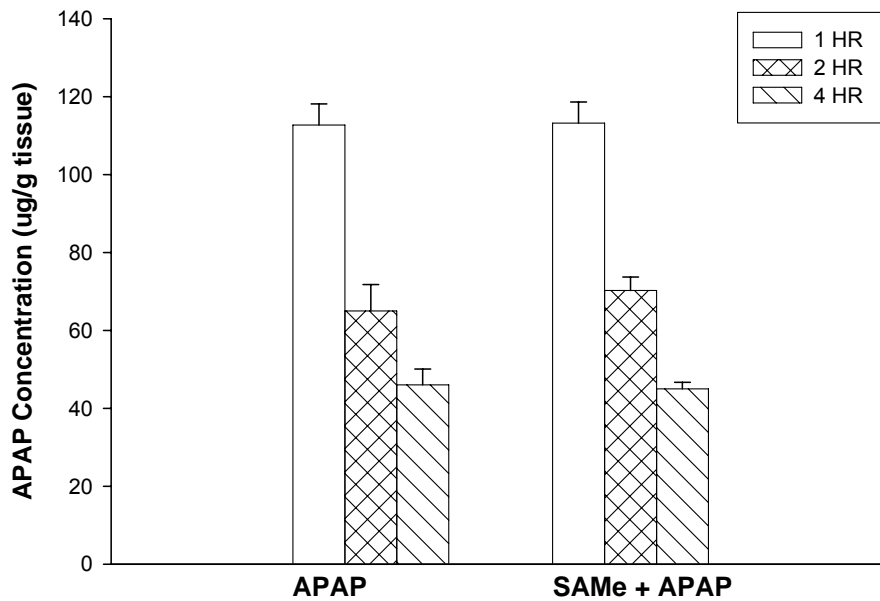


FIGURE 63. HPLC data of APAP concentration in liver.

Levels were measured 1, 2 and 4 hours after APAP injection. Values are expressed as $\mu\text{g/g}$ tissue. Groups are denoted as: acetaminophen treated (APAP) and mice treated with SAmE and APAP (SAmE + APAP). Values represent mean \pm SEM with $n = 4-5$ animals/group.

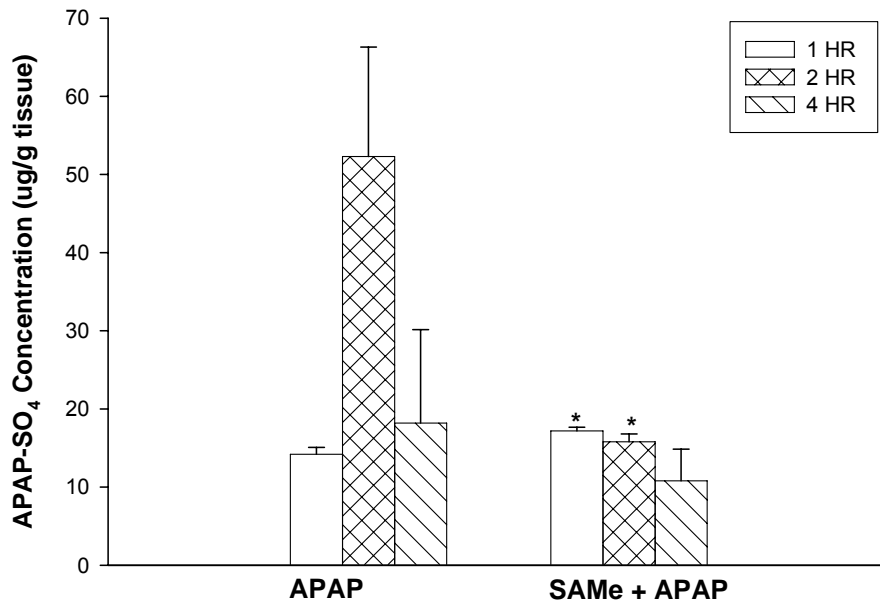


FIGURE 64. HPLC data of APAP-SO₄ concentration in liver.

Levels were measured 1, 2 and 4 hours after APAP injection. Values are expressed as $\mu\text{g/ml}$. Groups are denoted as: acetaminophen treated (APAP) and mice treated with SAME and APAP (SAME + APAP). Values represent mean \pm SEM with $n = 4-5$ animals/group. Groups with an asterisk (*) were statistically different ($p < 0.05$) from the other group at the same time frame.

4.6 Proteomics

As described previously, liver samples were prepared and shipped on ice to Dr. Serrine Lau at the University of Arizona. Dr. Lau confirmed our findings regarding protein carbonyls using an OxyBlot. The OxyBlot, similar to the results we received in our laboratory, showed a strong increase in carbonyl groups in the APAP group as compared to the control. The SAME group was similar to the control group and was significantly protected from APAP-induced protein oxidative modifications. Figure 65 shows the protein bands that were excised from the OxyBlot, trypsin digested, and analyzed on the LCQ ion-trap (Deca) LC/MS-MS at the University of Arizona. After trypsin digestion and LC/MS-MS analysis the data was analyzed with Turbo SEQUEST. Table 33 shows proteins that were identified in each band. The raw data was also analyzed by X!Tandem, a valid protein hit was required to be present in both the SEQUEST and X!Tandem data. A protein sequence database was created from Table 33 and used to search the raw data for 4-HNE adducts using PMOD. There were several 4-HNE adducts identified, two of which were confirmed using X!Tandem.

The two peptides with promising PMOD scores and confirmation using X!Tandem were then manually validated using SpecPlot (Figure 66 and 67). Both peptides showed excellent coverage of b and y ions and were both found with X!Tandem and PMOD, adding to the confidence of an accurate adducted peptide identification. Both proteins are compartmentalized in the mitochondria and recent literature suggests mitochondrial proteins are targets of APAP induced hepatocellular protein adduction, further validating our findings

(Masubuchi et al., 2005). These data, run in collaboration with Dr. Lau, support our hypothesis of SAME working via mechanisms other than GSH production. As reported here, SAME is also able to prevent protein modifications induced by APAP overdose.

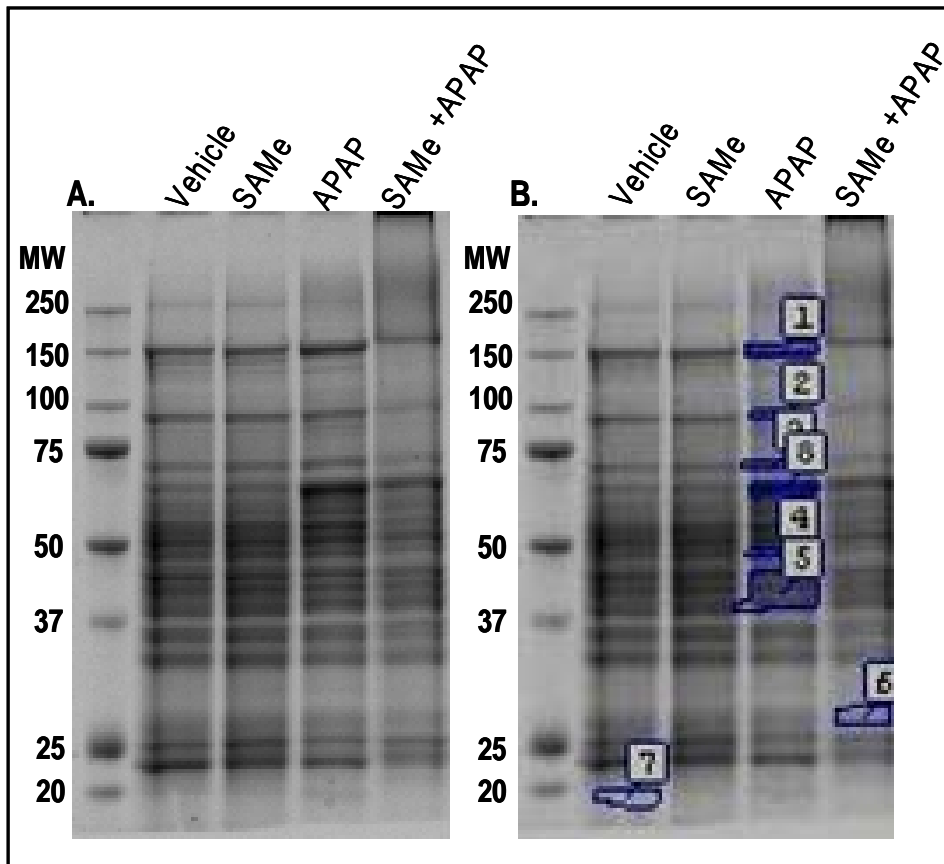


FIGURE 65. OxyBlot ran at the University of Arizona.

A) Gel electrophoresis of 40 μ g for all groups. The first lane was loaded with a molecular weight marker (kDa). B) Shows the protein bands excised for LC/MS-MS analysis from the same gel as in figure 67A.

Band	Protein	Accension number	Molecular weight	Num. of Peptides	% Coverage
1	GRP78 (glucose regulated protein precursor)	gj 2506545	72,422	3	7.6
2	Aldehyde Dehydrogenase	gj 24418394	98,709	14	18.2
	Sarcosine Dehydrogenase	gj 52000836	101,682	8	10.1
3	Transferrin	gj 20330802	76,724	14	27.0
	Heat Shock 70 Protein	gj 1661134	70,837	13	19.3
4	Tubulin alpha 2	gj 539933	50,036	3	3.8
	Carbamoyl-phosphate synthetase isoform 1	gj 82879179	164,618	14	35.0
5	Gent1 protein	gj 24657508	49,832	4	3.3
6	Dodecenoyl-Coenzyme A delta isomerase	gj 12836323	32,250	4	20.4
	Carbonic anhydrous III	gj 3198286	29,366	3	11.5
7	ADP-Ribosylation factor 2	gj 14714692	20,710	3	22.1
8	Gamma-Actin	gj 80956	41793	7	21.7

TABLE 33. Altered proteins post-APAP injection in male C57Bl/6 mice.

