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Comparative evaluation of N-acetylcysteine and N-acetylcysteine amide in acetaminophen-induced oxidative stress

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COMPARATIVE EVALUATION OF N-ACETYLCYSTEINE AND N-ACETYLCYSTEINE AMIDE IN ACETAMINOPHEN-INDUCED OXIDATIVE STRESS

by

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ABSTRACT

Acetaminophen (APAP) is the most widely used pharmaceutical analgesic-antipyretic agent in the world, but its toxicity is a common cause of drug-induced hepatotoxicity. With APAP toxicity, cellular glutathione (GSH) is depleted. This results in the availability of N-acetyl-p-benzoquinone imine (NAPQI), is a toxic metabolite of APAP that binds to cellular macromolecules, which leads to cell necrosis. N-acetyl cysteine (NAC), a GSH precursor, is the only approved antidote for an acetaminophen overdose. It is a negatively charged molecule that diminishes its penetration into the cells, thereby requiring fairly high doses that increase the severity of side effects. In addition, oral and I.V. administration of NAC in a hospital setting is laborious and costly. Recently, NACA, an amide form of NAC, which is neutral at physiological pH has been developed to improve NAC's bioavailability. Therefore, in this study, we conducted an investigation to determine the mechanism of APAP-induced hepatotoxicity. We also evaluated the hepatoprotective effectiveness of NACA and compared it with NAC in the hepatic cell line, HepaRG. This comparison was based on several oxidative stress parameters, including the levels of intracellular reactive oxygen species, GSH, various antioxidant enzyme activities, and lactate dehydrogenase levels. In conclusion, NACA protected HepaRG cells against damage induced by acetaminophen toxicity and may, therefore, be a more useful antidote than NAC (the only approved antidote).

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Finally, I would like to dedicate this thesis to my parents, Naeem Khayat and Shadia Salamah; my husband, Yamin Mirdad: and my sisters and brother, Ajwaa, Alaa, and Ahmed Khayat for their encouragement and support. And last but not the least, I would like to present this thesis to my twins, Aasser and Alin, without their love, this work would never have been possible.

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LIST OF ABBREVIATIONS

APAP	N-acetyl p-aminophenol
BHT	Butylated hydroxytoluene
CAR	Constitutive androstane receptor
COX	Cyclooxygenase enzyme
CYP_{450}	Cytochrome P_{450}
DCHF-DA	2, 7 Dichlorofluorescin diacetate
DMSO	Dimethyl ssulfoxide
FDA	Food and drug administration
GR	Glutathione reductase
GSH	Glutathione
GSSG	Glutathione disulfide
GST	Glutathione S transferase
HPLC	High performance liquid chromatography
IV	Intravenous
LDH	Lactate dehydrogenase
MDA	Malondialdehyde
MPT	Mitochondrial membrane permeability transition
NAC	N-acetyl cysteine
NACA	N-acetyl cysteine amide
NAPQI	N-acetyl-p-benzoquinone imine
NSAIDs	Non-steroidal anti-inflammatory drugs

OTC	Over-the-counter
ROS	Reactive oxygen species
TBA	Thiobarbituric acid
TCA	Trichloroacetic acid

1. INTRODUCTION

Acetaminophen, also known as paracetamol in Britain, is derived from the chemical compound named para-acetylaminophenol (APAP). It is an analgesic and antipyretic medication. APAP is one of the most commonly used over- the- counter drugs in the United States[1]. The US Food and Drug Administration (FDA) approved acetaminophen in 1950 and as an over- the- counter medication in 1985[2]. In 2005, consumers purchased more than 28 billion doses of products containing APAP, and the hydrocodone–acetaminophen combination product being the most frequently prescribed drug [2]. More than 200 million people take APAP each year, and about 200 die each year of fulminant hepatic failure from APAP overdosage[3]. It is considered safe at therapeutic doses but, when overdosed, APAP produces a centrilobular hepatic necrosis that can be fatal[4]. In 2009, the FDA required that nonprescription and prescription APAP-containing medications provide information regarding the risks of APAP-induced hepatotoxicity[5, 6].

N-Acetyl cysteine (NAC) is the N-acetyl derivative of the amino acid, L-cysteine, which is a glutathione precursor and the only approved antidote for the hepatotoxicity induced by an acetaminophen overdose[7]. One drawback of NAC is its poor bioavailability due to a negative charge at physiological pH (7.35-7.45), so that it cannot readily pass through the cell membrane[8]. The purpose of this project was to evaluate the potential protective and antioxidant effects of N-acetylcysteine amide (NACA) on acetaminophen toxicity. It is a modified form of NAC to improve the bioavailability. Moreover, a comparison was made of the roles of NAC and NACA in protecting liver cells from APAP toxicity.

Therefore, our goals are as follows:

GOAL I: To explore the antidote role of NACA in APAP poisoning: This goal was accomplished by using an *in vitro* model. The pro-oxidant and antioxidant status, antioxidant enzymes, reduced and oxidized glutathione (GSH and GSSG respectively) levels were measured. Malondialdehyde (MDA), a stable byproduct of lipid peroxidation and a commonly used oxidative stress indicator, was also measured

GOAL II: To determine whether NACA is better than NAC in treating APAP-induced hepatotoxicity: Cells were pretreated with NAC or NACA; then, a toxic dose of APAP was administered. Cell viability and oxidative stress parameters mentioned in Goal I were measured and compared.

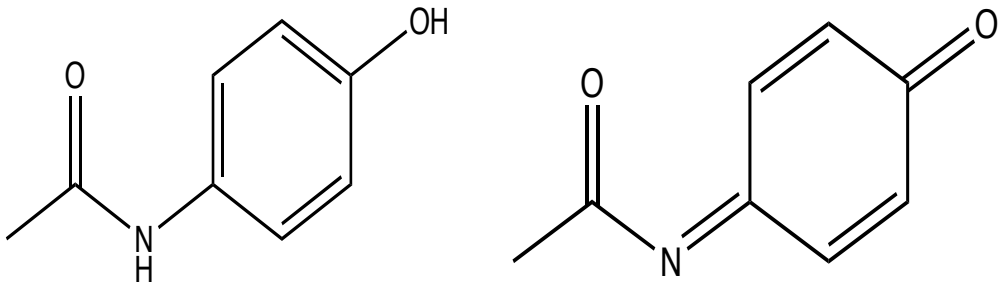
2. LITERATURE REVIEW

2.1. MECHANISM OF HEPATOTOXICITY INDUCED BY ACETAMINOPHEN OVERDOSE

2.1.1. Acetaminophen Mechanism of Action. Acetaminophen (APAP) is one of the world's most commonly used analgesic antipyretic medications. It has a unique activity that inhibits cyclooxygenase 3 (COX-3) isoform, the cyclooxygenase enzyme that produces prostaglandins that are responsible for pain, fever, and inflammation. COX-1 and COX-2 are the targets of non-steroidal anti-inflammatory drugs (NSAIDs). COX-1 is expressed in most tissues and plays an essential role in maintaining the integrity of the stomach mucosal lining, while COX-2 expression is induced by inflammation. Selective NSAIDs for COX-2 have been developed to avoid the development of ulcers by some non-selective NSAIDs, including aspirin. COX-3, a COX enzyme isoform encoded by the COX-1 gene, contains additional 30-34 amino acids, making COX-3 sensitive to selective inhibition by analgesic antipyretic medication. It has weak anti-inflammatory activity and is expressed selectively in the brain[9, 10]. The nuclear receptor, the constitutive androstane receptor (CAR) that is activated by APAP, induces the expression of three cytochrome P_{450} enzymes that play a role in APAP metabolism and toxicity[11].

2.1.2. Acetaminophen Mechanism of Toxicity. Drug-induced hepatotoxicity is one of the major causes of the withdrawal of new drugs from the market[12]. The liver is the target organ for APAP toxicity because this is where it is detoxified. At therapeutic doses, APAP is safe, with around 90-95 % of the APAP being glucuronidated or sulfated in the liver and then excreted[13]. The remaining 5-10% are metabolized by cytochromes P_{450} (CYP450) (including CYP1A2, CYP3A4, and mainly CYP2E1) to the electrophilic

intermediate N-acetyl-p-benzoquinoneimine (NAPQI) [13] (Figure 2.1). NAPQI is toxic, but it can be neutralized by conjugation with glutathione (GSH) to form a GSH- adduct which is mainly excreted into the bile[14]. However, after an overdose of acetaminophen, the formation of NAPQI exceeds the detoxification capacity of GSH, which results in covalent binding, particularly the sulfhydryl group on cysteine of the cellular proteins[15, 16](Figures 2.2 and 2.3). The subsequent mitochondrial dysfunction leads to the inhibition of mitochondrial respiration, ATP depletion, and formation of reactive oxygen and peroxynitrite inside the mitochondria[15]. Oxidant stress is involved in the activation of the c-jun-N-terminal kinase (JNK) pathway. It eventually triggers the opening of the mitochondrial membrane permeability transition (MPT) pore, resulting in collapse of the mitochondrial membrane potential[15]. Furthermore, fragmentation of DNA has been observed, preventing cell recovery and regeneration, contributing to necrotic cell death in acetaminophen toxicity. [14,17, 18].



Acetaminophen (APAP) N-acetyl-p-benzoquinoneimine (NAPQI)

