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CONTRIBUTION OF NEW THIOL ANTIOXIDANT IN THE TREATMENT OF

ACETAMINOPHEN TOXICITY

by

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A DISSERTATION

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ABSTRACT

Acetaminophen (N-acetyl-p-aminophenol, APAP) is one of the most widely used over-the-counter antipyretic analgesic medications. It is safe at therapeutic doses, but an overdose can result in severe hepato-nephrotoxicity, a leading cause of drug-induced acute liver failure in the U.S. Although a few different mechanisms have been proposed for APAP-induced toxicity, a significant amount of evidence has pointed to the potential involvement of oxidative stress in acetaminophen toxicity. Depletion of glutathione (GSH) is one of the initiating steps in APAP-induced toxicity; therefore, one strategy for restricting organ damage is to restore GSH levels by using GSH prodrugs like N-acetylcysteine (NAC). Although NAC is the treatment of choice for APAP-induced toxicity, fairly high doses and longer treatment times are required due to its poor bioavailability. In addition, oral and IV administration of NAC in a hospital setting are laborious and costly. With limited therapeutic options, other than NAC, it is important to develop therapeutic alternatives to effectively protect against APAP-induced toxicity and to improve treatment outcomes and prevent death. Therefore, we studied the protective effects of N-acetylcysteine amide (NACA), a novel antioxidant with higher bioavailability, and compared it with NAC in APAP-induced toxicity in C57BL/6 mice. Our results showed that a lower dose of NACA is better than NAC in combating oxidative stress and protecting against APAP-induced damage. The higher efficiency of NACA, in protecting against APAP-induced toxicity, suggests that NACA can be developed into a promising therapeutic option for treatment of an APAP overdose.

ACKNOWLEDGEMENTS

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LIST OF ABBREVIATIONS

Symbol	Description
AA	Arachidonic acid
ALT	Alamine transaminase
AM404	N-arachidonoylphenolamine
APAP	Acetaminophen
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
BBB	Blood brain barrier
BHT	Butylated hydroxytoluene
BLM	Bleomycin
BSO	L-buthionine-sulfoximine
BUN	Blood urea nitrogen
CAT	Catalase
CK	Creatine kinase
CNS	Central nervous system
COX	Cyclooxcygenase
Cys	Cyestine
CYP	Cytochrome P450
DMSO	Dimethyl sulfoxide
EGTA	Ethylene glycol tetraacetic acid
FAAH	Fatty acid amide hydrolase

FDA	Food and drug administration
G-6-PD	Glucose 6-phosphate deficiency
GDH	Glutamate dehydrogenase
GPx	Glutathione peroxidase
GR	Glutathione reductase
GS	Glutathione synthertase
GSH	Glutathione
GSSG	Glutathione disulfide
GST	Glutathione S-transferase enzymes
H&E	Hematoxylin and eosin
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV	Human immunodeficiency virus
HPLC	High performance liquid chromatography
i.p.	Intraperitoneal
IV	Intravenous
JNK	c-jun-N-terminal kinase
MDA	Malondialdehyde
METH	Methamphetamine
MPT	Mitochondrial permeability transition
NAC	N-acetylcysteine
NACA	N-acetylcysteine amide
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate

NAPQI	N-acetyl-p-benzoquinoneimine
NFT	Nitrofurantoin
NMDA	N-methyl-D-aspartate
NPM	(1-pyrenyl) maleimide
NSAIDs	Nonsteroidal anti-inflammatory drugs
OTC	Over-the-counter
PBS	Phosphate buffered saline
PCr	Phosphocreatine
PGES	Prostaglandin endoperoxidase synthetase
PGH_2	Prostaglandin H
PGHS	Prostaglandin H synthase
PO	Orally
PUFA	Polyunsaturated fatty acid
POX	Peroxidase
ROS	Reactive oxygen species
SBB	Serine borate buffer
SOD	Superoxide dismutase
TBA	Thiobarbituric acid
t-BHP	Ter-butyl hydroperoxide
TCA	Trichloroacetic acid
UDPG	uridine diphosphate-glucuronic acid
y-GCS	*y*-glutamylcysteine synthetase
y-GT	*y*-glutamyl transpeptidase

1. INTRODUCTION

Acetaminophen (APAP), also known as paracetamol in Britain, is derived from the chemical compound named para-acetylaminophenol.[1] It is an analgesic and antipyretic drug that is widely used by approximately 43 million adults in the USA every week. [2] It is safe at a therapeutic dose and is available over-the-counter as a single formula and in combination with other medications, as well as by prescription when it is combined with opioids.[2] However, an overdose results in hepatic toxicity, the most common cause of drug-induced liver injury,[3-6] that has led to nearly 80,000 emergency room visits and around 30,000 hospitalizations annually in the USA.[7, 8] The first case of APAP hepatotoxicity was reported in 1966 and concerns about APAP toxicity have been ongoing since then.[9] In fact, the Food and Drug Administration (FDA) requested that manufacturers limit the amount of APAP in combination products to no more than 325 mg in each tablet.[2]

Increased attention is being given to APAP toxicity, in the medical setting, as over doses of APAP appear to be common in the USA, despite the known risks of toxicity. Without immediate treatment of an overdose, APAP toxicity leads to liver and kidney failure and death. N-acetylcysteine (NAC), a GSH precursor, [10] is currently the only FDA approved antidote for this toxicity. NAC restores GSH levels by reducing extracellular cystine to cysteine, a GSH precursor, and increases the synthesis of GSH, thus helping in the detoxification of the reactive metabolite and the scavenging of free radicals.[11-13] Additionally, NAC supplies mitochondrial energy substrates in the Krebs cycle and restores hepatic adenosine triphosphate (ATP) levels by providing excess amino acid (not needed for GSH synthesis) and uses it as energy substrates. [11-13]

Thereby, NAC helps to maintain a balance between reactive species and the antioxidant defense system in the body. However, high doses of NAC are required due to its low bioavailability since the carboxyl group loses its proton at a physiological pH of 7.35-7.45, making NAC negatively charged. [14] This impedes the passing of NAC through the biological membranes, which increases the risk of side effects and the costs of treatment. Martello et al. [15] reported a cost analysis that compared actual costs for patients who received oral or IV NAC.[16] Overall health care cost (median cost) of IV NAC was reported as $7,607.82, versus $18,287.63 for oral NAC, with a median length of stay of 5 days versus 7 days, respectively. The decreased cost of treatment and a shorter hospital stay have been used to justify approval of the use of an IV NAC. In addition to that, the side effects that accompany a high dose of IV NAC are similar to those of a clinical presentation of true anaphylaxis that includes rash, pruritus, angioedema, bronchospasm, and (rarely) hypotension. This may require discontinuation or delay in providing treatment. [17] Oral administration of NAC has been associated with vomiting and diarrhea, along with an unpleasant odor. Therefore, it would be of great value to introduce a safe and more effective drug that requires a shorter treatment time without these adverse side effects. Although, many investigators are exploring and endeavoring to introduce different compounds that can be active against the toxicity induced by APAP, most of those compounds are administered prior to APAP toxicity [18-25],which does not give an exact picture of their antidotal effect.

N-acetylcysteine amide (NACA) is a modified form of NAC in which the carboxyl group is replaced with an amide that increases its lipophilicity.[14] This allows NACA to easily cross cell membranes and to require lower doses than NAC, which may

overcome the adverse side effects of NAC. The enhanced ability of NACA over NAC to penetrate cells was shown in my previously published study using HepaRG as the hepatic cell line.[26] NACA is a thiol antioxidant that enhances cellular antioxidant defense mechanisms and acts as a precursor to GSH. It can also promote intracellular detoxification and act directly as a free radical scavenger. Promising results with NACA, in treating various oxidative stress-related disorders, [27-31] as well as its encouraging effects on toxicity induced by APAP in the HepaRG cell line, inspired us to investigate these effects in an animal model.

The purpose of this study was to investigate and determine the therapeutic effect of NACA against APAP-induced toxicity of various organs in an animal model, and to compare that with the effect of NAC. In this study, I used a mouse rather than a rat model for the experiment because Mitchell McGill and his group showed that rats are more resistant to APAP hepatotoxicity than mice.[32] This is due to a reduced mitochondrial protein binding that limits mitochondrial dysfunction, oxidative stress, and peroxynitrite formation and, therefore, does not show the exact picture of the toxicity induced by APAP. Accordingly, I used C57BL/6 mice, a well-known strain, that is commonly used for studies of APAP toxicity since the mechanisms of its toxicity are well known.[33]

2. LITERATURE REVIEW

2.1. HISTORY OF ACETAMINOPHEN

Acetaminophen (APAP) was discovered by chance and Mata Jozwial *et al*. published an account of that discovery in their paper. "*In the '80s of the 19th century, two young doctors at the University of Strasburg, in order to eradicate worms, by mistake dispensed acetanilide to a patient instead of naphthalene. They noticed that the drug had a small impact on intestinal parasites; however, it significantly decreased high temperature. Young doctors - Arnold Chan and Paul Heppa - quickly published their discovery and acetanilide was introduced into medical practice in 1886 under the name of antifebrin. Soon it appeared that, although the production of this drug was very cheap, acetanilide could not be used as an antipyretic medicament due to its high toxicity, the most alarming of which was methemoglobinemia. This resulted in a great deal of research on less toxic derivatives of acetanilide. Phenacetin and N-acetyl-p-aminophenol appeared to be the most satisfying compounds, which had been earlier synthesized by Harmon Northrop Morse in 1878. The first clinical trials with those two acetanilide derivatives were performed by a German pharmacologist Joseph von Mering. On the basis of the obtained results, a faulty conclusion was drawn that paracetamol was characterized by high toxicity similar to acetanilide, therefore phenacetin was the first derivative to be introduced into medical practice in 1887. Phenacetin was widely used in analgesic mixtures until the time when it was associated with the development of analgesic nephropathy after a prolonged usage. In Poland, phenacetin was used as a*

component of very popular and available everywhere analgesic ëítablets with the crossíí. In fact, acetaminophen became popular in 1948 when Bernard Brodie and Julius Axelrod demonstrated that paracetamol was the main active metabolite of acetanilide and phenacetin was responsible for their analgesic and antipyretic action and that methemoglobinemia was induced by another metabolite, phenylhydroxyl- amine. That discovery revolutionized the pharmaceutical market of analgesic drugs and since then paracetamol has started its staggering career. "[34] Now, it is included in the WHO Model List of Essential Medicines.

2.2. ACETAMINOPHEN FACTS AND TOXICITY SIGNIFICANCE

The Food and Drug Administration (FDA) approved the use of APAP in 1950 and as an over-the-counter medication in 1985.[35] In 2005, consumers purchased more than 28 billion doses of products containing APAP, with the hydrocodone–acetaminophen combination product being the most frequently prescribed drug [35], and 165,000 cases of overdose being reported in the United States alone.[36] It is widely used by approximately 43 million adults in this country every week, [2] leading to nearly 80,000 emergency room visits and around 30,000 hospitalizations annually.[7, 8] The first case of APAP hepatotoxicity was reported in 1966 and concerns about APAP toxicity have been ongoing since then.[9] The kidney is the second target organ of APAP toxicity, after the liver.[37] Studies have reported renal dysfunction in 1%-2% of the patients who overdosed on APAP. [36] In 2009, the FDA required that nonprescription and prescription APAP-containing medications provide information regarding the risks of APAP-induced

hepatotoxicity.[38, 39] In addition, they recently requested that manufacturers limit the amount of APAP in combination products to no more than 325 mg in each tablet. [2]

2.3. ACETAMINOPHEN MECHANISM OF ACTION

Despite the widespread use of APAP for over 60 years in the United States, its mechanism of action has not been fully understood until now. The primary mechanism of action is believed to be similar to that of nonsteroidal anti-inflammatory drugs (NSAIDs), by inhibiting the conversion of arachidonic acid (AA) into prostaglandin H (PGH_2), a local hormone that is associated with pain, fever, and inflammation.[40] This conversion is catalyzed by prostaglandin H synthase (PGHS), referred to as cyclooxygenase (COX), which has two isoenzymes (COX-1 and COX-2). PGHS is a bifunctional enzyme and possesses two different enzymatic activities: cyclooxygenase and peroxidase (POX). The conversion of AA to PGH2 involves two reactions: cyclization of AA to unstable 15-hydroxyperoxide (PGG2) with the involvement of a cyclooxygenase component, and double oxidation in positions 9 and 11; whereas the reduction of the PGG2 molecule to its 15-hydroxy analogue (unstable structure of PGH2) takes place due to peroxidase activity of PGHS (POX). APAP does inhibit COX activity, although its mechanism is different from that of NSAIDs. APAP does not appear to act as a traditional NSAID, and selective COX-2 inhibitors inhibit cyclooxygenase by competing with arachidonic acid for the active site of the enzyme. APAP reduces the enzyme to an inactive form by acting as a factor that reduces a ferryl protoporphyrin IX radical cation within the peroxidase site of the PGHS enzyme. The selectivity of paracetamol appears to be based on peroxide tone. It should not be termed a selective COX-2 inhibitor, although it often appears to be one. [34]

A third COX isozyme (COX-3), a COX enzyme isoform encoded by the COX-1 gen, contains additional 30-34 amino acids. Although COX-3 is inhibited by APAP, it does not function in human organisms. For this reason, COX-3 has been discounted as a cause of APAP-induced analgesia in humans. [34, 41]

Other studies have suggested that APAP is acting by modulation of the body's endocannabinoid system. APAP deacetylates to p-aminophenol that reacts with arachidonic acid (AA) by fatty acid amide hydrolase (FAAH), resulting in the formation of active metabolite N-arachidonoylphenolamine (AM404).[34] AM404 acts indirectly to increase the activity of the endocannabinoid system. These substances can both modulate a serotonergic descending pain pathway and lower body temperature. At the same time, AM404 has been found to inhibit COX-1 and COX-2 enzymes at different concentrations. This may be used to explain the inhibitory action of APAP on COX enzymes in the CNS as well.[42, 43] Other investigators have suggested inhibition of nitrogen oxide formation as a mechanism of analgesic action by APAP. APAP directly inhibits N-methyl-D-aspartate (NMDA) receptors, blocks substance P-dependent synthesis of nitric oxide (an important neurotransmitter in the nociceptive processes) through the L-arginine-nitric oxide pathway, and reduces nociception.[34, 40]

2.4. ACETAMINOPHEN TOXICITY

2.4.1. Acetaminophen Mechanism of Toxicity. The liver is the target organ for APAP toxicity because this is where it is detoxified (Figure 2.1). It is noted that drug-induced hepatotoxicity is one of the major causes for withdrawal of drugs from the market.[44] Although the exact mechanism of the action of APAP is unclear, and remains

to be discovered, its metabolism is well known and characterized (Figure 2.2). APAP is mainly metabolized by glucuronidation, sulfation, and oxidation by cytochrome P450 (CYP) to the reactive metabolite N-acetyl-p-benzoquinoneimine (NAPQI) [45-47] (Figure 2.3). At therapeutic doses, APAP is safe, with around 90-95% of the APAP being glucuronidated or sulfated in the liver, and then excreted. A small fraction of APAP remains unchanged and is also excreted in the urine. The remaining 5% -10%, metabolized by CYP (including CYP1A2, CYP3A4, and mainly CYP2E1) to the electrophilic intermediate NAPQI, is toxic, but it can be neutralized by conjugation with glutathione (GSH) to form GSH-adduct which is mainly excreted in bile. However, after an overdose of APAP, the formation of NAPQI exceeds the detoxification capacity of GSH, which leads to NAPQI accumulation.

NAPQI has been known as the toxic metabolite of APAP, since the early 1980s, through the two-electron oxidation of APAP to a reactive quinone. CYP2E1 is a major isozyme that is responsible for NAPQI formation and is largely concentrated in the centrilobular region of the liver that corresponds to the site of APAP-induced toxicity.[48] Walubo *et al*. showed that inhibition of CYP3A4, 2E1, and 1A2, with a cocktail of ketoconazole, isoniazid, and caffeine, effectively prevents APAP toxicity in rats.[49] CYP2E1 knockout mice were found to be less susceptible to APAP-induced toxicity.[50] Toxicity of NAPQI was evaluated by dosing the hepatic (HepaRG) cell line with NAPQI and evaluating cell viability, which showed a drastic decrease in cell viability.[26] NAPQI is rapidly conjugated at the 3 position by GSH and excreted in the bile. This conjugation can proceed in either an enzymatic or non-enzymatic pathway. The enzymatic reaction is

catalyzed by the glutathione S-transferase enzymes (GST) that are heavily localized in the centrilobular region of the liver, although they are found throughout the liver.[48]

NAPQI is an electrophilic compound that reacts with the nucleophilic sulphydryl group. An overdose of APAP results in the excess formation of NAPQI, which binds to the protein following a massive decrease in the amount of GSH.[51-53] Although cysteine is the major target for binding, lysine binding has been reported after APAP toxicity. This protein adduct can be measured in samples from intoxicated patients. Protein binding (mainly to mitochondrial protein) leads to mitochondrial dysfunction, which is reported to be a main step in toxicity.[54] NAPQI binding results in the inhibition of both complexes I and II in the mitochondrial respiratory chain, which disrupts the proton gradient that is necessary for the production of adenosine triphosphate (ATP). At the same time, NAPQI directly adducts ATP synthase, which leads to the inhibition of ATP production as well.[55] As a subsequent event, reactive oxygen and peroxynitrite are formed inside the mitochondria, as a result of mitochondria dysfunction, and GSH depletion proceeds to ROS generation as well.[56-58] The production of peroxynitrite leads to protein nitration and is a precursor to mitochondrial permeability transition (MPT). Protein nitration is predominantly localized to the centrilobular region of the liver, which is consistent with APAP toxicity. Peroxynitrite also directly induces mitochondrial damage, as indicated by reduction of intact mitochondrial DNA and nitrotyrosine protein adducts localized to the mitochondria following APAP toxicity. Oxidant stress is involved in the activation of the c-jun-N-terminal kinase (JNK) pathway.[59, 60] It eventually triggers the opening of the MPT pore, resulting in collapse of the mitochondrial membrane potential. Furthermore, fragmentation of DNA has been

observed, preventing cell recovery and regeneration, and contributing to necrotic cell death in APAP toxicity. Inhibition of JNK by the inhibitor leflunomide reduces mitochondrial cytochrome c release following an APAP overdose (Figure 2.4).

The kidney is the second organ affected by an APAP overdose (Figure 2.5). It receives a large supply of blood and contains specialized transport processes for concentrating and secreting drugs, as well as containing an active enzyme system to metabolically activate drugs.[61, 62] The mechanism of toxicity in the kidney is less clearly understood, as compared to the liver, although several potential mechanisms of renal toxicity are proposed in the literature.

When an overdose of APAP is administered, the metabolic pathways in the liver become saturated, which allows large amounts of unmetabolized APAP to reach the kidneys.[22] APAP-nephrotoxicity is due to metabolic activation of APAP by renal P450, a similar mechanism proposed for hepatotoxicity.[63-66] The severity of renal damage is significantly reduced by administration of piperonyl butoxide (a CYP inhibitor). [67] Another study showed that, when an animal is pre-treated with 3-methylcholanthrene (CYP induced in the liver only), APAP-induced hepatic necrosis occurred, while the kidneys were not affected, which suggested that the toxic metabolite was formed *in situ* in the kidneys.[68] Another possible mechanism of APAP toxicity is related to prostaglandin endoperoxidase synthetase (PGES). [67-69] PGES is an enzyme found in the kidneys that activates APAP to NAPQI in the medulla, where it is more pronounced, in contrast to CYP, which plays a more important role in the cortex. NAPQI conjugates to the protein of the proximal tubule that may initiate cell death. Moreover, N-deacetylation of APAP produces aminophenol, which is a nephrotoxic metabolite that could start redox

cycling in the kidney.[67, 69, 70] NAPQI-GSH conjugate is excreted into bile, reabsorbed in the small intestine, and transported to the kidneys, where it is degraded by Ƴ-GT to NAPQI-cysteine. It was proposed that NAPQI-cysteine could form a reactive intermediate formed by C-S-lyase, instead of being conjugated to an acetyl group and excreted in the urine as the mercapturic acid conjugate.[71]
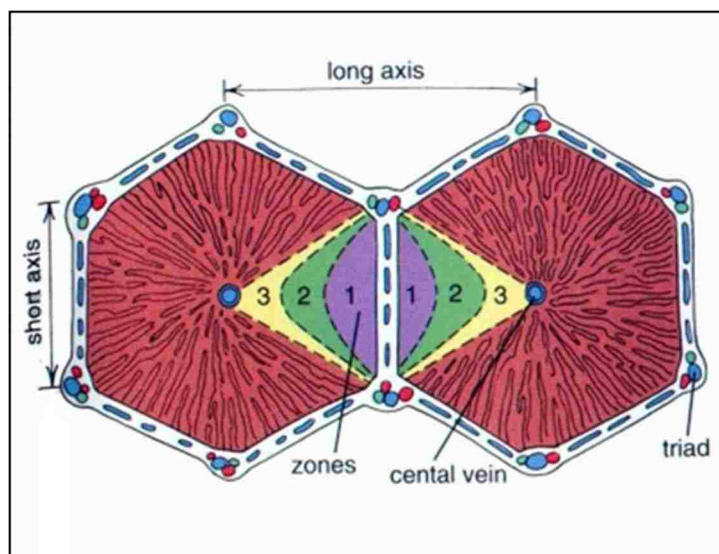
Figure 2.1. Localization of APAP toxicity in the centrilobular region (zone 3) of a liver cell
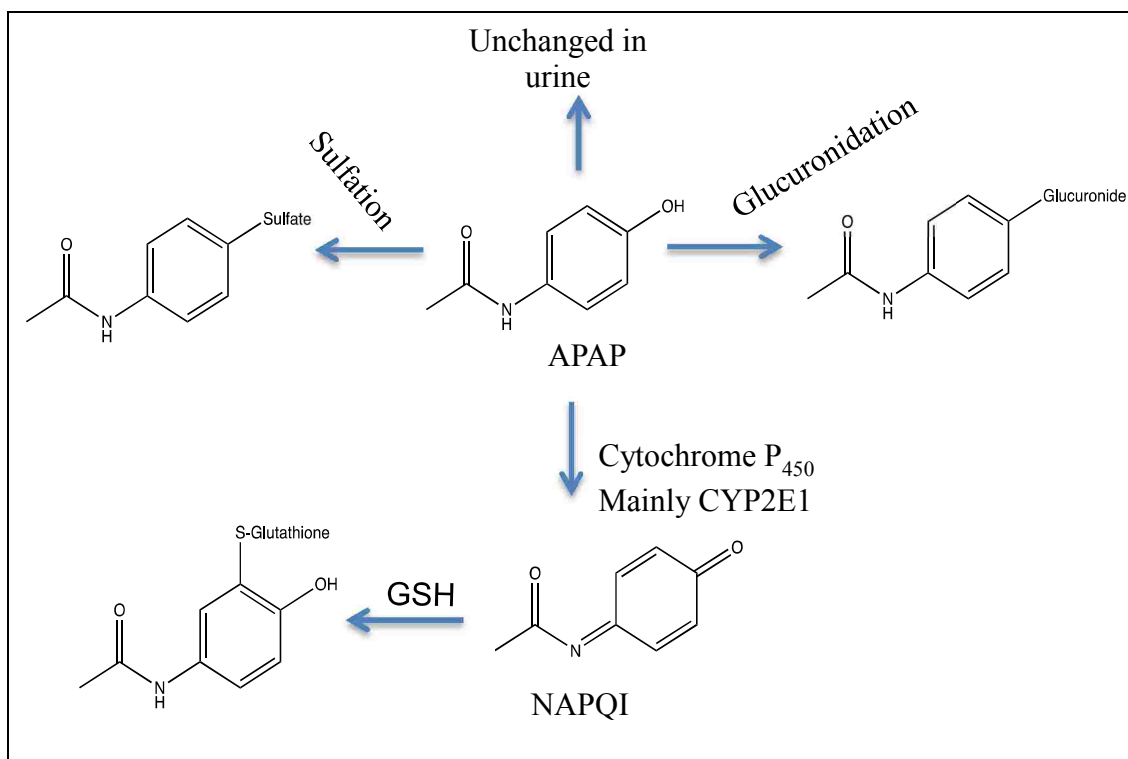
Figure 2.2. Metabolism of acetaminophen (APAP)