Shows proteins identified in each band excised. Each protein was required to have a minimum of 3 peptides identified from SEQUEST with reasonable spectra in order to be accepted. Also, the raw data was analyzed using X!Tandem, the above table represents proteins fitting the initial criteria as well as being represented in both SEQUEST and X!Tandem.

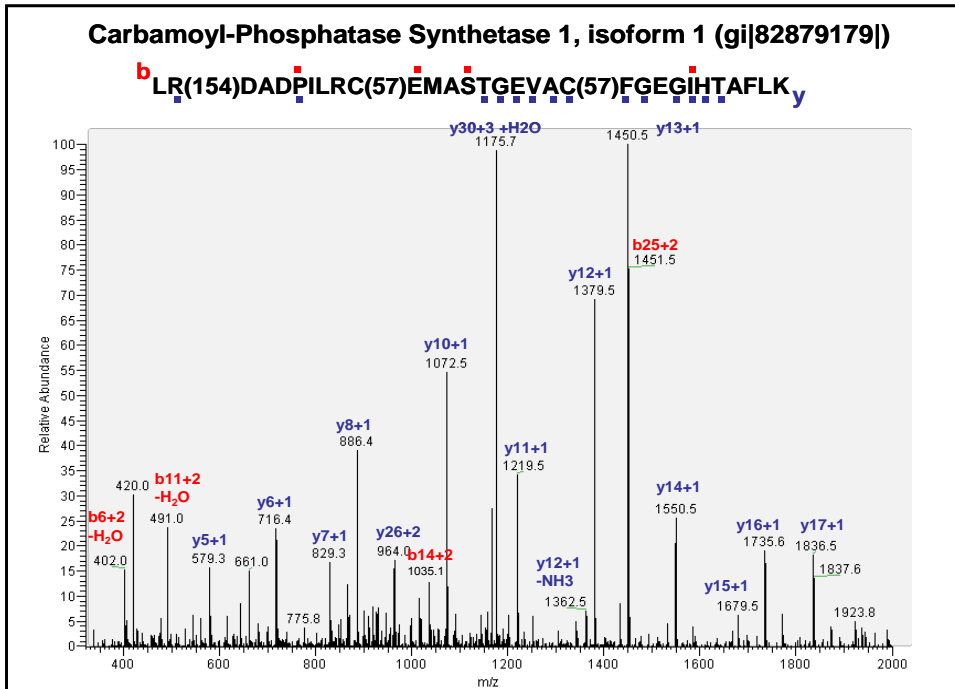


FIGURE 66. Manual validation of an adducted peptide from Carbamoyl-Phosphate Synthetase 1, isoform 1.

This protein is localized in the mitochondria. The second amino acid in this peptide (R) is adducted with a mass of 154 which corresponds with an addition of 4-HNE. The addition of 57 to the two cysteines is iodoacetamide and is a product of the reduction and alkylation step prior to the trypsin digestion. The dots above or below the amino acids of the peptide above represents ions identified within the spectrum.

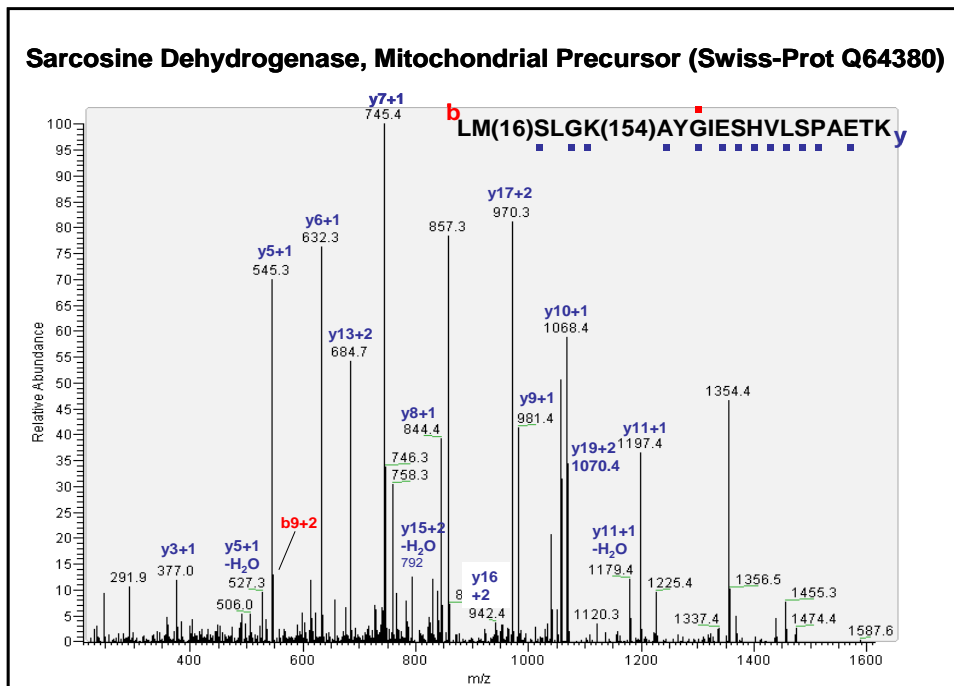


FIGURE 67. Manual validation of an adducted peptide from Sarcosine Dehydrogenase, mitochondrial precursor.

This protein is localized in the mitochondria. The sixth amino acid in this peptide (K) is adducted with a mass of 154 which corresponds with an addition of 4-HNE. The addition of 16 to the second amino acid (M) implies an oxidation of the sulfur on the side chain of the methionine. The dots above or below the amino acids of the peptide above represents ions identified within the spectrum.

CHAPTER V

Summary and Conclusion

5.1 General Toxicity

Physiological Changes Upon APAP Treatment

It is known that acetaminophen (APAP) is harmful if taken in excessive doses. Studies have been conducted involving doses of APAP up to 1000 mg/kg (Bray et al., 1992). Several treatment modalities have been investigated as antidotes for APAP toxicity and include N-acetylcysteine (NAC), vitamin E, melatonin (Sener et al., 2003) and S-Adenosyl-L-methionine (SAME) (Valentovic et al., 2004; Stramentinoli et al., 1979). However in past studies, the end result was typically animal survival (Stramentinoli et al., 1979; Bray et al., 1992). We wanted to look further into the mechanistic properties of not only APAP, but also how SAME (and NAC) attenuates APAP-induced hepatotoxicity.

Previous studies have shown APAP to be toxic to the liver; however the mechanism of APAP is not well characterized. The goal of this study was to help characterize APAP toxicity in an in vivo model and develop a hypothesis into potential treatments, such as S-Adenosyl-L-methionine (SAME), to attenuate APAP-induced hepatic toxicity. In order to make any substantial conclusions about the mechanism of APAP or by which the treatment worked, APAP toxicity was first characterized in our mouse model.

The dose of APAP used in this study ranged from 250 to 500 mg/kg. These doses were chosen based on the following: (1) the concentration of 250 mg/kg was the lowest dose used, but was determined to still yield toxic effects in the liver; (2) the 500 mg/kg SAME dose was chosen based on past literature (Bray et al., 1992; Stramentinoli et al., 1979) investigating APAP-induced liver injury.

APAP was directly toxic to the mouse liver via a dose- and time-dependent mechanism. APAP produced a loss of cell viability and increased oxidative stress as illustrated by the histology slides, increased plasma ALT values, and depleted glutathione (GSH) levels. This effect was seen at all doses of APAP. These results and initial characterization of SAME protection of APAP toxicity have been published by our laboratory (Valentovic et al., 2004). GSH levels were depleted up to 90% 4 hours post-injection in APAP dosed animals. These GSH values are comparable to what has been seen in prior studies (Arnaiz et al., 1995; Liu et al., 1999). APAP also induced toxicity in a time-dependent manner. Stronger indications of damage, i.e. higher plasma ALT values, lower GSH levels and histological alterations, were seen in livers excised with longer post-APAP injection times. However, damage was seen as early as 1 hour post-APAP injection. While the time-dependent toxicity of APAP has been reported, this is the first study to document hepatic damage within 60 minutes of APAP administration. These data demonstrate the rapidity with which APAP causes damage to the liver. This information also reiterates the importance of knowing the exact mechanism of APAP-induced hepatotoxicity and ways to

improve on treatments given to patients in desperate need of an antidote. These studies investigated a potential new remedy for APAP-overdose cases. By directly comparing the therapeutic effects to animals dosed with only APAP and by showing the benefits of SAME vs. NAC, we have provided new information regarding the treatment of APAP overdose cases.

5.2 Treatments Just Prior to APAP

The role of SAME in attenuating APAP-induced hepatic toxicity has been postulated to include production of GSH. To date, there have been many publications involving different dosing regimens of SAME just prior to different doses of APAP. However, in all of these previous studies, the primary parameter measured was survival rate. Previous studies had compared survival rates 21 and 48 hours after APAP treatment and have used mortality as an endpoint. SAME pretreatment (Stramentinoli et al., 1979; Bray et al., 1992) in these studies was assessed only by hepatic function and morphology in surviving animals 21 and 48 hours after APAP treatment. Evaluation at this late stage does not provide an accurate measurement of the effects of SAME on APAP-mediated GSH depletion, which is thought to be critical to the development of toxicity. Consequently, our current understanding of the effects of SAME on APAP toxicity at the onset of hepatic necrosis is not well known. In addition, these earlier studies evaluated only the effect of SAME on APAP toxicity and did not assess the effect of SAME alone on the liver (Bray et al., 1992).