Figure 2. 1. Structures of Acetaminophen and its Active Metabolite NAPQI

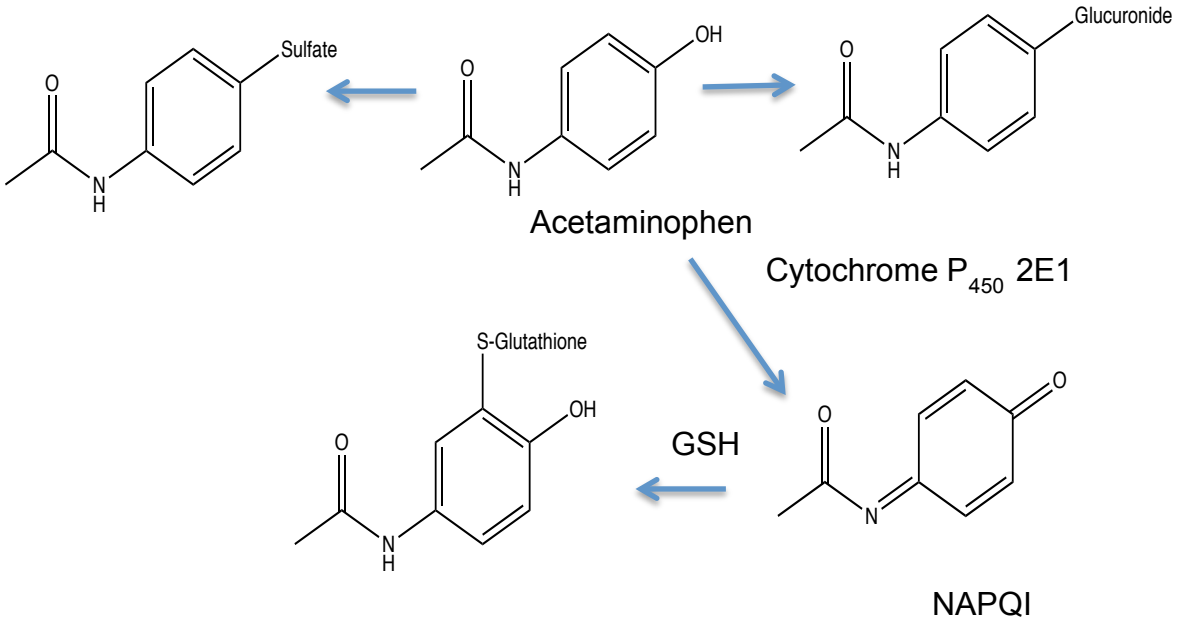


Figure 2.2. Metabolism of Acetaminophen

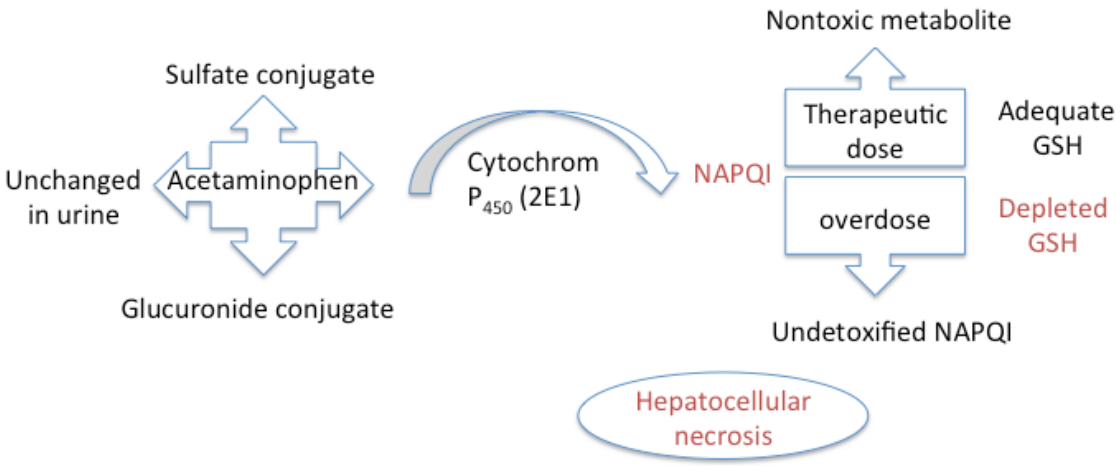


Figure 2.3. Mechanism of Toxicity

2.2. GLUTATHIONE

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[19] (Figure 2.4). Intracellularly >98% of the tripeptide is kept in its reduced state by glutathione reductase (GR) enzyme with the remainder as glutathione disulfide and glutathione conjugates (GS-R). 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 with them both enzymatically and nonenzymatically [19]. GSH plays a major role in APAP detoxification since it spontaneously reacts with the active toxic metabolite of the acetaminophen (NAPQI) or is catalyzed by glutathione S transferase (GST) [19]. An APAP overdose causes severe GSH depletion in the liver, allowing NAPQI to attach to protein (mainly mitochondrial protein) leading to oxidative stress, which ends in hepatocyte death.

2.3. OXIDATIVE STRESS

Oxidative stress**,** which takes place when the balance between antioxidants and free radicals is broken, is generally induced by drugs and environmental toxins[19]. In the case of APAP toxicity, there is a severe depletion in GSH, functional deterioration, and reactive oxygen formation[20]. Oxidative stress is known to be involved in the propagation of cell injury induced by APAP toxicity[20].

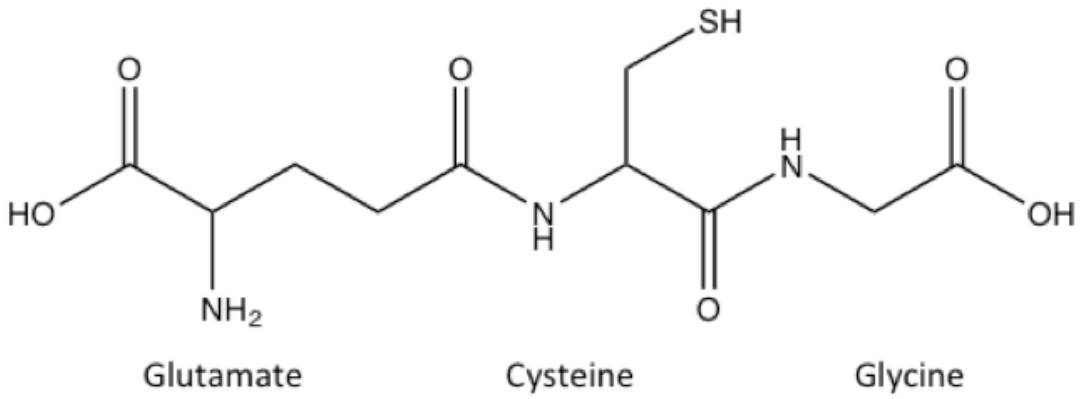


Figure 2.4. Glutathione Structure

2.4. N-ACETYLCYSTEINE

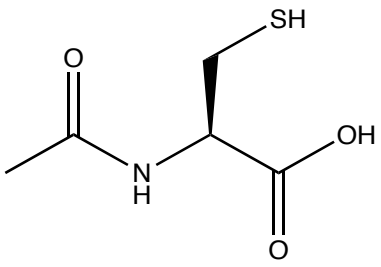
N-acetylcysteine (NAC) is the drug of choice to treat APAP poisoning[20] (Figure 2.5). The use of NAC for the treatment of APAP poisoning originated in England in the 1970's[21]. NAC functions to replenish glutathione (GSH) stores, the body's natural defense against endogenously generated reactive oxygen species and toxic acetaminophen metabolite (NAPQI), by providing an important GSH precursor, cysteine via the acetylation in the liver[12]. Although, NAC should be given as early as possible, it may still be of value 48 hours or more after ingestion. It may be given orally (140 mg/kg by mouth or nasogastric tube diluted to 5% solution, followed by 70 mg//kg by mouth every 4 hours for 17 doses)[22]. NAC has few side effects (occasional nausea, vomiting, rare urticaria, or bronchospasm), and may also be administered intravenously (loading dose is 150 mg/kg in 5% dextrose over 15 minutes; maintenance dose is 50 mg/kg, given over 4 hours, followed by 100 mg/kg administered over 16 hours)[22]. Allergic reactions may be successfully treated by discontinuing the NAC administration, antihistamines, and epinephrine for bronchspasm.[21, 22] The United States Food and Drug Administration (FDA) approved it for oral administration in 1985 and intravenous administration in 2004[23]. APAP levels provide the basis for determining the need to initiate or continue treatment with NAC. These levels should be plotted in a nomogram and measured within 4 hours, or as soon as possible[24]. Levels obtained before 4 hours cannot be plotted in the nomogram[24] (Figure 2.6). 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, making its passage through the biological membrane difficult[8].

2.5. N-ACETYLCYSTEINE AMIDE

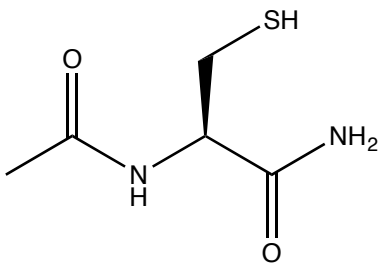
N-acetylcysteine amide (NACA) is a modified form of NAC that has an amide group instead of a carboxyl group of NAC to improve the cell membrane's permeability[8] (Figure 2.5). It hydrolyzes to give cysteine, a precursor for GSH. NACA has been shown to overcome the drawbacks of NAC[8] and has many advantages over it. NACA is more membrane permeable than NAC, and owing to its neutral charge at physiological pH, it acts as a carrier of NAC. It is effective at a lower concentration, which eliminates the pro-oxidant effects of NAC that occur at higher concentrations[8].

The antioxidant and free radical scavenging abilities of NACA are equal to or are an improvement over those of NAC. NACA has higher membrane permeability and is, therefore, effective at a lower concentration

A large number of studies have been undertaken to evaluate the effectiveness of NACA as an antioxidant and free radical scavenger[30-33]. In this study, we compared the effect of NACA to NAC, the only approved antidote for APAP toxicity.



N-acetylcysteine (NAC)



N-acetylcysteine amide (NACA)