Acetaminophen (APAP)　　　　N-acetyl-p-benzoquinoneimine (NAPQI)
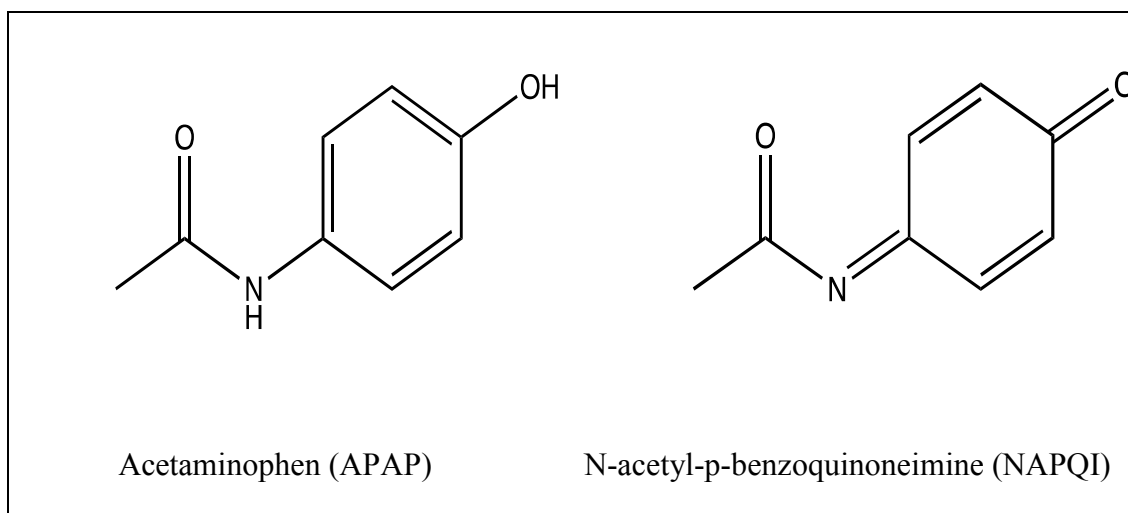
Figure 2.3. Chemical structures of APAP and its active metabolite NAPQI
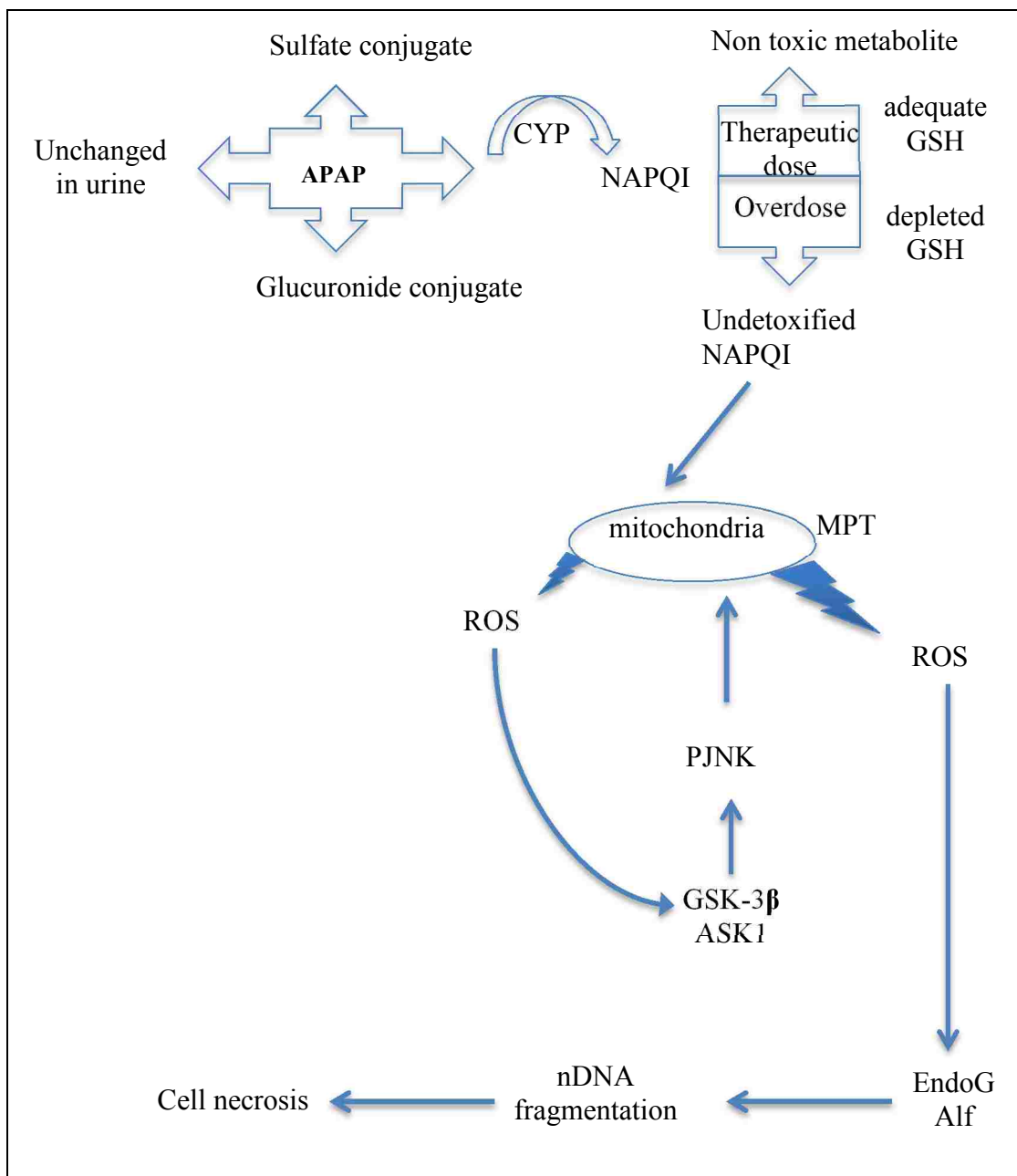
Figure 2.4. Mechanism of toxicity of acetaminophen (APAP)
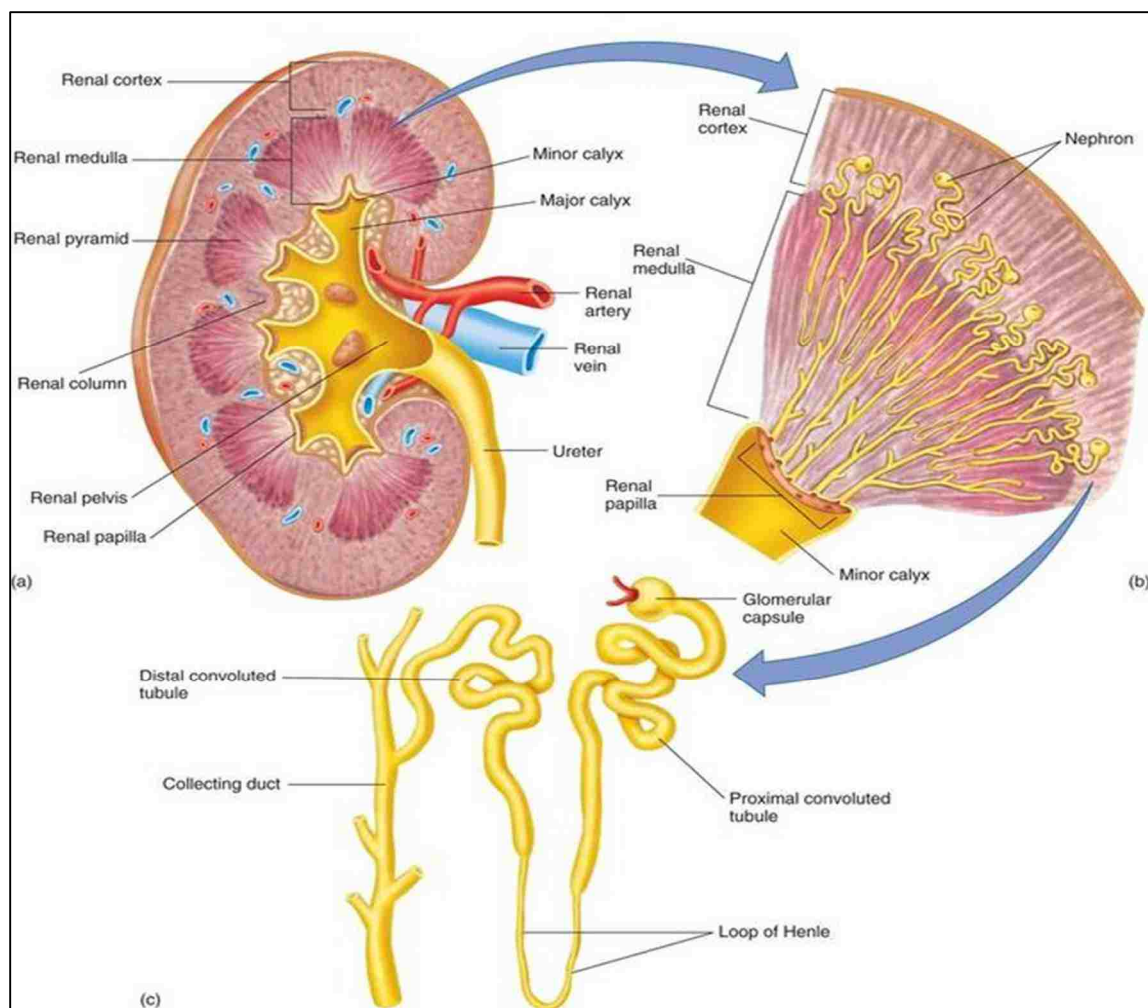
Figure 2.5. Site of toxicity of acetaminophen (APAP) in the kidney

2.4.2. Oxidative Stress in Acetaminophen Toxicity. Oxidative stress takes place when the balance between antioxidants and free radicals is broken; drugs and environmental toxins generally induce that. It is known to be involved in the propagation of cell injury induced by an APAP overdose and is regarded as a mechanism of hepato-renal toxicity.[72-81] Metabolism of APAP to NAPQI and its detoxification by GSH lead to severe depletion of GSH (stored in the liver and kidneys), thereby inducing ROS. In addition, a consequence of that event is excessive NAPQI binding to mitochondrial

protein. This leads to mitochondrial dysfunction that results in generation of reactive oxygen/nitrogen species as well. A high level of ROS is known to cause impairment of the antioxidant defense system that subsequently leads to oxidative stress. Oxidative stress, in turn, induces various deleterious actions, including lipid peroxidation, protein oxidation, and DNA fragmentation. Many investigators, who have evaluated the effects of natural antioxidants in preventing or treating APAP toxicity, have shown positive effects which confirm the role of oxidative stress.[20-24, 33, 80]

Glutathione (GSH) is a tripeptide with a gamma peptide linkage between the amine group of cysteine, which is attached by normal peptide linkage to a glycine, and the carboxyl group of the glutamate side-chain.[82] It is synthesized by the action of the two-enzymatic processes, γ-glutamylcysteine synthetase (γ-GCS) to link the amino acids of glutamate and cysteine together through a gamma peptide bond to form γ-glutamylcysteine. This is followed by further reaction of γ-glutamylcysteine with glycine to form glutathione, which is catalyzed by γ-glutathione synthetase (Figure 2.6). γ-GCS is the rate limiting step and it depends on the availability of cysteine, the sulfur amino acid precursor. GSH has a sulfhydryl group and acts as a reducing agent and antioxidant. It is converted to its oxidized form, glutathione disulfide (GSSG), when it reacts directly with free radicals (Figure 2.7). GSSG can be reduced back to GSH by a nicotinamide adenine dinucleotide phosphate (NADPH) dependent enzyme, glutathione reductase (GR). [82] The ratio of GSH/GSSG is a determinant of the redox status of biological systems, so any defect in the level of GSSG to GSH has a pronounced effect on this ratio. GSH is known to be involved either directly or indirectly in a number of biological phenomena and is mainly responsible for maintaining cellular redox status in endothelial cells. GSH

scavenges free radicals (R$^{\bullet}$) and other reactive oxygen species (ROS), and neutralizes toxic metabolites by condensing them enzymatically and non-enzymatically. GSH plays a major role in APAP detoxification since it spontaneously and rapidly reacts with the active toxic metabolite of the APAP (NAPQI) (k=3.2 $\times 10^4$ M^{-1} s^{-1} at pH 7.0) or is catalyzed by glutathione S transferase (GST).[83] An APAP overdose causes severe GSH depletion, allowing NAPQI to attach to protein (mainly mitochondrial protein), leading to oxidative stress, which ends in hepatocyte death. An i.v. administration of GSH after an APAP overdose results in accelerated recovery of the mitochondrial GSH content.[13] The elevated levels of mitochondrial GSH effectively scavenge reactive oxygen and peroxynitrite, which reduces APAP-induced liver injury and promotes regeneration. Glutathione-related enzymes, GPX and GR, that affect the conversion of GSSG to GSH (e.g., regeneration of GSH), are inhibited in the APAP-treated group, as well as other antioxidant enzymes (SOD, CAT, and GST) that play an important role in maintaining redox homeostasis under normal physiological conditions (Figure 2.8). This affects radical quenching capacity and results in oxidative stress.[21, 48, 70, 75]

Lipid peroxidation shows when the antioxidant defense system fails to prevent excess formation of free radicals. It affects the physicochemical properties of membrane lipid bilayers, including decreases in membrane fluidity and inactivation of membrane bound enzymes that result in severe cellular dysfunction.[82] A lipid peroxidation chain reaction is initiated by a hydroxyl radical combining with one hydrogen from a methylene carbon on a polyunsaturated fatty acid (PUFA) to form water and a lipid radical. Due to the presence of many double bonds between carbon atoms, the abstraction of the hydrogen atom is easier and the resulting molecule is more stable. The lipid radical

is not a very stable molecule, so it reacts readily with molecular oxygen, thereby creating a peroxyl radical. This radical is also an unstable species that reacts with another free fatty acid, producing a different lipid radical that combines with the abstracted hydrogen to form hydroperoxides. This cycle continues, as the new fatty acid radical reacts in the same way. The lipid hydroperoxides decompose in the presence of metals, such as iron or copper, to form toxic aldehydes, such as 4-hydroxy-2-nonenal (4-HNE) and malondialdehyde (MDA). The APAP toxicity research community showed the role of lipid peroxidation in the aggravation of hepatic and renal toxicity.[20, 22, 33, 36, 63]
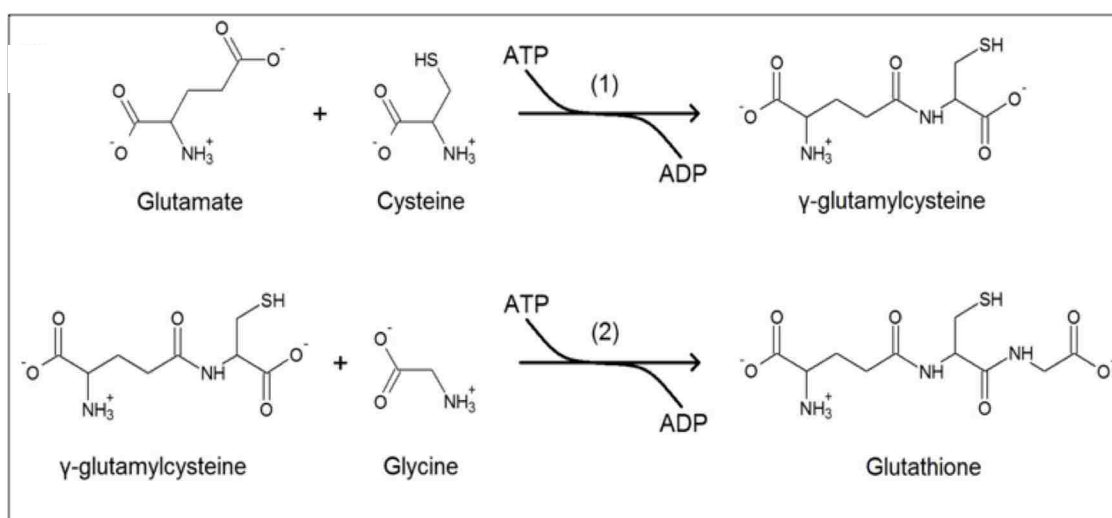
Figure 2.6. Synthesis of glutathione (GSH)

In step (1) ϒ-glutamylcysteine synthetase. (2) glutathione synthetase.
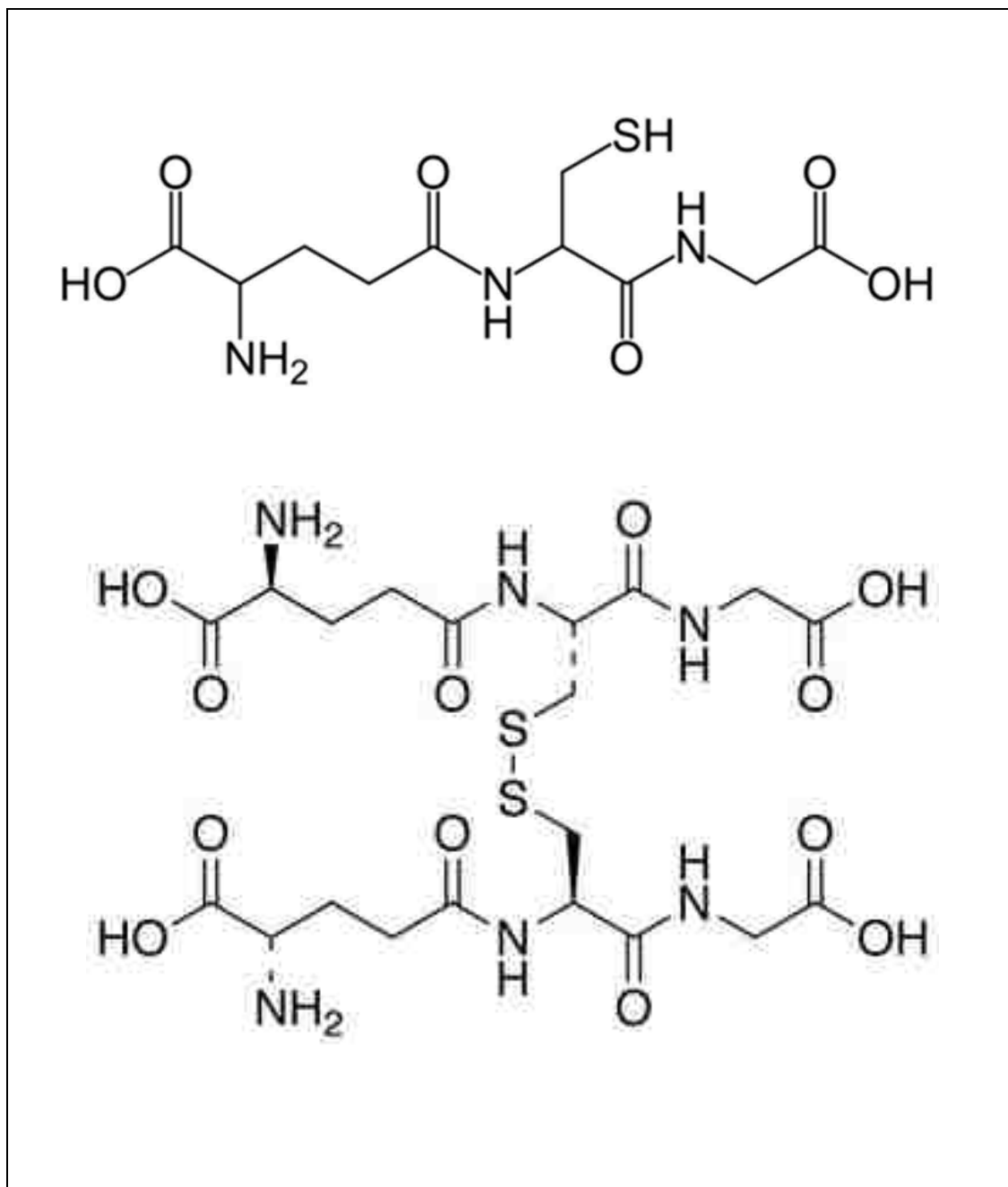
Figure 2.7. Structure of glutathione (GSH) on top, and glutathione disulfide (GSSG) at bottom
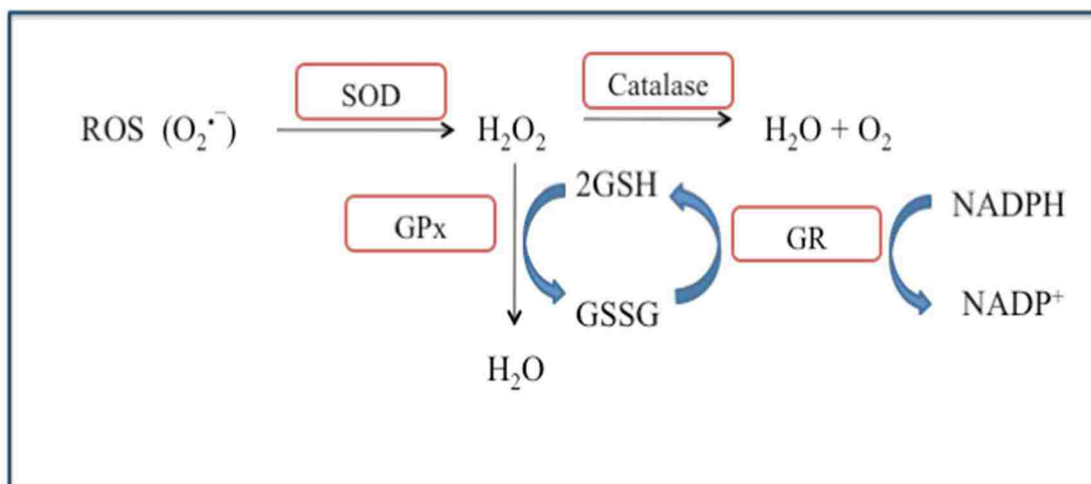
Figure 2.8. Antioxidant enzymes.

2.4.3. Contributing Factors in Acetaminophen Toxicity. Factors that contribute to increased risk of APAP toxicity are generally due to one or both of the following two mechanisms:

A) Depletion of GSH: as in the cases of malnutrition, eating disorders (such as anorexia nervosa), HIV infection, and cystic fibrosis.[84, 85] GSH is the first defensive line against the toxicity induced by APAP. NAPQI, which is the toxic metabolite of APAP, is detoxified by conjugation with GSH. [45-47] Therefore, a depleted GSH level, due to any disorder, means a defect in the defensive pool against toxicity, which increases the risk of toxicity. Several cases of hepatotoxicity have been reported from therapeutic doses of APAP when it is administered to fasting patients or to those who are not eating well.[84]

B) Induction of CYP: as in chronic alcohol use and use of drugs that induce an enzyme (mainly CYP2EA and CYP3A4 isoenzymes), such as phenytoin, barbiturate, and carbamazepine, that increase the risk of toxicity.[85] Inductions of CYP, that metabolizes APAP to the toxic metabolite (NAPQI), results in the

accumulation of NAPQI to levels that exceed the detoxification capacity of GSH. Genetic variations in CYP and APAP metabolism are documented, and it is well known that some patients are resistant to APAP toxicity.[86]

2.4.4. Role of Alcohol in Acetaminophen Toxicity. Chronic alcohol abuse is recorded in about 50% of the patients with APAP toxicity.[84] The underlying mechanism is related to the induction of CYP2E1,[87] and results in an increase in the production of NAPQI to a level that exceeds the availability of GSH for conjugation and detoxification. One reviewer suggested that the late presentation of those chronic alcohol patients was the main reason for the increased susceptibility to toxicity.[86] Although two of the largest published patient cases of APAP hepatotoxicity did not show a significant link between alcohol and APAP toxicity,[85] the FDA has required an alcohol warning on the label of all formulas containing APAP, by stating that patients should ask their doctor if they consume three or more alcoholic drinks per day.[84]

Chronic alcoholism is often associated with poor nutrition (fasting state). Fasting affects APAP metabolism by decreasing the metabolism in the glucuronidation pathway, which enhances toxicity.[85] APAP is metabolized mainly to acetaminophen glucuronide (50% – 65% of APAP) by glucuronusyltransferase. Glucuronide is provided by uridine diphosphate-glucuronic acid (UDPG), which is derived from glucose 1-phosphate, that is derived from either glycogen or glucose 6-phosphate so that it is dependent on glucose reserves. There is a ten-fold difference in the glucose levels during a fasting state in rats than in the fed state, which is explained by the glucostat function of the liver. Liver metabolic pathways are directed to provide glucose to other organs by performing gluconeogenesis, thereby reducing the amount of glucose precursor available for

glucuronidation. The amount of the enzyme CYP, which increases directly by fasting (or because of diminished glucuronidation) is responsible for NAPQI (toxic metabolite) formation. At the same time, it decreases the availability of GSH, the precursor for NAPQI detoxification, which leads to the accumulation of NAPQI and results in toxicity.[88] Alcohol can potentiate the fasting effect on APAP toxicity by inducing CYP_{450} (mainly CYP2E1) as well (Figure 2.9).
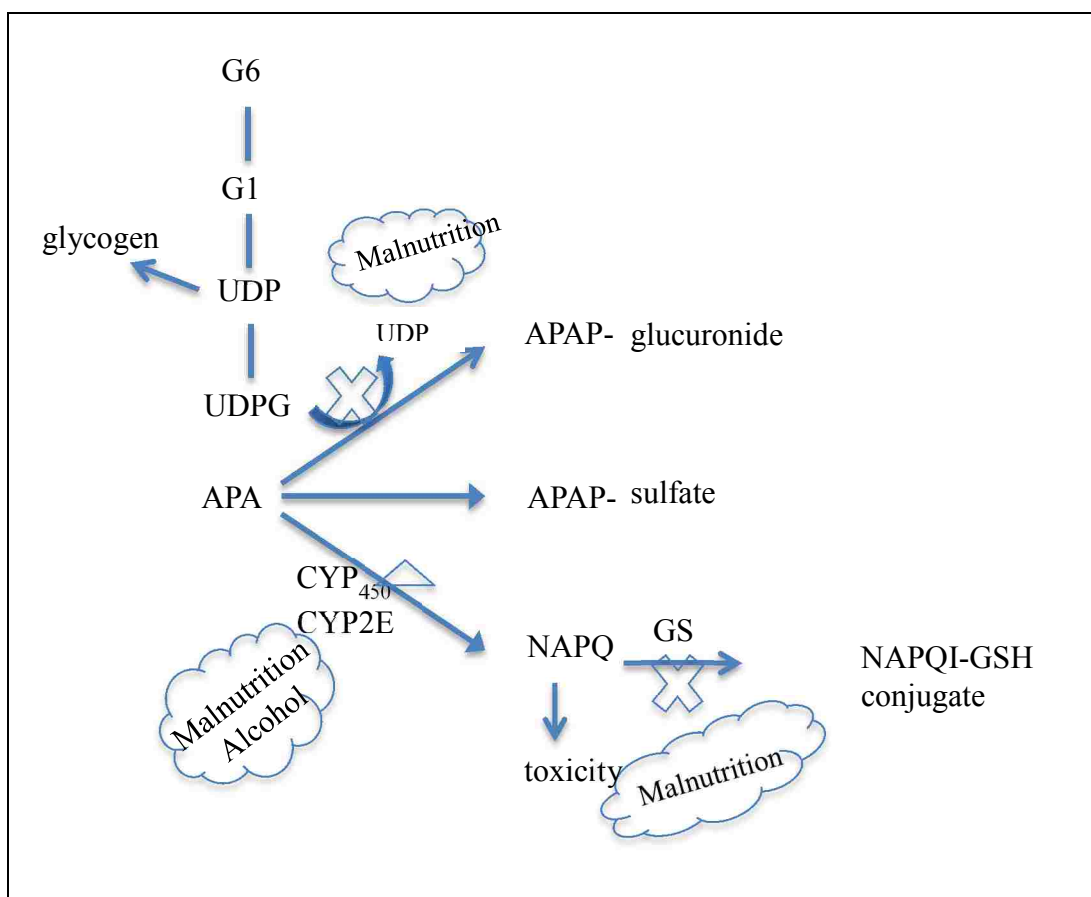
Figure 2.9. Role of alcohol and malnutrition in acetaminophen (APAP) toxicity

⋈　Inhibitory effect
△　Induction effect

2.4.5. Prevention of Acetaminophen Toxicity. Responding to concerns about the high incidence of APAP toxicity, the FDA has announced many warnings over the years and has insisted that manufacturers adhere to various restrictions in their formulas to prevent and decrease further incidences of this toxicity. This intervention has included restricting tablet strength, limiting combination formulas, improving labeling and, most important, enhancing efforts for effective public education.[34] Unfortunately, some consumers believe that OTC products are extremely safe and are not likely to lead to serious toxicity. They do not read labels or follow directions for use. They are not aware that APAP can cause serious liver injury, and some people (e.g., certain users of alcohol and medications that induce CYP, and individuals with liver disease) may be more susceptible to hepatic injury. Because some patients may not get adequate pain relief after taking the recommended dosage of APAP, they may take more than the recommended amount, or use other products that also contain APAP. They should be aware that the symptoms of an APAP overdose may not appear for up to 3 days, so they may continue to take APAP and suffer increased damage. The symptoms of liver injury may mimic the condition that they are treating (e.g., flu symptoms). [89]

2.5. TREATMENT OPTIONS FOR ACETAMINOPHEN TOXICITY

2.5.1. Approved Option for Acetaminophen Toxicity Treatment (N-acetylcysteine).

2.5.1.1. N-acetylcysteine mechanism of protection. N-acetylcysteine (NAC) is the drug of choice to treat APAP poisoning. The use of NAC in the treatment of APAP poisoning originated in England in the 1970's. [90] NAC helps reduce the effects of APAP

toxicity in different ways.[11-13] It functions to help replenish GSH stores and, in conjunction with NAPQI, acts as a detoxification agent for that toxic metabolite by providing an important GSH precursor, cysteine. The initial and rate-limiting step in GSH synthesis involves y-GCS that depends on cysteine availability. Although cysteine is normally available in low concentrations in the liver, its properties of rapid catabolism and poor solubility in aqueous media make direct administration of cysteine impossible.[17] Since depletion of GSH below a critical threshold concentration is required for APAP-induced toxicity, the main way that NAC helps protect against toxicity is by stimulating GSH synthesis. However, in order to provide effective protection by this method, the NAC has to be administered during the metabolism phase of APAP. NAC treatment accelerates the recovery of mitochondrial GSH and scavenges reactive oxygen and nitrogen species. Mitochondrial dysfunction is a consequence of oxidative stress induced by mitochondrial respiration inhibition and enhanced formation of reactive species into the mitochondrial (due to NAPQI binding to mitochondrial protein). This is the main step in APAP toxicity, so the mechanism of NAC helps reduce the toxicity and promotes regeneration.[11-13] NAC treatment, in addition to stimulating the synthesis of GSH, the body's natural defense against endogenously generated reactive oxygen species and toxic acetaminophen metabolite (NAPQI), provides excess amino acids that are not needed for GSH synthesis as an energy substrate for the Krebs cycle to maintain a hepatic ATP level.[11-13]

 2.5.1.2. N-acetylcysteine dosing and treatment protocol. The Food and Drug Administration (FDA) approved NAC for oral administration in 1985 (140 mg/kg by mouth or nasogastric tube diluted to 5% solution, followed by 70 mg//kg by mouth every

4 h for 17 doses) and intravenous (IV) administration in 2004 (loading dose is 150 mg/kg in 5% dextrose over 15 minutes; maintenance dose is 50 mg/kg, given over 4 h, followed by 100 mg/kg administered over 16 h). [91] Although oral and IV formulation are equally effective when administered 8-10 h after an APAP overdose, the IV route is preferred because of increased patient tolerance, a shorter treatment period, and cost effectiveness.[16]

APAP levels provide a basis for determining the need to initiate or continue treatment with NAC. These levels should be plotted in a nomogram and measured at 4 h, or as soon as possible [91] (Figure 2.10). Levels obtained before 4 h cannot be plotted in the nomogram.[91] In the UK, the standard treatment nomogram is called 200-line. It is drawn from an APAP concentration of 200 mg/L at 4 h to 30 mg/L at 15 h. Concentrations above the nomogram line are associated with a high risk of toxicity that indicates a requirement for NAC treatment.[92] If a patient is at high risk for toxicity due to other risk factors (malnutrition, chronic ethanol consumption, or taking any CYP inducer medication), then treatment should be more aggressive and follow a nomogram drawn at 50% of the standard treatment line (called 100-line). In the U.S. and Australia, a 150-line is drawn intermediate to the UK standard, and high risk nomogram lines are used to determine the need for NAC treatment, while in Denmark, NAC is routinely administered to all patients after an APAP overdose, irrespective of the serum level of APAP.[17]