We wanted to further investigate the abilities of SAME as a hepatoprotectant for APAP hepatotoxicity. The first experiments were designed to establish the protective threshold SAME could provide against APAP-induced liver damage. Pre-treatment experiments using SAME (and NAC) were utilized to help establish the baseline of protection against APAP-induced hepatotoxicity and provide information regarding the time-and dose-dependent attenuation of liver damage caused by APAP. The effect of different doses and time after administration of SAME on APAP-induced hepatotoxicity had not been reported. Furthermore, studies have not been published examining the time course for SAME's hepatoprotective effects for APAP. During our initial studies involving SAME in our laboratory, we used 1000 mg/kg of SAME just prior to 500 mg/kg APAP. We found that this large dose did attenuate the APAP-induced damage, but as we further explored the properties of SAME, we found that half of our initial dose worked just as well. We also found that doses as small as 250 mg/kg APAP can still elicit severe damage in the mouse liver thus decreasing the possibility of drug-induced mortality of the mice.

Data collected regarding the abilities of SAME to attenuate damage caused by APAP overdose when given just prior to APAP strengthened the theory that SAME does produce GSH. However, since the degree of restoration of GSH levels was small relative to reversal of hepatotoxicity, the experiments raised the question of whether SAME might operate through pathways not involving GSH production. These data show not only increased GSH production in animals dosed with SAME just prior to APAP vs. animals given just APAP, but

the treatment also lowered plasma ALT values, lessened histological changes decreased protein carbonyl and 4HNE-adducted protein levels as well as prevented rises in liver weights.

Effect of 1000 mg/kg or 500 mg/kg SAME just prior to 500 mg/kg APAP dose

Considerable variability exists within the literature for the reported dose of SAME that will reduce APAP toxicity.. Initial studies (Stramentinoli et al., 1979) and many thereafter (Song et al., 2004) used doses of SAME up to 1000 mg/kg. For our studies we used an initial dose of 1000 mg/kg.

We found that SAME protected mice from APAP hepatic toxicity at both 1000 and 500 mg/kg dosings. Mild hepatic degeneration begins in the centrilobular region as early as 1 hour after APAP injection and at 4 hours, livers have necrotic and degenerative changes in the midzonal and periportal regions. This finding was also reported by Placke and colleagues (1987). Centrilobular necrosis is more severe at 6 and 8 hours after APAP injection (Placke et al., 1987; Esteban et al., 1993). SAME pretreatment prior to APAP injection protected against hepatotoxicity as indicated by the absence of centrilobular necrosis 4 hours after APAP treatment. Stramentinoli et al. (1979) reported that centrilobular necrosis was very limited 24 hours after APAP injection in SAME pretreated mice. The late assessment of histology, however, was done only in surviving animals as APAP had a 43% incidence of mortality and SAME pretreatment reduced mortality to 8%.

APAP hepatotoxicity requires cytochrome P450 mediated biotransformation of acetaminophen to the toxic metabolite, *N*-acetyl-*p*-benzoquinone imine (NAPQI) (Mitchell et al., 1973b). Detoxification of APAP's toxic metabolite requires adequate levels of GSH (Walker et al., 1982; Potter and Hinson, 1986). Hepatic damage ensues when GSH levels become depleted. It is well documented that GSH levels rapidly fall in mice treated with APAP and are decreased below control values within 1 hour of APAP administration (Hinson et al., 1981). GSH levels are depressed in mice by 78% within 2 hours by an oral dose of 600 mg/kg APAP (Placke et al., 1987). Bray et al. (1992) noted plasma GSH levels were decreased by APAP 2 hours after injection and in surviving animals returned to near normal levels within 9 hours. Plasma GSH levels were less extensively diminished in APAP animals pretreated with SAmE at 2 hours after APAP but values were not given for any other time period. In our studies, 1000 or 500 mg/kg SAmE administration just prior to 500 mg/kg APAP injection provided partial protection from the depletion in hepatic GSH when measured 2 and 4 hours after APAP injection. A lower dose (500 mg/kg) of SAmE did not provide the same level of protection of hepatic GSH but was able to maintain higher GSH levels than APAP treated groups. In addition, the lower dose of SAmE (500 mg/kg) was able to provide an increase in baseline hepatic GSH. The higher dose of SAmE (1000 mg/kg) did not increase baseline GSH levels. Although it would be expected that baseline GSH levels should be increased by agents that are precursors for GSH, the response to some agents is variable in the liver. Previous studies with NAC, the antidote for APAP toxicity, showed that

NAC does not increase baseline GSH levels when administered at a dose sufficient to lower hepatic toxicity in mice (Sener et al., 2003) and rats (Raza et al., 2003). Sener and associates reported a decrease in hepatic APAP induced toxicity but not a change in baseline hepatic GSH levels with NAC treatments. A similar effect was noted when NAC was administered to Wistar rats (Raza et al., 2003). This may also be part of the mechanism of GSH production: if GSH is not needed, then it is simply not produced.

SAMe pretreatment prior to APAP injection further prevented a rise in hepatic lipid peroxidation 2 and 4 hours after APAP injection. In our studies, APAP alone markedly increased lipid peroxidation. The rise in lipid peroxidation by APAP was in agreement with reports by other laboratories that noted APAP increased lipid peroxidation in mice (Ozdemirler et al., 1994). The mechanism for SAMe protection against the hepatotoxic effect of APAP is not completely known, but quite possibly involves GSH. Studies have shown that supplementation with SAMe can reverse hepatic damage in humans associated with liver diseases including alcoholic liver and intrahepatic cholestasis (Frezza et al., 1990; Lieber, 1997, 2002). SAMe pretreatment of baboons or mice reduced alcohol induced liver damage by preventing GSH depletion (Lieber et al., 1990; Song et al., 2003). SAMe is a substrate for transmethylation reactions to form SAH and homocysteine (Finkelstein, 1990). The trans-sulfuration pathway then converts homocysteine to cysteine, a substrate for GSH synthesis (Lu, 2000). It is plausible that the protection by SAMe against APAP

hepatotoxicity is mediated by a similar mechanism and therefore can better explain the protection seen with this experiment.

Effect of 500 mg/kg SAME just prior to 400 mg/kg APAP dose

After the initial results with 500 and 1000 mg/kg of SAME with the fed animals, we found that 500 mg/kg protected just as well, if not better than, 1000 mg/kg SAME. For the remaining experiments reported in this thesis we used 500 mg/kg SAME. We also decided to fast the animals overnight (1700-0900 h). We found that after 7-10 days in the animal facilities, the mice are known to start establishing dominance with their cage-mates. This can affect eating habits of the other mice if not rectified. To eliminate the possibility of all the animals not eating the same amounts the night before, food was removed. This added extra assurance that we were getting the most consistent results possible and by fasting the animals we were able to better establish the true effects of SAME on the liver. By eliminating confounding factors, such as food, we could better establish the abilities of SAME to produce GSH and attenuate APAP-induced hepatotoxicity.

We also lowered the amount of APAP dose from 500 mg/kg to 400 mg/kg. Since we had already established that 500 mg/kg APAP in fed animals induced severe hepatic toxicity, we postulated that less APAP would be needed in fasted animals. It was decided that we would lower the dose to 400 mg/kg APAP and measure the new toxicity levels with GSH, lipid peroxidation, plasma ALT and protein assays. We also had Dr. Betts Carpenter examine morphology by light

microscopy to check for centrilobular necrosis and verify that APAP was toxic in the animals at this level.

The APAP-induced hepatic toxicity was confirmed with our measurable assays and we now had a new treatment dose to examine the effects of both APAP and SAME on attenuating APAP-induced toxic effects. This particular data set explored very early effects of toxicity seen 1 hour post-APAP injection. We were not only able to document the therapeutic effects of SAME attenuating APAP-induced liver injury, but we were able to display some of the early showings of toxicity and how it evolves into more dramatic injury 4 hours post-APAP overdose.

With 400 mg/kg APAP, SAME was able to prevent hepatotoxicity as indicated by all parameters. Stramentinoli et al. (1979) confirmed the beneficial effects of SAME when given just prior to APAP by percent of animals that survived. In our study, toxicity started to occur within 1 hour post-APAP treatment and SAME reversed certain indicators of hepatotoxicity. The only protection that was not seen was a statistically significant rise in GSH levels. This was most likely due to the short time frame post-SAME treatment. At 4 hours post-APAP, GSH levels began to increase in animals given SAME just prior to APAP treatment compared to the animals not pre-treated with SAME. Earlier time frames did not give the animal adequate time to replace GSH that was lost due to oxidation by NAPQI.

Effect of 500 mg/kg SAME just prior to 300 mg/kg APAP dose

In order to establish a threshold dose for APAP-induced hepatotoxicity we lowered the dose to 300 mg/kg APAP. We had continually received consistent results regarding the initial treatments, but we also wanted to discover a threshold for toxicity regarding APAP in mice. As expected, larger amounts of APAP can sometimes affect the mice differently. Some are able to handle larger doses, such as 1000 mg/kg APAP, better than others. The end results when using larger amounts of APAP usually contain many inconsistencies when trying to measure different toxicological parameters and establish statistically significant differences.