Figure 2.5. N-Acetylcysteine and N-Acetylcystine Amide Structures

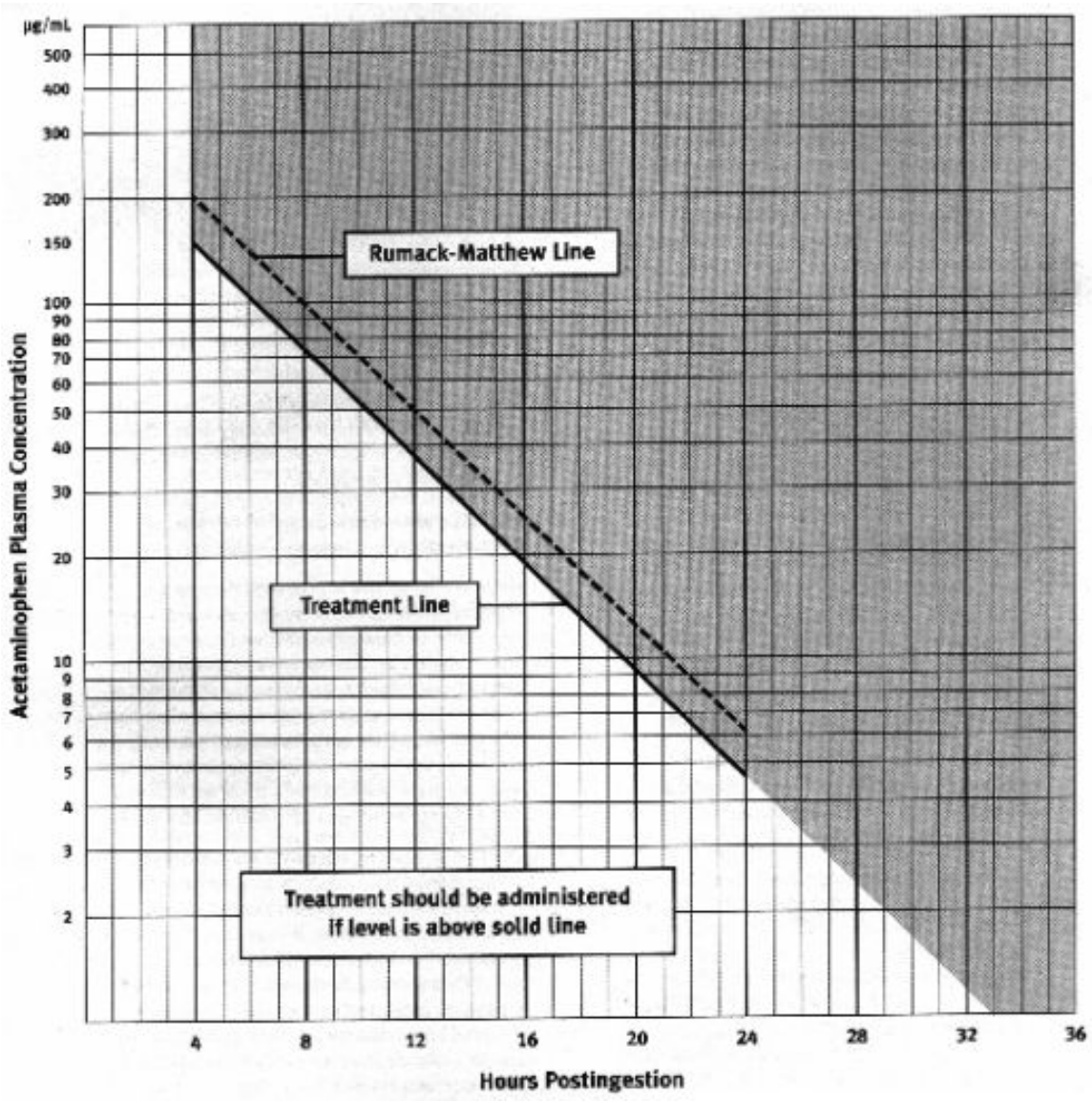


Figure 2.6. Acetaminophen Nomogram

http://labmed.ucsf.edu/labmanual/db/data/tests/5.html

3. EXPERIMENTATION

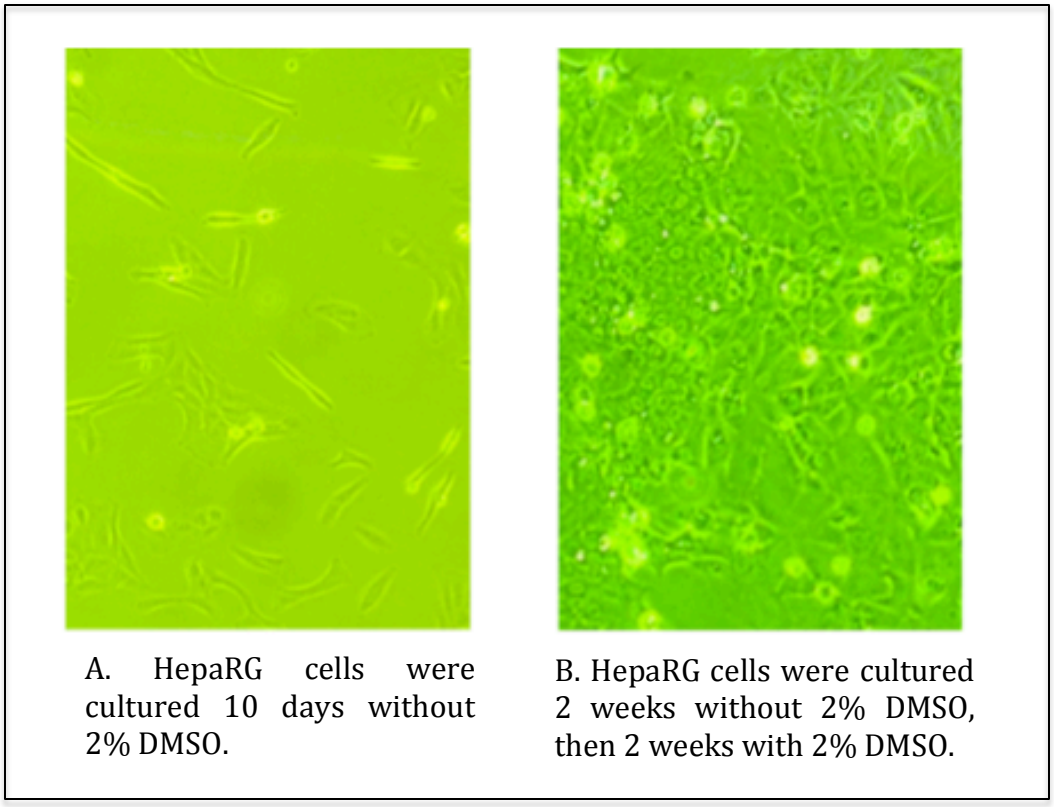
3.1. CHEMICALS

High performance liquid chromatography (HPLC) grade solvents were purchased from Fisher Scientific. A lactate dehydrogenase assay kit was purchased from Promega. All other chemicals were purchased from Sigma Aldrich.

3.2. CELL CULTURE

The HepaRG liver cell line, which was isolated and cultured from a hepatoma in a female patient with cirrhosis, subsequent to hepatitis C virus infection[29], was used and obtained from Invitrogen (Figure 3.1). When seeded at low density, these cells had undifferentiated morphology and actively divided. After the flask was full, typical hepatocyte-like colonies formed and were surrounded by biliary epithelial-like cells[30.] HepaRG cells expressed various cytochrome P_{450} subtype enzymes (1A2, 2B6, 2C9, 2E1, and 3A4), nuclear receptors as constitutive androstane receptors (CAR) and, others[31]. The HepaRG cells were seeded in a 75 cm^2 flask with a full medium consisting of William's E medium supplemented by 10%FCS, 100U penicillin, 100ug/ml streptomycin, 5ug/ml insulin, and hydrocortisone, until the flask was full. Then a differentiated medium induced differentiation of the hepatocyte-like cells into more granular cells closely resembling the primary hepatocyte (2% DMSO to the full medium). The medium was renewed every other day for two more weeks. After that, the medium was switched to a DMSO-free medium for one day, and the cells were ready for the experiment[32, 33]. HepaRG cells are more differentiated than any other hepatic cell line because they are

derived from hepatocarcinoma (HepG2 from hepatoblastoma), they also show limited chromosomal rearrangement, and they recover the character of able to differentiate in both hepatocyte and biliary epithelial cells, when seeded at low density[34].



A. HepaRG cells were cultured 10 days without 2% DMSO.

B. HepaRG cells were cultured 2 weeks without 2% DMSO, then 2 weeks with 2% DMSO.

Figure 3. 1. HepaRG Morphology

3.3. CALCEIN AM ASSAY

The Calcein AM Assay determined cell viability by seeding the cells in 96-well tissue culture plates in which the Calcein AM is a non-fluorescent, hydrophobic compound that easily permeated intact live cells. The hydrolysis of Calcein AM by intracellular esterases produced calcein, a hydrophilic, strongly fluorescent compound that was well retained in the cell cytoplasm. The fluorescence on the fluorescence plate reader was measured with an excitation wavelength at 485 nm and an emission wavelength of 530 nm. The fluorescent signal generated from the assay was proportional to the number of living cells.

To determine APAP toxicity, after seeding HepaRG in the 96-well plates for 24 hours, cells were exposed to different concentrations of acetaminophen (5,10,15, 20, and 25 mM) for different time periods (1, 3, 6,12, 24, and 48 hours) and the cytotoxicity was quantified by the Calcein AM Assay.

For NACA and NAC toxicity studies, HepaRG cells were incubated with different concentrations of NACA and NAC (0.5, 0.75, 1, 1.5, 5, and 10 mM) for 24 hours. The cytotoxicity was quantified by the Calcein AM Assay.

In order to determine the concentrations of NAC and NACA, that provide antidotes for toxicity of APAP, HepaRG cells were exposed to different concentrations of NAC and NACA (0.25, 0.5, 0.75, and 1 mM) and then the cells were dosed with 20mM APAP for 24 hours to induce the toxicity.

3.4. REACTIVE OXYGEN SPECIES (ROS) MEASUREMENT

The cellular ROS accumulation was measured using 2', 7'-dichlorofluorescin diacetate (DCHF-DA). DCHF-DA is a nonfluorescent compound that can be enzymatically converted to the highly fluorescent compound, DCF, in the presence of ROS. HepaRG cells were seeded in a 96-well plate at a density of 1 $x10^4$ /well. After 24 hours, they were treated with 40 uM of DCHF-DA in a dark environment for 1 hour. Then, they were treated with 20mM APAP and the DCF fluorescence was measured at 485 nm excitation and 520 nm emissions during several time periods (1, 3, 6, 12, and 24 hours). To compare the protective effect of NAC and NACA against ROS generation, we followed the same procedure except that the HepaRG cells were pre-dosed with 0.25 mM NAC or NACA for 2 hours, and then with 20mM APAP for 12 hours.

3.5. GLUTATHIONE MEASUREMENT

Cellular levels of glutathione (GSH) were determined by using the method developed in our laboratory. Cells were seeded in 75 cm^2 flasks to measure the GSH. The flasks were incubated for 2 hours with NAC or NACA (0.25mM) in a serum-free medium, then in a fresh medium containing APAP (20mM). After the incubation period, cells were removed from the cultures and homogenized in a serine borate buffer (100 mM Tris-HCl, 10 mM boric acid, 5 mM L-serine, 1 mM DETAPAC, pH 7.5). Fifty microliters of the diluted cell homogenate were added to 200 microliters of HPLC water and 750 microliters of NPM (1 mM in acetonitrile). NPM reacted with free sulfhydryl groups to form fluorescent derivatives which yielded fluorescent adducts that could be detected fluorimetrically (A-ex = 330 nm, A-em= 376 nm). After incubation at room

temperature for 5 minutes, the samples were then acidified with 10 microliters of 2N HCl to stop the reaction. The derivatized samples were filtered through a 0.45 microliter acrodisc and then injected onto a HPLC column.

3.6. GLUTATHIONE DISULFIDE MEASUREMENT

Cellular levels of glutathione disulfide (GSSG) were determined by using the same method used for measuring GSH. First, total GSH was measured after converting GSSG to GSH. Then, free GSH level was subtracted from the total level and divided by two to determine the GSSG level. Fifty microliters of the same diluted cell homogenate, was used to measure the GSH level. Ninety-five microliters of 2 mg/ml of NADPH in nanopure water and 5 microliters of 2-units/ml-glutathione reductase were added to reduce GSSG. An aliquot with 100 microliters of treated samples and 150 microliters of water was immediately derivatized with 750 microliters of 1.0 mM NPM. NPM reacts with free sulfhydryl groups to form fluorescent derivatives which yield fluorescent adducts that can be detected fluorimetrically and represent the total GSH level. After incubation at room temperature for 5 minutes, the samples were then acidified with 10 microliters of 2N HCl to stop the reaction. The derivatized samples were filtered through a 0.45 microliter acrodisc and then injected onto an HPLC column. The GSH level was subtracted from the total GSH, and then divided by 2 to determine the GSSG level.

The HPLC system consisted of a Finnigan™ SpectraSYSTEM SCM1000 vacuum membrane degasser, a Finnigan™ SpectraSYSTEM P2000 gradient pump, a Finnigan™ SpectraSYSTEM AS3000 Autosampler, and a Finnigan™ SpectraSYSTEM FL3000 fluorescence detector (A-ex=330 nm and A-em=376 nm) (Thermo Electron Corp.,

Austin, TX, USA). The HPLC column (Astec, Whipany, NJ) was 100x4.6 mm I. D. and was packed with C_{18} packing material. Quantitation of the peaks from the HPLC system was performed with a Chromatopac Model CR 601 integrator (Shimadzu). The mobile phase was 30% water, 70% acetonitrile, 1ml/L acetic acid, and 1ml/L phosphoric acid. The NPM derivatives were eluted from the column isocratically at a flow rate of 1.0 ml/min.

3.7. PROTEIN DETERMINATION

Protein levels were determined by the Bradford method with Coomassie Blue (Bio-Rad). Concentrated Coomassie Blue (Bio-Rad) was diluted 1:5 (v/v) with distilled water. One milliliter of the diluted reagent was added to 25 microliters of the homogenized cells in a serine-borate buffer (pH 7.5). The mixture was incubated at room temperature for 5 minutes and the optical density was measured at 595 nm.

3.8. MALONDIALDEHYDE MEASUREMENT

To prepare the solution for malondialdehyde (MDA) measurement, 350 microliters of cell homogenate, 100 microliters of 500 ppm BHT (butylated hydroxytoluene), and 550 microliters of 10% TCA (trichloroacetic acid) were combined and boiled for 30 minutes. The tubes were cooled on ice and centrifuged for 5 min at 2500 RPM. Five hundred (500) microliters of the supernatant were removed and 500 microliters of TBA (thiobarbituric acid) were added. From this solution, 500 microliters were removed, added to 1.0 ml of n-butanol, vortexes, and centrifuged for 5 minutes at 1000 RPM to facilitate a phase separation. Pipette 200ul from the top layer was then

transferred to a 96-well plate for analysis. Fluorescence was then measured (ex. 510 nm and em. 590 nm).

3.9. LACTATE DEHYDROGENASE MEASUREMENT

Lactate dehydrogenase measurement (LDH) in the media determines the membrane integrity. We used the LDH assay kit (Promega), which quantitatively measures LDH, a stable cytosolic enzyme that is released upon cell lysis. The LDH released into the cultured medium was measured within 30 minutes by the coupled enzymatic assay that resulted in the conversion of tetrazolium salt into a red formazan product. The amount of color formed was proportional to the number of lysed cells. Visible wavelength absorbance data were collected using a 96-well plate reader. HepaRG cells were seeded in the 96-well plate with a density of 10^4 per well in four groups that were control; APAP (20mM); NAC or NACA (0.25mM) pretreatment, followed by APAP (20mM). After incubation for the desired test period, 50 microliter of the supernatant was transferred to another well and 50 microliter of the reconstituted Substrate Mix (provided in the kit) was added. The plate was covered (protected from light) and incubated at room temperature for 30 minutes. After the incubation, 50 microliter of the Stop Solution (provided in the kit) was added to each well of the plate and absorbance at 490 nm was recorded.

3.10. GLUTATHIONE REDUCTASE MEASUREMENT

To measure glutathione reductase (GR) activity**,** cell homogenates were diluted in 50 mM of phosphate buffer with 1 mM of EDTA (pH=7.8). 800 microliters of

homogenate, 40 microliters of GSSG, and 160 microliters of NADPH were taken. This method measured the exponentially decreasing level of NADPH at 340 nm in the presence of cell homogenates.