2.5.1.3. N-acetylcysteine drawbacks. The main drawbacks of NAC are the requirement for high doses and a long treatment course, due to poor bioavailability. Its carboxyl group loses its proton at physiological pH, making the compound negatively

charged, and making its passage through the biological membrane difficult.[14] High doses of NAC are associated with side effects such as occasional nausea, vomiting, rare urticaria, or bronchospasm (with oral administration), and it may also lead to an anaphylactic reaction with intravenous administration.[93, 94] The mechanism is related to a NAC concentration, that is dependent on histamine release, but it is different from the true anaphylaxis which is independent of tryptase and immunoglobulin E.[17] Discontinuing NAC administration, antihistamines, and epinephrine for bronchospasm may successfully treat these effects. Some studies have shown that prolonged use of NAC can delay liver recovery after APAP toxicity by impairing liver regeneration due to reduced NF-κB DNA binding and decreased expression of cyclin D1 protein in the liver tissue.[16]

http://labmed.ucsf.edu/labmanual/db/data/tests/5.html
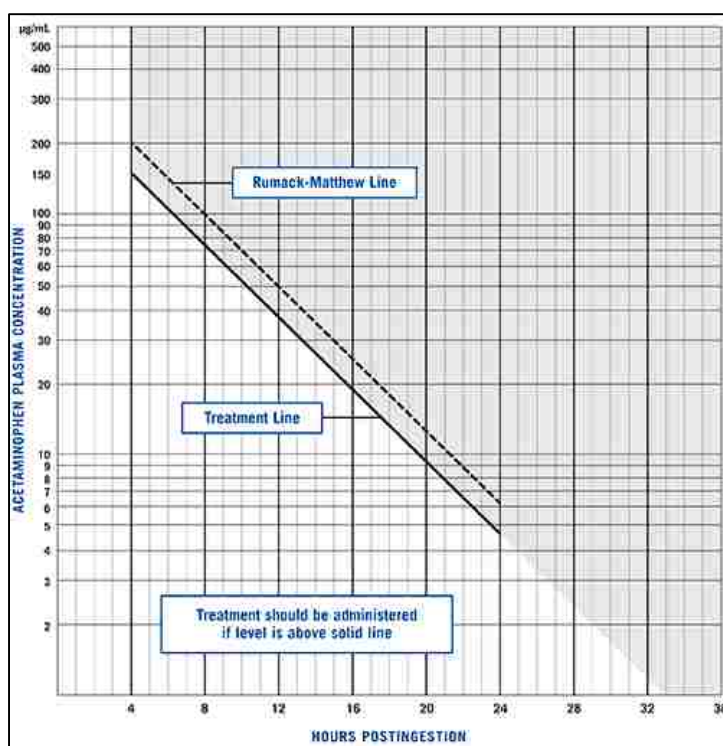
Figure 2.10. Acetaminophen (APAP) nomogram

2.5.2. Proposed Option for Acetaminophen Toxicity Treatment

(N-acetylcysteine amide). N-acetylcysteine amide (NACA) is a modified form of NAC that has an amide group instead of a carboxyl group of NAC that increases its lipophilicity, allowing it to cross cell membrane easily [14] (Figure 2.11). NACA hydrolyzes to give cysteine, a precursor for GSH, which is the major component in defending against APAP toxicity. NACA has been shown to, not only overcome the drawbacks of NAC, but to have many advantages over it.[14] NACA is more membrane permeable than NAC and, owing to its neutral charge at the physiological pH, NAC is negatively charged at physiological pH that limits its ability to cross cell membranes and, therefore, requires higher doses and longer treatment times. The nausea and vomiting, caused when NAC is administered orally, and the anaphylactic reactions that follow its IV administration, could be overcome by using a lower dose of NACA, which has higher bioavailability, and eliminates the pro-oxidant effects of NAC that occur with higher concentrations.[14] The greater membrane permeability of NACA (over NAC) was addressed by Grinberg L *et al.* by using human red blood cells as a model system in a study where NACA was found to be five times more potent than NAC, suggesting better membrane permeability of NACA.[28] It acts as a carrier of NAC and acts directly as a free radical scavenger. The antioxidant and free radical scavenging abilities of NACA are equal to, or are an improvement over, those of NAC.[14] Ates *et al.* concluded from their study that amide derivatization had enhanced the antioxidant property of NAC.[14] The authors also demonstrated in cell-free assays that the thiol-disulfide exchange between NACA and oxidized GSH (GSSG) was a mechanism of GSH regeneration.[28]

Several studies have been undertaken to evaluate the effectiveness of NACA, as it can cross the blood brain barrier as well as biological membranes as an antioxidant and free radical scavenger, with promising results being reported.[95, 96]
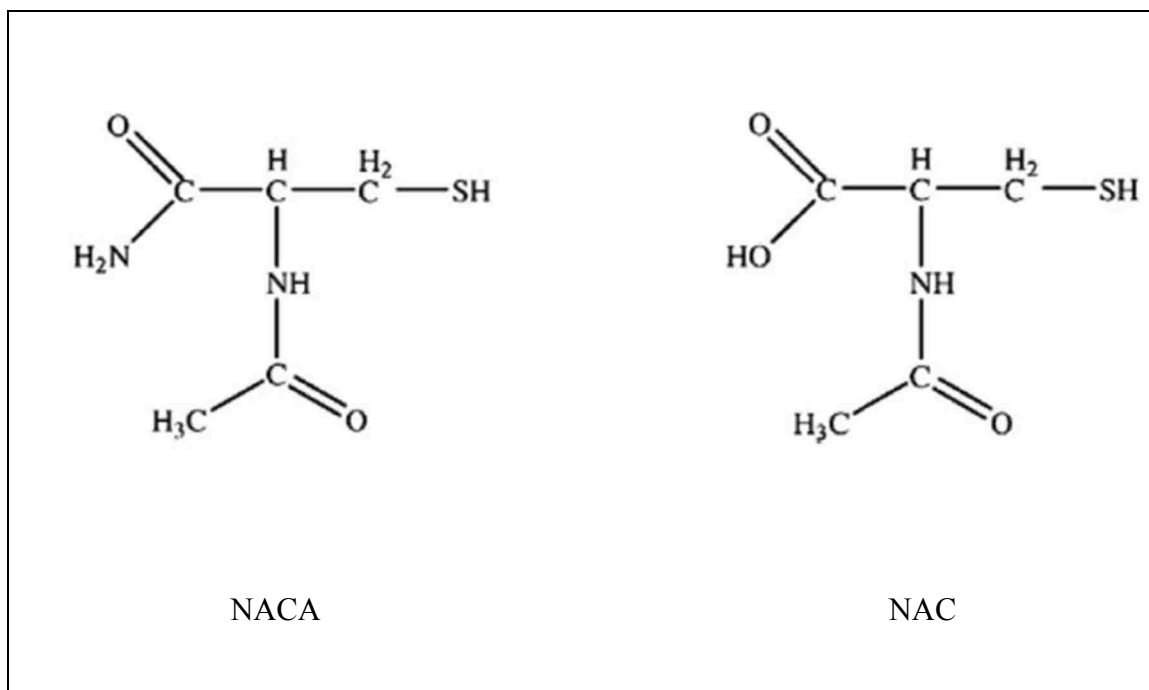
Figure 2.11. Chemical structures of N-acetylcysteine (NAC) and N-acetylcysteine amide (NACA)

2.5.2.1. Application of N-acetylcysteine amide. Depletion of GSH renders cells particularly vulnerable to oxidative stress. The resulting damage is the key step in the onset and progression of many diseases. The protective effects of NACA as an antioxidant have been demonstrated both *in vitro* and *in vivo* using different cell lines and rodent animal models, respectively. I have summarized the role of NACA in various disorders, including neurotoxicity, hematological disorder, metal ion toxicity, radiation-induced toxicity, eye-related disorders, and medicinal drug-induced toxicity.

2.5.2.1.1. Neurotoxicity

2.5.2.1.1.1. Glutamate-induced toxicity. The protective effects of NACA and the mechanism behind its protection against glutamate-induced cytotoxicity in the neuronal cell line, PC12, were investigated.[97] Incubation of PC12 cells with glutamate resulted in reduction of GSH and cysteine levels, when compared to the control group. Reduced levels of cysteine indicated that the presence of excess glutamate inhibited cystine uptake, which led to decreased GSH levels. Depletion of intracellular GSH may also be due to glutamate-induced accumulation of ROS within the cell. NACA treatment was able to increase GSH and cysteine levels and to effectively reverse the inhibitory action of glutamate. It was demonstrated that NACA protects PC12 cells against glutamate-induced cytotoxicity by preventing glutamate-induced loss of cellular GSH and inhibiting lipid peroxides.

2.5.2.1.1.2. Human immunodeficiency virus 1 (HIV-1)-associated neurotoxicity. The oxidative stress induced by HIV-1 proteins, gp120, and Tat, *in vitro* was studied in an immortalized cell line of rat brain endothelial RBE4 cells.[98] Exposure to gp120 and Tat proteins depleted intracellular GSH, enhanced MDA levels, and reduced CAT, GPx, and GR activities in cultured rat brain endothelial cells, indicating that HIV proteins induce oxidative stress in RBE4 cells. NACA treatment did reverse the increased caspase-3 activity following gp120 and Tat exposure. The oxidative stress induced by HIV-1 viral proteins was effectively blocked by NACA, and its effectiveness was further evaluated in an *in vivo* model.[99] Pretreatment of the animals (in the gp120+Tat+METH group) with NACA significantly increased the GSH levels, indicating

that the antioxidant NACA was able to partially abrogate oxidative stress-induced damage in these animals.

2.5.2.1.1.3. Methamphetamine (METH) induced toxicity. The authors observed that METH causes oxidative stress to BBB cells, as demonstrated by decreased intracellular GSH, increased MDA levels, and intracellular ROS production, as well as decreased GPx activity.[31] In addition, METH treatment also alters the integrity of the BBB by increasing the permeability of the cells. These toxic effects of METH were reversed, however, by pretreatment of the cells with NACA. This antioxidant restored the levels of GSH, and scavenged the ROS produced by treatment with METH, thereby maintaining the permeability of the BBB.[100]

2.5.2.1.2. Hematological disorder

2.5.2.1.2.1. β-thalassemic patients. NACA, to a greater extent than NAC, effectively attenuated the oxidative stress on blood cells *in vitro* in β-thalassemic patients, as well as *in vivo* in mice.[101] It increased GSH and reduced ROS levels in RBCs, platelets, and polymorphonuclear leukocytes. In addition, NACA reduced RBC lysis and phagocytosis by macrophages more effectively than NAC did.

2.5.2.1.3. Radiation-induced cytotoxicity. The search for more effective radioprotectors has intensified recently due to increased use of ionizing radiation in radiotherapy, for the treatment of malignant tumors, which induces oxidative damage to normal cells. The highly toxic hydroxyl radicals, produced by ionizing radiation, attacked the DNA molecules, causing single and double strand breaks. Additionally, the ROS also caused lipid peroxidation and protein oxidation. These deleterious changes negatively impacted cellular functions, resulting in cell death and, thereby, lowered cell viability.

NACA demonstrated better ability for preventing radiation-induced cytotoxicity and lipid peroxidation (as compared to NAC) in *in vitro* by using CHO cells.[102] Based on those results the authors concluded that NACA appears to be an ideal radioprotector. It offers good protection against radiation damage.

2.5.2.1.4. Metal ion toxicity

2.5.2.1.4.1. Lead. Lead is an ubiquitous environmental toxicant. Severe and acute lead poisoning can cause encephalopathy, convulsions, a coma, and even death. Despite several efforts to reduce lead levels in the environment, lead exposure continues to be a major public health problem, particularly in urban areas in the U.S., as well as in third world countries. Oxidative stress is possibly involved in lead toxicity. Lead crosses the BBB and accumulates in astrocytes, where it may impair cell function and perturb glial-neuronal interactions. Therefore, antioxidants that cannot cross the BBB may not be effective against lead-induced neurotoxicity. NACA, which is able to cross the BBB, might be a potential therapeutic for use in treatment of lead-induced neurotoxicity. Furthermore, NACA was found to be more effective in lowering blood lead levels, when compared to NAC. This may be attributed to the greater permeability of NACA (than that of NAC). In addition, it was more effective than NAC at counteracting lead-induced hepatotoxicity. Considering current chelation therapy and the toxic effects of chelators, the authors suggest that NACA, which is multi-functional, may be effectively included in the treatment of lead poisoning.[103]

2.5.2.1.4.2. Manganese. Manganese (Mn) is an essential trace element required for normal cellular functioning. However, overexposure of Mn can be neurotoxic, resulting in the development of manganism, a syndrome that resembles Parkinson's

disease. Although the pathogenetic basis of this disorder is unclear, several studies have indicated that it is mainly associated with oxidative stress and mitochondrial energy failure. Pretreatment with NACA protects against Mn-induced toxicity by inhibiting lipid peroxidation, scavenging ROS, and preserving intracellular GSH and mitochondrial membrane potential.[104]

2.5.2.1.5. Eye disorders. Cataracts, the most common cause of blindness worldwide, develop as a result of the progressive loss of transparency of the lens. It has been demonstrated that oxidative stress plays an important role in its pathogenesis. Supplemental NACA injections during L-buthionine-sulfoximine treatment (BSO, a GSH synthesis inhibitor that induces cataracts) were found to prevent cataract formation in most of the rat pups in the NACA+BSO group. NACA could confer a protective effect by providing a substrate for the generation of GSH and the ability to maintain antioxidant levels within the lens, possibly, through disulfide-exchange mechanisms.[105] Further, NACA has also been shown to ameliorate *ter*-butyl hydroperoxide (tBHP)-induced oxidative stress in retinal epithelial cells.[106]

2.5.2.1.6. Medicinal drug-induced toxicity. The purpose of medicinal drugs is to cure certain diseases, or to relieve some symptoms. Unfortunately, many clinical medicinal drugs have some adverse effects, especially in cases of improper use and/or an overdose. Some of these are believed to have adverse effects by inducing oxidative stress. Therefore, antioxidants have been studied to determine if they alleviate the toxicity induced by these medicinal drugs.

2.5.2.1.6.1. Bleomycin-induced oxidative stress. Bleomycin (BLM), a glycopeptide antibiotic from Streptomyces verticillus, is an effective antineoplastic drug.

However, its clinical use is restricted due to a wide range of associated toxicities, especially pulmonary toxicity. Oxidative stress has been implicated as an important factor in the development of BLM-induced pulmonary toxicity. NACA was determined to have a protective role against BLM-induced toxicity by inhibiting lipid peroxidation, scavenging ROS, and preserving intracellular GSH and mitochondrial membrane potential by using the A549 cell as a cell model.[30]

2.5.2.1.6.2. Nitrofurantoin-induced oxidative stress. Nitrofurantoin (NFT) is an antibiotic that is commonly used for treatment of urinary tract infection and other infections caused by Gram-negative bacteria (such as E. coli). However, it is contraindicated in oxidation-sensitive patients with glucose-6-phosphate (G-6-PD) deficiency or related genetic disorders due to the risk of intravascular hemolysis. It is well known that NFT causes oxidative stress by generating superoxide anion via a redox cycle that occurs in the liver. Results indicate that NFT causes oxidative stress in this cell line (HepaRG) and NACA protects cells from this oxidative damage (data not published).

2.5.2.1.6.3. Doxorubicin-induced oxidative stress. Doxorubicin is a potent and broad-spectrum antineoplastic agent. However, its lengthy treatment term may be associated with irreversible cardiomyopathic changes and consequent congestive heart failure. The cardiotoxicity is believed to be caused by oxidative stress.[107] The generation of free radicals may lead to dysfunction of the mitochondria in cardiac cells, interference with cell calcium regulation, and bioenergetics failure. NACA shows a protective effect against its toxicity by using H9c2 cardiomyocytes. NACA reduces the level of lipid peroxidation, ROS, and increases both GSH and GSH/GSSG ratio levels. It restores the antioxidant enzymes levels as well.[108]

2.5.2.1.6.4. Iohexol-induced oxidative stress. Iohexol is one of the most widely used contrast agent. Usage of radiocontrast media in radiological procedures has been reported to cause contrast-induced nephropathy via a poorly understood mechanism. However, oxidative stress has been proposed as one of the possible mechanisms. NACA shows a protective effect against apoptosis of renal proximal tubular epithelial cells, which were induced by iohexol.[109]

3. EXPERIMENTAL DESIGN

The experiments were performed with male C57BL/6 mice (Charles River laboratories), that were 7 to 9 weeks old, were housed in an environmentally controlled room with a 12 h light/dark cycle, and allowed free access to food and tap water. The animals were acclimatized for a minimum of 1 week before the experiment and drugs were administered i.p. at the indicated dose in phosphate buffered saline (PBS).

The effects of NACA as an antidote for APAP toxicity were determined by two different experiments. The potential for antidote activity was the focus of Experiment 1 and the protective effects of NACA in APAP-induced oxidative organ injury were evaluated in Experiment 2 by measuring numerous oxidative stress parameters (detailed below). The proposed mechanism of protection of NACA is shown in Figure 3.1.

3.1. EXPERIMENT 1: EXPERIMENTAL DESIGN FOR SURVIVAL STUDY

The animals were divided into four groups (n=40) as PBS, APAP, APAP+NAC, and APAP+NACA. NAC or NACA (106 mg/kg) was administered 1.5 h post to APAP administration (500 mg/kg) and then every 12 h for 72 h. The animals were monitored for a week and body weight was recorded every day. (Table 3.1)

Table 3.1. Experimental design for survival study

Group (n=10)	Treatment (all in i.p)
Control	PBS
APAP	APAP (500 mg/kg)

Table 3.1. Experimental design for survival study (cont.)

APAP+ NAC	APAP (500 mg/kg) then after 1.5 h NAC (106 mg/kg) and repeat NAC dosing q12 h for 72 h
APAP+ NACA	APAP (500 mg/kg) then after 1.5 h NACA (106 mg/kg) and repeat NACA dosing q12 h for 72 h

3.2. EXPERIMENT 2: EXPERIMENTAL DESIGN FOR OXIDATIVE STRESS STUDIES

The protective effects of NACA in APAP-induced oxidative organ injury were performed with the animals being divided into six groups (n=36) as PBS, NAC, NACA, APAP, APAP+NAC, and APAP+NACA (Table 3.2). Food was withdrawn 12-15 h prior to treatment. NAC or NACA (106 mg/kg) was administered 1.5 h post to APAP administration (300 or 400 mg/kg) and the mice were sacrificed 4 or 24 h after APAP injection; blood was then drawn and centrifuged to obtain serum. Liver, kidney, brain, and spleen samples were removed and a portion from each lobe of the liver was fixed in 10% phosphate-buffered formalin for histology or kept in a mitochondrial isolation buffer to isolate the mitochondria. The rest of the livers, kidneys, brains, and spleens were immediately frozen on liquid nitrogen and kept at -80°C until analysis.

As the toxicity of APAP is dose-and-time dependent, according to results published previously based on using cell and animal models, we studied this toxicity by using two different doses of APAP (300 and 400 mg/kg) for 4 h treatment periods. We evaluated this toxicity at two different time points (4 h and 24 h), as well as by using a fixed dose (300 mg/kg). The effects of NAC and NACA on the toxicity induced by different doses of APAP and different treatment times were evaluated by conducting some of the experiments mentioned in chapter 4.

Table 3.2. Experimental design for oxidative stress studies

Group (n=6)	Treatment (NAC and NACA will be given 1.5 h later than PBS or APAP, all in i.p.)
Control	PBS
NAC only	PBS + NAC (106 mg/kg)
NACA only	PBS + NACA (106 mg/kg)
APAP only	PBS + APAP
APAP+NAC	APAP + NAC (106 mg/kg)
APAP+NACA	APAP + NACA (106 mg/kg)
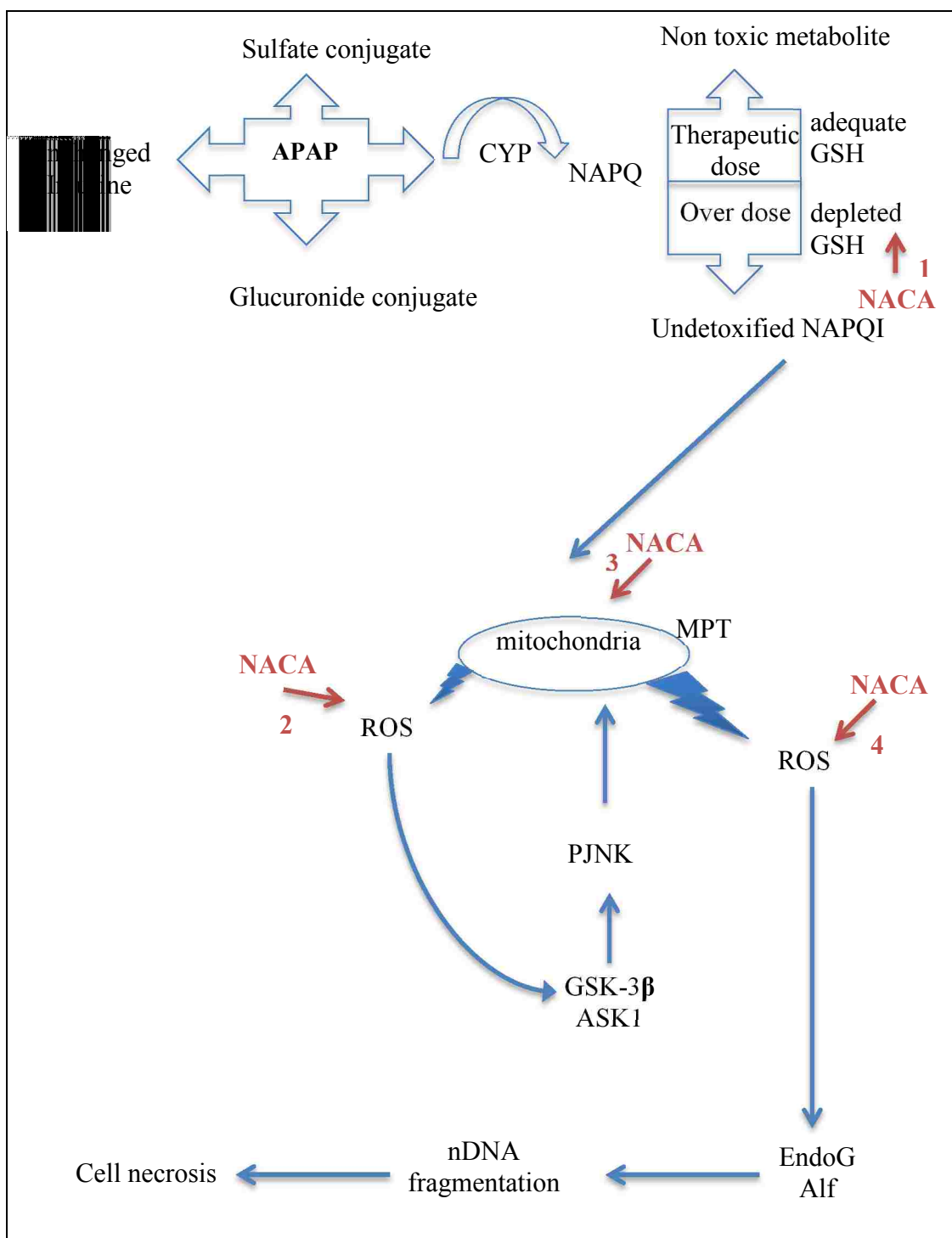
Figure 3.1. Proposed mechanism of protection of N-acetylcysteine amide (NACA)

In 1: NACA provides cysteine that helps in GSH synthesis; 2 and 4: NACA scavenges the reactive oxygen species; 3: NACA provides excess cysteine as energy substrate for the Krebs cycle to maintain hepatic ATP.

4. METHODS

4.1. HPLC METHODS

4.1.1. Intracellular Glutathione (GSH). Intracellular GSH content was determined by reverse phase HPLC, according to the method developed in our laboratory.[110] The samples were homogenized in serine borate buffer (SBB). Fifty microliters of this homogenate were added to 200 µl of HPLC grade water and 750 µl of NPM (1 mM in acetonitrile). The resulting solutions were incubated at room temperature for 5 min. The reaction was stopped by adding 10 µl of 2 N HCl. The samples were then filtered through a 0.45 µm filter (Advantec MFS, Inc. Dulin, CA, USA) and injected onto the HPLC system (Figure 4.1). 5 µl of the sample were injected for analysis using a Thermo Finnigan TM Spectra SYSTEM SCM1000 Vacuum Membrane Degasser, Finnigan TM SpectraSYSTEM P2000 Gradient Pump, Finnigan TM SpectraSYSTEM AS3000 Autosampler, and FinniganTM SpectraSYSTEM FL3000 Fluorescence Detector (λex=330 nm and λem=376 nm). The HPLC column was a Reliasil ODS-1 C18 column (Column Engineering, Ontario, CA, USA). The mobile phase was 70% acetonitrile and 30% water and was adjusted to a pH of 2.5 through the addition of 1 ml/L of both acetic and o-phosphoric acids. The NPM derivatives were eluted from the column isocratically at a flow rate of 1 ml/min; an example of the chromatogram is shown in Figure 4.2.

4.1.2. Total Glutathione (GSH) and Glutathione Disulfide (GSSG). Total glutathione content was determined by reverse phase HPLC. Samples were homogenized in SBB. Fifty microliters of this homogenate were added to 60 µl of NADPH (2 mg/ml) in nanopure water and 20 µl of 1 unit/ml glutathione reductase were added to reduce

GSSG. After 3 min of incubation at room temperature, the treated samples were diluted with 120 μl H$_2$O, and then immediately derivatized with 750 μl of 1.0 mM NPM. These samples were analyzed as detailed for the determination of GSH using reverse phase HPLC. Data from the original GSH levels and the total GSH levels in each sample were subsequently used to calculate the levels of GSSG present in each sample.
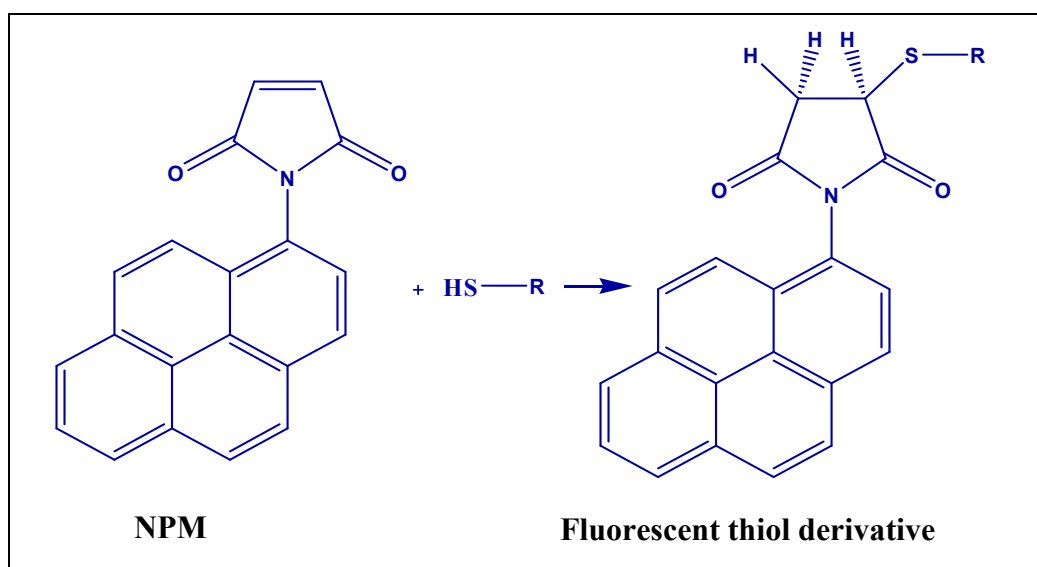
Figure 4.1. Reaction of (1-pyrenyl) maleimide (NPM) with SH group to produce fluorescent thiol derivative
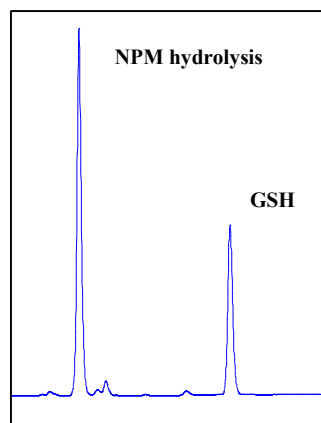
Figure 4.2. Chromatogram of GSH

4.1.3. Lipid Peroxidation. Malondialdehyde (MDA) is a thiobarbituric acid reactive substance. The extent of lipid peroxidation was determined as described by Shi *et al*.[108] Briefly, the samples were homogenized in SBB. To 0.350 ml of the homogenate, 0.550 ml of 10% trichloroacetic acid (TCA) and 0.100 ml of 500-ppm butylated hydroxytoluene (BHT) in methanol were added. The samples were then heated in a boiling water bath for 30 min. After cooling on ice, the samples were centrifuged. The supernatant fractions were mixed 1:1 with saturated thiobarbituric acid (TBA). The samples were again heated in a boiling water bath for 30 min. After cooling on ice, 0.50 ml of each sample was extracted with 1 ml of n-butanol and centrifuged to facilitate the separation phases. The resulting organic layers were first filtered through a 0.45 μm filter and transferred to HPLC vials for analysis. Fluorescence was then measured (ex. 515 nm and em. 550 nm). (Figure 4.3.)
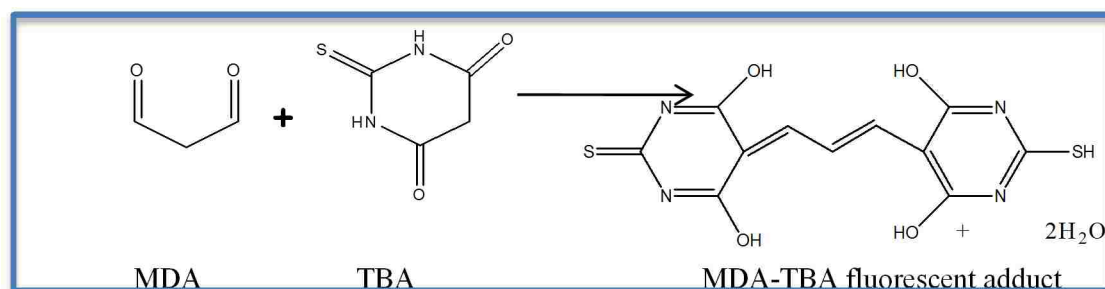
Figure 4.3. The reaction of malondialdehyde (MDA) with thiobarbituric acid (TBA) to form MDA-TBA fluorescent adduct

4.1.4. Depletion of thiol antioxidants by NAPQI. Cell-free experiments were performed to determine the effect of NAPQI on GSH, NAC, and NACA levels. This was assayed by comparison of GSH, NAC, and NACA levels in the presence and absence of NAPQI. RP-HPLC with fluorometric detection of free thiols was used to quantify

unbound thiol levels. 5 µM aqueous solutions of GSH, NAC and NACA (triplicates of each) were prepared. This group served as a control. Then, identical solutions were prepared, except NAPQI was added to each for a final concentration of 10 µM NAPQI. Both sets were incubated for 30 minutes at room temperature. Then, each was derivatized with NPM, which reacts with only free sulfhydryl groups to form fluorescent derivatives.