In our laboratory, we confirmed that a 1 hour post-APAP 300 mg/kg injection resulted in toxicity within the liver as defined by: increased liver weights, plasma ALT values increased and histological damage was seen around the centrilobular region. We also found that this toxicity increased in a time-dependent manner. As previously reported (Valentovic et al., 2004), GSH levels are markedly decreased 1 hour after APAP injection and continue to be significantly lower than the VEH group 4 hours post-APAP injection. These decreases were also seen with 300 mg/kg APAP dosing 1, 2 and 4 hours post-injection. What was interesting was the continued increase in GSH stores in animals dosed with SAME just prior to APAP as compared to those dosed only with APAP. We have shown the continuous increase in GSH stores from 1 hour post-APAP injection to 4 hours post-injection. These data confirmed our belief of

protective effects of SAME, including antioxidant behavior, are based on a time-dependent manner.

Effect of 500 mg/kg SAME just prior to 250 mg/kg APAP dose

In our last set of experiments we used 250 mg/kg APAP. This is the lowest amount of APAP-induced toxicity data established. Previous studies did not, for the most part, identify APAP overdose-induced effects on GSH levels, liver weights, histological changes or other parameters. It had already been established in our laboratory that 500, 400 and 300 mg/kg APAP were toxic. It had also been established that SAME can attenuate the levels of extreme toxicity of APAP at a dose of 500 mg/kg within 1 hour of induced toxicity. These data furthered our understanding of both the effects of lower amounts of APAP in the body as well as the benefits of SAME when given just prior to the APAP dose. Abilities of SAME, when given just prior to 250 mg/kg APAP, included, but are not limited to, lowering plasma ALT levels, decreasing oxidative stress as measured by 4-hydroxy-2-nonenal (4HNE) adducted proteins and protein carbonyls, increasing GSH stores and attenuating APAP-induced increases in liver weight.

Effect of 204 mg/kg NAC just prior to 300 mg/kg APAP dose

Abilities of SAME to attenuate APAP-induced hepatic toxicity needed to be directly compared to a therapeutic agent that is currently used to treat APAP overdose cases: N-acetylcysteine (NAC). In the United States, as with other countries such as the United Kingdom and Australia (James et al., 2003), NAC is

the drug of choice in treating patients who have voluntarily or involuntarily overdosed on APAP. The treatment regimen of NAC is quite vigorous (over 17 doses including a loading dose) when treating overdose patients. However, we wanted to directly compare the effects seen with SAME vs. the protective effects of NAC. To do this, NAC was administered at the same concentration as SAME, 1.25 mmol (204 mg/kg).

Data has been published on the benefits of NAC (Lawson et al., 1999; James et al., 2003; Prescott, 1981). Therefore, our results seen when NAC was given to APAP-injected mice were quite surprising. Liver weights started to increase only 1 hour post-APAP. At 4 hours post-APAP, animals dosed with only APAP yielded a statistically significant rise in liver weight. However, unlike the studies when animals were given SAME just prior to APAP, whenever NAC was dosed just prior to APAP, there was not a decrease in liver weight. NAC was also unable to prevent a rise in plasma ALT values 4 hours post-APAP. There was no statistically significant difference between the APAP group and the NAC + APAP group. Histology showed moderate damage around the centrilobular region in groups pretreated with NAC just prior to APAP and decreased GSH levels were not reversed. OxyBlot and 4HNE gels also portrayed the same conclusions: NAC, at the same mmol dose of SAME, is not able to prevent damage caused by 300 mg/kg APAP. To summarize, this dose of NAC is not equivalent to an antidote dose given to patients who have overdosed and SAME is more potent than NAC in our model. These observations are the first to truly

investigate the possibility that SAME may protect better than today's APAP overdose antidote: NAC.

Effect of 500 mg/kg SAME just prior to 1000 mg/kg DEM

Diethyl maleate (DEM) is a known GSH depleting agent that directly binds to GSH (Casey et al., 2002). Consequently once DEM is bound to GSH, the GSH can not provide any type of antioxidant support or protection. However, new GSH can be produced provided cysteine is available. DEM has no effect on the enzymes involved in glutathione synthesis and GSH can occur after DEM treatment (Casey et al., 2002; Hosoya et al., 2002).

This fact is illustrated in our time-dependent studies with DEM. By fasting the animals prior to DEM treatment, they have no substrate available to make new GSH and we can, therefore, study the effects of SAME on the production of new GSH. With the effects of DEM in mind, we designed these studies to investigate the time-dependent properties/abilities of SAME to produce new GSH. The treatment dosing and time frames mimic those done by Casey and colleagues (2002). Animals were treated as before: fasted the night prior to injection with free access to water, blood drawn and toxicity parameters measured. Toxicity parameters included liver weights to insure that DEM had no toxic effects at the treatment dose.

Baseline GSH levels were established in VEH, DEM and SAME + DEM treatment groups. GSH levels found in VEH and DEM groups were comparable to values published by Bray and Rosengren, 2000. As expected, GSH levels

were not markedly increased in SAME + DEM groups until 4 hours post-DEM. Animals that were treated with SAME + DEM showed no statistically different changes in GSH values 2 hours post-DEM. At 4 hours post-DEM, it was found that animals dosed with SAME just prior to DEM yielded approximately a 50% increase in GSH levels when directly compared to animals given only DEM. This supports data found in our earlier studies: SAME is in fact a time-dependent treatment that does elicit protective effects, but some of which (such as GSH production) are not detectable until 4 hours after treatment. It also reiterates the theory of SAME's protective ability to produce new GSH.

This experiment enabled us to answer questions regarding the abilities of SAME to produce new GSH. It also strengthened the SAME mechanism published by Lu (1999) regarding GSH production using SAME. New questions were also derived from this experiment. These questions include: (1) how do these GSH levels compare with NAC + DEM when NAC is given at a same mmol dose and (2) due to the time frame needed to produce a statistically significant rise in GSH levels, what other protective pathways is SAME working by to provide protection earlier on?

Effect of 204 mg/kg NAC just prior to 1000 mg/kg DEM

To directly compare the properties of SAME in production of GSH, we once again had to treat the animals with a known producer of GSH: NAC. The known pathway of NAC in production of GSH as shown in Figure 39, has been published (Lawson et al., 1999). NAC is a producer of cysteine, the rate-limiting

compound in the production of GSH. We wanted to conduct a study that involved a direct comparison of utilizing the production of cysteine in the time-dependent GSH production using NAC vs. SAmE. Through this experiment we were able to directly compare the capabilities of both SAmE and NAC in the production of new GSH.

As described above, animals were dosed and toxicity parameters (liver weights) were checked to insure DEM had no toxic effects besides depleting GSH levels. Just as before, DEM significantly reduced GSH levels only 2 hours post-injection and these values remained depleted 4 hours post-DEM injection. However, unlike what we recorded with SAmE involving DEM, NAC was not as hepatoprotective and unable to elicit increases in GSH levels 2 and 4 hours post-DEM injections. Values of GSH remain depleted and statistically similar to animals given only DEM at both time frames.

The ability of SAmE to modulate an increase in GSH at a faster rate than NAC strengthened our initial data and further provided evidence that SAmE, when given in a same mmol dose as NAC, is more potent and protects better than NAC with regards to GSH production. These data also raise the question of whether GSH production is a significant factor involving the ability of SAmE to provide protection against APAP-induced hepatotoxicity. We have shown abilities of SAmE that included reversing histological alterations around the centrilobular region of the liver, decreasing ALT values, preventing rises in liver weights and lowering concentrations of protein carbonyls and 4HNE-adducted

proteins. This new information now documents the potential of SAME vs. NAC in providing support to a stressed liver by providing GSH at a faster rate.

Effect of 250 mg/kg SAME + 102 mg/kg NAC just prior to 300 mg/kg APAP dose

Our data on the effect of SAME vs. NAC suggest that the two treatments may work via different mechanisms. NAC mechanism has been well documented (Lauterburg et al., 1983; Marzullo, 2005) and one potential pathway of SAME (Lu, 1998) involves a very similar mechanism. Yet, to our knowledge no known data had been published on a therapeutic cocktail involving both SAME and NAC. Other antioxidant treatments have been studied, some of which include vitamin E and melatonin (Sener et al., 2003). We had seen promising protection by SAME and a potential of NAC on a same mmol basis.

Treatment dosings were chosen based on our earlier studies as previously described. To draw direct conclusions between each of the treatments, we had to keep the same mmol concentration of doses. Consequently, the doses of both SAME and NAC were cut in half to yield the same final concentration. Beyond this, animals were treated the same as before and all measurable parameters were carried out.

The SAME + NAC combination experiments gave unexpected results. Despite NAC not yielding any protective effects with our previous studies involving 204 mg/kg just prior to APAP, we had expected to see some protection. The combination therapy was unable to prevent APAP-induced liver toxicity and increased ALT values. GSH levels were depleted at both 1 and 4 hours post-

APAP injection. The combination treatment when given just prior to APAP, was unable to increase GSH levels when directly compared to the APAP group. In fact, results seen were very similar to animals dosed with NAC only just prior to APAP. The inability of SAME + NAC to attenuate APAP-induced hepatic toxicity were also seen in the protein carbonyl and 4HNE-adducted protein levels. In all parameters measured, the only positive result seen was lipid peroxidation values. The SAME + NAC predose was able to attenuate APAP-induced increases in TBARS. This was the only protection that was recorded for the SAME + NAC combination treatment.

We wanted to address the question of whether a combination treatment would yield similar or even synergistic effects. We expected that if the two drugs did in fact work via the same mechanism, then we should see similar results with respect to GSH production, decreases in plasma ALT values, lowered liver weights, etc. Also, if the two drugs worked via same mechanisms, when given together there could be a possibility of synergistic behavior. Ultimately, this experiment was designed to help better establish the main mechanism of SAME utilization during APAP-induced liver damage. Results from this experiment helped us answer the question of whether GSH production was the main and/or only protective pathway of SAME regarding attenuation of APAP-induced liver damage. After the completion of this study, our initial beliefs were supported: SAME probably is working via other mechanisms besides GSH production. The lowered mmol concentrations of SAME (0.625 mmol/kg) and NAC (0.625 mmol/kg) when combined together yielded poor protective results. These data

illustrate the hypothesis that if the two drugs worked via the same mechanism, we should have at least seen results comparative of earlier experiments.