3.11. TOXICITY INDUCED BY N-ACETYL-P-BENZOQUINONE IMINE

HepaRG cells were seeded 10^4 cells per well into a 96-well plate in four groups that were control; NAPQI (250 microM); NAC or NACA (0.25mM) pretreatment, followed by NAPQI (250 micro M) [43]. Calcein AM Assay measured cell viability 24 hours after NAPQI treatment. NAPQI solubilized in 0.1% DMSO.

3.12. THE EFFECT OF N-ACETYL-P-BENZOQUINONE IMINE ON GLUTATHIONE LEVEL

The effect of NAPQI on GSH levels were assayed by measuring GSH level by using the same method explained in section 5. The solutions with various GSH final concentrations were prepared as 167, 333, 667, 1000, 1333 and 1667 mM. This group sited as a control. Then, 83 uM NAPQI was added in different set of test tubes that had the same concentrations of GSH used in the control group. They were incubated for 2 hours at room temperature. After the incubation period, they were derivatized by using 750 microliters of NPM (1 mM in acetonitrile). NPM reacted with free sulfhydryl groups to form fluorescent derivatives which yielded fluorescent adducts that could be detected fluorimetrically (A-ex = 330 nm, A-em= 3 76 nm). After incubation at room temperature for 5 minutes, the samples were then acidified with 10 microliters of 2N HCl to stop the reaction. The derivatized samples were filtered through a 0.45 microliter acrodisc and then injected onto a HPLC column to determine GSH levels.

3.13. STATISTICAL ANALYSIS

The data was given as the Mean ± SD. The one-way analysis of variance (ANOVA) and Tukey's multiple comparison tests were used to analyze the significance of the differences between the control and the experimental groups. Values of $p < 0.05$ were considered significant.

4. RESULTS

4.1. CYTOTOXICITY OF ACETAMINOPHEN, N-ACETYLCYSTEINE AND N ACETYLCYSTEINE AMIDE IN HepaRG CELL LINE

A Calcein AM Cell Viability Assay was used to assess the cytotoxicity of APAP, NAC, and NACA. The 24-hours APAP-induced toxicity in HepaRG cells was concentration dependent in the range of 5 mM to 25 mM. At 20 mM of APAP, cell viability was reduced to 60% of that in the control group (Figure 4.1). In this study, a dose of a 20 mM concentration of APAP induced toxicity as shown in the results and supported by previous studies.[11, 37] The APAP- induced toxicity was time-dependent in a range of 1- 48 hours (Figure 4.2). To assess the cytotoxicity caused by NACA and NAC, the HepaRG cells were incubated for 24 hours with different concentrations of NACA or NAC (0.5, 0.75, 1, 1.5, 5, and 10 mM). The NACA and NAC induced toxicity in HepaRG cells above 1mM concentration (Figure 4.3).

4.2. THE PROTECTIVE EFFECT OF N-ACETYLCYSTEINE AND N-ACETYLCYSTEINE AMIDE ON HEPATOTOXICITY INDUCED BY ACETAMINOPHEN

To study the protective effects of NAC and NACA on APAP-induced toxicity, HepaRG cells were pretreated for 2 hours with several concentrations of NAC or NACA (0.25, 0.5, 0.75, and 1mM), followed by incubation with 20mM APAP for 24 hours. The cell viability was then measured using the Calcein AM Assay. There was a significant increase in the cell viability in the 0.25 mM NAC or NACA pretreatment group (Figures 4.4 and 4.5). 0.25 mM of NACA or NAC, a non-toxic level, was chosen for subsequent experiments to study the protective effects in APAP-induced cytotoxicity (Figure 4.6).

4.3. GENERATION OF REACTIVE OXYGEN SPECIES IN ACETAMINOPHEN INDUCED CYTOTOXICITY AND THE EFFECT OF N-ACETYLCYSTEINE AND N-ACETYLCYSTEINE AMIDE

Reactive oxygen species (ROS) levels were significantly increased by time as measured by DCF fluorescence at several time points (1, 3, 6, 12, 24, and 48 hours) after treatment of HepaRG with 20mM APAP (Figure 4.7). In the 20 mM APAP treatment group, ROS was significantly increased over the control by 800% at 12 hours. The ROS level was reduced back to control level with 0.25 Mm of NAC, and 0.25mM of NACA reduced it even more (Figure 4.8).

4.4. GLUATHIONE, GLUTATHIONE DISULFIDE AND GLUATHIONE TO GLUTATHIONE DISULFIDE RATIO IN ACETAMINOPHEN TOXICITY AND THE PROTECTIVE EFFECT OF N-ACETYLCYSTEINE AND N ACETYLCYSTEINE AMIDE

Glutathione (GSH) level was significantly decreased after treatment of HepaRG cells with 20mM APAP for 12 hours to 48% of the control. NAC and NACA pre-treatment restored the level of GSH to 55% and 76% of the control respectively (Figure 4.9). GSH level was significantly decreased after 24 hours treatment to 14% of the control. NAC and NACA pre-treatment restored the level of GSH by 19% and 22% of the control, respectively (Figure 4.10).

There is no significant difference in oxidized glutathione (GSSG) levels after treatment of HepaRG with 20mM APAP for 12 hours, but there is a significant difference after 24 hours of treatment (Figure 4.11). The GSSG levels increased to 420% of the control after 24 hours treatment. NAC and NACA pre-treatment decreased the levels of GSSG to 289% and 204% of the control, respectively (Figure 4.12).

The GSH/GSSG ratio was significantly decreased to 37% of the control in the 20mM APAP treatment group for 12 hours. NAC pre-treatment increased this ratio to 65% of the control, while NACA increased it to the nearly control levels (Figure 4.13).

4.5. GLUTATHIONE REDUCTASE ACTIVITY IN ACETAMINOPHEN TOXICITY AND THE PROTECTIVE EFFECT OF N-ACETYLCYSTEINE AND N-ACETYLCYSTEINE AMIDE

The enzymatic activity of GR was not significantly reduced in the APAP treatment group at 12 hours (Figure 4.14), but it was significantly reduced to 28% of the control at 24 hours. NAC and NACA pre-treatment significantly restored the activity of GR by 57% and 70% of the control respectively (Figure 4.15).

4.6. LIPID PEROXIDATION IN ACETAMINOPHEN TOXICITY AND THE EFFECT OF N-ACETYLCYSTEINE AND N-ACETYLCYSTEINE AMIDE

Malondialdehyde (MDA) is the end products of lipid peroxidation. The MDA in the sample is reacted with thiobarbituric acid (TBA) to generate the MDA-TBA adduct. The MDA-TBA adduct can be easily quantified fluorometrically. MDA was not significantly increased in the APAP treatment group at 12 hours (data not shown), but it was significantly increased to 592% of the control at 24 hours. NAC and NACA pre-treatment reduced the MDA levels to 302% and 263% of the control respectively, these reductions were statistically significant (Figure 4.16).

4.7. PROTECTIVE EFFECT OF N-ACETYLCYSTEINE AND N-ACETYLCYSTEINE AMIDE ON ACETAMINOPHEN-INDUCED CELL NECROSIS

Cell death was assessed by the extent of lactate dehydrogenase (LDH) release in the culture medium. LDH increased significantly in the APAP treatment group to 265% of the control in a 12 hours treatment time and to 370% of the control in a 24 hours treatment time. NAC decreased LDH to 237% of the control whereas NACA decreased LDH to 194% of the control in a 12 hours treatment time. The LDH levels were decreased to 349% of control in NAC pre-treatment group and 330% of the control in the NACA pre-treatment group for 24 hours treatment time (Figure 4.17 and 4.18).

4.8. PROTECTIVE EFFECT OF N-ACETYLCYSTEINE AND N ACETYLCYSTEINE AMIDE ON TOXICITY INDUCED BY N-ACETYL-P BENZOQUINONE IMINE

Cell viability was significantly reduced in NAPQI treatment group by 52% of the control. NAC pre-treatment increased it by 58% of the control while NACA significantly increased it by 73% of the control (Figure 4.19).

4.9. THE EFFECT OF N-ACETYL-P-BENZOQUINONE IMINE ON GLUTATHIONE LEVELS

In order to determine whether APAP-induced oxidative stress is due to depletion of GSH by NAPQI, known concentrations of GSH were incubated with NAPQI in a cell-free environment for 2 hours at room temperature. Then, the samples were derivatized with NPM to measure GSH levels in the absence and presence of NAPQI. Areas under the GSH peaks were tabulated (Table4.1). The GSH peaks of the first four concentrations (167, 333, 667, 1000 mM) could not be detected, which indicated that the GSH in these

concentrations was possibly bound to NAPQI by the –SH functional group in its cysteine residue. However, NAPQI was not able to block free sulfhydryls in the last two higher concentrations of GSH (1333 and 1667 mM) (Table4.1).

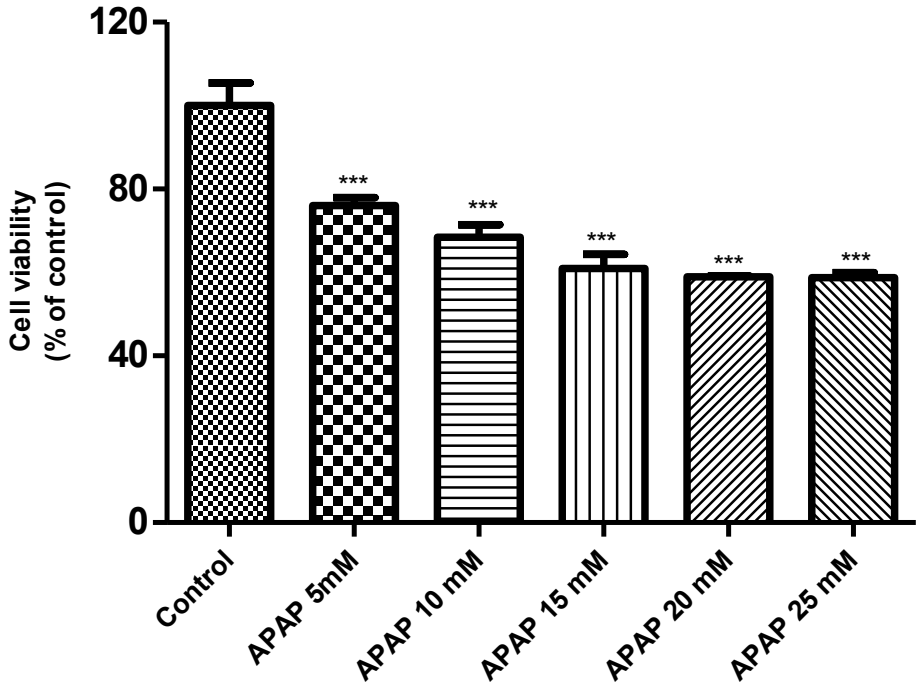


Figure 4.1. Cytotoxicity: Dose Dependence Response of Acetaminophen. HepaRG cells were treated with various concentrations of APAP (5, 10, 15, 20,and 25 mM) for 24 hours. The cell viability was measured by the Calcein AM Assay. The results represent the average of three experiments. * $p < 0.05$ compared to control.

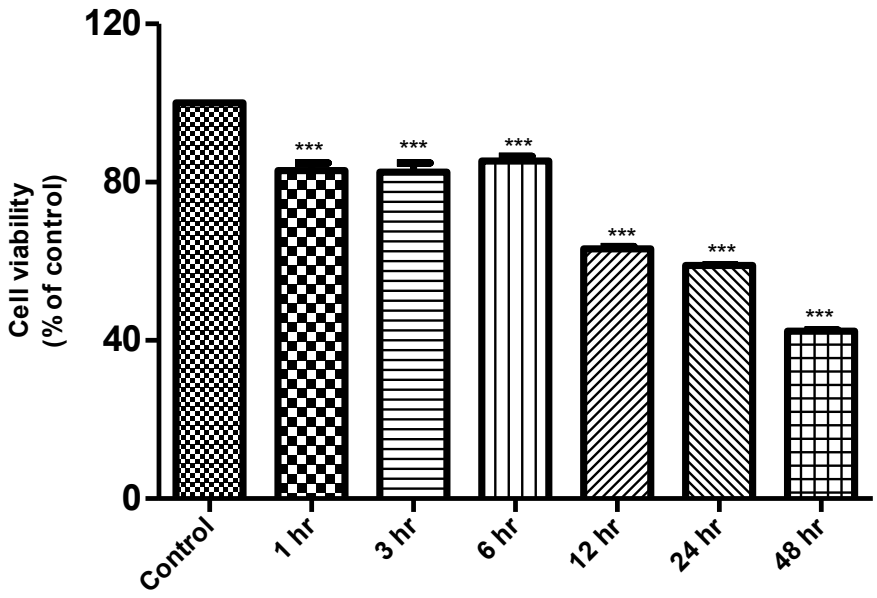


Figure 4.2. Cytotoxicity: Time Dependence Response of Acetaminophen. HepaRG cells were treated with 20 mM of APAP at various time points 1, 3, 6, 12, 24, and 48 hours. The cell viability was measured by the Calcein AM Assay. The results represent the average of three experiments. * $p < 0.05$ compared to control.