4.2. SPECTROPHOTOMETRIC METHODS

4.2.1. Serum Alanine Aminotransferase (ALT). To get the serum, blood was first allowed to clot for 30 min at room temperature, and then it was centrifuged at 2000 ×g for 15 minutes at 4°C. The top yellow serum layer was pipetted off without disturbing the white buffy layer. ALT was measured (using a kit-Cayman Chemical) by adding 150 ul of substrate, 20 ul of cofactor, and 20 ul of sample in a 96-well plate, and incubating at 37°C. After15 min, reactions were initiated by adding 20 ul of ATP initiator and immediately reading the plate at 340 nm, once every min for 5 min (Figure 4.4.)
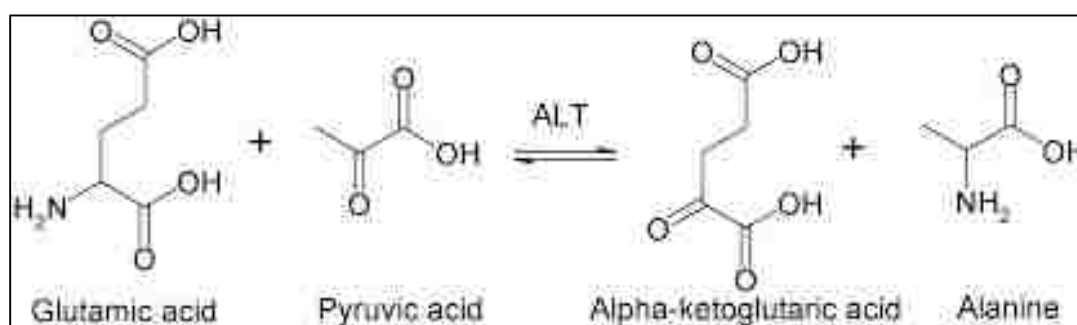
Figure 4.4. The activity of alanine transaminase (ALT).

4.2.2. Glutamate Dehydrogenase (GDH). GDH was determined as described by McGill *et al.* [46] Briefly, aliquots with 10–100 µl of serum were mixed in 700 µl of 200

mM imidazole buffer with 25 mM of ammonium acetate, 200 μM of NADH, 100 μM of ADP, and 0.05% bovine serum albumin, pH 8.0. The disappearance of NADH was monitored at 340 nm to obtain a baseline reading, and then 50 μl of a 2 mM α-ketoglutarate solution were added to begin the GDH reaction. The baseline activity was then subtracted from the GDH activity (Figure 4.5.).
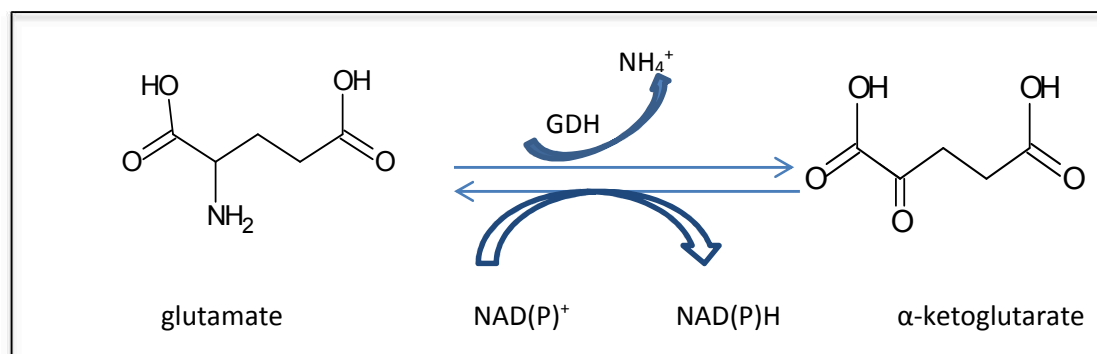
Figure 4.5. Activity of glutamate dehydrogenase enzyme (GDH)

4.2.3. Glutathione Reductase (GR) Activity. GR is the enzyme responsible for recycling GSSG into GSH via a reduction mechanism, utilizing both GSSG and NADPH as a substrate. The oxidation of NADPH to $NADP^+$ is accompanied by a decrease in absorbance at 340 nm, providing a spectrophotometric means for monitoring the enzyme activity of GR. The activity of GR is determined by adding homogenate to a solution containing both GSSG and NADPH, and then recording the absorbance as a function of time at 340 nm. The rate of decrease in the A340 is directly proportional to the GR activity in the sample. (Figure 4.6.)
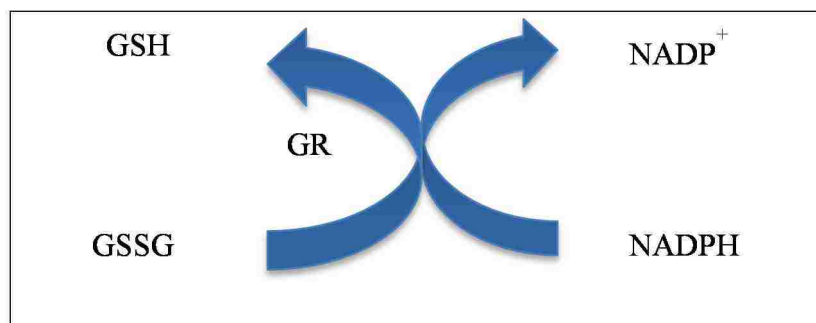
Figure 4.6. Activity of glutathione reductase enzyme (GR)

4.2.4. Creatine Kinase (CK) Activity. Creatine kinase activity was determined in the serum by using a kit (Pointe scientific Inc.) and by initially preparing the working reagent by mixing five parts of R1 with one part of R2. Then, 1 ml of this reagent mix was pipetted into an appropriate tube and pre-warmed at 37°C for 5 min. 25 ul of a sample were transferred to this reagent, mixed, and incubated at 37°C for 2 min. A reading was recorded after 2 min after the spectrophotometer was zeroed with water at 340 nm. The sample tube was returned to 37°C and a reading was repeated every min for 2 min. (Figure 4.7.)
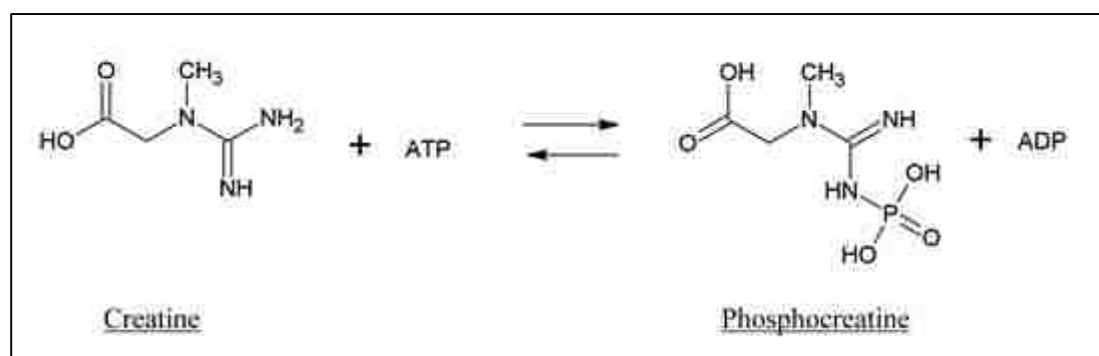
Figure 4.7. Activity of creatine kinase

4.2.5. Blood Urea Nitrogen Level (BUN). BUN level was determined in the serum by using a kit (Pointe scientific Inc.). The procedure was started by preparing a working reagent by mixing five parts of R1 with one part of R2. Then, 1 ml of this reagent mix was pipetted into an appropriate tube and pre-warmed at 37°C. After that, 10 ul of calibrator, control, or sample were added to the tube and immediately placed in the spectrophotometer. The rereading was recorded after 30 sec and then after 60 sec (the spectrophotometer was zeroed with water at 340 nm).

4.2.6. Protein Determination. Protein levels of the samples were measured by the Bradford method.[111] Concentrated Coomassie blue was diluted 1:5 with HPLC water. 20 ul of the sample or standard were then added to 1 ml of this diluted dye, the solution was then vortexed, and allowed to stand at room temperature for 5 min. The absorbance was then measured at 595 nm by using a UV spectrophotometer. Bovine serum albumin was used as the protein standard.

4.3. ISOLATION OF MITOCHONDRIA

Liver samples were homogenized in a mitochondrial isolation buffer (215 mM of Mannitol, 75 mM of Sucrose, 0.1 % BSA, 1 mM of EGTA, and 20 mM of HEPES). The samples were spun at 1,300 g for 3 min at 4°. Supernatant was poured into a new tube and spun at 13,000 g for 10 min. The pellets were re-suspended in 10% v/v DMSO/Isolation buffer and saved in a temperature of -80°C until analysis.[112]

4.4. HISTOLOGY

Formalin fixed liver samples were embedded in paraffin and 5-μm sections were cut. Replicate sections were stained with Hematoxylin and Eosin (H&E) and

evaluated by a pathologist in a blind study. Three liver sections with five fields of 20X view were evaluated for hemorrhage, hepatocellular vacuolization, and hepatocellular necrosis, scored from 0 (no change) to 4 (severe change). These values were used to determine a total lesion score for each animal, scaled from 0 (no change) to 16 (severe change). The number of liver samples was three per treatment group.

4.5. STATISTICAL ANALYSIS

All reported values were represented as the mean ± S.D. (n=3-6). Statistical analysis was performed using GraphPad Prism software (GraphPad, San Diego, CA). Statistical significance was ascertained by one-way analysis of variance, followed by Tukey's multiple comparison tests. Values of $p<0.05$ were considered significant. Survival data were analyzed using the Kaplan-Meier method, and the log-rank test was used to compare statistical significances.

5. SURVIVAL STUDY

5.1. RESULTS

5.1.1. Potential Antidotal Effect of N-acetylcysteine or N-acetylcysteine amide in Acetaminophen Intoxicated Animals (Survival Study). APAP dosing decreased the survival percentage to 30% (as compared with 100% for the control) after 4 days of APAP intoxication, and maintained that percentage until the end of the week. Post treatment with NACA increased the survival to 100%, which was the same as that of the control group for the entire period. However, NAC was not as effective as NACA because it increased the survival rate to only 60% of the control by the end of the observation period (Figure 5.1). There was a significant increase in liver GSH in all of the treated groups, following the survival observation period. It had dramatically increased in the APAP-only treated group and less in the NAC-treated group, with the least in the NACA-treated group (which was closest to that of the control), as shown in Figure 5.2.

5.1.2. The Effect of N-acetylcysteine or N –acetylcysteine amide on the Weight of the Animals Intoxicated with Acetaminophen. Individual body weights of all treatment groups were compared during the observation period. These weights showed significant decreases in the APAP-treated group, and post treatment with NAC did not help. NACA treatment, however, was able to increase the body weights to levels that were close to those of the control group (Figure 5.3).
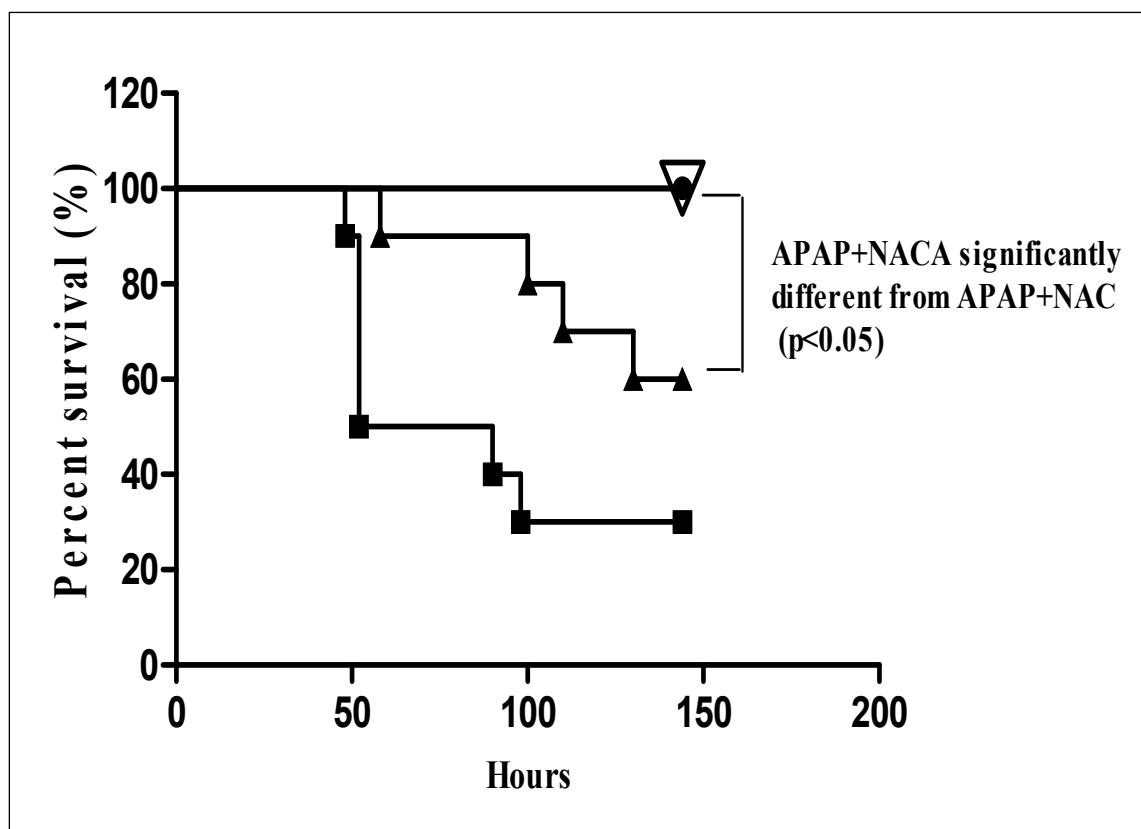
Figure 5.1. Survival curve for APAP toxicity for a period of one week

C57BL/6 mice were intoxicated by a lethal dose of APAP (500 mg/kg), followed by NAC/NACA (106 mg/kg) at 1.5 h post APAP, and then every 12 h up to 72 h (n=10). Circle represents control group, square represents APAP group, triangle represents APAP+ NAC group, and inverted triangle represents APAP+ NACA group. A significant difference (p= 0.0291) was observed between the APAP+NAC group and the APAP+NACA group.
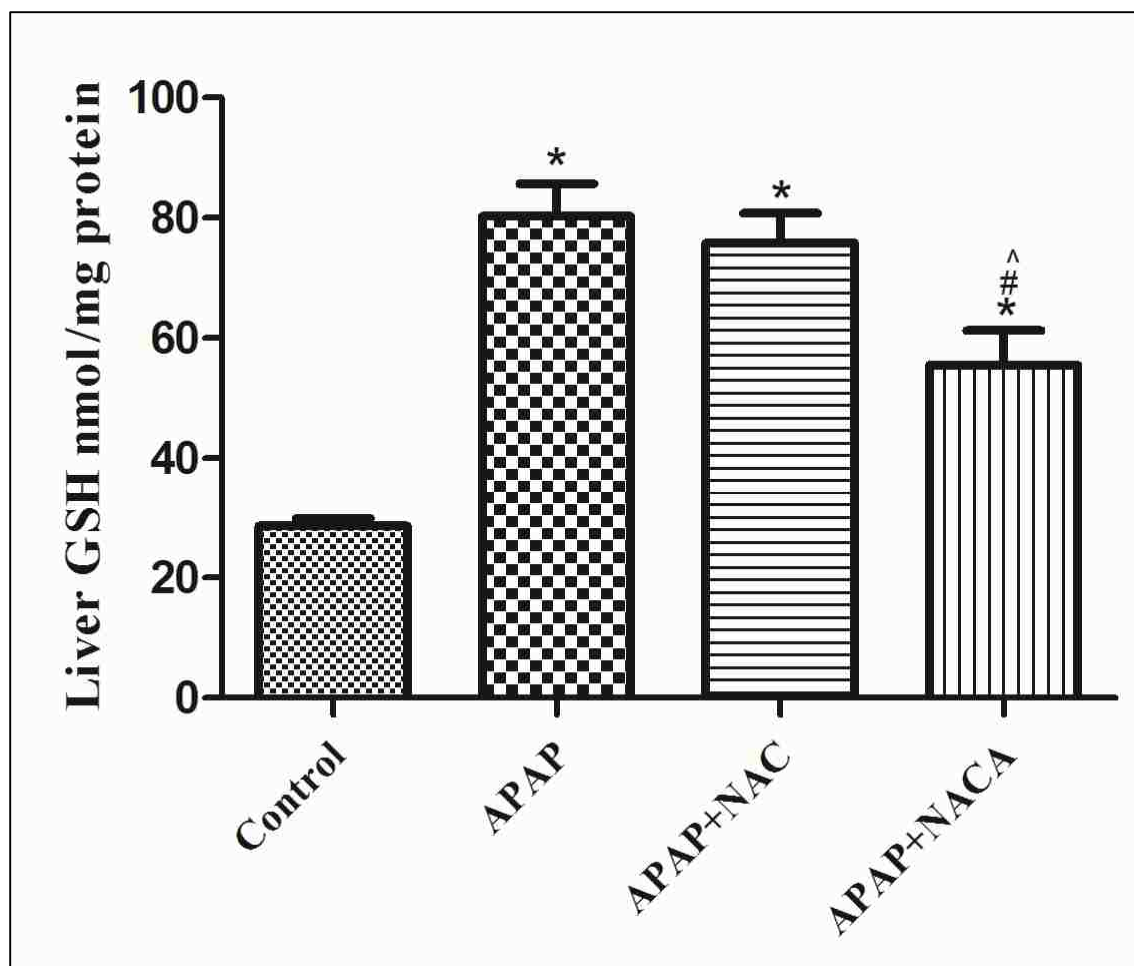
Figure 5.2. Liver glutathione level (GSH) 18 days post acetaminophen (APAP) injection

GSH level of liver sample of animals who lived after the observation period of the survival study in which C57BL/6 mice were intoxicated by a lethal dose of APAP (500 mg/kg), followed by NAC/NACA (106 mg/kg) at 1.5 h post APAP, and then every 12 h up to 72 h. Control (n=10), APAP (n=3), APAP+NAC (n=6), and APAP+NACA (n=10). Values represent mean ±SD (n=6) (*: different from the control group, #: different from the APAP group, and ^: different from APAP+ NAC group) p< 0.05.
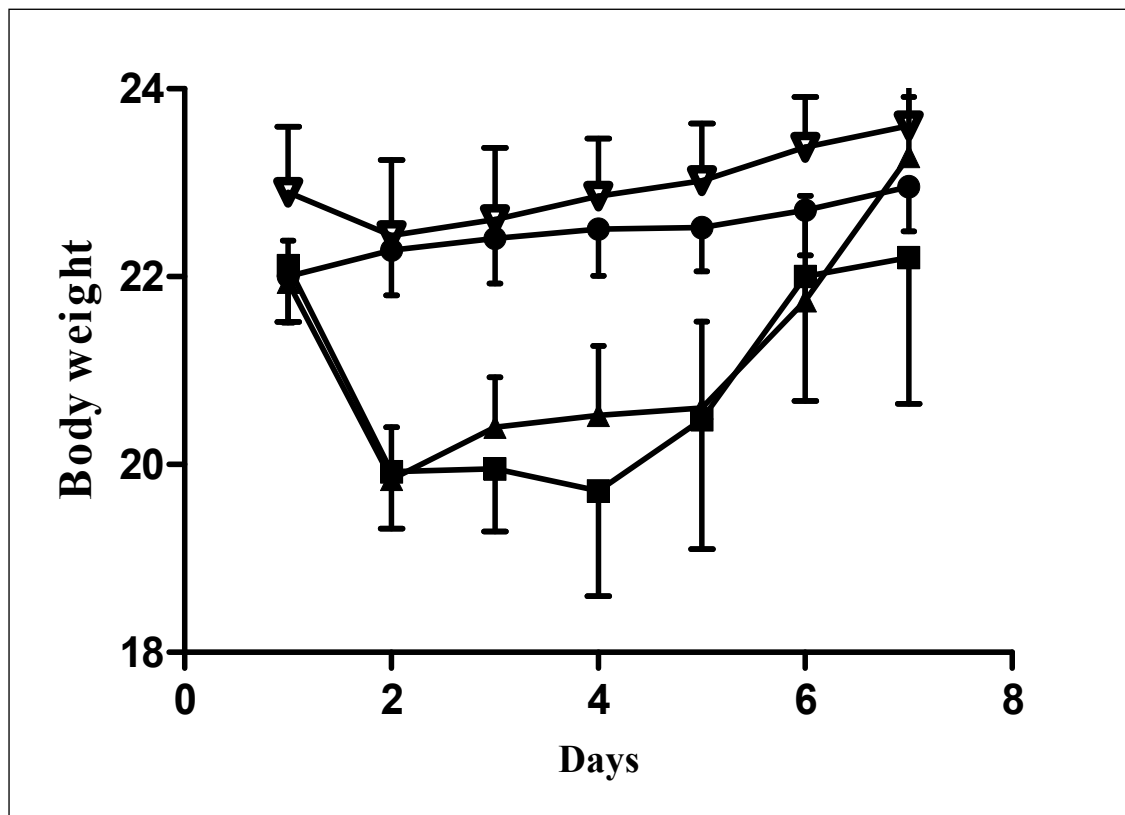
Figure 5.3. The effect of N-acetylcysteine or N-acetylcysteine amide on the weight of mice injected by APAP for a week

C57BL/6 mice were intoxicated by a lethal dose of APAP (500 mg/kg), followed by NAC/NACA (106 mg/kg) at 1.5 h post APAP, and then every 12 h up to 72 h (n=10). Circle represents control group, square represents APAP group, triangle represents APAP+ NAC group, and inverted triangle represents APAP+ NACA group.

5.2. DISCUSSION

An APAP overdose leads to the damage of various organs (mainly the liver and a second organ, the kidney), which results in high mortality rates, as reported in studies by many investigators.[113-117] The rescue potential of NAC and NACA was studied by conducting a survival study and weight monitoring. NAC is the only known antidote for APAP toxicity on the market today. In this study, mortality was not observed until 48 h after APAP dosing, mainly because of the nutritional status of the animals since they were fed before dosing with APAP. Fasting is known to deplete GSH and, therefore, the hepatotoxicity induced by APAP increases, which also increases the mortality rate. This result is consistent with other reported results that were conducted under the same conditions.[118] After 48 h, we observed a significant decrease in the survival percentage of the APAP-treated group, which had reduced to about 30% by the end of the observation period. NACA was better than NAC at increasing the survival percentage to coincide with that of the control group. The drastic increase in the liver GSH of the mice in the APAP-treated group, that were alive at the end of the study, indicated that the defending mechanism of the liver had overcome the toxicity by regenerating GSH. The drastic increase in GSH in the APAP-treated group (when the body was trying to overcome toxicity by producing more GSH) is a natural defense mechanism of the body. In the NAC-treated group, the increase in GSH was less than that in the APAP-only treated group, while that in the NACA-treated group was even less and was close to the control level, indicating less toxicity in this group.

Many researchers reported losses in body weight that were caused by an overdose of APAP.[119, 120] Adjuwon *et al*.[119] explained these weight loses by relating them to suppression of appetite and subsequent increases in the metabolic state associated

with hypoglycemia, which is associated with APAP-induced hepatotoxicity. Literature review showed metabolic complications of an APAP overdose that included hypoglycemia. Adeneye A. *et al.*[66] related the weight loss in their study to the greater urinary volume recorded in the APAP-treated group that indicated renal lesion and nephrotoxicity, which is explained in detail in chapter 7. NACA was able to maintain animal weights and survival percentages at values similar to those of the control.

APAP intoxication led to multi-system failure in the mice (discussed in detail in chapters 6, 7, and 8), leading to attenuation in the weights of the mice and increases in the mortality percentages. NACA was able to rescue the mice and maintain their survival percentages on a par with those of the control group. These results indicated that NACA is far better than NAC at protecting against APAP-induced toxicity.

6. HEPATOTOXICITY

6.1. RESULTS

6.1.1 Effect of NAC/NACA on Intracellular Levels of GSH, GSSG, and GSH/GSSG. As depletion of intracellular GSH is a first step in APAP-induced toxicity, we studied the effect of APAP on GSH levels. An APAP overdose caused a dose-dependent toxicity. I studied APAP-induced toxicity at two different doses of APAP: 300 mg/kg and 400 mg/kg. Furthermore, I chose to study toxicity at two different time points for the 300 mg/kg dose of APAP to gain further insight into the regeneration mechanism of GSH as reported in literature. The GSH level reduced to 25% of the control with the dosing of 300 mg/kg APAP at 4 h post APAP injection (Figure 6.1 A). However, there was no significant change in the level of GSSG at this dose and time point (Figure 6.1 B). The GSH/GSSG ratio decreased to around 60% of the control, which is a better indicator of oxidative stress (Figure 6.1 C). Furthermore, treatment with NACA resulted in a significant increase in the GSH level, along with a significant decrease in the level of GSSG. However, NAC was not effective at restoring these levels.

A 24-h treatment time with 300 mg/kg of APAP did not cause any change in the GSH level (Figure 6.2 A), but the GSSG level increased by about two fold compared to that of the control (Figure 6.2 B). This elevation in the GSSG level resulted in a considerable reduction in the GSH/GSSG ratio to around 62% of the control level (Figure 6.2 C). Interestingly, NAC and NACA were equally effective at decreasing the GSSG level, which led to increases in the GSH/GSSG ratio.

To study the effect of a dose of APAP on toxicity, I studied the oxidative stress parameters at 4 h post of an APAP dose of 400 mg/kg as well. The results indicated that GSH levels decrease significantly to approximately 22% of the control after treatment with 400 mg/kg at 4 h post of an APAP dose (Figure 6.3A), with a concomitant increase in the levels of GSSG (oxidized form of GSH; Figure 6.3 B). Although the decrease in GSH, with a 400 mg/kg dose of APAP, is comparable to that with a 300 mg/kg dose of APAP, there is an increase in GSSG with the 400 mg/kg dose, which leads to a further reduction in the GSH/GSSG ratio to 16% of control (Figure 6.3 C). However, the 300 mg/kg APAP dose leads to a GSH/GSSG ratio of 60% of control. Interestingly, the treatment with NAC was not effective at increasing the level of GSH or the GSH/GSSG ratio and significantly decreasing the level of GSSG. However, the NACA-treatment group showed a significant increase in the GSH level (approximately 66.5% of the control), in addition to a significant decrease in GSSG, leading to a considerable increase in the ratio of GSH/GSSG (60.5% of the control). Since significant toxicity was observed with a 400 mg/kg dose of APAP, I chose this dose and time point for further studies.