Positive Control: High dose of NAC

We have used NAC in our mouse studies at doses much lower than those used therapeutically in humans. The doses of NAC given in emergency room situations involving cases of APAP overdose are quite large: 140 mg/kg loading dose followed by 70 mg/kg doses every 4 hours for a total of at least 17 doses. The therapeutic effects of NAC have been shown. However when directly compared on a mmol basis to SAMe, NAC was unable to provide any statistically significant protection in our laboratory. To confirm the protective effects of NAC in our mouse model as well as dismiss any questions of the abilities of NAC in our laboratory, we chose a dose amount (1200 mg/kg) similar to concentrations previously reported to attenuate APAP (James et al., 2003).

When we used 1200 mg/kg NAC we found excellent protection in all measure of toxicological parameters measured. NAC prevented rises in liver weight, plasma ALT values, prevented histological alterations induced by APAP and it prevented rises in levels of oxidized proteins as shown by Western blotting. These results confirmed that a high dose of NAC does in fact prevent APAP-induced hepatic toxicity in our laboratory.

Effect of 3.84 mg/kg vitamin B₆ + 500 mg/kg SAME just prior to 300 mg/kg APAP

Lu (1998) hypothesized that vitamin B₆ plays a vital role in the production of SAME into GSH. In fact, Lu states that vitamin B₆ is a rate-limiting factor when it comes to the proposed SAME mechanism. Therefore, we tested the effect of vitamin B₆ + SAME on protection from APAP-induced hepatotoxicity. A vitamin B₆ dose comparable to what a human recommended daily dosage was used.

Results showed limited protection with respect to vitamin B₆ + SAME just prior to APAP. At 1 hour post-APAP injection there was no notable protection seen in any of the measured assays. GSH levels as well as plasma ALT values were very similar in the vitamin B₆ + SAME + APAP group when directly compared to the APAP group 1 hour post-injection. At 4 hours post-APAP injection, some protection was seen especially in the GSH levels and lipid peroxidation values as well as liver weights. While limited response was seen early on, 4 hours post-APAP injection recorded an increase in GSH levels of over 50% in animals given the pretreatment as compared to those dosed only with APAP. Plasma ALT values were also diminished (almost 50%), but the protection seen with respect to SAME were not as promising. TBAR production, as measured by the lipid peroxidation assay, yielded excellent protection 4 hours post-injection in animals dosed with vitamin B₆ + SAME just prior to APAP. Protection by vitamin B₆ + SAME yielded the best results of all treatments in the lipid peroxidation assay.

These data are the results testing the hypothesis first proposed by Lu (1998) that vitamin B₆ may be an important factor in the production of GSH from

SAMe. Our results were not sufficient to prove or disprove this hypothesis. Further studies involving vitamin B₆ and SAMe are needed. These studies would examine the beneficial effects of vitamin B₆ when administered after APAP. The inconclusive data recorded most likely was the result of the SAMe mechanism itself. If GSH was not immediately needed, excess SAMe and vitamin B₆ were probably eliminated from the body or used elsewhere. No GSH can be produced if the ratio of SAH:SAMe favors SAH. The mechanism as portrayed by Lu (1998) is reversed and no GSH is made. Instead, the parent compound of the SAH, SAMe, remains. Further investigation using this treatment cocktail would need to be done to strengthen these claims. The investigation should include a post-APAP treatment. Only then could the protective effects of vitamin B₆ + SAMe be represented in this model.

5.3 Treatments Post-APAP

Effect of 250 mg/kg APAP 1 hour prior to 500 mg/kg SAMe dose

We have established a role of SAMe in protecting the liver from APAP-induced hepatic toxicity. However, to be effective as a therapeutic agent, SAMe needs to be administered after APAP overdose. To illustrate the potential therapeutic benefits of SAMe, animals were given an overdose of APAP (250 mg/kg) and 1 hour later a treatment of 500 mg/kg SAMe was injected, i.p.

Severe hepatic stress was illustrated 4 hours post-APAP injection by increased liver weights, high plasma ALT values, severe centrilobular necrosis and decreased GSH levels. Animals given SAMe 1 hour post-APAP showed

almost 100% recovery in all parameters measured. Liver weights were normal, plasma ALT values were significantly decreased and there was no centrilobular necrosis present. The most remarkable protection seen was the rise in GSH levels. These levels had completely recovered and were statistically similar ($p < 0.05$) to the VEH and SAME groups.

These data portrayed some remarkable results. They not only showed the abilities of SAME to attenuate APAP-induced hepatic stress, but they also reinforced the hypothesis of one potential mechanism of SAME (Lu, 1998). We hypothesize injection of SAME after the APAP-induced depletion of GSH allowed more efficient use of this substrate for the production and thus restoration of GSH levels. This need was so strong that the GSH-producing mechanism of SAME remained in a feed-forward manner until enough GSH was produced and reversal and/or prevention of damage was carried out. We were now seeing even stronger protection, probably based on the circumstance of a desperate need for GSH production due to the APAP overdose. However, the possibilities of other potential protective mechanisms still present itself with these results. While GSH is a strong antioxidant, we did see positive results with SAME in the pre-treatment experiments when GSH levels were not as high.

Effect of 250 mg/kg APAP 1 hour prior to 204 mg/kg NAC dose

We had been able to directly compare the protective benefits of SAME and NAC throughout the experiments thus far, therefore to fully quantify the results seen with the SAME post-treatment, a study was conducted to display the

abilities of NAC to attenuate APAP-induced toxicities. The treatment of NAC was injected 1 hour post-APAP at a similar mmol concentration of SAME.

As expected, similar results regarding hepatic induced stress given by 250 mg/kg APAP was recorded. NAC treatments were able to attenuate the APAP-induced hepatic toxicity, but in some cases not as well as SAME. For instance, liver weights were not reduced when the NAC post-treatment was given. Furthermore, histological slides showed signs of centrilobular necrosis still present. Both liver weight and histological values were similar to the VEH when SAME was given as treatment for APAP overdose in the mice.

Consequently, NAC was able to significantly decrease plasma ALT values and increase hepatic GSH levels. Even though GSH levels were increased with the NAC post-treatment, the values were not as high as those shown in the SAME post-treatment. These data force the question of which treatment should be elicited post-APAP induced hepatic toxicity. Data shown thus far have suggested that, under our experimental conditions, SAME post-treatment is more effective than NAC in reversing APAP-induced hepatotoxicity. Further studies involving these treatments, as well as higher concentrations of both treatments should be conducted to fully illustrate the capabilities of SAME vs. NAC.

5.4 Mitochondrial Isolation: Treatments Post-APAP

Effect of 250 mg/kg APAP 1 hour prior to 500 mg/kg SAME dose

It is known that cytosolic GSH is the first line of defense against toxicants such as NAPQI and that under severe stress, mitochondrial GSH can become

depleted. However, the effect of SAME on mitochondrial GSH is not well known. To establish a better understanding of the protective abilities of SAME as well as try to create a proposed mechanism by which SAME elicits its protective effects, this experiment was designed to show the aptitude of APAP to deplete mitochondrial GSH and the possible ability of SAME to prevent (or restore) this depletion.

Mitochondrial isolation was done similarly to Lash, 1999. After isolation, LDH assays confirmed that mitochondria components were separate from other cytosolic components.

APAP decreased GSH levels, but GSH levels in both the supernatant and the mitochondria displayed no protection with SAME when the treatment was given post-APAP. One explanation for this lack of effect by SAME on increasing mitochondrial GSH levels could be the short post-treatment time frame. Since GSH cannot be produced in the mitochondria, it has to be carried in via cellular transport. This process requires time and energy. Whenever the cell is under some sort of stress (such as APAP overdose), GSH production is a first priority. Our previous data showed that SAME was capable of restoring total cellular GSH levels in APAP-treated animals. Another possibility could be the protein carriers involved with GSH shipment into the mitochondria were altered. The protein carriers are designed to recognize GSH and allow binding. If the GSH is conjugated or the protein carriers altered, GSH would possibly be unable to get into the mitochondria. We have illustrated with our proteomic studies some proteins that become changed after a large dose of APAP. Further investigation

regarding mitochondrial GSH concentrations will need to be investigated using longer post-APAP time periods to determine the reason of why SAME was not increase mitochondrial GSH levels.

Nitrate and nitrite production are typically used as markers of nitric oxide (NO) production. These analytes by themselves are unable to address the possible adverse effects associated with its reaction with free radical species in vivo. Active NO metabolites can react with superoxide to form peroxynitrite, a powerful oxidant and nitrating agent. Subsequent reaction of peroxynitrite with proteins results in nitrotyrosine formation. As a stable end product of peroxynitrite mediated oxidation/nitration, nitrotyrosine can be used as a surrogate index of NO dependent damage in vivo.

Nitrotyrosine levels in the mitochondria were analyzed using Western blotting as another measure of the oxidative effects within the mitochondria produced by APAP. Initial results suggest that SAME reduces the amount of nitrotyrosine-adducted proteins in APAP treated mice. SAME had no effect on nitrosylation of proteins when compared to VEH. Despite the inability of SAME to produce GSH within 3 hours of treatment (4 hours post-APAP), it was able to decrease the levels of nitrotyrosine within the mitochondria. This too strengthens our hypothesis that SAME is working via other mechanisms other than GSH production. This, to our knowledge, is the first study conducted investigating the effects of SAME protection within the liver mitochondria post-APAP overdose.