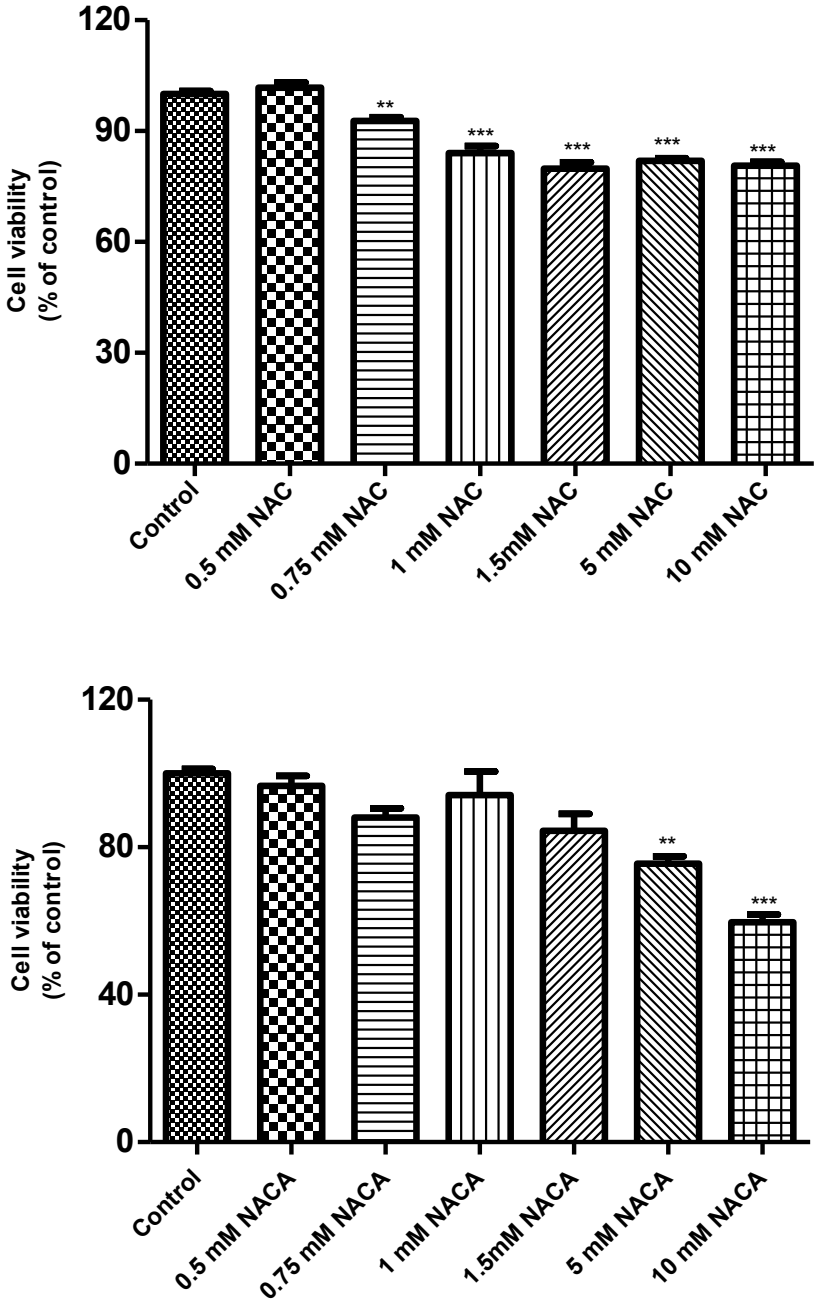


Figure 4.3. Cytotoxicity of NAC and NACA. HepaRG cells were treated with various concentrations of NAC or NACA (0.5, 0.75, 1, 1.5, 5, and 10 mM) for 24 hours. The cell viability was measured by the Calcein AM Assay. The results represent the average of three experiments. * $p < 0.05$ compared to control.

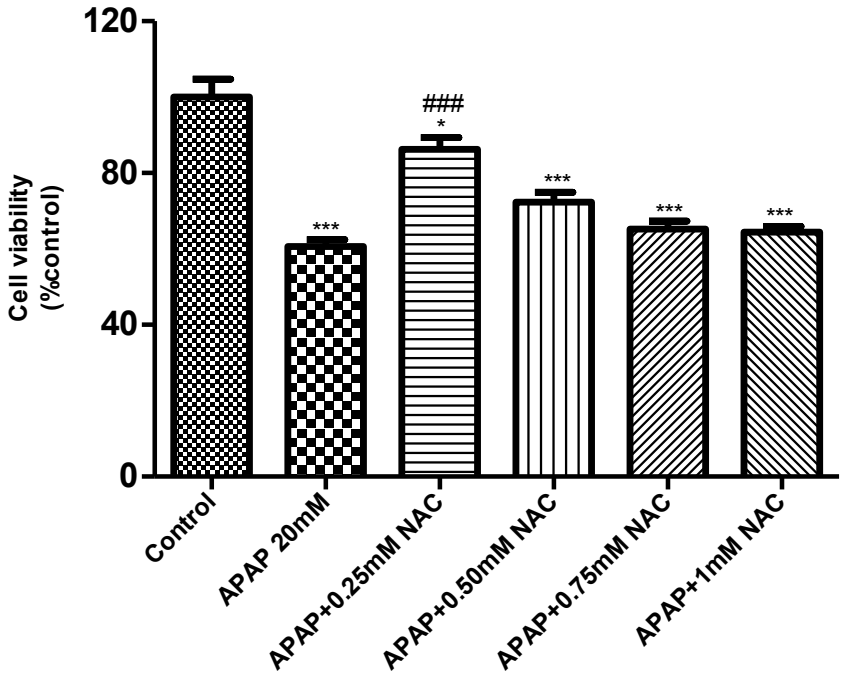


Figure 4.4. Protective Effect of NAC. HepaRG cells were pretreated with various concentrations of NAC (0.25, 0.5, 0.75 and 1mM) for 2 hours, followed by APAP for 24 hours. The cell viability was measured by the Calcein AM Assay. The results represent the average of three experiments.* $p < 0.05$ compared to control and # $p < 0.05$ compared to APAP treated group.

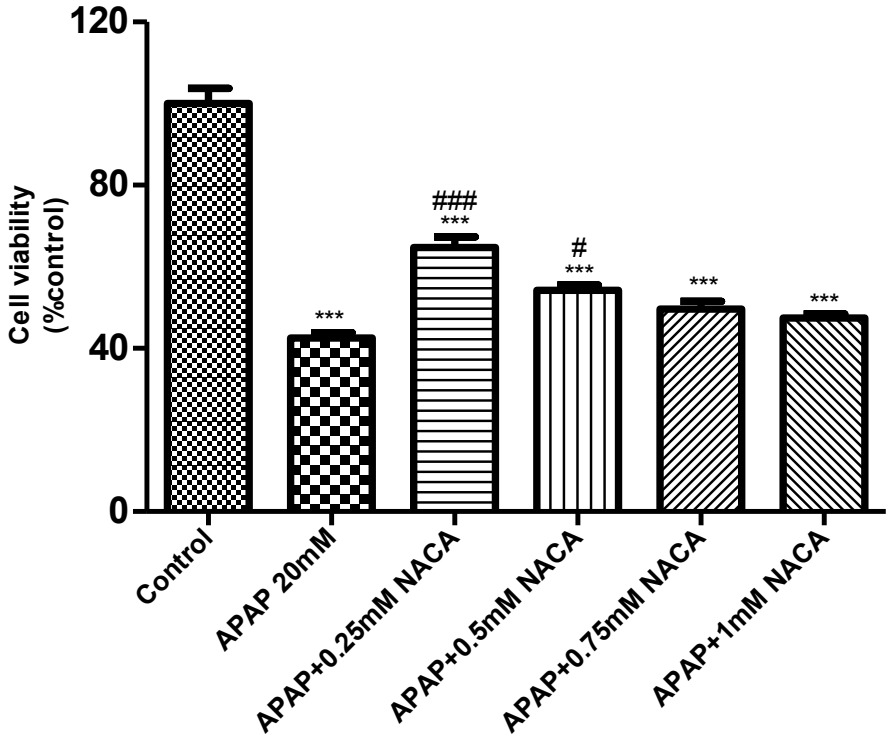


Figure 4.5. Protective Effect of NACA. HepaRG cells were pretreated with various concentrations of NACA (0.25, 0.5, 0.75 and 1mM) for 2 hours, followed by APAP for 24 hours. The cell viability was measured by the Calcein AM Assay. The results represent the average of three experiments.*$p < 0.05$ compared to control and # $p < 0.05$ compared to APAP treated group.

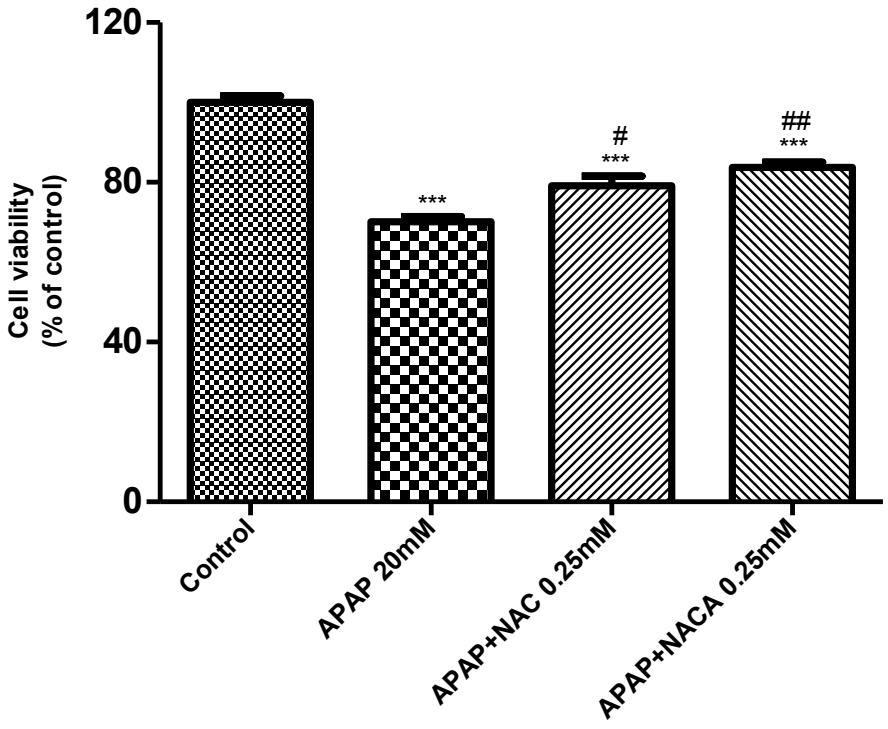


Figure 4.6. Protective Effect of NAC and NACA. HepaRG cells were pretreated with 0.25 mM of NAC or NACA for 2 hours followed by APAP for 12 hours. The cell viability was measured by the Calcein AM Assay. The results represent the average of three experiments.* $p < 0.05$ compared to control and # $p < 0.05$ compared to APAP treated group.