6.1.2 Effect of NAC/NACA on Mitochondrial Levels of GSH, GSSG, and GSH/GSSG. An APAP dose of 400 mg/kg led to a significant reduction in mitochondrial GSH (Figure 6.4 A) at 4 h post APAP injection with no change in the GSSG level (Figure 6.4 B). The ratio of GSH/GSSG was reduced to around 4% of the control (Figure 6.4 C). NACA treatment was successful in restoring GSH levels and the GSH/GSSG ratio to around 87% and 78% of the control, respectively. However, NAC was not able to restore the depleted levels of GSH after APAP dosing.

6.1.3 Depletion of thiol antioxidants by NAPQI. Many investigators have pointed to the formation of the GSH-NAPQI conjugate as the critical step in detoxification of NAPQI at therapeutic doses of APAP. A cell-free experiment was performed to determine whether excessive amounts of NAPQI could be bound in the presence of sufficient quantities of GSH or another thiol antioxidant. The proportion of the unbound NACA was determined to be significantly different from the unbound NAC and unbound GSH ($p < 0.01$) (Figure 6.5). This suggests that in the presence of NAPQI, a higher proportion of NACA reacts with NAPQI, compared to the other thiols.

6.1.4 Effect of NAC/NACA on the Activity of GR. GR activity was reduced to 57% of the control after 400 mg/kg of APAP treatment for 4 h. NACA treatment restored the GR activity to a level that was not very different from that of the control, while NAC was not able to significantly increase the activity of GR (Figure 6.6 A).

Administration of 300 mg/kg of APAP for 4 h reduced the level of GR enzyme to 84.5% of the control, and neither NAC nor NACA was not be able to restore this level back to the control level (Figure 6.6 B). However, a 24-h treatment period of 300 mg/kg APAP dosing increased the GR level to 54% of the control. Post treatment with NAC or NACA had the same effect by reducing the level back to that of the control (Figure 6.6 C).

6.1.5 Protective Effect of NAC/NACA on Lipid Peroxidation. MDA, an index of lipid peroxidation, increased significantly after treatment with 400 mg/kg of APAP and with 300 mg/kg of APAP by 145% or 70% of the control, respectively, at 4 h post APAP injection. Increasing the dose of APAP resulted in increased MDA levels. NAC was effective at decreasing the level of MDA; however, it was not very different from the

control or the APAP group. Furthermore, NACA was better than NAC at decreasing the MDA levels significantly when compared with the APAP treated group (Figures 6.7 A and B). MDA levels were elevated in the APAP group (53% higher than control) at 24 h post a 300 mg/kg dose of APAP. However, NAC and NACA acted equally to reduce this elevation close to control (Figure 6.7 C).

6.1.6 Protective Effect of NAC/NACA on ALT Activity. Activity of ALT, a liver function enzyme, which was measured in the serum at 4 h post APAP treatment (400 mg/kg), showed a 100-fold increase over that of the control level. Treatment with NACA significantly reduced this increase in ALT activity to about 70% of that of the APAP-treated group, while NAC treatment did not reduce it significantly (Table 6.1 A).

An APAP dose of 300 mg/kg resulted in a 10-fold increase in ALT activity compared to that of the control at 4 h post APAP dose. Both NAC and NACA were able to reduce this elevation significantly, but NACA was better than NAC at reducing it (Table 6.1B). However, at 24 h post, an APAP dose of 300 mg/kg led to around a 2.5-fold elevation in ALT activity. NAC and NACA acted equally to greatly reduce this elevation (Table 6.1 C).

6.1.7 Protective Effect of NAC/NACA on GDH Activity. GDH activity increased 25-fold from that of the control, after APAP treatment (400 mg/kg for 4 h). NACA treatment, however, significantly reduced the GDH activity to 15 times that of the control group, while NAC was unable to significantly reduce the activity (Table 6.1 A).

An APAP dose of 300 mg/kg for 4 h resulted in an increase in GDH activity to approximately six times the activity of the control. Both NAC and NACA were able to reduce this elevation significantly, but NACA was better than NAC at reducing it (Table

6.1B). While APAP dosing for 24 h led to around a four-fold elevation of GDH, both NAC and NACA were able to reduce the levels considerably (Table 6.1 C).

6.1.8 Effect of NAC/NACA on Protecting Liver Tissue (Macroscopic and Microscopic Evaluation). It has been previously shown that APAP toxicity can induce centrilobular necrosis. This is widely thought to be due to the abundant distribution of CYP in this region, which is responsible for APAP metabolism.[121] In this study, mice treated with APAP (400 mg/kg) had small, multifocal pale areas of tissue necrosis throughout all lobes of their livers, while the livers of control mice and those in the APAP+NACA group looked grossly normal (Figure 6.8). Histologically, liver sections from mice in the control group treated with PBS were within normal limits and hepatocellular damage was not observed (Figure 6.9 A, 6.10 A, and 6.11 A). However, liver sections from mice in the APAP-treated group (400 mg/kg) showed severe diffuse coagulative necrosis around the central veins (Figure 6.9 B). Necrotic areas were characterized by swollen, vacuolated, eosinophilic hepatocytes consistent with previously described APAP toxicity.[24, 122] Liver sections from mice in the APAP+NAC treated group had mild, centrilobular to midzonal hepatocellular changes characterized by one to two medium sized vacuoles to clusters of several small vacuoles (Figure 6.9 C arrows) with rare clusters of one to three infiltrating mononuclear cells. In contrast, mice in the APAP+NACA group had only occasional multifocal, centrilobular to midzonal areas of mild hepatocellular vacuolization characterized by clusters of several small vacuoles (Figure 6.9 D arrow heads). Necrosis and hemorrhage were not observed in either the APAP+NAC or APAP+NACA groups. Liver sections of mice treated with 300 mg/kg APAP for 4h did not show any necrosis (Figure 6.10 B), while the 24h treatment showed

mild necrosis (Figure 6.11 B). NAC or NACA treated liver sections in both treatment groups were within normal limits and hepatocellular damage was not observed (Figure 6.10 and Figure 6.11 C and D). To quantitatively assess hepatocellular damage, five 20X fields of view of three histologic sections of liver per mouse were scored for severity of hemorrhage, hepatocellular vacuolization, and hepatocellular necrosis and a total lesion score, ranging from 0 (no damage) to 16 (severe damage), for each mouse was determined. Hepatocellular damage for the APAP (400 mg/kg at 4h), was the most severe and was statistically different from the PBS, APAP+NAC and APAP+NACA treated groups. However, 300 mg/kg treatment of APAP for 4h and 24h was not able to induce hepatocellular damage statistically different from the control. No statistical differences were found between the PBS or APAP+NAC and APAP+NACA treated groups in all dosing designs (Figure 6.12).
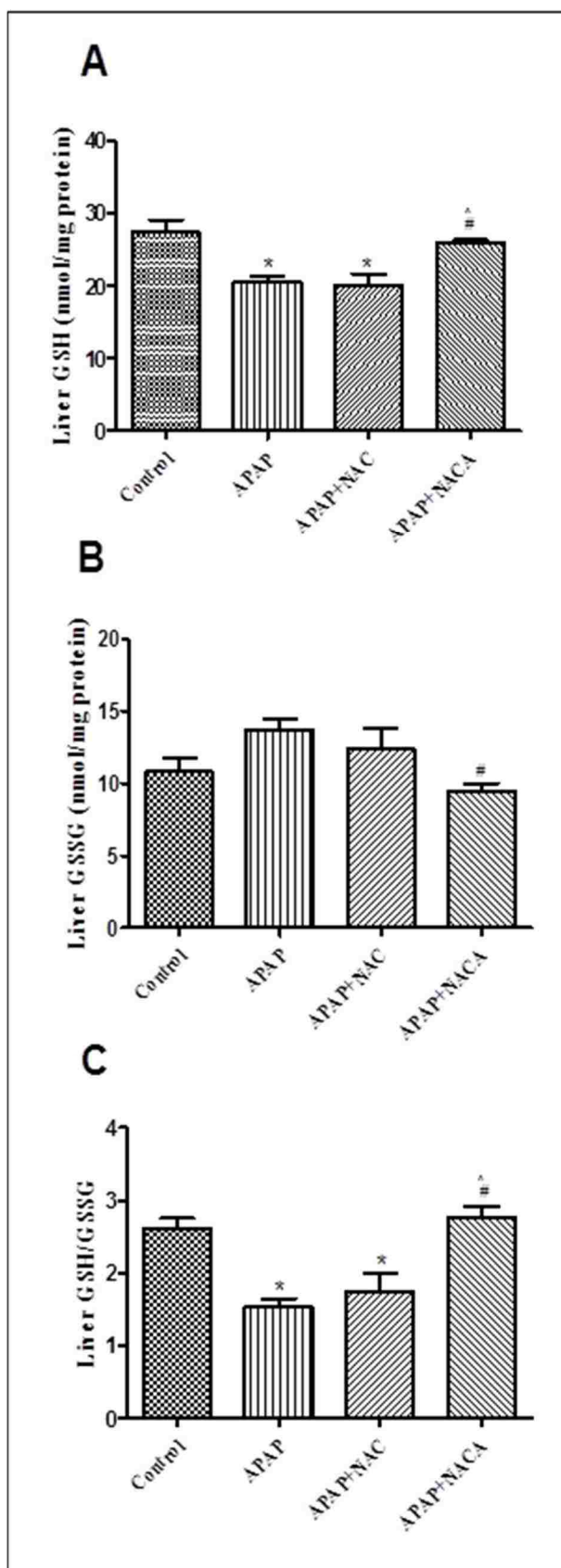
Figure 6.1. Liver glutathione (GSH), glutathione disulfide (GSSG), and GSH/GSSG ratio levels of C57BL/6 mice injected with APAP (300 mg/kg) for 4 h

(A) GSH, (B) GSSG, and (C) GSH/GSSG ratio. Post treatment with NAC or NACA (106 mg/kg) at 1.5 h after APAP treatment in all treatment groups. Values represent mean ±SD (n=6) (*: different from the control group, #: different from the APAP group, and ^: different from APAP+ NAC group) $p < 0.05$.
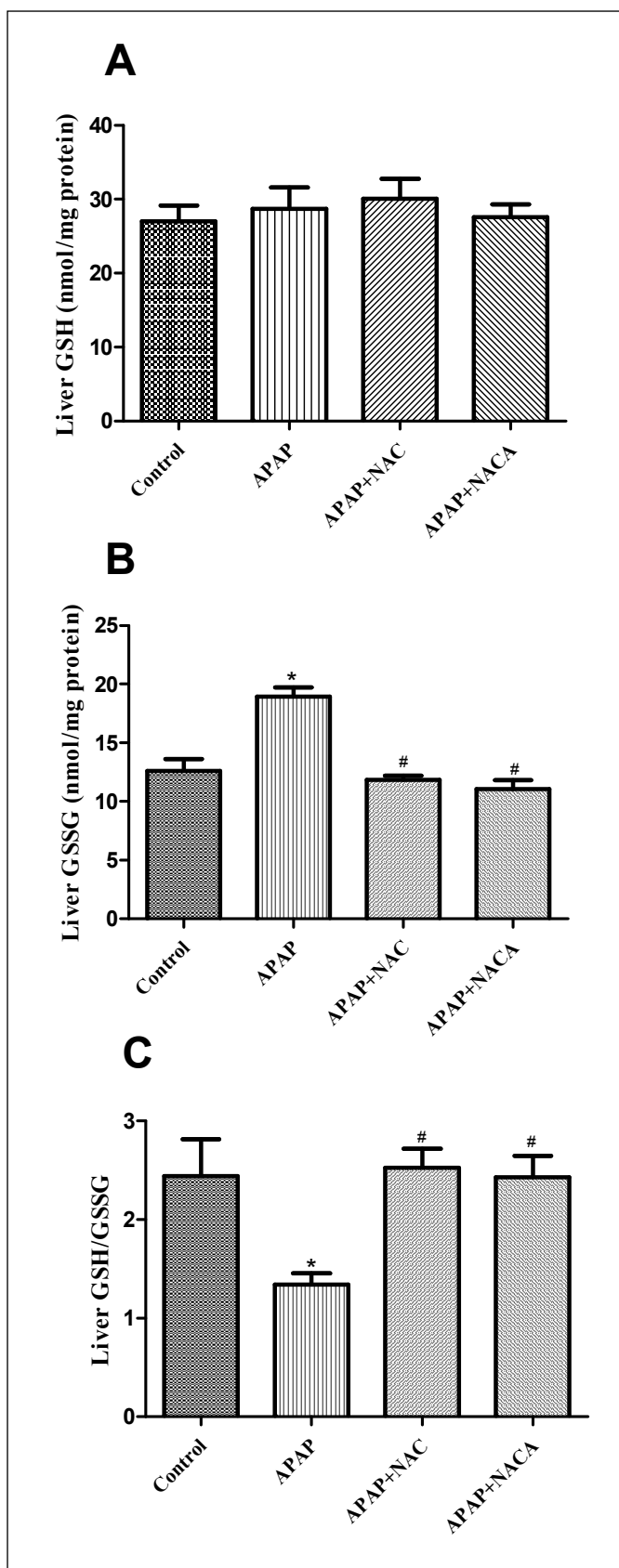
Figure 6.2. Liver glutathione (GSH), glutathione disulfide (GSSG), and GSH/GSSG ratio levels of C57BL/6 mice injected with APAP (300 mg/kg) for 24 h

(A) GSH, (B) GSSG, and (C) GSH/GSSG ratio. Post treatment with NAC or NACA (106 mg/kg) at 1.5 h after APAP treatment in all treatment groups. Values represent mean ±SD (n=6) (*: different from the control group, #: different from the APAP group, and ^: different from APAP+ NAC group) $p < 0.05$.
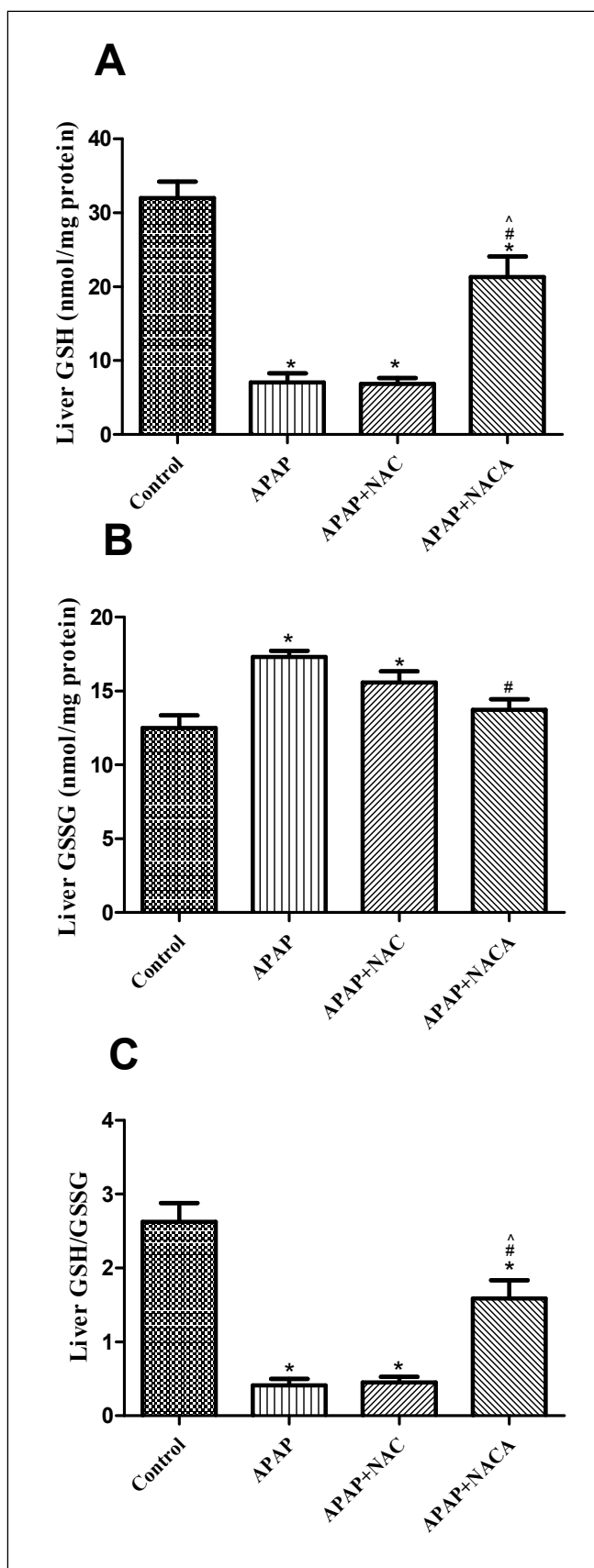
Figure 6.3. Liver glutathione (GSH), glutathione disulfide (GSSG), and GSH/GSSG ratio levels of C57BL/6 mice injected with APAP (400 mg/kg) for 4 h

(A) GSH, (B) GSSG, and (C) GSH/GSSG ratio. Post treatment with NAC or NACA (106 mg/kg) at 1.5 h after APAP treatment in all treatment groups. Values represent mean ±SD (n=6) (*: different from the control group, #: different from the APAP group, and ^: different from APAP+ NAC group) $p < 0.05$.
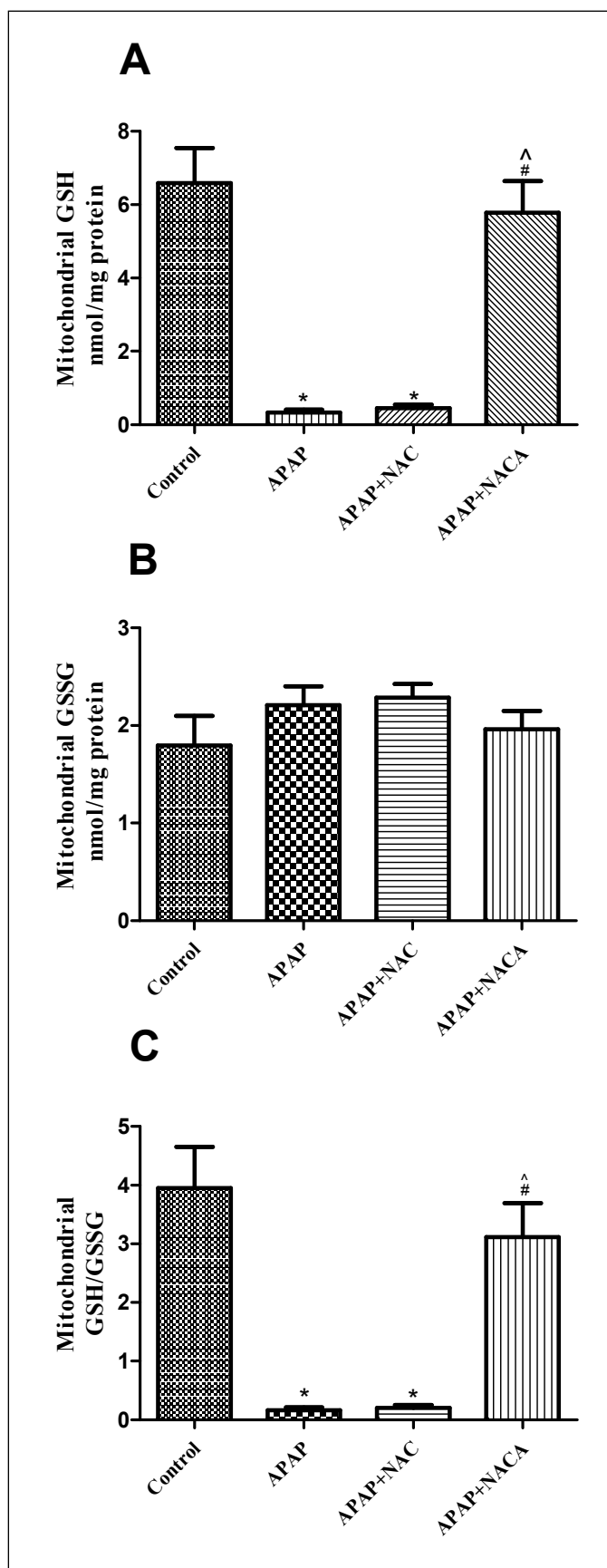
Figure 6.4. Liver's mitochondrial glutathione (GSH), glutathione disulfide (GSSG), and GSH/GSSG ratio levels of C57BL/6 mice injected with APAP (400 mg/kg) for 4 h

(A) GSH, (B) GSSG, and (C) GSH/GSSG ratio. Post treatment with NAC or NACA (106 mg/kg) at 1.5 h after APAP treatment in all treatment groups. Values represent mean ±SD (n=6) (*: different from the control group, #: different from the APAP group, and ^: different from APAP+ NAC group) $p < 0.05$.
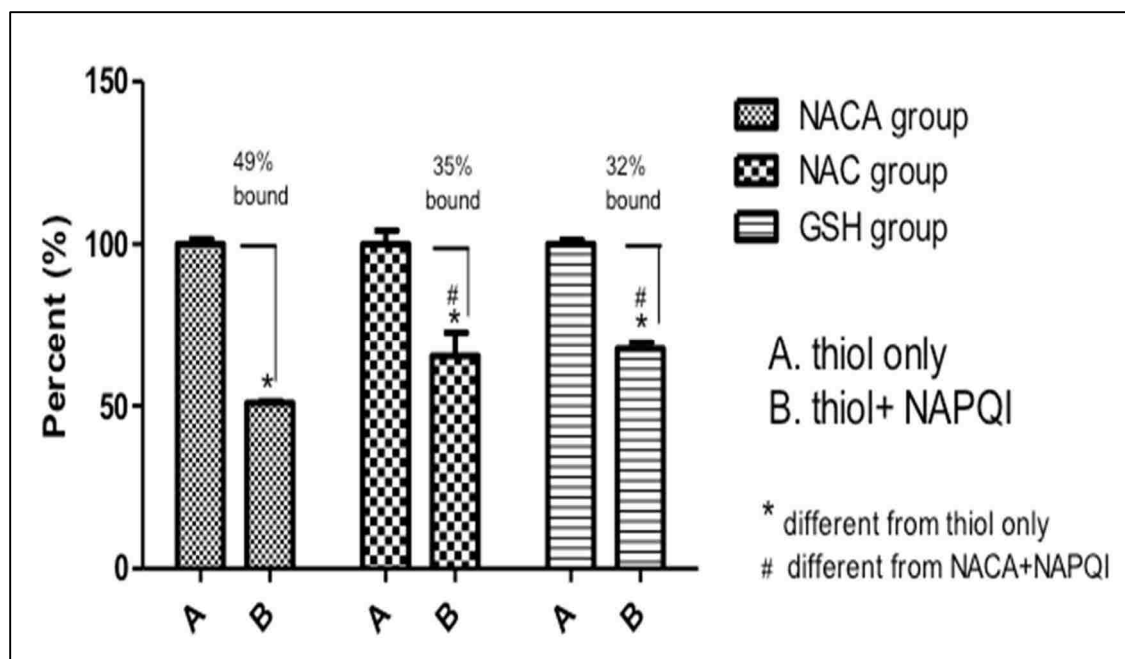
Figure 6.5. Depletion of thiol antioxidants by NAPQI.

Cell-free experiment in which 5 μM aqueous solutions of GSH, NAC and NACA (triplicates of each) were prepared. The decrease in levels of the given thiol after addition of 10 μM NAPQI suggest the formation of NAPQI conjugates, and therefore greater detoxification capacity. Our results indicate NACA levels dropped more than GSH or NAC in the presence of NAPQI. (* and # $p < 0.01$)
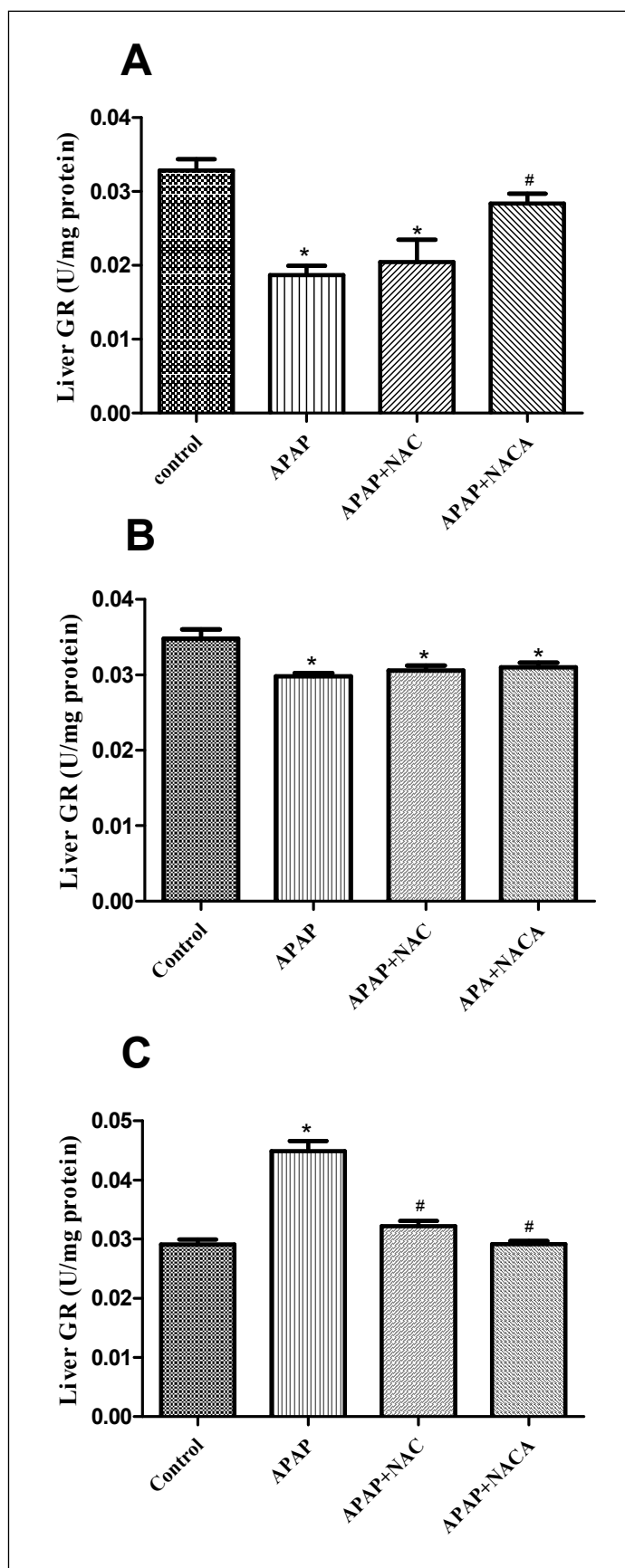
Figure 6.6. Liver glutathione reductase (GR) activity

C57BL/6 mice injected with (A) APAP (400 mg/kg) for 4 h, (B) APAP (300 mg/kg) for 4 h, and (C) APAP (300 mg/kg) for 24 h. Post treatment with NAC or NACA (106 mg/kg) at 1.5 h after APAP treatment in all treatment groups. Values represent mean ±SD (n=6) (*: different from the control group, #: different from the APAP group, and ^: different from APAP+ NAC group) p<0.05.
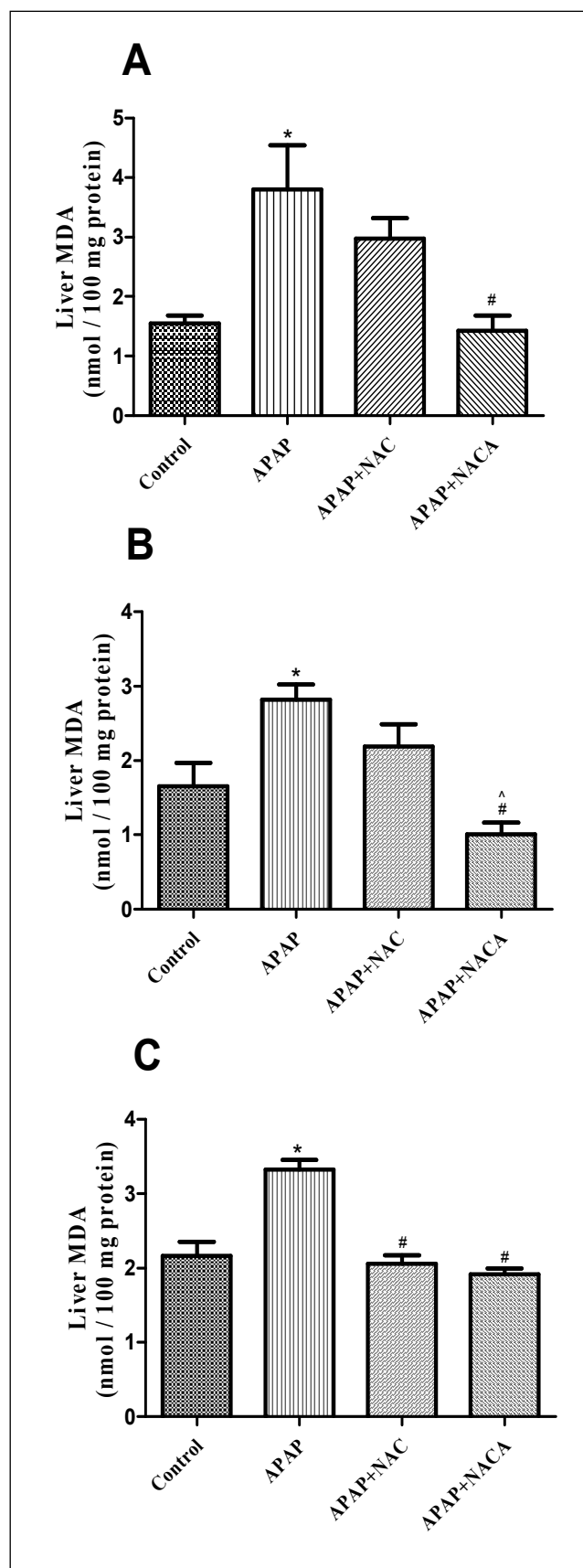
Figure 6.7. Liver malondialdehyde (MDA) levels

C57BL/6 mice injected with (A) APAP (400 mg/kg) for 4 h, (B) APAP (300 mg/kg) for 4 h, and (C) APAP (300 mg/kg) for 24 h. Post treatment with NAC or NACA (106 mg/kg) at 1.5 h after APAP treatment in all treatment groups. Values represent mean ±SD (n=6) (*: different from the control group, #: different from the APAP group, and ^: different from APAP+ NAC group) p<0.05.