Effect of 250 mg/kg APAP 1 hour prior to 204 mg/kg NAC dose

To illustrate and directly compare SAME to NAC, a similar study was conducted involving mitochondrial isolation and NAC (1.25 mmol/kg). To our knowledge, a study including the involvement of NAC and the protection of mitochondrial GSH has not been done. Furthermore, a direct comparison of NAC vs. SAME investigating protection/production of mitochondrial GSH has never been reported.

Similar to SAME, NAC did not reverse the APAP-induced depletion of GSH in the mitochondria. Comparably to SAME, additional time may be required in order for NAC to replenish mitochondrial GSH. It is not known how long newly produced GSH takes to get into the mitochondria after APAP-induced depletion. Therefore, just as with SAME, further investigation into the effects on mitochondrial GSH with NAC and/or SAME need to be done to better understand this principle.

Nitrotyrosine levels in the mitochondria post NAC + APAP treatment were also analyzed using Western blotting. In contrast to the effect of SAME, NAC did not reduce the amount of nitrotyrosine-adducted proteins when given 1 hour post-APAP. NAC had no effect in producing nitrosylated proteins when compared to VEH. These data are supportive of the hypothesis that SAME may in fact attenuate severe hepatic damage following APAP overdose more efficiently than NAC by another mechanism.

5.5 Summary For Pre-Treatments

Effect of 500 mg/kg SAME, 204 mg/kg NAC, 250 mg/kg SAME + 102 mg/kg NAC or 500 mg/kg SAME + 3.84 mg/kg Vitamin B₆ just prior to 300 mg/kg APAP

The overall summary for each treatment showed some protection in at least one of the measurable parameters conducted. Some of the treatments showed matching protective abilities, while some treatments showed a much better ability to attenuate APAP-induced hepatic toxicity. Table 34 summarizes the results of the pretreatment studies and figures 68 and 69 show a summary of OxyBlot and 4HNE blots of the treatments. All animal preparations were identical and can be reviewed in the previous results sections. Direct comparisons can be drawn for each of the treatments and are the same on a mmol basis.

From the results in the table and the Western blots (FIGURES 68 AND 69), conclusions can be drawn on the benefits of each pretreatment in protecting against APAP-induced hepatic toxicity. From these data, as well as the gels shown in the results section, we can see that on a mmol basis SAME protects better than all other studied treatments. Despite being the drug treatment of choice for APAP overdose cases, NAC did not yield as many protective effects as did SAME. NAC and SAME do possibly work via different mechanisms, but these mechanisms do not produce synergistic protection when given together. Vitamin B₆ may very well be a rate limiting factor in the production of GSH from SAME, but further studies must be conducted using a vitamin B₆ + SAME mixture post-APAP treatment to address this hypothesis.

Treatment	GSH	Liver Weight	ALT
NAC	↑ 30%	↓ 6%	No protection
SAMe	↑ 53%	↓ 8%	↓ 63%
SAMe + NAC	↑ 30%	↓ 7%	No protection
SAMe + Vitamin B ₆	↑ 41%	↓ 8%	↓ 46%

TABLE 34. Summary of pretreatment protection seen 4 hours post APAP.

These results summarize the protective effects seen of each of the major parameters measured. As seen in the table, SAMe produced the most favorable protective effects of all other treatments. These results were calculated based on percent changes of APAP vs. Pretreatment + APAP within their experimental data set. Values were rounded to the nearest whole number.

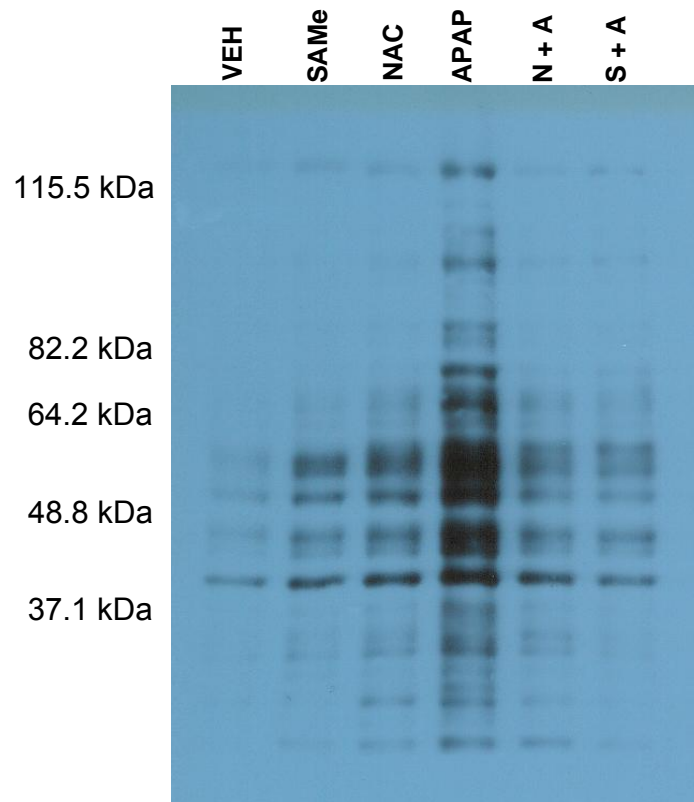


FIGURE 68. OxyBlot of samples 4 hours post-APAP injection.

Lanes are denoted as: Lane 1 vehicle, Lane 2 SAMe, Lane 3 NAC, Lane 4 APAP, Lane 5 NAC 1 hour post-APAP (N + A) and Lane 6 SAMe 1 hour post-APAP (S + A). SAMe was administered as 500 mg/kg and NAC was given as 204 mg/kg, i.p. APAP was injected i.p. at a dose of 250 mg/kg (15 ml/kg). VEH animals were injected i.p. with water (15 ml/kg).

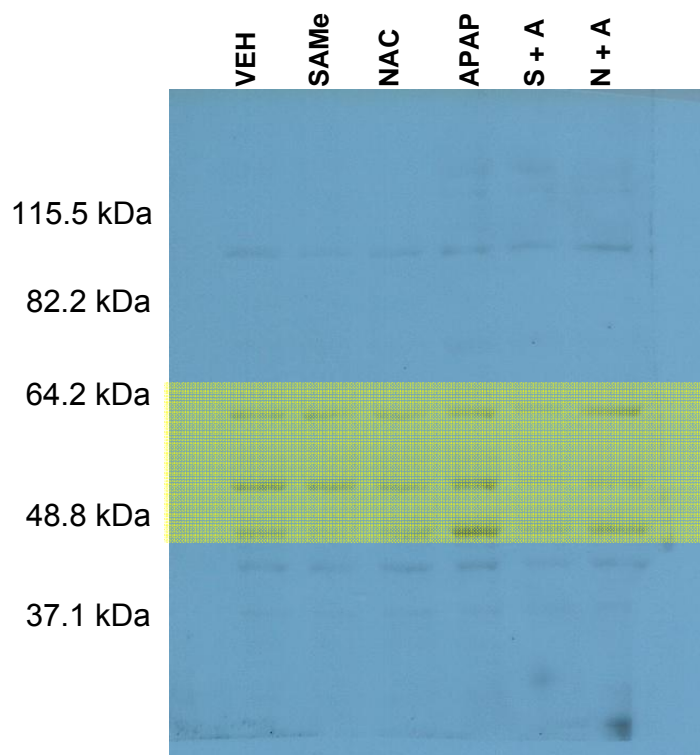


FIGURE 69. 4HNE Blot of samples 4 hours post-APAP injection.

Lanes are denoted as: Lane 1 VEH, Lane 2 SAMe, Lane 3 NAC, Lane 4 APAP, Lane 5 SAMe 1 hour post-APAP and Lane 6 NAC 1 hour post-APAP. SAMe was administered as 500 mg/kg and NAC was given as 204 mg/kg, i.p. APAP was injected i.p. at a dose of 250 mg/kg (15 ml/kg). VEH animals were injected i.p. with water (15 ml/kg). Highlighted region represents area of greatest differences.

5.6 Summary For Post-Treatments

Effect of 500 mg/kg SAME or 204 mg/kg NAC 1 hour after 300 mg/kg APAP

SAMe and NAC protected against APAP-induced hepatic toxicity. Both SAMe and NAC reduced plasma ALT levels, reduced oxidative stress and significantly increased GSH levels. However, SAMe was able to protect to a greater extent than NAC. SAMe produced higher amounts of GSH and decreased oxidized proteins more than NAC. These data imply that SAMe is possibly more potent than NAC and provides better protection against APAP-induced oxidative stress. 4-Hydroxy-2-nonenal and protein carbonyls have been extensively studied and are known to cause severe damage (Renes et al., 2000; Schaur, 2003; Yang et al., 2003), with high protein carbonyl concentration possibly leading to Alzheimer's disease (Picklo et al., 2002). SAMe, as discussed previously in the results section, provides a larger area of protection with regards to these parameters than does NAC.

The mechanism of GSH production by SAMe against APAP-induced oxidative stress is supported by the data published by Lu (1998). However, this research yielded some very interesting results forcing the question of possibilities of other mechanisms regarding SAMe. It is very possible that the mechanism of SAMe is further delineated to produce other protective effects as displayed in the results of this study. This hypothesis is made stronger by the other protective effects given by SAMe: lower liver weight, reversal of centrilobular necrosis, decreased plasma ALT values, increased GSH levels, decreased nitrosylated-protein levels within the mitochondria and decreased protein oxidation. It is

unlikely that all of these protective effects stem from GSH production by SAME. This hypothesis can be based on the results seen in the pre-treatment studies. GSH production in animals dosed with SAME just prior to APAP was not as significant as the post-treatment studies, but there was still protection seen. Further work is required to elucidate non-GSH dependent mechanisms by which SAME protects against APAP-induced liver damage.