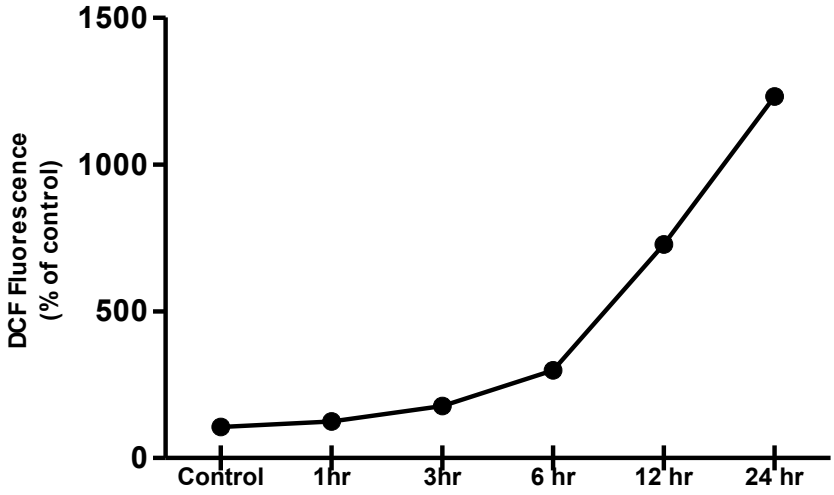


Figure 4.7. ROS Generation in APAP-Induced Cytotoxicity Over Time. The ROS generation was measured by DCF fluorescence at several time points (1, 3, 6,12, 24, and 48 hours) after treating HepaRG with 20mM of APAP. The results represent the average of three experiments.

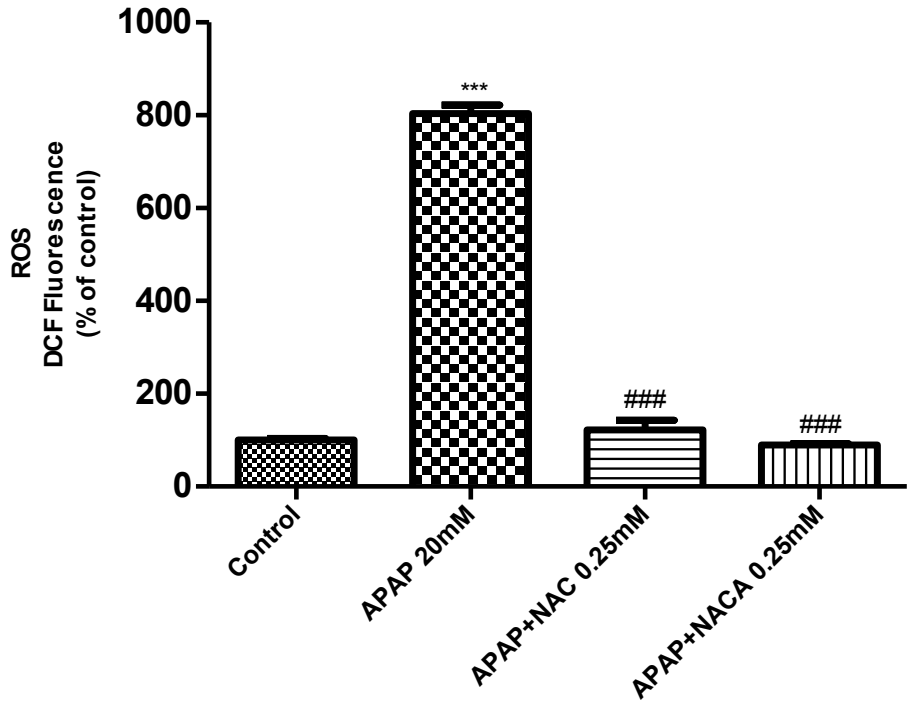


Figure 4.8. Protective Effect of NAC and NACA in ROS Generation. HepaRG cells were pretreated with 0.25mM of NAC or NACA for 2 hours followed by APAP for 12 hours. The ROS was measured by DCF fluorescence. The results represent the average of three experiments. * $p < 0.05$ compared to control and # $p < 0.05$ compared to APAP treated group.

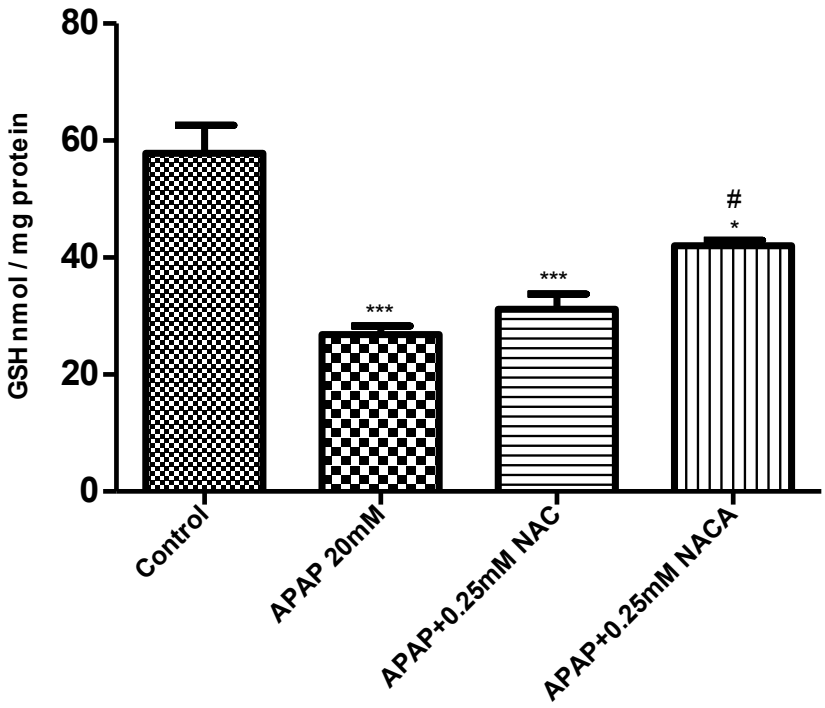


Figure 4.9. GSH Levels After APAP Overdose and The Protective Effect of NAC and NACA (12 hours). HepaRG cells were pretreated with 0.25mM of NAC or NACA for 2 hours followed by APAP for 12 hours. The GSH levels were then measured. The results represent the average of three experiments. * $p < 0.05$ compared to control and # $p < 0.05$ compared to APAP treated group.

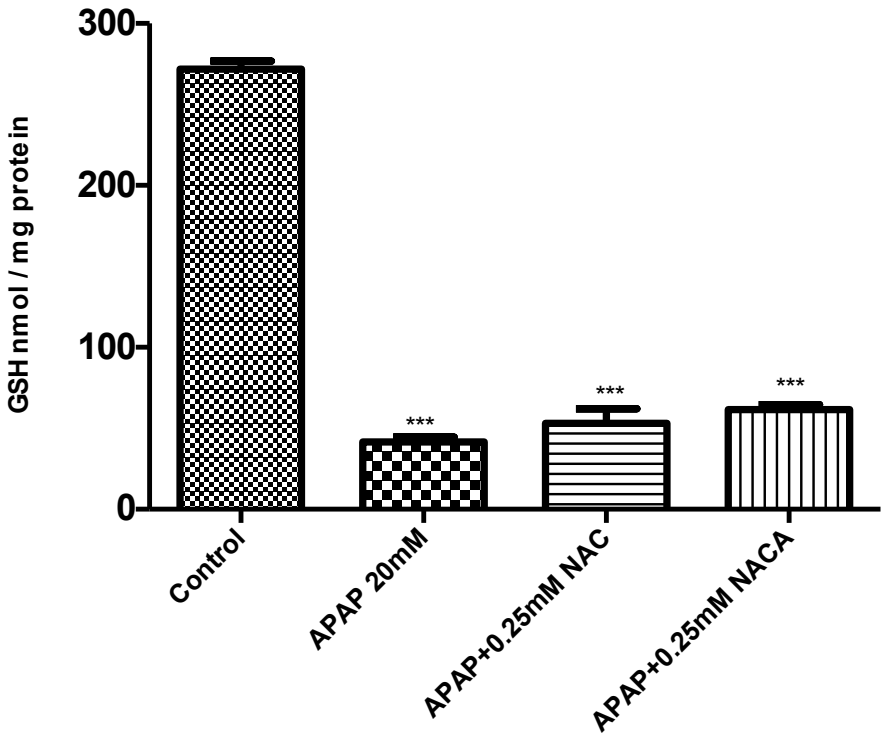


Figure 4.10. GSH Levels After APAP Overdose and the Protective Effect of NAC and NACA (24 hours). HepaRG cells were pretreated with 0.25mM of NAC or NACA for 2 hours followed by APAP for 24 hours. The GSH was measured. The results represent the average of three experiments. * $p < 0.05$ compared to control.

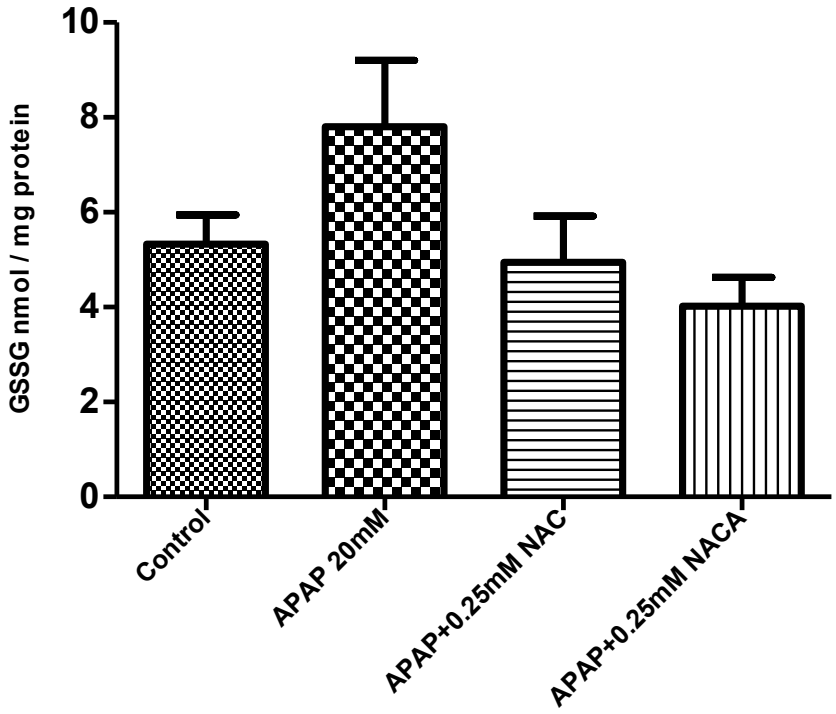


Figure 4.11. GSSG Levels After APAP Overdose and The Protective Effect of NAC and NACA (12 hours). HepaRG cells were pretreated with 0.25mM of NAC or NACA for 2 hours followed by APAP for 12 hours. The GSSG levels were measured. The results represent the average of three experiments. There were no significant differences among groups.