Table 6.1. Serum levels of alanine aminotransferase (ALT) and glutamate dehydrogenase (GDH)

C57BL/6 mice injected with (A) APAP (400 mg/kg) for 4 h, (B) APAP (300mg/kg) for 4 h, and (C) APAP (300 mg/kg) for 24 h. Post treatment with NAC or NACA (106 mg/kg) at 1.5 h after APAP treatment in all treatment groups. Values represent mean ±SD (n=6) (*: different from the control group, #: different from the APAP group, and ^: different from APAP+ NAC group) $p < 0.05$.

		Control	APAP	APAP+NAC	APAP + NACA
A	ALT	0.010±0.004	0.939±0.130*	0.857±0.126*	0.704±0.139$^{*\#}$
	GDH	6.906±2.181	169.7±35.44*	138.3±17.61*	105.7±6.240$^{*\#}$
B	ALT	0.017±0.005	0.112±0.021*	0.078±0.016$^{*\#}$	0.040±0.009$^{*\#}$
	GDH	6.590±1.789	38.63±3.453*	31.94±2.994$^{*\#}$	21.35±1.863$^{*\#\wedge}$
C	ALT	0.021±0.006	0.056±0.023*	0.016±0.002$^{\#}$	0.013±0.002$^{\#}$
	GDH	5.088±3.279	19.73±3.662*	14.42±1.504*	12.50±1.201$^{*\#}$
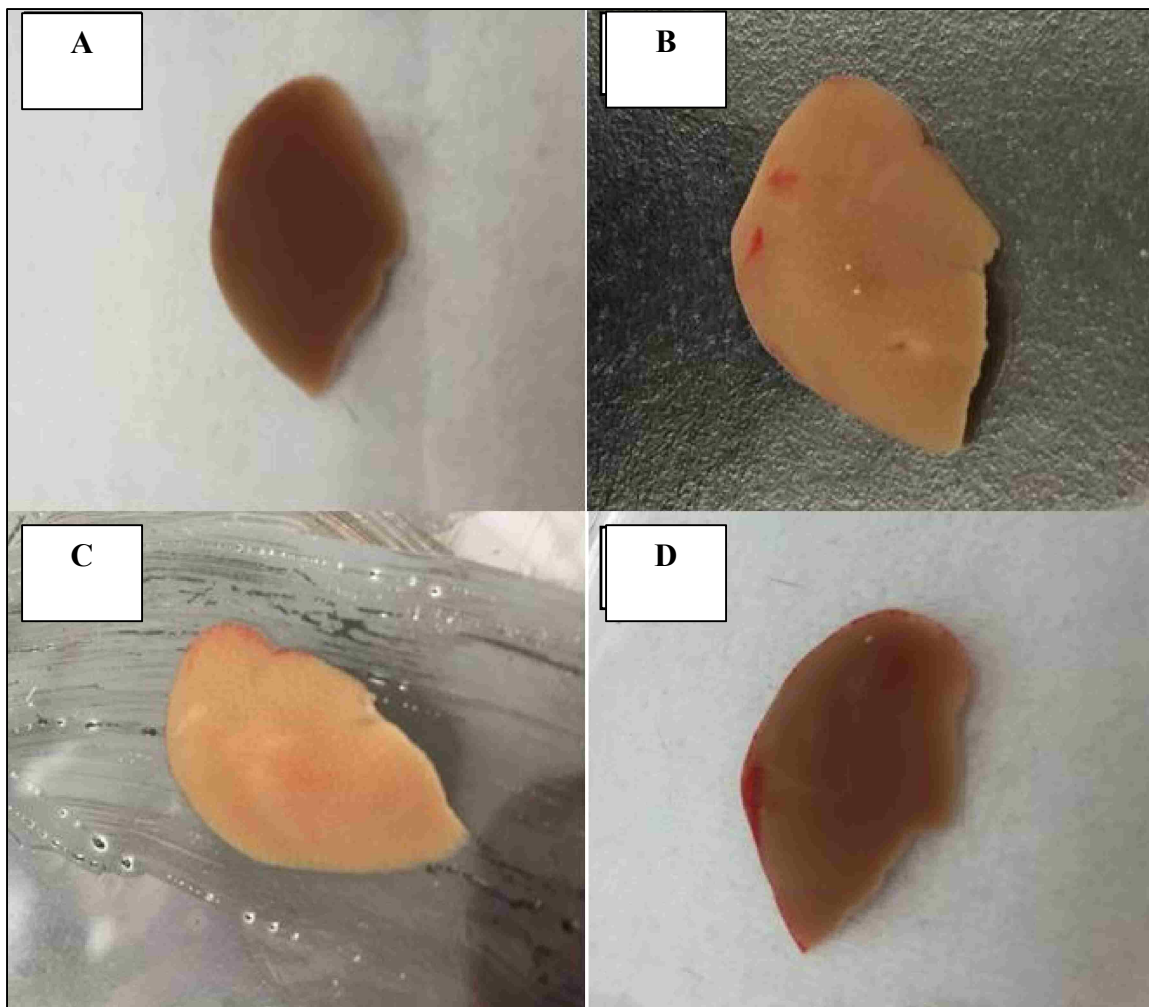
Figure 6.8. Macroscopic assessment of liver lobes from untreated and treated C57BL/6 mice with APAP (400 mg/kg for 4 h)

(A) Control group, which was dosed with PBS only; (B) APAP (400 mg/kg) 4 h only group; (C) APAP + NAC (106 mg/kg) group; and (D) APAP + NACA (106 mg/kg) group.
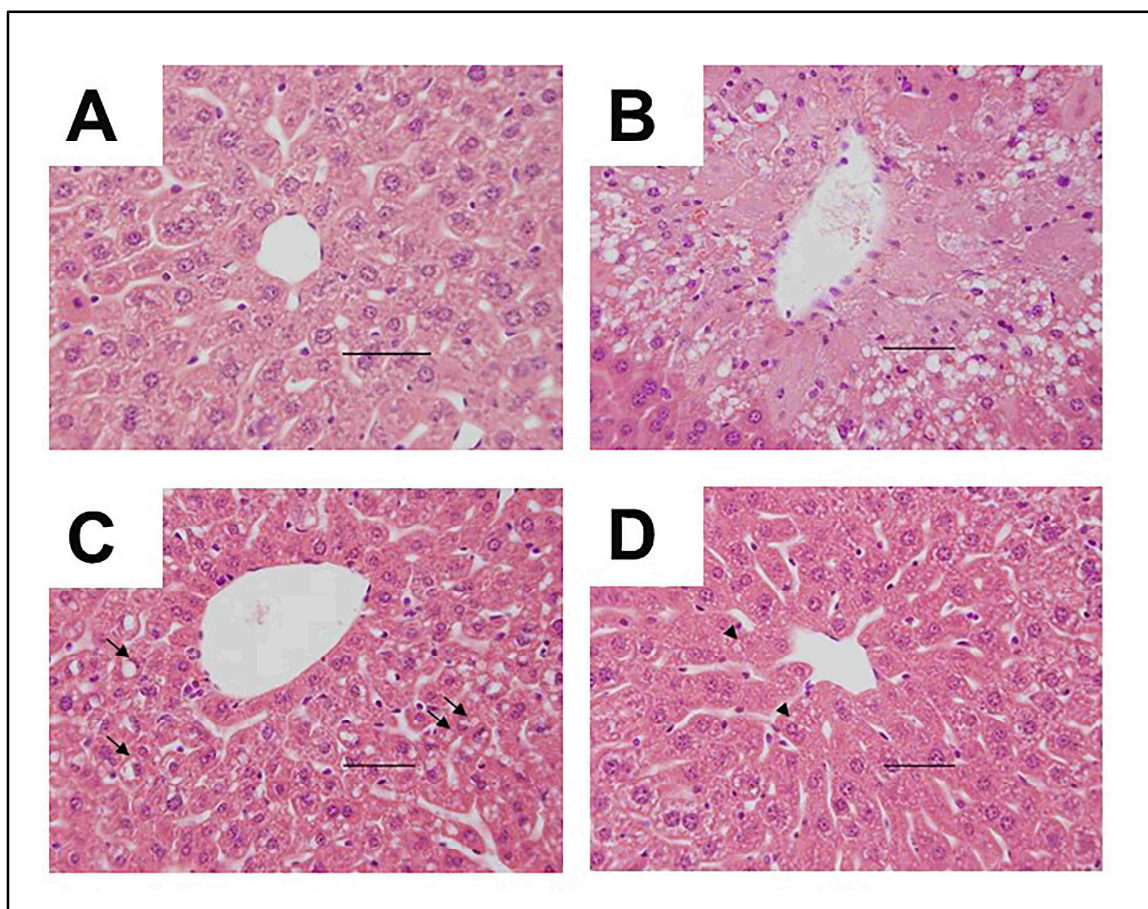
Figure 6.9. Histopathologic assessment of liver sections from untreated and treated C57BL/6 mice with APAP (400 mg/kg for 4 h)

(A) Control group, which were dosed with PBS only; (B) APAP (400 mg/kg) 4 h only group; (C) APAP + NAC (106 mg/kg) group, arrows indicate regions of medium to small hepatocellular cytoplasmic vacuoles; and (D) APAP + NACA (106 mg/kg) group arrow heads indicate regions of small hepatocellular cytoplasmic vacuoles. Scale bar = 50 µm.
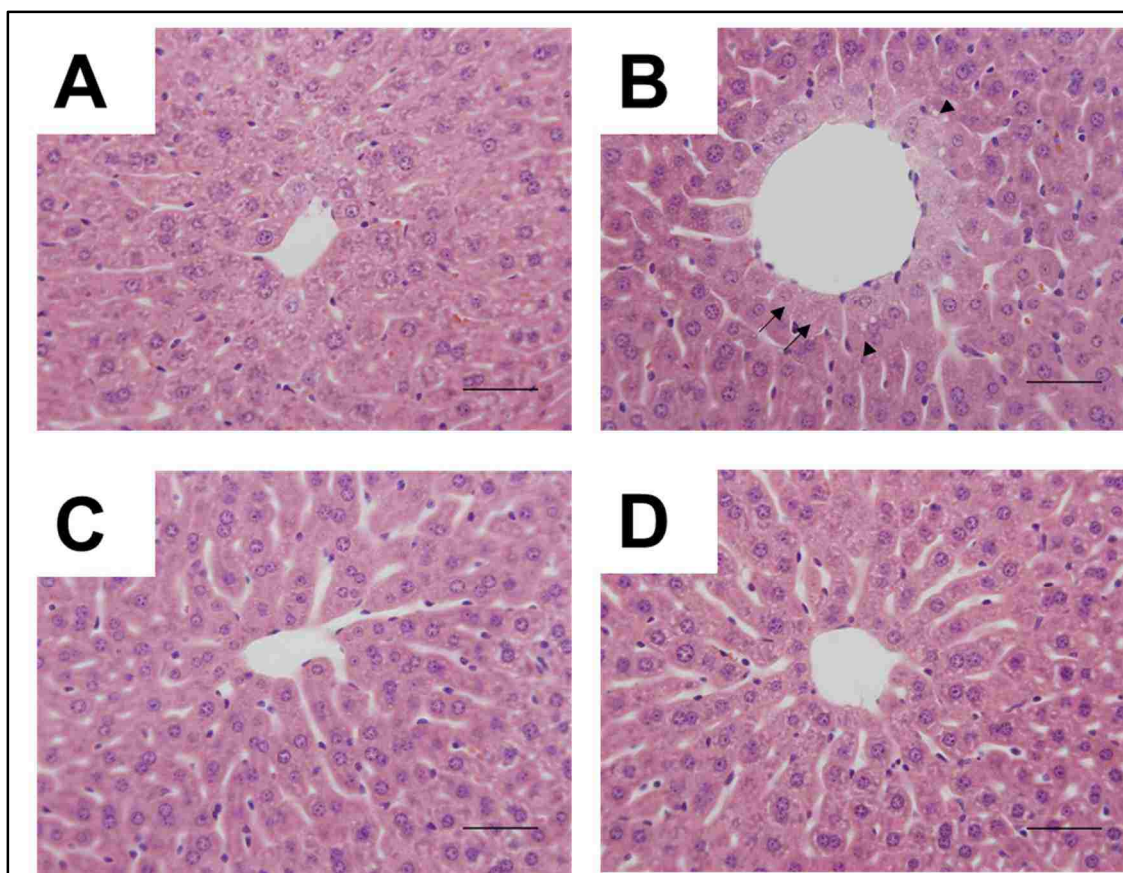
Figure 6.10. Histopathologic assessment of liver sections from untreated and treated C57BL/6 mice with APAP (300 mg/kg for 4 h)

(A) Control group, which were dosed with PBS only; (B) APAP (400 mg/kg) 4 h only group, cellular necrosis noted by arrows and cytoplasmic vacuoles noted by arrow heads; (C) APAP + NAC (106 mg/kg) group; and (D) APAP + NACA (106 mg/kg). Scale bar = 50 μm.
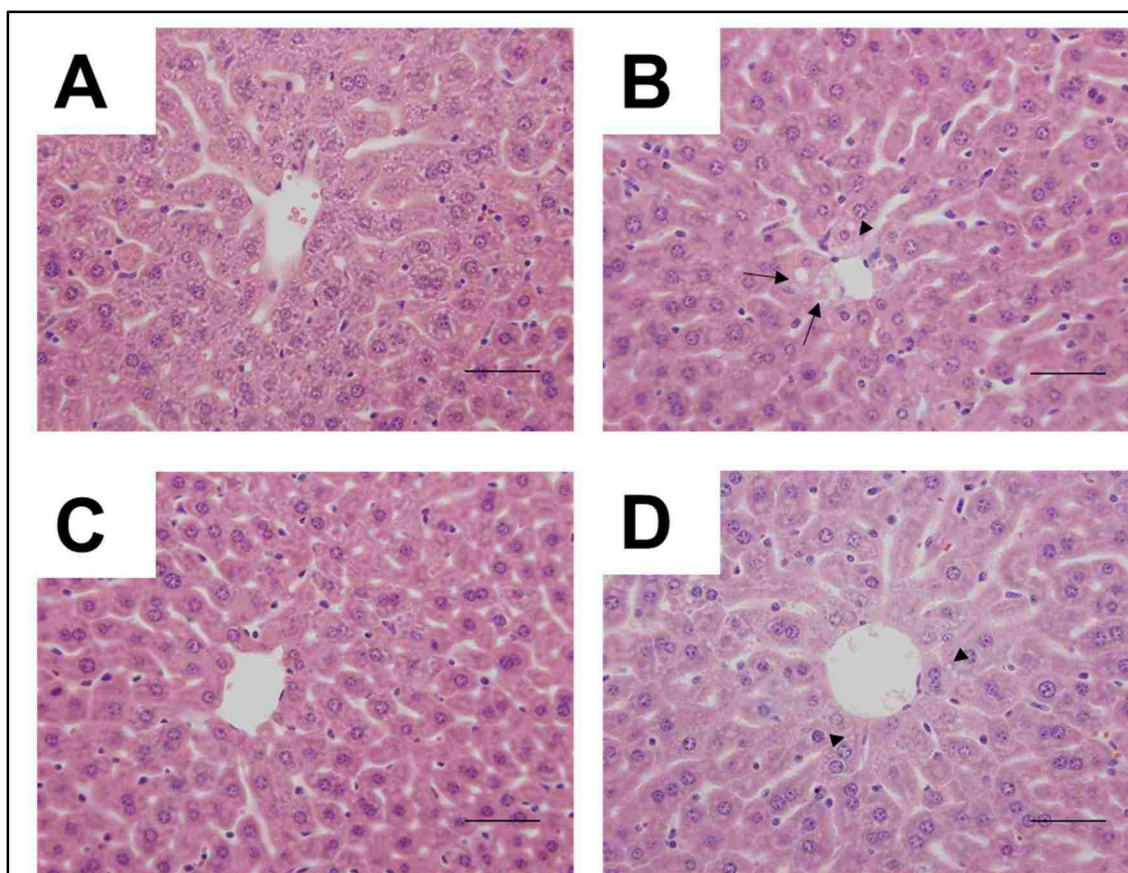
Figure 6.11. Histopathologic assessment of liver sections from untreated and treated C57BL/6 mice with APAP (300 mg/kg for 24 h)

(A) Control group, which were dosed with PBS only; (B) APAP (400 mg/kg) 4 h only group, cellular necrosis noted by arrows and cytoplasmic vacuoles noted by arrow heads; (C) APAP + NAC (106 mg/kg) group; and (D) APAP + NACA (106 mg/kg). Scale bar = 50 μm.
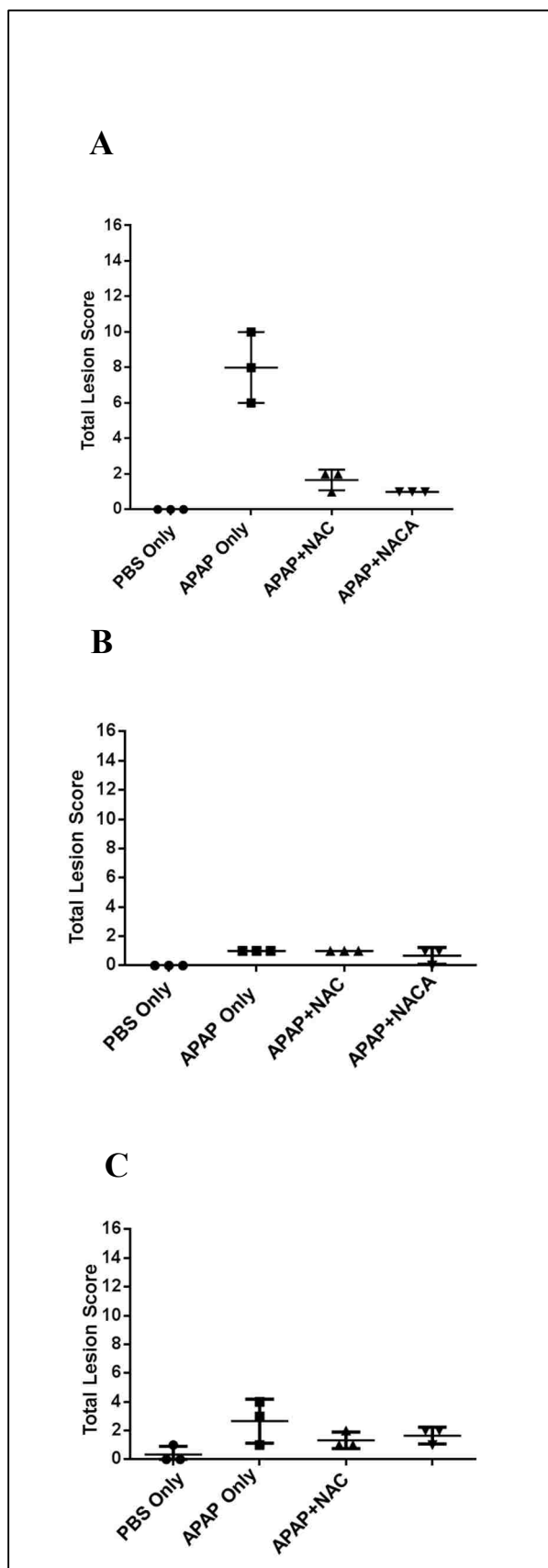
Figure 6.12. Histopathologic hepatic lesion scores from untreated and treated C57BL/6 mice (n=3)

(A) APAP (400 mg/kg for 4 h), (B) APAP (300 mg/kg for 4 h), and (C) APAP (300 mg/kg for 24 h). Post treatment with NAC or NACA (106 mg/kg) at 1.5 h after APAP treatment in all treatment groups. Bar represents mean ±SD lesion score per group. (#: different from APAP group) p<0.05.

6.2 DISCUSSION

APAP toxicity is one of the most common causes of drug-induced hepatotoxicity worldwide that lead to excessive treatment and high hospitalization costs every year. Oxidative stress has been hypothesized by many researchers to play an important role in the etiology of APAP toxicity.[121-123] It is defined as an imbalance between the production of reactive oxygen species (ROS) and endogenous antioxidants and repair capacity. Depletion of GSH by approximately 70% is the initial step in APAP hepatotoxicity, [51, 52] which is explained by the conjugation of GSH with the electrophilic toxic metabolite of APAP (NAPQI). Based on the mechanism of toxicity, the treatment focus is on a compound that acts as an antioxidant and stimulates GSH synthesis. The only approved antidote for APAP toxicity, NAC, relies on restoring the GSH level and antioxidant properties to restrict liver damage. NAC has quite a narrow therapeutic window and, because of its poor bioavailability, larger doses and longer treatment times are required.[24] In a clinical setting, NAC is administered at 150 mg/kg over 15 min, followed by 50 mg/kg over 4 h, and then 100 mg/kg over 16 h, so the total dose is 300 mg/kg.[17] For animal studies, 300 - 1200 mg/kg (high doses) are reported as an effective range for NAC dosing.[13, 124] Chieko Saito *et al*.[13], Marcus V. Terneus *et al*.[125], and Francesco Di Pierro *et al* [126] reported a direct dose response relationship using NAC and observed that a larger dose of NAC was sufficient to supply cysteine for GSH synthesis, promote recovery of the liver, and primarily maintain mitochondrial GSH levels. It improved mitochondrial bioenergetics as well. However, larger doses of NAC are associated with increased risk of side effects. Therefore, we studied the protective effects of NACA (which has higher bioavailability than NAC) against APAP-induced

hepatotoxicity and compared its effects with those of NAC. The serum levels of ALT and GDH were the main indices used to detect liver and mitochondria injury, respectively, while the levels of GSH, GSSG, and MDA were used as indicators of oxidative stress. In this study, I evaluated the role of NACA in counteracting the hepatotoxicity in an *in vivo* model, and these findings were compared with the effects of NAC.

My study show a significant decrease in the GSH level in the liver after a toxic dose of APAP, which is regarded as a first step in toxicity and induction of oxidative stress. This reduction in the level of GSH, after toxic administration of APAP, may have been due to its conjugation with NAPQI during the detoxification pathway. My results are in line with previous studies that reported a decrease in GSH levels following APAP treatment.[124-129] In addition to decreases in GSH, increases in intracellular GSSG have been reported during the recovery phase of cellular GSH content after APAP treatment.[57, 60, 72] A decrease in GSH, in conjunction with an increase in GSSG, following APAP treatment, is consistent with previous reports.[24, 125] Post-treatment with NACA significantly increased GSH in the APAP-treated group, while NAC did not, indicating that NACA was significantly better than NAC. These results indicated that NAC did not replenish the GSH levels at the dose given, which could be attributed to the lower bioavailability of NAC, when compared to that of NACA. My results are in line with one other study that compared two different doses of NAC (1.25 mmol/kg vs. 7.35 mmol/kg) in APAP-induced toxicity and reported no enhancement in GSH levels when 1.25 mmol/kg of NAC were administered to C57BL/6 mice.[125] Furthermore, in my study, I used 106 mg/kg, which corresponds to 0.65 mmol/kg of NAC, a dose that is much lower than that used by Terneus *et al*.[125] The protective effects of NACA can

possibly be explained by its ability to supply cysteine for GSH biosynthesis, in addition to reducing extracellular cystine to cysteine through conversion of GSSG to GSH by a nonenzymatic thiol disulfide exchange, and by restoring GR activity.[28] Changes in levels of either GSH or GSSG, or both, affect the GSH/GSSG ratio. GSH/GSSG represents a clear picture of the oxidative state in the body and is used as a good indicator of oxidative stress.[130] In agreement with previous studies, my results show a dose-dependent decrease, [121] as well as the effect of time, [22] in the GSH level. Dose-dependent effects can be explained by increased production of NAPQI, which concomitantly depletes GSH by conjugation. The low dose of APAP (300 mg/kg) did not affect the level of GSSG; however, a significant reduction in GSH also resulted in a significant reduction in the GSH/GSSG ratio. A drastic decrease in the ratio of GSH/GSSG post a 400 mg/kg dose of APAP could be attributed to the higher concentration of NAPQI, resulting in further depletion of GSH. Furthermore, binding of excess NAPQI to mitochondrial proteins leads to increased production of free radicals which, in turn, may be reduced by GSH resulting in a higher concentration of GSSG. By increasing the period of exposure to APAP from 4 h to 24 h, the GSH level was restored to the control level, which is consistent with a previous study that showed a similar decrease in hepatic GSH at 4 h and a regeneration at 24 h after APAP administration.[22] Marel R. *et al.* showed in their paper an increased level of GSH, instead of a decrease, after an exposure of APAP for 24 h, and they explained that as an attempt to restore the lost oxidative balance.[19] The disruption of GSH/GSSG ratio at 24 h post APAP injection is attributed to the higher GSSG levels and is not due to disruption of the GSH levels. NAC and NACA acted equally to restore this GSH/GSSG ratio, possibly by directly scavenging ROS and, thereby, lowering the

formation of GSSG. The low bioavailability of NAC was overcome by restoration of the hepatic GSH level in the 24 h design study.

Many studies have shown that treatment of rodents with different doses and durations of NAC decreased the hepatotoxicity induced by APAP to various levels of severity. Chieko Saito *et al*. showed in their paper the positive effect of using a high dose of NAC (318 mg/kg) for increasing GSH and ATP levels and for decreasing ALT activity, in comparison with a low dose of NAC (106 mg/kg), with both being measured 6 h after APAP dosing.[13] In addition, Marcus V. Terneus *et al*.[125] reported that a high dose of NAC (1200 mg/kg) was able to restore the GSH level back to the control level, while a low dose (204 mg/kg) did not induce any change in the GSH levels when measured 4 h after APAP injection. These studies support my hypothesis that, NACA treatment, because of its high bioavailability, we will have the benefits provided by a high dose of NAC without actually using such a high dose.

GR is a key antioxidant enzyme involved in the maintenance of cellular GSH homeostasis under normal physiological conditions. It reduces GSSG to GSH, which allows cells to detoxify more NAPQI and free radicals. A reduction in the activity of GR was observed with APAP treatment and is documented in literature.[131, 132] Some studies relate this impairment in the activity of antioxidant enzymes to excessive production of NAPQI, resulting in generation and accumulation of ROS and concomitant loss of GSH, which directly affects the activity of GSH-related enzymes.[133, 134] Furthermore, the reduced activity of GR could also be attributed to a decrease in its cofactor, NADPH, which has reportedly been used for the reduction of NAPQI to APAP.[12, 135] Decreased activity of GR has a deleterious effect, resulting in accumulation of GSSG that leads to

further oxidative stress. My studies show a dose response effect of APAP on GR activity. The reduction in GR activity could be attributed to the oxidation of crucial sulfhydryl groups in GR, which are important for GR activity. Post-treatment with NACA significantly restored GR activity; however, a similar dose of NAC was not effective. After a 24-h exposure time the tissue might be defending against oxidative stress by de novo GSH synthesis, as well as regeneration via reduction of GSSG to GSH by enhancing the activity of GR. The enhancement in GR activity could be attributed to an enhanced supply of NADPH as well as reduction of crucial sulfhydryl groups in GR. However, this enhancement in GR activity might not be sufficient to convert all of the GSSG back to GSH.