5.7 HPLC Analysis

Investigation into the production and quantification of particular APAP metabolites was studied to better diagram the mechanisms of both SAME and APAP. We had shown protective capabilities by SAME for APAP toxicity, but it became apparent that other possible mechanisms SAME attenuation may exist. To explore this possibility, we utilized HPLC protocols to identify effects of SAME on APAP metabolism. If SAME was able to affect the production of particular APAP metabolites, we could therefore draw a stronger conclusion to our belief of other protective pathways of SAME.

Techniques to isolate and quantitate the metabolites of these two drugs have been developed (Aboul-Enein and Abu-Zaid, 2001; Dietze et al., 1997; Esteban et al., 1992; Esteban et al., 1993; Wang 2001; Fischer et al., 1981; Hamedani et al., 1993; Jensen et al., 2004; Miners et al., 1984; Vertzoni et al., 2003; Wang and Cederbaum, 2006; Moldéus, 1978), but none had looked at the

effects of SAME on APAP metabolites. We chose to use the methods as published in Vertzoni et al. (2003) and Moldéus (1978).

Results from the HPLC showed differences in APAP and APAP-SO₄ concentrations in both the liver and plasma at specific time frames. Whenever SAME was given as a pre-dose to APAP, we saw higher concentrations of APAP and APAP-SO₄ in the plasma (as compared to animals given only APAP). On the other hand, we saw higher concentrations of APAP-SO₄ in the liver when SAME was given just prior to APAP (when directly compared to animals dosed with only APAP). These statistical differences ($p < 0.05$) allow us to further question the abilities of SAME on attenuating APAP-induced hepatic toxicity. For instance, we know that SAME produces GSH, but what we do not know are the effects of SAME on particular enzymes such as CYP2E1, the enzyme responsible for the production of NAPQI. There is also no published data documenting the effects of SAME on other protective mechanisms in the body involved with attenuating APAP overdoses. These other protective mechanisms include glucuronidation and sulfation of APAP.

Investigation of metabolites produced when APAP is injected and directly comparing those results to animals dosed with both SAME + APAP will help us to further understand the metabolic processes of both SAME and APAP. It will also help strengthen our hypothesis that SAME works via other mechanisms besides GSH production. This data does in fact support our hypothesis that SAME may be involved in effecting the activity of CYP2E1, the enzyme responsible for the

production of the toxic APAP metabolite NAPQI, and production of other metabolites involved with APAP, such as APAP-Glucuronide and APAP-sulfate.

This HPLC data shows that SAME is affecting APAP metabolism (higher concentrations of APAP in the plasma) and the production of sulfate, as shown by the APAP-sulfate conjugation concentration increases in the liver and plasma. Further investigation regarding metabolites produced during APAP overdose, as well as the metabolites produced during SAME treatment + APAP, will help to further define these possible mechanisms and answer the questions involved protective effects seen with SAME.

5.8 Proteomics

The limitation of this set of experiments was that analysis was performed on individual bands from a gel. While this experiment allowed us to achieve better coverage of proteins identified, it did not allow us to identify all of the adducted proteins. Table 33 gives just a glimpse of some of the altered proteins that we have identified during APAP overdose.

Future experiments will involve multidimensional protein identification technology (MuDPIT). For this procedure, the Dr. Lau at the University of Arizona, will run a gel as previously described, but instead of digesting a select number of bands, the entire lane will be digested and then loaded on the mass spectrometer for MuDPIT analysis. Then by using X!Tandem, PMOD, and SpecPlot we will be able to globally identify and validate 4HNE adducts that are

unique to the APAP treated group as well as those unaltered after SAME + APAP administration. Additionally, we will also begin searching for NAPQI and other APAP metabolites adducts on liver proteins using a similar approach. These future studies will help identify some of the pathways taken during APAP overdose. It will also, of course, help describe some of the effects of SAME regarding the prevention of protein alterations during APAP-induced damage.

5.9 Future Studies

The current study was designed to address many aspects involving the mechanistic properties of SAME, including the ability of SAME to attenuate forms of APAP-induced hepatic toxicity. This study also reproduced previously published data showing that SAME produces GSH (Lu, 1998). However, this investigation has also raised other questions involving SAME. For instance, our data indicate that SAME works, at least in part, via other non-GSH dependent mechanisms. These questions that have arisen include other antioxidative properties of SAME. For instance, how is SAME preventing the production of protein carbonyls and 4HNE adducted proteins when given just prior or 1 hour post-APAP? How does a dosing of SAME effect the production of certain metabolites produced when APAP is injected (i.p.)? Why are there protective effects seen within 1 hour of SAME treatment just prior to APAP injection, but GSH production is not statistically significant? Also, questions involving protection seen using SAME to decrease plasma ALT values and liver weights when given just prior or post-APAP forces the question of other mechanistic

properties of SAME. It is because of the data collected during this study that these questions have been asked. While our data does in part support that of Lu (1998) and others (Stramentinoli, 1979), it also raises the possibility that GSH production may not be the only mechanism by which SAME protects the liver against APAP-induced effects. Further support for this conclusion is seen involving studies using NAC. Even though NAC was able to protect the liver, to some extent, against APAP-induced hepatic toxicities, and was able to restore GSH levels, it was not as effective as SAME when given at the same equimolar dose. This not only suggests that SAME is more potent than NAC, but SAME may also be working via different mechanisms to elicit more protective effects. These questions, as well as future studies, are discussed below.

As stated in a paper published by Lu (1998), vitamin B₆ is of vital importance in the production of GSH from SAME. We tried to demonstrate the dependence of SAME on vitamin B₆ in our mouse model, but were unable to show a significant difference with adding vitamin B₆ vs. SAME by itself. The probable reason for these results is that GSH stores had not yet been completely depleted and there was not an immediate need for new GSH to be produced. We now know that GSH production is time-dependent when involving SAME. A better protocol for demonstrating the limiting effects of vitamin B₆ on SAME-dependent GSH production might be the recovery model as described previously. In a recovery study animals are first dosed with APAP, then 1 hour post-APAP they are injected with SAME + vitamin B₆. This experiment could

prove the hypothesis of Lu (1998) as well as what we have previously stated: if there is no direct and/or immediate need for GSH, no GSH is produced.

We saw a change in mitochondrial GSH 4 hours post-APAP injection. The level of GSH within the mitochondria, as well as the cytosol, yielded significant decreases after APAP was administered. Both SAME and NAC were unable to prevent the decrease of GSH within the mitochondria. However, 3 hours after administering SAME (4 hours post-APAP), there was a trend of higher levels of GSH within the mitochondria. This data is enough to question the involvement of SAME in protecting the mitochondria from damage elicited by APAP overdose. Further investigation involving longer time frames could produce even more information regarding this protective effect. As mentioned earlier, GSH is not produced within the mitochondria. Instead, any GSH found in the mitochondria has been brought in from the cytosol. Measurements of GSH levels after longer time frames post-APAP would illustrate another possible protective ability of SAME. This type of study would give a pharmacokinetic view of SAME production of GSH and the time frame for the GSH to get into the mitochondria.

Proteomics is a rapidly growing field with new technologies advancing very quickly. With the availability of this new technology comes opportunity to better the understanding of how APAP elicits its damaging effects and how SAME is able to attenuate and/or reverse this damage. There have been several articles published discussing different approaches and methods of isolating proteins (Craven et al., 2002; Isozaki et al., 2002; O'Connell and Stults, 1997; Shimazaki et al., 2004; Wang et al., 2003; Wu et al., 2005) as well as results

from effects of certain toxins, such as cadmium (Chrestensen et al., 2000), on protein modification. More importantly for our study, there have also been some reports on APAP-induced alterations in proteins (Fountoulakis et al., 2000; Ruepp et al., 2002; Su et al., 2005; Sumioka et al., 2004) as well as effects of SAME on proteins in the liver (Yang et al., 2004). However, there is no information regarding the effects of SAME on altered proteins caused by APAP overdose. This study will carry the protein carbonyl and 4HNE data further. We already know that SAME can decrease the concentrations of both protein carbonyls and oxidized proteins; this study is meant to try and identify the specific proteins SAME protects.

This set of proteomic experiments will continue to be completed in collaboration with Dr. Serrine Lau, Director, Southwest Environmental Health Sciences Center, University of Arizona. Samples are first tested in our laboratory for protein concentration, protein carbonyl alteration and 4HNE values. Dr. Lau and associates then test the samples for specific protein changes. To date, we have identified several proteins altered by APAP overdose. We have also identified these same proteins to remain unchanged/protected whenever SAME is given just prior to APAP. Further research into the role of proteins modified by APAP will produce a better understanding of toxicity and how SAME can protect the liver against this APAP-induced damage. For example, SAME may reduce APAP mediated effects on mitochondrial proteins or enzymes that are involved in repair or regeneration. In this situation, SAME would act through a mechanism

different from the current antidote, NAC and this would be a novel finding with great potential for clinical relevance.

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Appendix

Buffer Preparation

Krebs-Ringer Buffer

(126.4 mM NaCl; 6.0 mM Na₂HPO₄; 5.2 mM KCl; 1.3 mM MgSO₄; 1.0 mM CaCl₂)

Into approximately 900 ml of deionized distilled water, the following salts were added: 7.39 g NaCl; 852 mg Na₂HPO₄; 385 mg KCl; 318 mg MgSO₄; 149 mg CaCl₂. The pH was adjusted to 7.4 with HCl. The volume was brought to 1 L with deionized distilled water, followed by a final pH check. Prior to incubation the buffer was oxygenated for 45 min.