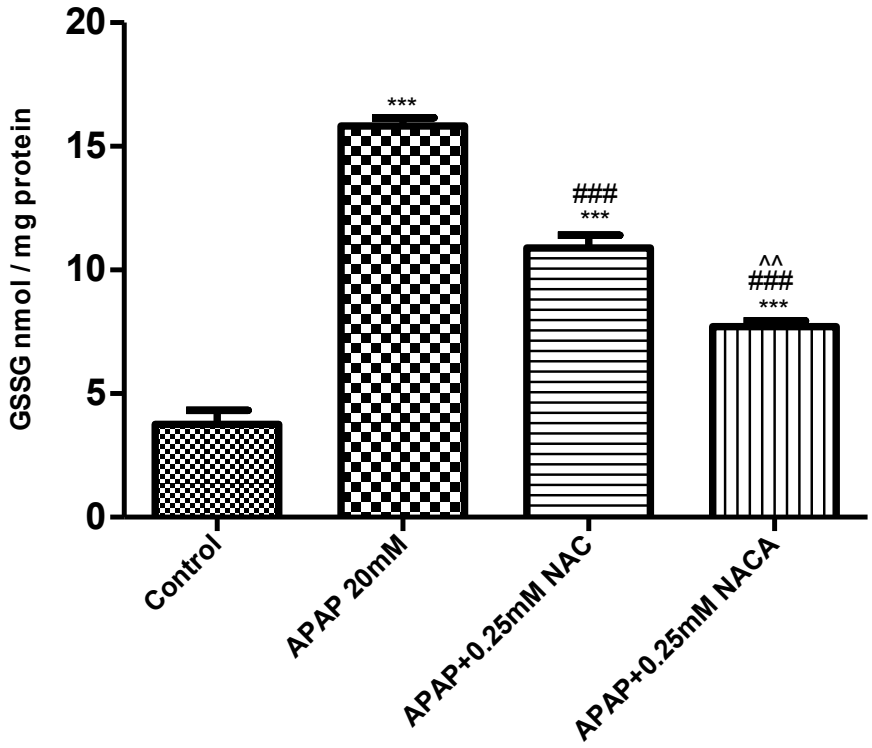


Figure 4.12. GSSG Levels After APAP Overdose and the Protective Effect of NAC and NACA (24 hours). HepaRG cells were pretreated with 0.25mM of NAC or NACA for 2 hours followed by APAP for 24 hours. The GSSG was measured. The results represent the average of three experiments. * $p < 0.05$ compared to control, # $p < 0.05$ compared to APAP treated group and ^ $P < 0.05$ compared with APAP + 0.25 mM NAC.

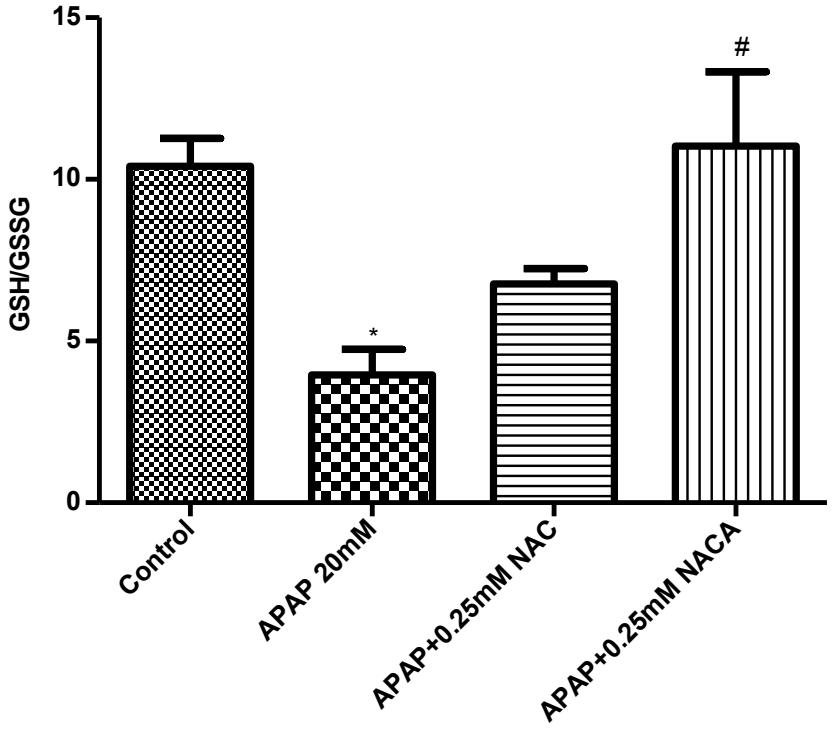


Figure 4.13. GSH/GSSG After APAP Overdose and Protective Effect of NAC and NACA (12 hours). HepaRG cells were pretreated with 0.25mM of NAC or NACA for 2 hours followed by APAP for 12 hours. The GSH/GSSG ratios were calculated by using GSH and GSSG results shown in Figures 4. 9. and 4.10. The results represent the average of three measurements. *$p < 0.05$ compared to control and # $p < 0.05$ compared to APAP treated group.

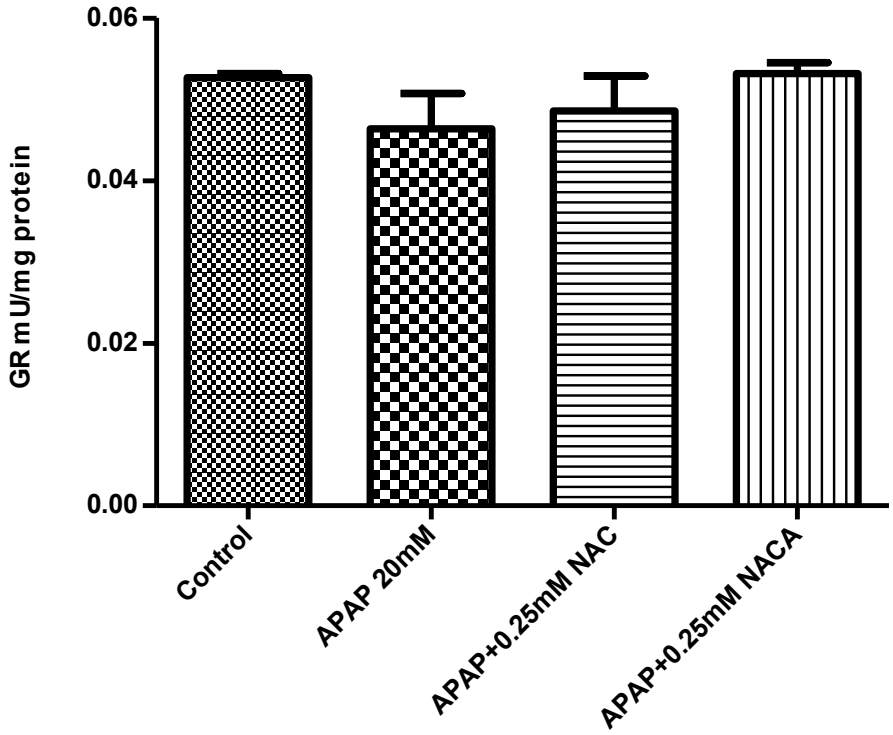


Figure 4.14. Glutathione Reductase (12 hours). HepaRG cells were pretreated with 0.25 mM of NAC or NACA for 2 hours followed by APAP for 12 hours. The GR was measured and the results indicated that there were no statistical significances among groups. The results represent at least the average of three experiments.

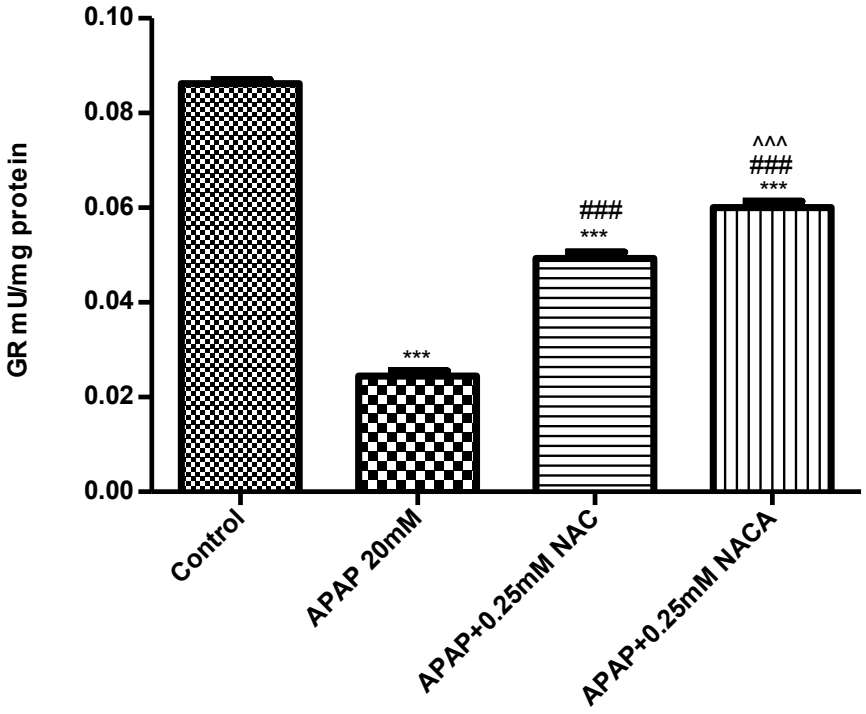


Figure 4.15. Glutathione Reductase Level After Acetaminophen Toxicity and the Protective Effect of NAC and NACA (24 hours). HepaRG cells were pretreated with 0.25 mM of NAC or NACA for 2 hours followed by APAP for 24 hours. The GR was measured. The results represent the average of three experiments. *$p < 0.05$ compared to control, # $p < 0.05$ compared to APAP treated group and ^ $p < 0.05$ compared with APAP + 0.25 mM NAC.

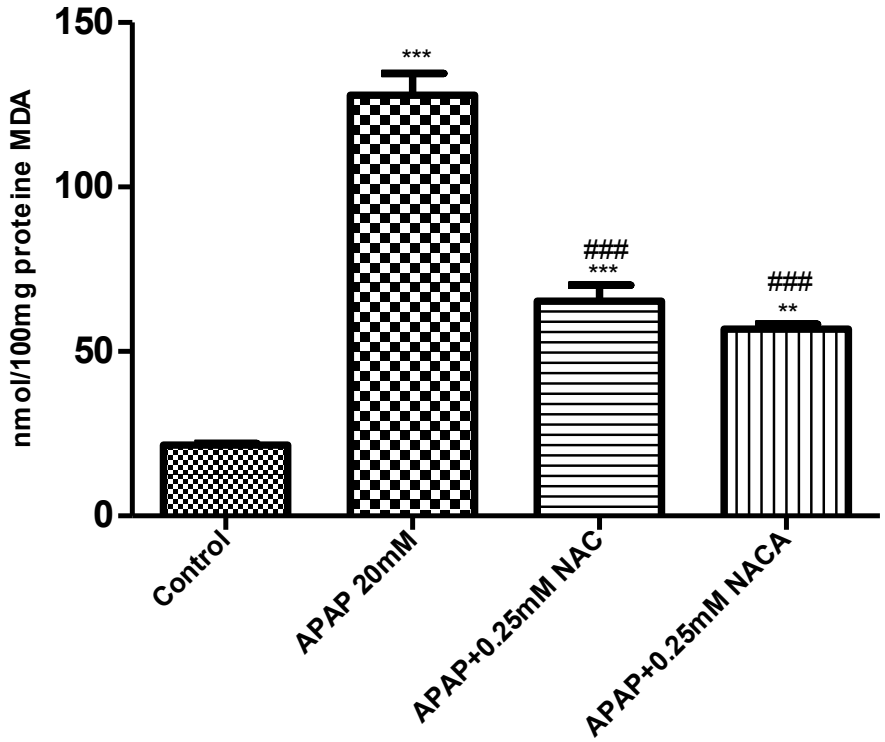


Figure 4.16. Malondialdehyde Level After Acetaminophen Toxicity and The Protective Effect of NAC and NACA. HepaRG cells were pretreated with 0.25 mM of NAC or NACA for 2 hours followed by APAP for 24 hours. The MDA was measured. The results represent the average of three experiments. *$p < 0.05$ compared to control, # $p < 0.05$ compared to APAP treated group and ^ $p < 0.05$ compared with APAP + 0.25 mM NAC.