Depletion of GSH sets off a cascade of further oxidative damage. Excess NAPQI binds to cellular protein (mainly mitochondrial protein) that leads to mitochondrial dysfunction that inhibits oxidative phosphorylation, and depletes adenosine triphosphate (ATP), that leads to mitochondrial oxidative stress.[58, 132] NAPQI binding to mitochondrial proteins and mitochondrial dysfunction are the central mechanisms of toxicity. The severe depletion of mitochondrial GSH reported here is in line with the literature.[13, 73, 113] The role of mitochondrial oxidative stress in APAP toxicity is documented, and evidence for superoxide and peroxynitrite selective formation in the mitochondria is reported in the literature, [58] which supports my premise that a significant reduction in the GSH/GSSG ratio indicates oxidative stress.[130] NAC has a limited capacity to increase the GSH level, but administration of NACA at an equimolar concentration is able to restore the depleted mitochondrial GSH level, which scavenged the free radicals (as confirmed by significant restoration of the GSH/GSSG ratio) that

may have attenuated cell injury. However, scavenging of the free radicals is not the only NAC mechanism that protects against APAP toxicity in the mitochondria. Excess cysteine that is not needed in GSH synthesis is degraded and used as an energy substrate in the TCA cycle to support mitochondrial energy production.[13] To further support my results of mitochondrial oxidative stress and dysfunction, I measured the levels of serum GDH (an enzyme of the mitochondrial matrix that is largely expressed in the liver). I observed elevated levels of GDH, which is in agreement with previous studies and indicates mitochondrial damage.[32, 136] Post-treatment with NACA is able to significantly decrease GDH levels while NAC cannot. An equidose of NACA is better than that of NAC in providing a sufficient amount of GSH to overcome protein binding. My previous paper reported that mitochondrial dysfunction was detected in a HepaRG cell model after APAP toxicity, [26] which is supported by other studies as well.[32, 46, 132]

The liver tissue contains a large amount of polyunsaturated fatty acids (PUFAs), which are susceptible to peroxidative damage.[137] NAPQI binding to a sulfhydryl group of proteins and the overproduction of free radicals in APAP toxicity may have prompted lipid peroxidation and depletion of antioxidant enzymes and, consequently, an increase in the MDA content. MDA is a biomarker of lipid peroxidation and its elevation during APAP toxicity is reported in the literature.[20, 22, 33, 121] Lipid peroxidation is a multi-step process requiring the initiation of a chain reaction and propagation. This peroxidation affects the membrane fluidity which results in severe cellular dysfunction and complete destruction of the cell membrane and cell death.[129, 138] Glutathione peroxidase (GPx) plays a major role in defense mechanism by reducing lipid peroxidation. GPx inhibits lipid peroxidation by reducing lipid peroxides to their corresponding alcohols in the

presence of GSH as a substrate.[48] A reduced level of GSH, as a consequence of a high level of NAPQI, may have affected GPx levels, and led to the accumulation of MDA.[75] However, post-treatment with NACA interrupted this chain reaction of lipid peroxidation by supplying an adequate amount of GSH (GSH precursor), a substrate for GPx, to effectively reduce MDA levels and scavenge the very reactive hydroxyl and lipid peroxyl radicals. Interestingly, NAC was not able to supply an adequate amount of GSH at this low dose.

The end point in the hepatotoxicity induced by APAP is the necrosis in the hepatic cells, which was confirmed by histological and macroscopic evaluation and by measuring liver function enzymes. ALT is the enzyme present in hepatocyte cytoplasm; however, damaged hepatocytes lead to increases in the level of ALT in the serum. This is a reliable marker and a sensitive indicator of liver injury because of its 90% presence in the liver.[76, 119, 139] My results showed a very large increase in ALT activity in the APAP-treated group, which was consistent with previous studies in which researchers reported elevations in serum transaminases following a toxic dose of APAP.[57, 116, 125] This is a significant criterion for assessing the positive effects of any hepatoprotective drugs that can preserve normal physiological functions, assure membrane stability that may have been disturbed, and promote regeneration of liver cells.[131] NACA successfully restored the transaminase enzyme to normal levels, stabilized the membrane, and allowed regeneration of liver cells. NAC, however, significantly restored this enzyme after administration of a lower dose of APAP (300 mg/kg), but was not able to do this with administration of a low dose (106 mg/kg) after administration of 400 mg/kg of APAP.

To gain further insight into the protective role of NAC/NACA, histological evaluations were performed. Evaluations showed centrilobular necrosis upon APAP treatment. This was consistent with literature reports of APAP toxicity.[23-25] Interestingly, I found that livers of the NAC- and NACA-treated mice had histologic lesion scores similar to the untreated control group, despite having higher levels of the serum ALT. Although serum ALT activity is considered a highly specific and sensitive marker of hepatotoxicity, it may have given us a false positive result. Furthermore, to explain the discrepancy in our ALT activity data with histological data, an additional biomarker, GDH activity, was used which improved specificity for liver function, as compared to the use of serum ALT activity alone. Serum GDH activity is more specific for the liver and would eliminate the risk of false results.[140]

Although, histopathology is a valuable tool that is frequently used to microscopically visualize cellular damage, the ability to adequately assess cellular damage utilizing this method depends on the amount of tissue injured, sample size, and tissue sectioning during histopathological preparation. This could have resulted in the discrepancy between our biochemical and histological data. Therefore, histopathologic assessment is commonly used in conjunction with other molecular based tests. As detection of serum ALT and GDH assays is independent of tissue sample size and sectioning, these assays may be more sensitive to changes in hepatocellular injury. Furthermore, for this study, a small portion of the liver samples was evaluated for histopathologic lesions and, therefore, the ALT and GDH assays may have been more representative of the molecular hepatocellular changes that occurred in the treatment groups. Despite elevations in ALT and GDH, as compared to that of normal control

animals, the NACA-treated group was statistically lower than the NAC-treated group, indicating better hepatocellular protection by NACA. Furthermore, the discrepancy in the histological data due to small sample size can be explained by the acinus concept. According to the acinar unit concept,[141] the gradient of blood supply and the presence of CYP in zone 3 determined the extent of damage within a single section of the same specimen of the liver. This gradient would make the histological evaluation less sensitive than that of the biochemical assay of a small sample size.

The maximum toxicity of APAP reported by investigators was at 4 to 6 h after administration; then, by 24 h, the liver started to regenerate.[22] My results were in agreement with that, starting from the level of GSH that was earlier depleted as a result of NAPQI formation, until the end point of toxicity (the level of ALT that indicates cell necrosis), which showed a maximum toxicity at 4 h compared to that at 24 h. That explains the equal effectiveness of NAC and NACA at 24 h, when a smaller dose was needed to counteract toxicity. However, NACA appeared to be more potent than NAC in reversing oxidative damages induced by APAP at 4 h.

The disadvantage of NAC's low bioavailability was more pronounced after 4 h of APAP administration, and this low dose (106 mg/kg) helped to highlight the advantage of using NACA. NACA was very efficient in scavenging free radicals, stimulating antioxidant enzyme, providing cysteine to stimulate ϒ-glutamylcysteine synthetase (the rate limiting step in GSH synthesis), all of these with this low dose.

In summary, this study clearly demonstrates that APAP hepatotoxicity was alleviated and the animals were rescued by NACA treatment. My data show that, at a dose of 106 mg/kg, NACA is more effective than NAC at combating APAP-induced

toxicity. This effectiveness may be attributed to NACA's higher bioavailability, as compared to that of NAC. The data indicates that NACA was more effective than NAC at a lower dose and, therefore, may be developed into a potential antidote for APAP-induced toxicity, with potentially lower or no indication of the side effects that have traditionally been associated with higher doses of NAC. Furthermore, the higher bioavailability of NACA may also help widen the therapeutic window for the treatment of APAP-induced toxicity.

7. NEPHROTOXICITY

7.1. RESULTS

7.1.1 Effect of NAC/NACA on Intracellular Levels of GSH. Intracellular depletion of GSH is reported as a first step in APAP-induced toxicity, so we studied the effect of APAP on the level of GSH. The results showed a significant decrease in the GSH levels (to approximately 66% of the control) after treatment with 400 mg/kg of APAP for 4 h (Figure 7.1A). Interestingly, the NAC treatment group was not effective in significantly increasing the level of GSH at this low dose (106 mg/kg). However, the NACA treatment group showed a considerable increase in the GSH level to that of the control level.

The dose and time dependent toxicity of APAP were evaluated by using a lower dose (300 mg/kg) and two different sacrificing times (4 h and 24 h). The GSH level was reduced to 79% of the control with the dosing of 300 mg/kg APAP for 4 h (Figure 7.1 B). The effects of NAC and NACA with this dose were similar to the effects with a higher dose (400 mg/kg) in which NACA was able to show a positive effect in restoring the GSH level back to that of the control level, while NAC was not be able to make any significant change in this level at this dose (106 mg/kg). A 24 h treatment time with 300 mg/kg APAP depleted the GSH level to around 56.7% of the control level (Figure 7.1 C). NAC and NACA showed similar enhancement of GSH levels back to the control level.

7.1.2 Effect of NAC/NACA on Intracellular Levels of Cysteine. The cysteine level was depleted to around 48% of the control level in the APAP-treated group (400 mg/kg for 4 h). Post treatment with NACA was able to significantly restore the cysteine

level, as compared to the level in the APAP-treated only group. Post treatment with NAC was able to increase the cysteine level; however, it was not significant when compared with that of the APAP-only treated group (Figure 7.2).

7.1.3 Effect of NAC/NACA on Creatine Kinase Level. The creatine kinase level in the serum was significantly elevated after dosing with APAP for 4 h, indicating nephrotoxicity (Figure 7.3). NACA post treatment was able to greatly decrease this elevation, while NAC post treatment was not effective.

7.1.4 Effect of NAC/NACA on Blood Urea Nitrogen Level. The blood urea nitrogen level increased significantly following dosing with 400 mg/kg of APAP for 4 h. NACA post treatment was able to significantly decrease this elevation, while NAC post treatment was not able to show much difference in this level (Figure 7.4).
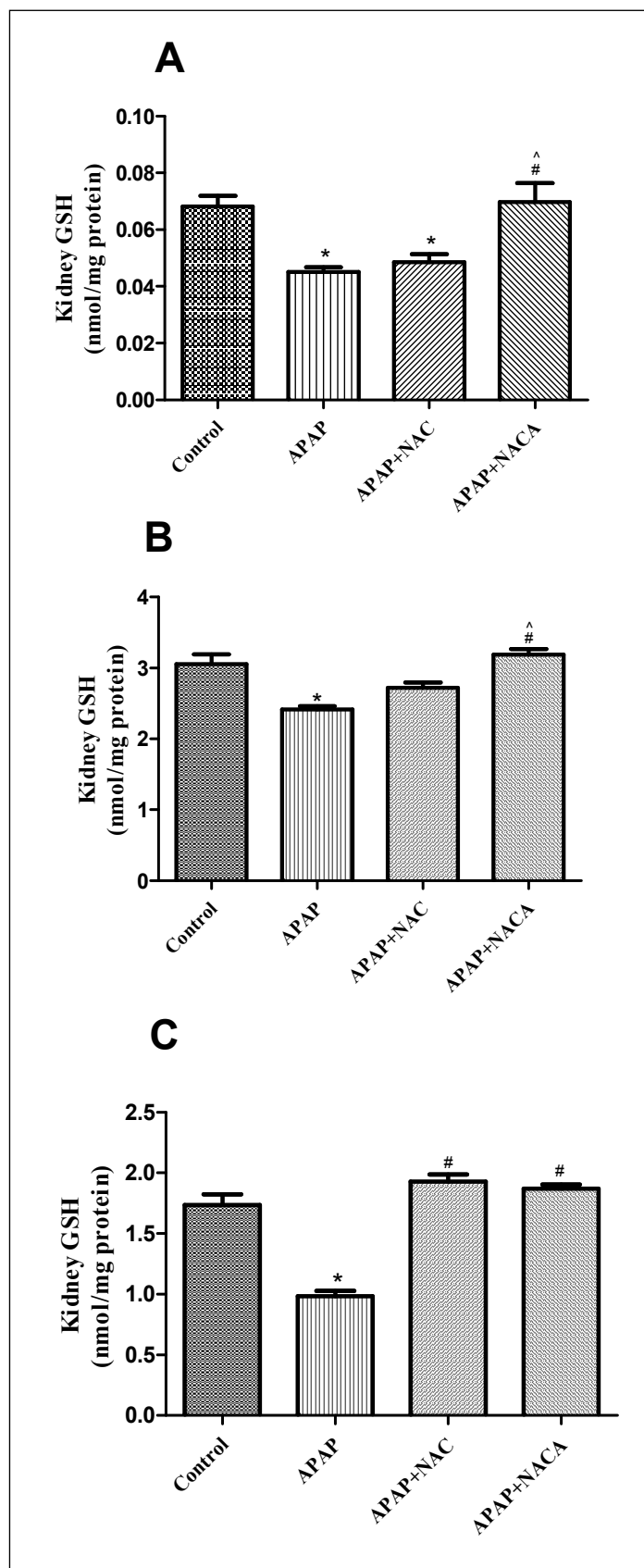
Figure 7.1. Kidney glutathione (GSH) levels

C57BL/6 mice injected with (A) APAP (400 mg/kg) for 4 h, (B) APAP (300 mg/kg) for 4 h, and (C) APAP (300 mg/kg) for 24 h. Post treatment with NAC or NACA (106 mg/kg) at 1.5 h after APAP treatment of all treatment groups. Values represent mean ±SD (n=6) (*: different from the control group, #: different from the APAP group, and ^: different from APAP+ NAC group) $p < 0.05$.
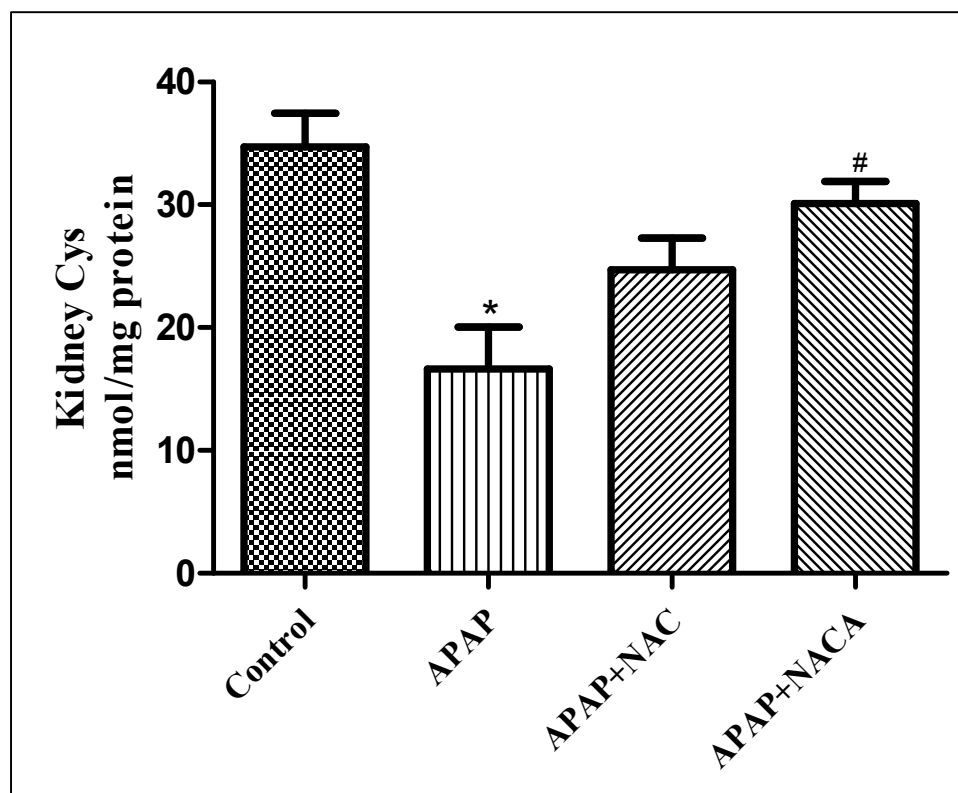
Figure 7.2. Kidney cyestine (Cys) levels

C57BL/6 mice injected with APAP (400 mg/kg) for 4 h. Post treatment with NAC or NACA (106 mg/kg) at 1.5 h after APAP treatment in all treatment groups. Values represent mean ±SD (n=6) (*: different from the control group, #: different from the APAP group) $p < 0.05$.
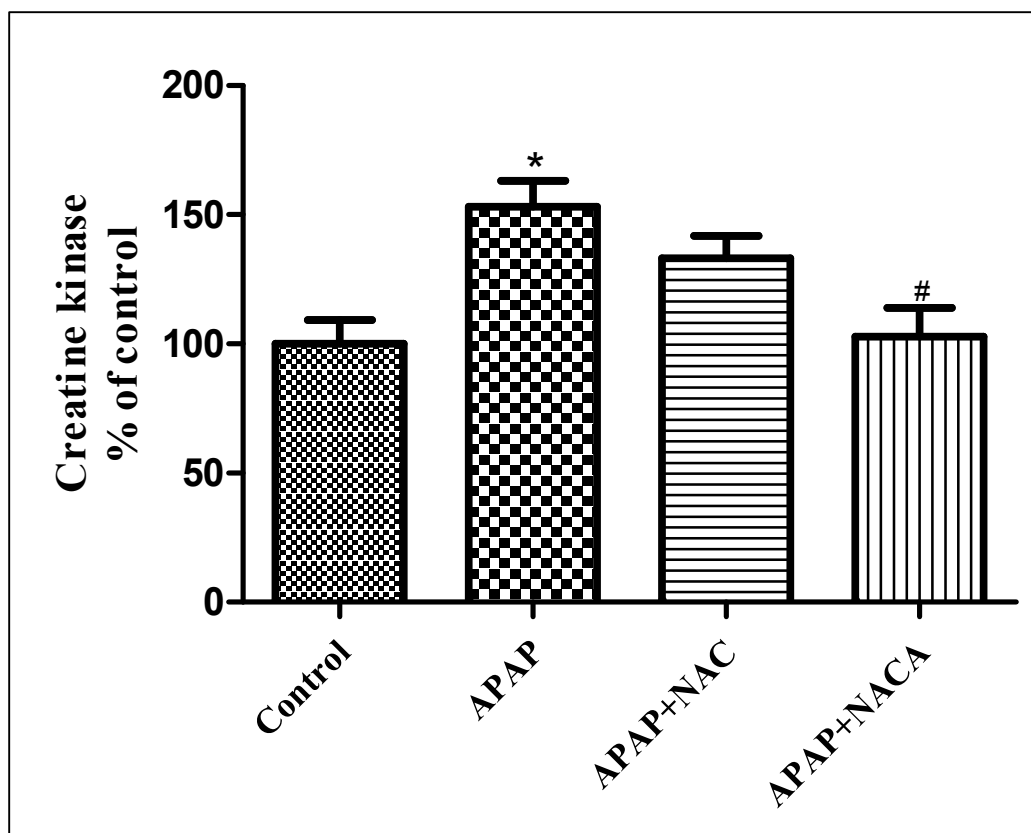
Figure 7.3. Serum creatine kinase activity

C57BL/6 mice injected with APAP (400 mg/kg) for 4 h, followed by treatment with NAC or NACA (106 mg/kg) at 1.5 h after APAP treatment. Values represent mean ±SD (n=6) (*: different from the control group, #: different from the APAP group) p<0.05.
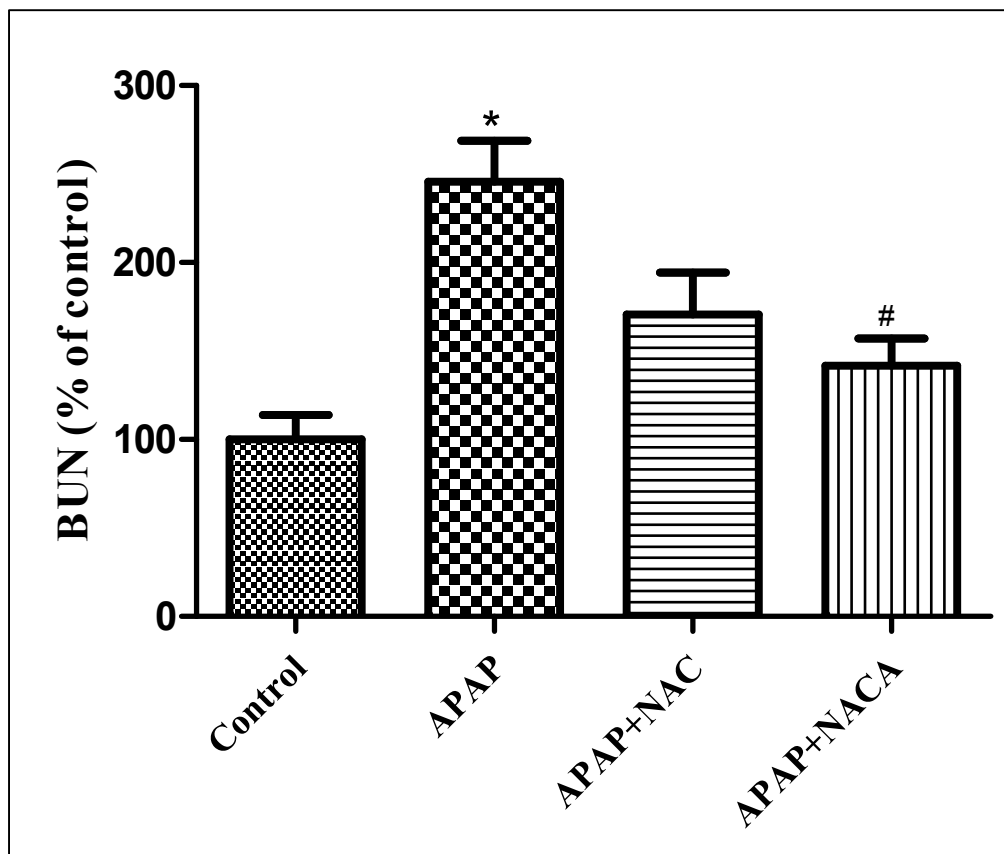
Figure 7.4. Serum blood urea nitrogen (BUN) level

C57BL/6 mice injected with APAP (400 mg/kg) for 4 h. Post treatment with NAC or NACA (106 mg/kg) at 1.5 h after APAP treatment in all treatment groups. Values represent mean ±SD (n=6) (*: different from the control group, #: different from the APAP group) $p < 0.05$.

7.2. DISSCUSION

The kidney is the second organ that may be affected by an APAP overdose and nephrotoxicity has been reported in many studies.[62-65] Although nephrotoxicity is less common than hepatotoxicity with an APAP overdose, many reports have associated the renal effect with severe hepatotoxicity,[37] and renal damage can occur, even in the absence of liver injury.[68, 70] Generation of ROS has been proposed as a mechanism by which many chemicals can induce nephrotoxicity, and oxidative stress remains as the most important cause in the case of APAP toxicity.[79-81] Studies have shown that an overdose of APAP induces lipid peroxidation and represses the antioxidant defense system of the kidney.[63, 79, 80] A mechanism, similar to that of hepatotoxicity, was proposed for the nephrotoxicity that is mainly related to the toxic metabolite of APAP (NAPQI), which binds covalently to proteins, causing cell death.[22, 63] The mechanism of toxicity in the kidney is less clearly understood, as compared to that in the liver.

Several potential mechanisms of renal toxicity are proposed in the literature. Those include the CYP pathway, prostaglandin synthase, deacetylase enzymes, and APAP-GSH conjugate excretion pathway (all of these pathways are explained in detail in section 2.4.1). NAC is the only approved antidote for APAP toxicity, and its mechanism of protection is based on the use of large doses to replenish GSH, that can scavenge NAPQI (if given early), or to recover GSH and scavenge the reactive oxygen and nitrogen radicals.[11] In addition, it provides excess amino acid to serve as energy substrates in mitochondria. NAC is not always effective in treating nephrotoxicity-induced by APAP, [67, 69, 70] although it is effective in cases of renal failure caused by toxic compounds such as gentamycin[142] and ifosfamide.[143] So, it is important to

introduce another compound which is effective against APAP-induced renal toxicity. NACA's effect in APAP-induced nephrotoxicity was biochemically evaluated in this study, and showed promising results.

In my results, NACA treatment effectively protected against nephrotoxicity induced by APAP overdoses in mice. This evidence was supported by significant improvement in the disturbed biochemical parameters. Administration of APAP-induced nephrotoxicity was manifested by a significant increase in the serum BUN [67, 70, 80] and creatine kinase enzyme activity (CK) [144] that indicated renal dysfunction (proved in many animal studies). CK catalyzed the conversion of creatine and utilized adenosine triphosphate (ATP) to create phosphocreatine (PCr) and adenosine diphosphate (ADP). Clinically, creatine kinase was assayed in blood tests as a marker of damage in CK-rich tissues, such as in a heart attack, severe muscle breakdown, and acute renal failure. Blood urea nitrogen that is found in liver protein is usually excreted in the urine. Renal diseases that are predicted based on the accumulation of BUN (where the rate of production exceeded the rate of clearance) make it a more reliable marker for renal function. However, these increases were reversed by the administration of NACA, while a similar dose of NAC was not effective.

It has been reported that intracellular GSH plays a significant role in APAP-induced nephrotoxicity.[63, 70, 79] The GSH level, which is significantly depleted following an APAP overdose, is used to detoxify NAPQI (toxic metabolite of APAP). The excess NAPQI then binds to cellular protein leading to renal injury. As a consequence of GSH depletion, there is a decrease in the detoxification of NAPQI (which increases its binding to macromolecules), in addition to serious harmful effects on cellular balance (which may

increase the toxic effects of the reactive metabolite). NAC, a GSH pro-drug approved for APAP hepatotoxicity, has not shown clear benefits for renal toxicity. Although some papers show that it might help in the treatment,[22] others indicate that it does not provide any benefit. [67, 69, 70] In this study, we show that NACA successfully increases the GSH level in kidneys.

ϒ-glutamyltranspeptidase (ϒ-GT) activity in the kidneys is much higher than that in the liver, so that GSH (as a substrate of ϒ-GT) is degraded more rapidly in the kidneys.[71, 145] This may explain the low GSH and high cysteine levels in the kidneys, where an APAP overdose led to significant decreases in the cysteine levels. This decrease might have resulted from the conjugation of the reactive metabolite with the SH group in the cysteine, [136] as supported by the study of L. J. Fischer *et al*. [146] This study shows a high concentration of cysteine conjugates in the kidneys. Furthermore, cysteine might be delivered to the blood under oxidative stress conditions that are induced by an APAP overdose. Daniela Giustarini *et al*.[147] show in their study that oxidative stress induces a reversible flux of cysteine from tissue to the blood. NACA post-treatment was able to restore this level back to the control level.

NACA may act as an antioxidant and scavenging agent for free radicals (which have a role in the pathogenesis of APAP-induced nephrotoxicity) and NAPQI, by directly binding with them and preventing their binding to cellular proteins. It may reduce GSSG back to GSH by non-enzymatic disulfide exchange and, thereby, increase the GSH level indirectly because the kidneys cannot synthesize GSH from its precursors, but takes it from circulation, when required.[70, 79, 144] In addition, NACA may help by providing more

cysteine in the cells, which may help in the detoxification process. Further studies in this area might provide insight into the protective role of NACA in APAP-induced nephrotoxicity.

8. OTHER ORGANS TOXICITY

8.1. BRAIN TOXICITY RESULTS

8.1.1 Effect of NAC/NACA on Intracellular Levels of GSH, GSSG, and GSH/GSSG. Dosing with 400 mg/kg of APAP did not affect the levels of GSH and GSSG and, thereby, the GSH/GSSG ratio (Figure 8.1). As the brain's GSH level was not affected by APAP dosing, post-treatment with NAC/NACA did not provide any benefit.

8.1.2 Effect of NAC/NACA on the Activity of GR. The GR levels of the brain were not significantly changed in any of the treatment groups, as the dosing of 400 mg/kg APAP did not have a noticeable effect (Figure 8.2).