Mitochondrial Isolation Buffer A

(225 mM sucrose; 3 mM KH₂PO₄; 5 mM MgCl₂; 20 mM KCl; 20 mM triethanolamine; 2 mM EGTA)

Into approximately 240 ml of deionized distilled water, the following salts were added: 19.25 g sucrose; 102 mg KH₂PO₄; 254 mg MgCl₂; 373 mg KCl; 746 mg EGTA. The pH was adjusted to 7.4 with HCl. The volume was brought to 250 ml with deionized distilled water, followed by a pH check.

Mitochondrial Isolation Buffer B

(225 mM sucrose; 3 mM KH₂PO₄; 5 mM MgCl₂; 20 mM KCl; 20 mM triethanolamine)

Into approximately 240 ml of deionized distilled water, the following salts were added: 19.25 g sucrose; 102 mg KH_2PO_4 ; 254 mg MgCl_2 ; 373 mg KCl. The pH was adjusted to 7.4 with HCl. The volume was brought to 250 ml with deionized distilled water, followed by a pH check.

Glutathione Determination

Total glutathione (GSH) levels were measured based on the methods of Anderson (1985). Following incubation, the tissues were rinsed in 3 ml of Krebs-Ringer buffer, blotted and weighed. Once weighed, the tissues were placed in 500 μl of 5% sulfosalicylic acid and homogenized followed by a rinse of the homogenizer shaft with an additional 500 μl of 5% sulfosalicylic acid. The mixture was centrifuged at 9000 g for 10 min at 4°C. The supernatant was decanted and saved. For GSH levels, 25 μl of supernatant was combined with 175 μl water and 700 μl of 0.3 mM NADPH. Following a 10-minute incubation at 30 °C, 100 μl of 6 mM DTNB and 100 μl of glutathione reductase (16 units/ml) were added. The samples were placed immediately into the spectrophotometer set at 412 nm and the absorbance was read at 0, 15, 30, 60, and 90 seconds. Values were calculated using a standard curve of reduced glutathione.

Glutathione disulfide (GSSG) levels were determined following 2-vinylpyridine derivatization (Griffith, 1980). For GSSG levels, 25 μl of supernatant was combined with 175 μl water. To this, 2 μl of triethylamine and 4 μl of 2-vinylpyridine were added and the mixture was incubated for 30 min at room

temperature. Following the incubation, 700 μ l of 0.3 mM NADPH was added with an additional 10-minute incubation at 30°C. After this incubation, 100 μ l of 6 mM DTNB and 100 μ l of glutathione reductase (16 units/ml) were added. The samples were placed immediately into the spectrophotometer set at 412 nm and the absorbance was read at 0, 15, 30, 60, and 90 seconds. Values were calculated using a standard curve of GSSG.

4-Hydroxynonenal-Adducted Protein

Tissues were weighed and homogenized in Krebs-Ringer Buffer on ice in a 2 ml total volume. Samples were stored at -80 °C until analysis. Samples were thawed on ice. Protein concentrations were determined for each sample (Bradford, 1976). For each sample 100 μ g protein equivalents were added, while H₂O was added to provide equal volumes. An equal volume of sample buffer was added and samples were boiled for 5 min. Samples and standards were loaded onto a polyacrylamide gel (12.5 % acrylamide) and electrophoresed at 65 volts for 17 hr. The gel was then transferred to a nitrocellulose membrane at 100 volts for 90 min. To verify efficiency of transfer, the membrane was placed in Ponceau S stain for visualization. The membrane was then rinsed with H₂O at room temperature under constant shaking to remove stain. The membrane was then blocked with milk at room temperature under constant shaking for 1 hr. Rabbit polyclonal antibody to (E)-4-Hydroxynonenal (anti-HNE PAb) (Alexis Biochemicals, ALX-210-767) in blocking buffer was added and incubated overnight at 40 °C under constant shaking. The membrane was then rinsed three

times in TBST. Secondary antibody, goat anti-rabbit linked with horseradish peroxidase, in blocking buffer was added and incubated at room temperature under constant shaking for 90 min. The membrane was then rinsed three times in TBST and once in TBS. Enhanced chemiluminescence substrate was added and the membrane was wrapped in Saran wrap. The membrane image was then transferred to Kodak Photographic film for visualization of 4hydroxynonelal-adducted proteins.

Mitochondrial Isolation

Isolate liver and rinse in 3 ml ice cold Buffer A. Blot and weigh livers, remove excess non-liver tissue. Add minced liver to 1.5 ml Buffer A and pour in ice cold Teflon homogenizer. Homogenize approximately 20 strokes. Adjust volume to 3 ml with Buffer A. Centrifuge total mixture at 600 xg for 10 minutes at 4°C. Remove and save supernatant. Centrifuge supernatant at 15,000 xg for 5 minutes at 4°C. Remove and dispose of fat layer on top of supernatant. Remove supernatant and store aliquots for GSH, LDH and protein assays. Rinse mitochondrial pellet with 250 µl of Buffer B. Re-suspend pellet in 1 ml of Buffer B per gram tissue weight. Aliquot and store samples for GSH, LDH and protein assays.

Curriculum Vitae

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EDUCATION: BS, Biology (summa cum laude, 3.8 GPA)
Coastal Carolina University, 2001

Ph. D., Pharmacology
Marshall University School of Medicine, 2006
(anticipated)

DISSERTATION TITLE: A Mechanistic Study of the Protective Effects of S-Adenosyl-L-methionine Against Hepatotoxicity of Acetaminophen.

CURRENT RESEARCH: S-Adenosylmethionine protection against acetaminophen induced hepatotoxicity

POSITIONS HELD/ EXPERIENCE IN RESEARCH:

Graduate Student, Department of Pharmacology, Marshall University School of Medicine, 2002-present

Teacher, Princeton Review, 2005 - present

Research Fellowship, National Science Foundation, University of Southern Alabama, 2001

PROFESSIONAL MEMBERSHIPS

Society of Toxicology

Graduate Student Organization, Biomedical Sciences, Marshall University School of Medicine

LECTURES AND SYMPOSIA

- Marshall University School of Medicine Research Day – Spring 2005 *Comparison of S-Adenosylmethionine (SAME) and N-Acetylcysteine (NAC) Effect on Acetaminophen Mediated Induction of Toxicity in Mice*
- Research Lecture, Fairmont State University – Spring 2006 *A Mechanistic Study in the Protective Effects of S-Adenosylmethionine Against Hepatotoxicity of Acetaminophen*
- Princeton Review, MCAT Preparatory Course – Spring 2005; Summer 2005; Spring 2006 *Atomic Structure; Periodic Trends and Bonding; Phases and Gases Solutions and Kinetics; Equilibrium; Acids and Bases; Thermodynamics; Redox and Electrochemistry*
- Medical Pharmacology (MSII classes of 2007 and 2008), Marshall University School of Medicine – Spring 2005; Spring 2006
- *Pharmacokinetics, Drug Interactions*
- Marshall University School of Medicine Research Day – Spring 2005 *The Effect of S-Adenosyl-L-methionine (SAME) on Mitochondrial Glutathione and Protein Carbonyls Following Acetaminophen (APAP) Treatment in Mice*
- BMS 680, Marshall University School of Medicine – Spring 2005 *Herbal Remedies, An Introduction to Today's Nutraceuticals*
- Seminar, Marshall University School of Medicine – Fall 2004 *S-Adenosyl-L-methionine (SAME) Attenuation of Acetaminophen (APAP) Induced Hepatotoxicity*
- BMS 680, Marshall University School of Medicine – Spring 2003 *Castor Beans Toxicity*
- Seminar, Marshall University School of Medicine – Fall 2003 *S-Adenosylmethionine attenuates acetaminophen hepatotoxicity in C57BL/6 mice*
- University of Southern Alabama REU Program – Fall 2001 *Effects of Nutrient Concentration and Pigment Composition on the Production of *Acartia tonsa* in the Northern Gulf of Mexico*

SERVICE

Graduate Student Mentoring Committee Student Representative, 2005 - 2006

Tutor for MSI students in Biochemistry, 2005 – 2006

Princeton Review MCAT Preparatory Class Instructor for Chemistry, 2005 - 2006

Graduate student mentor, 2004 - 2006

Volunteer tutor for undergraduate students in Organic Chemistry, Calculus, and Biology, 2000 – 2001

HONORS/ AWARDS

Keystone Symposia Travel Award, Keystone Symposia Victoria, BC, 2006

Finalist at 2006 Society of Toxicology Drug Discovery Specialty Section Research Award

Who's Who Among Students in American Universities & Colleges Award, 2006

Marshall University Biomedical Science's Presidential Fellowship Award, 2005

Elected President of Graduate Student Organization, Marshall University School of Medicine, 2005-present

The Chancellor's List Award, 2005, 2006

Society of Toxicology Travel Award, 2005

Marshall University Research Core Travel Award, Marshall University School of Medicine, 2005

Elected Secretary/Treasurer of Graduate Student Organization, Marshall University School of Medicine, 2004-2005

Biomedical Sciences Graduate Research Assistantship, Marshall University School of Medicine, 2002-current

National Science Merit Award, 1998; 1999

Presidential Scholarship, 1997; 1998; 1999; 2000

Tri Beta-Biology Honorary, 1997; 1998; 1999; 2000

PUBLICATIONS

Terneus MV, Kiningham KK, Carpenter AB, Sullivan S, Valentovic MA. Comparison of S-adenosyl-L-methionine and N-acetylcysteine protective effects of acetaminophen hepatic toxicity. *Journal of Pharmacology and Experimental Therapeutics*. In submission.

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Terneus M. Kurtoxin. *XPharm Pharmacology Reference*, 2004

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ABSTRACTS

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