8.1.3 Protective Effect of NAC/NACA on Lipid Peroxidation. None of the groups showed any significant change in the levels of MDA after a 400 mg/kg APAP dosing (Figure 8.3).
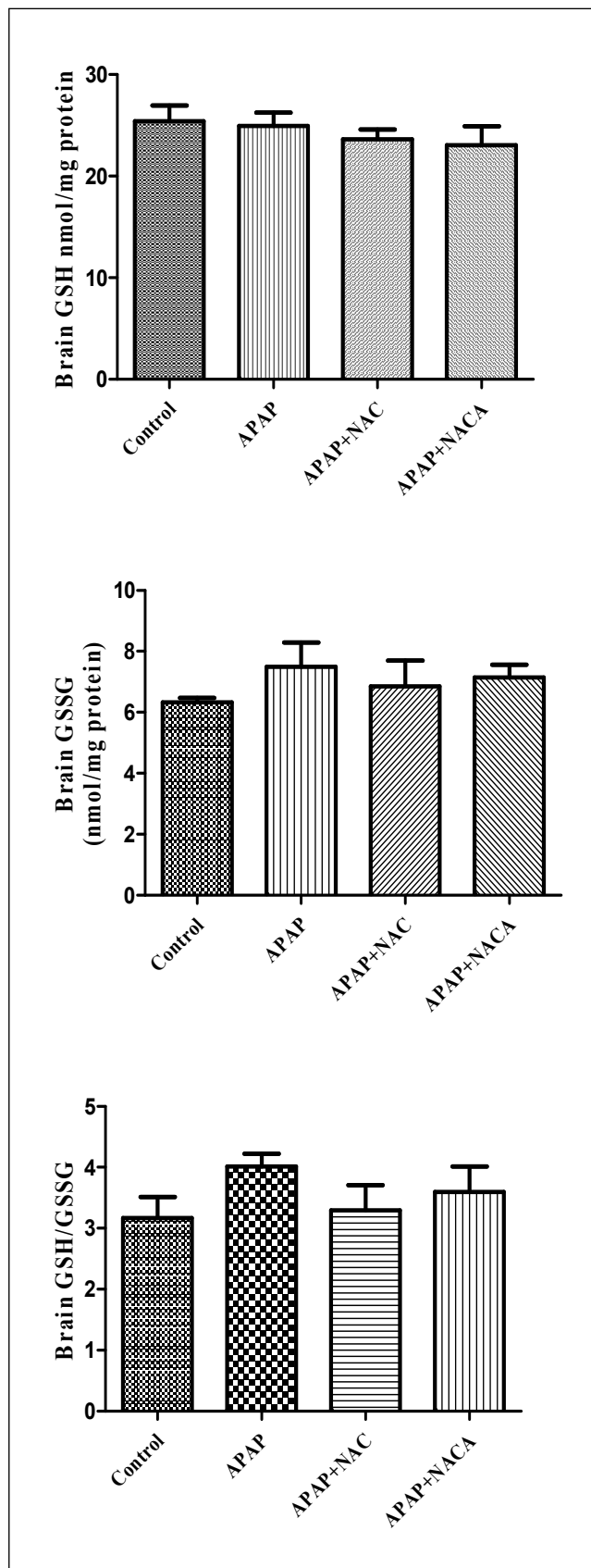
Figure 8.1. Brain glutathione (GSH), glutathione disulfide (GSSG), and GSH/GSSG ratio levels

(A) GSH, (B) GSSG, and (C) GSH/GSSG ratio levels in which C57BL/6 mice injected with APAP (400 mg/kg) for 4 h, followed by NAC or NACA (106 mg/kg) at 1.5 h after APAP treatment of all treatment groups. Values represent mean ±SD (n=6
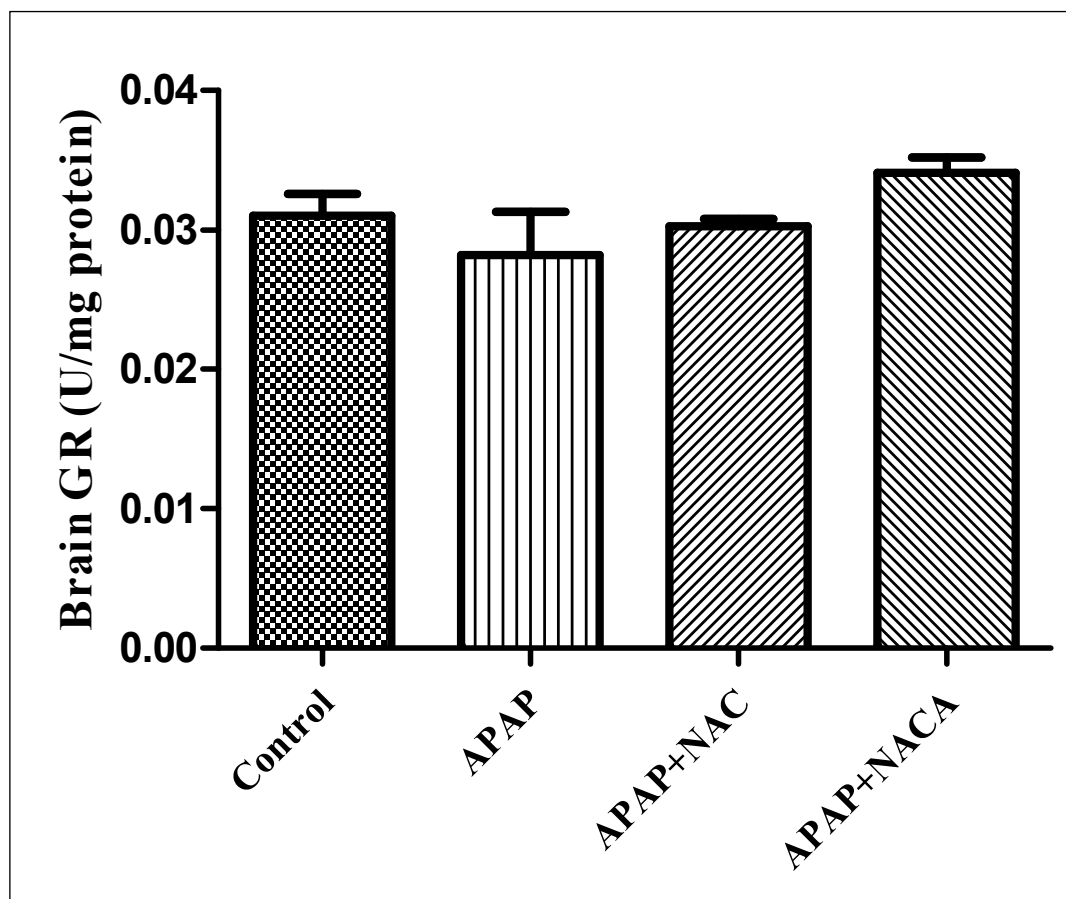
Figure 8.2. Brain glutathione reductase (GR) activity

C57BL/6 mice injected with APAP (400 mg/kg) for 4 h, followed by NAC or NACA (106 mg/kg) at 1.5 h after APAP treatment of all treatment groups. Values represent mean ±SD (n=6).
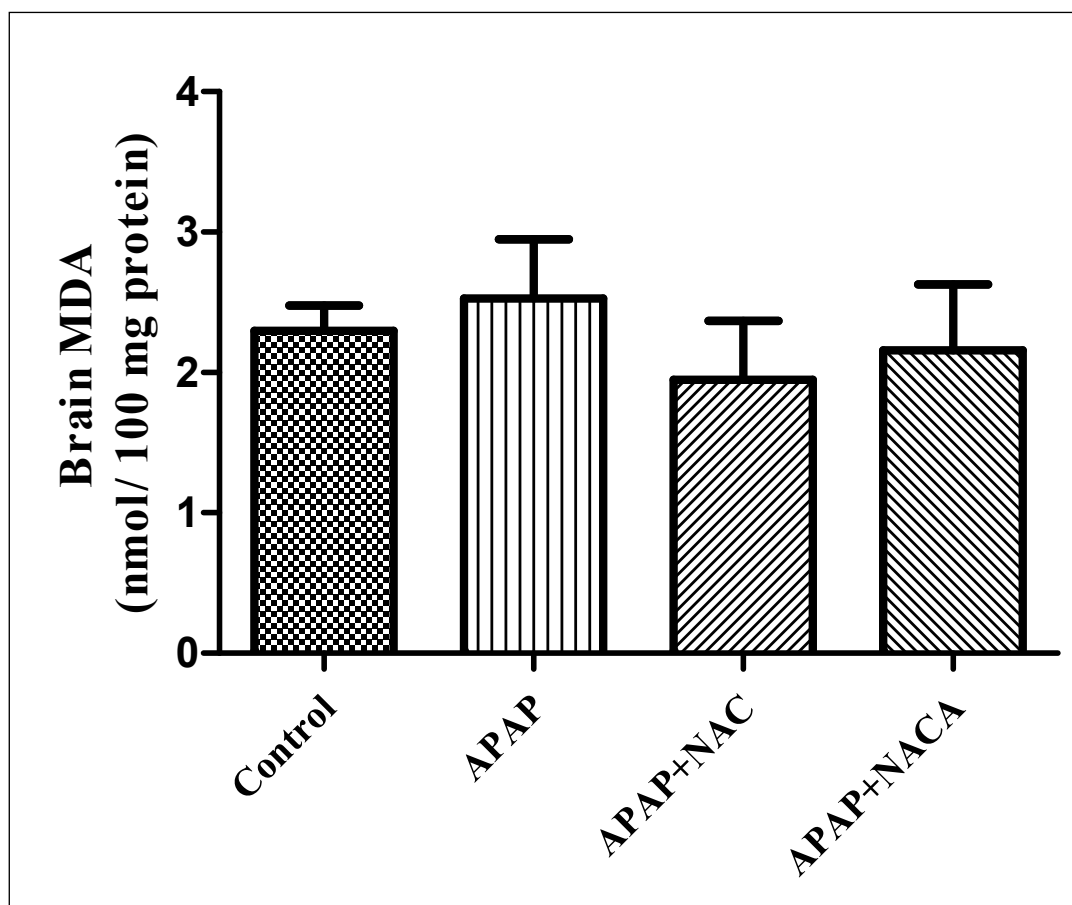
Figure 8.3. Brain malondialdehyde (MDA) levels

C57BL/6 mice injected with APAP (400 mg/kg) for 4 h, followed by NAC or NACA (106 mg/kg) at 1.5 h after APAP treatment of all treatment groups. Values represent mean ±SD (n=6).

8.2. SPLEEN TOXICITY RESULTS

8.2.1 Effect of NAC/NACA on Intracellular Levels of GSH, GSSG, and GSH/GSSG. Intracellular depletion of GSH was observed, which was reduced to 42.2% of the control level, following 400 mg/kg dosing of APAP. Interestingly, a low dose (106 mg/kg) of NAC was not effective in significantly increasing the levels of GSH. However, the NACA treatment group showed a major increase in the GSH level, as compared to that of the control. Interestingly no significant changes were observed in either the GSSG levels or the GSH/GSSG ratios of the treatment groups (Figure 8.4).

8.2.2 Effect of NAC/NACA on the Activity of GR. Dosing with 400 mg/kg of APAP showed a significant reduction in GR activity by approximately 70% of the control level. NACA post-treatment was able to restore the activity to that of the control, while NAC was not able to induce any major change in GR activity (Figure 8.5).

8.2.3 Protective Effect of NAC/NACA on Lipid Peroxidation. MDA levels were not affected in any of the treatment groups after dosing with 400 mg/kg of APAP (Figure 8.6).
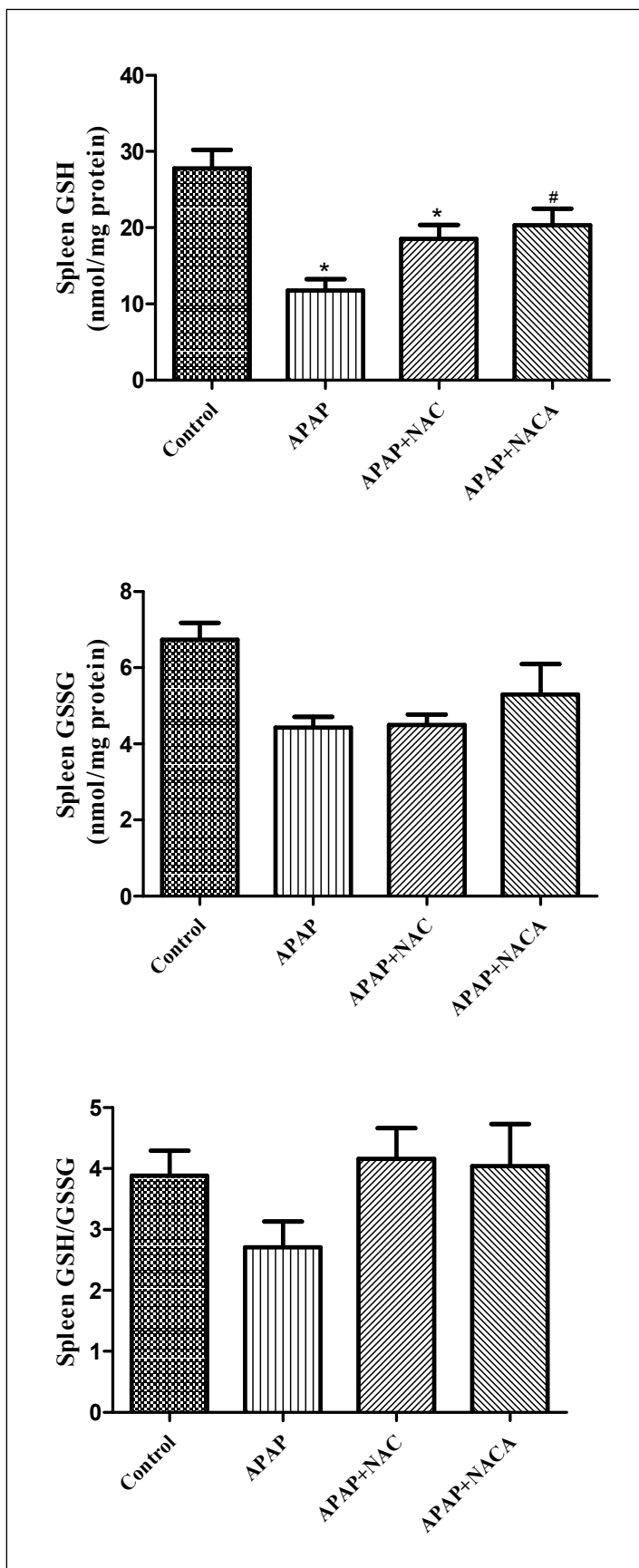
Figure 8.4. Spleen glutathione (GSH), glutathione disulfide (GSSG), and GSH/GSSG ratio levels

(A) GSH, (B) GSSG, and (C) GSH/GSSG ratio levels in which C57BL/6 mice injected with APAP (400 mg/kg) for 4 h, followed by NAC or NACA (106 mg/kg) at 1.5 h after APAP treatment of all treatment groups. Values represent mean ±SD (n=6).
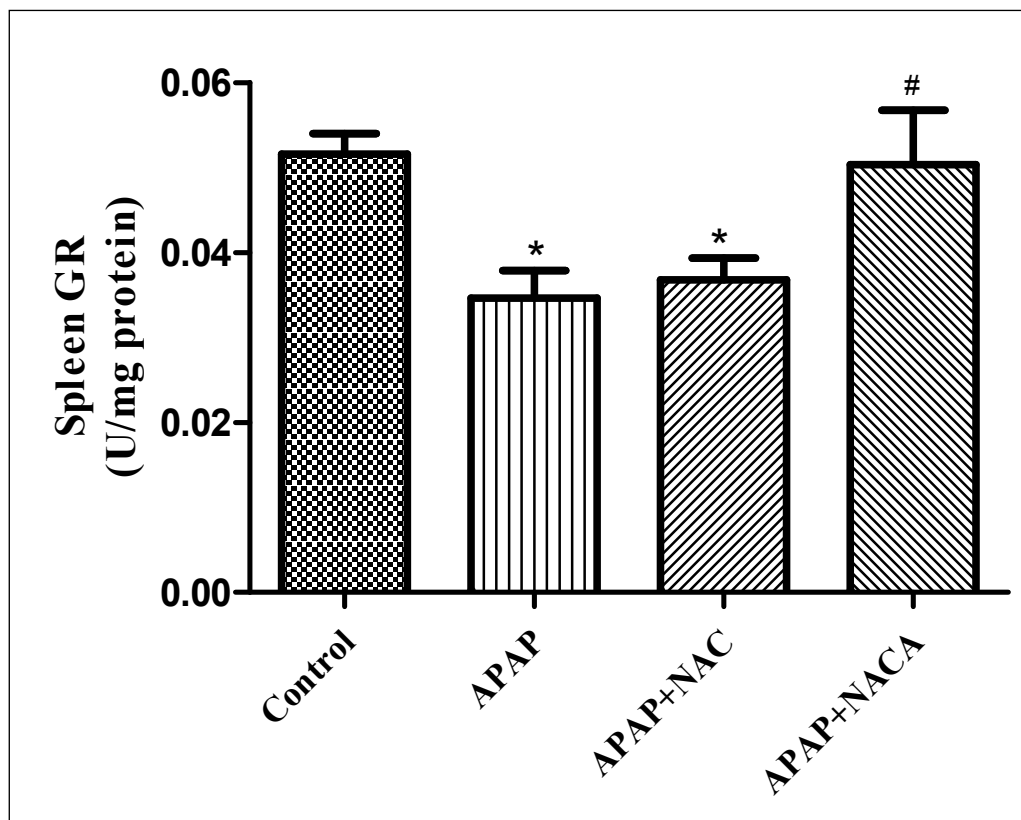
Figure 8.5. Spleen glutathione reductase (GR) activity

C57BL/6 mice injected with APAP (400 mg/kg) for 4 h, followed by NAC or NACA (106 mg/kg) at 1.5 h after APAP treatment of all treatment groups. Values represent mean ±SD (n=6) (*: different from the control group, #: different from the APAP group) $p < 0.05$.
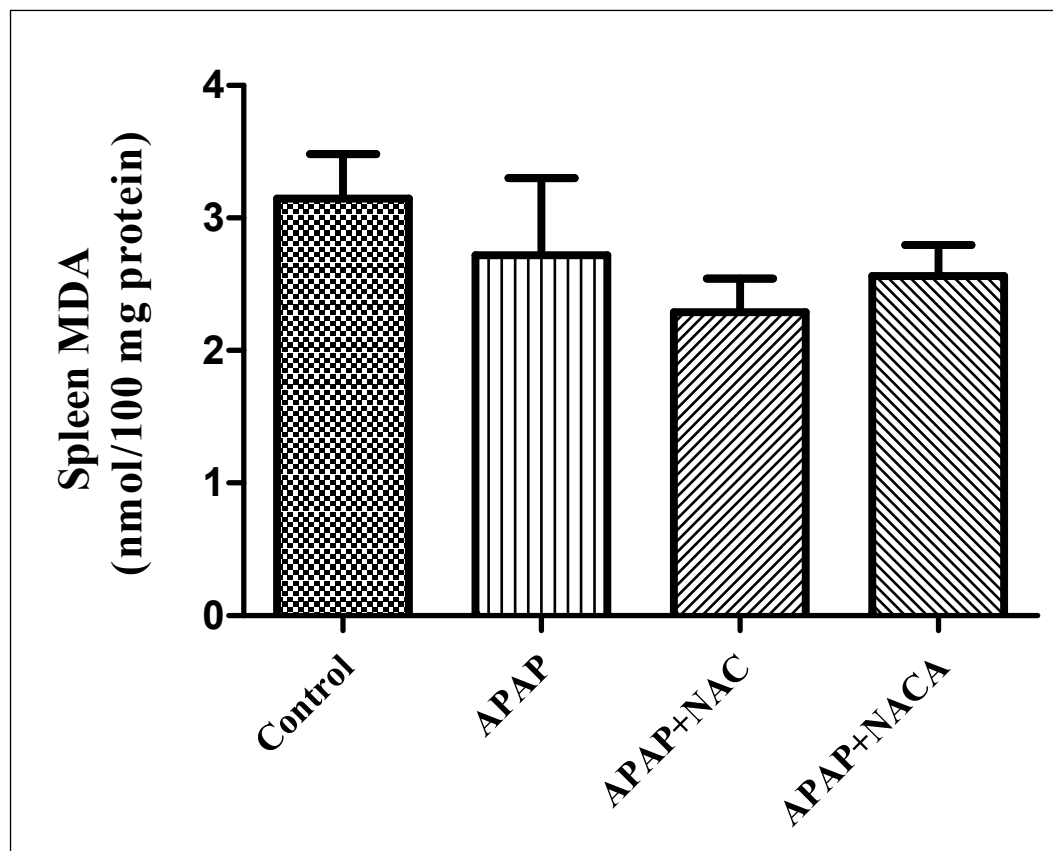
Figure 8.6. Spleen malondialdehyde (MDA) levels

C57BL/6 mice injected with APAP (400 mg/kg) for 4 h, followed by NAC or NACA (106 mg/kg) at 1.5 h after APAP treatment of all treatment groups. Values represent mean ±SD (n=6).

8.3. DISCUSSION

Although, APAP overdose is known to induce hepato- as well as nephrotoxicity, it is generally not considered to be toxic to other organs. However, since most epithelial tissues possess the enzymes necessary to produce the APAP toxic metabolite, extrahepatonephric, APAP toxicity needs to be evaluated. Liver dysfunction results in the development of hepatic encephalopathy by the accumulation of ammonia and formation of ROS, which affects the antioxidant capacity of the whole organism.[148] Brain tissue is highly susceptible to oxidative stress, owing to its high content of unsaturated fatty acids, high oxygen consumption, and poorly developed oxidative defense mechanisms.[149] The spleen is the largest peripheral lymphatic organ and plays an important role in adaptive immune response.

L. J. Fischer et al.[146] show in their research that the covalent binding of reactive metabolite of APAP was highest in the liver, followed by the extent of its presence in the kidneys (one-fifth of that in the liver), but it was very low in the brain and did not increase with time after an APAP dose. In another study, the authors reason that there is low covalent binding of NAPQI in the brain because of the relative low activity of CYP there (as compared to that in the liver), which activates APAP, even though the level of unchanged APAP is high in the brain (substrate for potential toxicity). [150] This is in contrast to several other studies that indicate that rats have an isoform of CYP (CYP2E1) that is responsible for NAPQI formation, which forms directly in brain tissue following APAP's crossing of the blood brain barrier.[149] Kamilla Blecharz-klin et al.[148] observed changes in the levels of neural amino acid in animals treated with APAP, which may be linked with a wide range of pharmacological effects of APAP on the central nervous

system, and may play a role in the pathology of some neurobehavioral disturbances. In contrast, Susan G *et al*. shows, by using immunohistochemical methods, that the brain and spleen do not exhibit APAP-induced toxicity or covalent binding and, also, do not express CYP2E1.[151] Spielberg and Gordon [152, 153] show that APAP could cause lymphocyte necrosis *in vitro* following drug activation by added microsomal enzymes. This is consistent with the observation of splenic lymphoid necrosis in the Michael E. Placke *et al*. study.[154] Another study, by Mary Jane Masson, [155] shows that a hepatotoxic dose of APAP is followed by a lymphocyte loss that may be due to apoptosis in the spleen, thymus, and hepatic draining lymph nodes. Literature review shows many controversial results of the effects of an APAP overdose on the spleen and brain.

In this study, APAP did not induce any noticeable toxicity in the brain although it significantly decreased the GSH and GR levels in the spleen. Michele Hinerasky[149] reports decreases in GSH levels associated with increases in lipid peroxidation in the brain but, in that study, higher doses of APAP were used to induce this neurotoxic effect than are used in this study.

It is possible that chronic administration of APAP, or its concurrent use with a modifying factor, may enhance its toxicity and have a role in spleen and brain toxicity. Findings from other papers suggest that the combination of APAP and ethanol may increase the inhibition of antibody production caused by ethanol alone.[156] They show that APAP, combined with ethanol, significantly enhances the reductions of phagocytic activity and circulating leukocyte counts caused by ethanol alone. Further studies are required wherein higher doses or longer exposure times may help clarify the immunological and neurological effects of an APAP overdose.

9. CONCLUSION

The most common painkiller (APAP) can be a "real" killer if it is not used properly. The primary toxicity of APAP results from drug metabolism in both the liver and extrahepatic tissue. Most of the drug is metabolized by glucuronidation and sulfation and only about 5% is metabolized by CYP to form NAPQI (the toxic metabolite). At therapeutic doses, NAPQI is reduced by GSH and is subsequently excreted as mercapturic acid. In the case of an overdose, GSH is depleted and more NAPQI is accumulated. These electrophilic intermediates then form adducts with cellular proteins that disrupt homeostasis and result in tissue necrosis and organ dysfunction. This has been demonstrated in both the liver and extrahepatic tissue in animal models. The mechanism of toxicity is well described for the liver, but is less clearly understood for the kidneys, which have several potential mechanisms.

The liver is the central organ in the metabolism of almost all drugs, and the kidneys, which contain active enzyme systems capable of metabolically activating drugs, are the site of excretion. Studies are being conducted throughout the world to search for and identify protective molecules that can provide maximum protection for the liver and kidneys, as well as for other organs, to assure that there are very few (or no) side effects to these crucial functions of the body. GSH serves as an important antioxidant for cells and its altered level is considered as an indicator of oxidative damage. The essential role of GSH in maintaining the redox status in a cell makes it an attractive potential therapeutic agent in oxidative stress-related conditions. The efficacy of the GSH prodrug, NACA, is increased due to its higher bioavailability which, in turn, would reduce the

toxicity associated with higher doses of NAC. Various studies indicate that NACA acts by restoring GSH levels, which allow cells to combat oxidative stress.

Although NAC is the treatment of choice in APAP-induced toxicity, fairly high doses and longer treatment times are required due to its poor bioavailability. This study demonstrates that NACA is better than NAC and has the potential to be developed into a viable therapeutic agent against APAP-induced toxicity. Thus, not only the effectiveness of NACA, but also the potential for reduced dosage and a wider therapeutic window might support its use for treating patients. As a result of growing interest, use of NACA for the treatment of diseases and conditions associated with oxidative stress was patented by Glenn A. Goldstein in 2013 (U.S. Patent No. 8,354,449 B2). [157]

10. FUTURE DIRECTION

Based on the results of this project, with regard to NACA's effect in APAP toxicity, a series of experiments should be performed in the future to gain further insight. This study has stimulated our thinking and guided us toward recommendations for the future direction of our research. These include:

- Evaluate the antidotal effect of NACA against APAP-induced toxicity, after delayed application, and compare findings with the effect of NAC.

    For this study, C57BL/6 mice will be divided into six groups (n=6): PBS, NAC, NACA, APAP, APAP+NAC, and APAP+NACA. The mice will be administered 400 mg/kg of APAP, followed by NAC/NACA (106 mg/kg) at different time points (1.5, 4, 6, 12, and 20 h). The animals will be sacrificed 24 h after APAP dosing. Blood will be drawn and centrifuged to get serum. The tissues will be frozen at -80°C for later analysis.

- Evaluate the antidotal effect of NACA against APAP-induced toxicity and compare it with NAC by using a protocol to mimic the one approved for NAC in clinical practice.

    For this study, C57BL/6 mice will be divided into six groups (n=6): PBS, NAC, NACA, APAP, APAP+NAC, and APAP+NACA. The mice will be administered 400 mg/kg of APAP (i.p), followed by p.o administration of NAC/NACA on a dosing schedule of 140 mg/kg at 1.5 h after an APAP dose, followed by 17 doses of 70 mg//kg each, every 4 h. The animals will be sacrificed 1 h after the last NAC/NACA dose. Blood will be drawn and centrifuged to get serum. The tissues will be frozen at -80°C for later analysis.

- Evaluate the effect of NACA on the regeneration of liver tissue over time and compare findings with the effect of NAC.

For this study, C57BL/6 mice will be divided into six groups (n=10): PBS, NAC, NACA, APAP, APAP+NAC, and APAP+NACA. The mice will be administered 400 mg/kg of APAP (i.p), followed by NAC/NACA (106 mg/kg) orally on a dosing schedule of 140 mg/kg 1.5 h after an APAP dose, followed by 17 doses of 70 mg//kg each, every 4 h. A blood sample will be drawn from the tail every day for 2 weeks to check the liver function enzymes (ALT and GDH). The samples will be withdrawn through a temporary cannula to reduce the pain and stress of the animals. If a vein is not visible, the tail will be dipped into warm water (40°C) and a local aesthetic cream will be applied to the surface of the tail 30 min before the experiment. Because of repeated blood sample collection, fluid replacement may be required. Lactated Ringer's solution (LRS) will be used, as recommended by the National Institutes of Health.

An alternative method to collect blood samples is the retro-orbital blood collection, in which anesthesia is required.

- Explore the nephrotoxicity induced by APAP by adding more parameters to evaluate the effects of NACA as GSSG, MDA, and antioxidant enzymes.

- Focus on brain and spleen toxicity induced by APAP.

In this study, the dose of APAP will be increased to 600 mg/kg, and the exposure time will be extended to 12 h. The redox status will be evaluated by measuring GSH, GSSG, MDA, and some antioxidant enzymes.

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