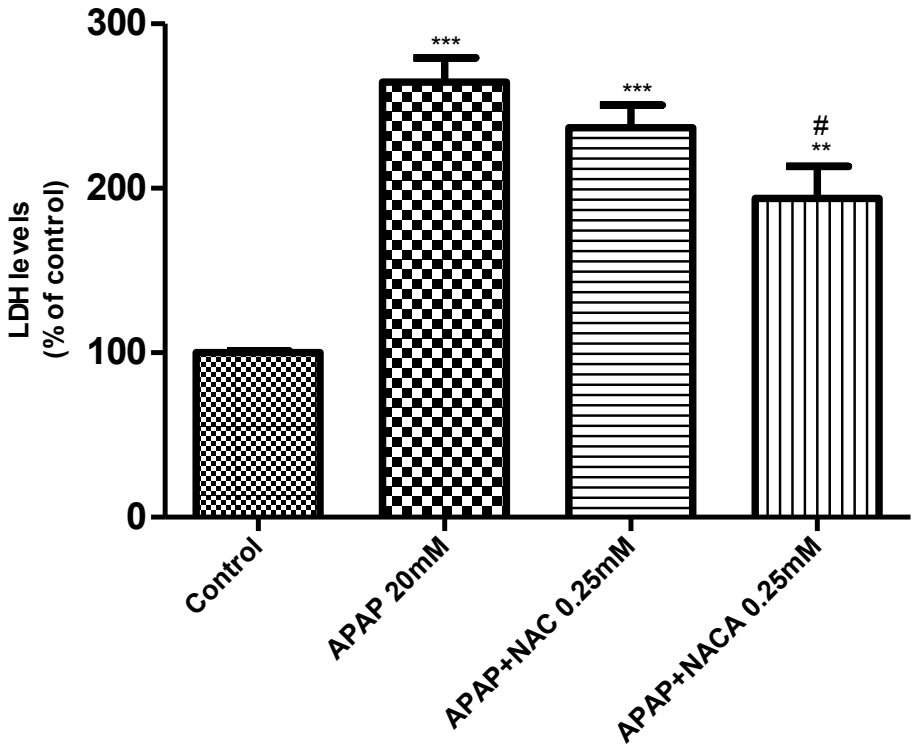


Figure 4.17. Lactate Dehydrogenase (LDH) Release in The Culture Medium and the Protective Effect of NAC and NACA (12 hours). LDH levels were measured after HepaRG cells were pretreated with 0.25mM of NAC or NACA for 2 hours followed by APAP for 12 hours. The results represent the average of three experiments. * $p< 0.05$ compared to control, # $p < 0.05$ compared to APAP treated group.

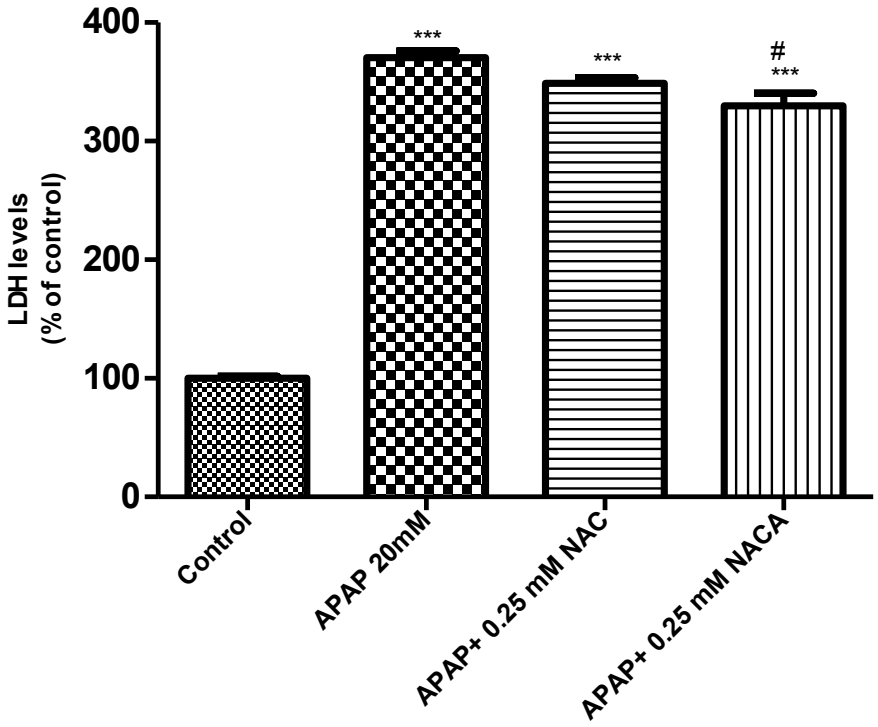


Figure 4.18. Lactate Dehydrogenase (LDH) Release in The Culture Medium and the Protective Effect of NAC and NACA (24 hours). LDH levels were measured after HepaRG cells were pretreated with 0.25mM of NAC or NACA for 2 hours followed by APAP for 24 hours. The results represent the average of three experiments. * $p < 0.05$ compared to control, # $p < 0.05$ compared to APAP treated group and ^ $p < 0.05$ compared with APAP + 0.25 mM NAC.

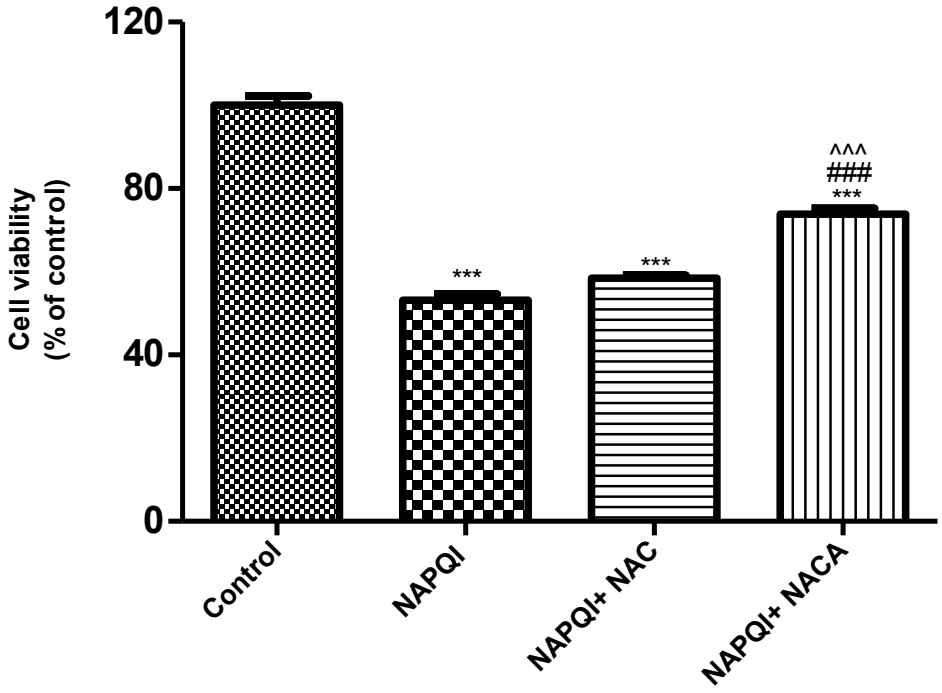


Figure 4.19. The Protective Effect of NAC and NACA on Toxicity Induced by NAPQI. HepaRG cells were pretreated with 0.25 mM of NAC or NACA for 2 hours followed by 250 microM NAPQI for 24 hours. The cell viability was measured by the Calcein AM Assay. The results represent the average of four experiments. $p < 0.05$ compared to control, # $p < 0.05$ compared to NAPQI treated group and ^ $p < 0.05$ compared with NAPQI + 0.25 mM NAC.

Table 4.1 Effect of NAPQI on GSH Levels

GSH nM	Peak area in the absence of NAPQI	Peak area in the presence of NAPQI
167	294852	0
333	1033447	0
667	2932180	0
1000	4066436	0
1333	5309778	725208
1667	6741048	1930765

Different concentrations of GSH indicated in the first column were prepared and areas under each concentration were determined by HPLC (second column). In a different set of test tubes, in addition to the same concentrations of GSH, 83 mM NAPQI was also added, and areas under the GSH peaks were determined (third column). As shown in Table1.1, the peak areas were only seen in higher concentrations of GSH in the presence of NAPQI.

5. DISCUSSION

APAP is a well-known analgesic antipyretic over-the-counter medication. At therapeutic doses, it is safe because 90-95% are metabolized and detoxified by glucuronidation and sulphation[13]. The remaining 5-10% are metabolized by cytochrome $P_{450,}$ mainly CYP 2E1 to form NAPQI, the toxic metabolite of APAP, which is detoxified by conjugation with GSH.[23] However, after an overdose of acetaminophen, glucuronidation and sulphation are saturated and the formation of NAPQI exceeds the detoxification capacity of GSH. This results in covalent binding, particularly with the sulfhydryl group on cysteine of the cellular proteins, which contributes to necrotic cell death[14]. The CYC 2E1, a major P_{450} isoform that is responsible for NAPQI formation, is induced by ethanol. Chronic ethanol consumption depletes liver mitochondrial GSH that increases the risk of APAP toxicity[35, 36].

NAC, a GSH precursor, is the only approved antidote for APAP toxicity. The main drawback of NAC is its poor bioavailability because of its carboxylic group, which loses its proton at physiological pH, making the compound negatively charged. This makes it unable to cross the cell membrane efficiently[26]. NAC is available in oral and intravenous forms, which show equal effectiveness when administered within 8-10 hours of an APAP overdose. Use of an IV is preferred because of the required shorter treatment course[37]. Prolonged treatment with NAC delays liver regeneration from APAP, as shown in many articles. This is explained by the reduction in two important factors in hepatic recovery, hepatic NFκB DNA binding and the expression of cyclin D1, the cell cycle protein [38, 39]. Researchers have recently introduced many chemicals with hepatoprotective and antidotal effects on APAP toxicity. Most of them are from natural products such as

Moringa oleifera[40, 41], Lupeol [42], Ozagrel hydrochloride[43], Rosa damascene Mill[44], 5-methoxypsoralen[45], ethyl pyruvate[46], and beta-carotene[47]. In this study, we investigated the protective effect of NACA against oxidative stress induced by an APAP overdose. Antioxidant and free radical scavenging properties of NACA have been tested and reported in many articles published from our lab[8, 25, 27, & 28]. NACA is a modified form of NAC that has an amide group, instead of a carboxyl group, which improves the membrane permeability and may shorten the treatment course[8]. We used the HepaRG cell line, which is a clinically relevant model for APAP-induced hepatotoxicity because of its expression of P_{450}, which is critical in the induction of APAP toxicity[48]. The main toxicity of APAP stems from its toxic metabolite, NAPQI, which is generated by the P_{450} system in HepaRG cells. Although this pathway is not the major detoxification pathway, the byproduct of this pathway (NAPQI) is very affinic to functionally important thiol groups and has a greater binding to mitochondrial proteins. The subsequent mitochondrial dysfunction led to inhibition of mitochondrial respiration, ATP depletion, and formation of ROS inside the mitochondria, which ended in necrotic liver cell death[49]. NAPQI causes significant GSH depletion and covalent links with many macromolecules, particularly the sulfhydryl group of cysteine in proteins, which leads to loss of its function[16]. Therefore, GSH pro-drugs have been the main antidote for APAP toxicity over the years. In this study, NACA has been used to restore GSH levels in APAP-exposed HepaRG cells.

APAP used alone significantly affected cell viability, ROS generation, GSH, GSSG, GR, MDA, and LDH levels, as compared with the control. NACA protected HepaRG from APAP-induced hepatotoxicity, because of its effect in decreasing ROS,

GSSG, MDA, and LDH. Moreover, NACA increased cell viability, GSH, GR, and GSH/GSSG at the same time. The results of this study show that the NACA group led to a significant increase in GSH levels, GSH/GSSG ratios, and a significant decrease in the LDH levels at a concentration of 0.25mM. The GSH/GSSG ratio has been shown to be the best indicator of oxidative stress and, therefore, NACA, due to its better cell permeability, was able to restore GSH by providing Cys and improving the cells' oxidative status at lower concentrations. However, the NAC group results were not statistically significant, which indicated that NAC was not as effective at a 0.25 mM concentration. Also, the GR results showed the same scenario, with a significant difference between the NAC and NACA groups. GR is an important antioxidant enzyme which is involved in reducing GSSG to GSH thereby protecting cells from oxidative damage.

In summary, there was a significant difference between the NAC and NACA groups in protecting cells against APAP-induced oxidative stress, which supports our conclusion that NACA acts more effectively. Therefore, our results indicate that NACA improves the antidote effect of NAC and can be used at a lower concentration.

6. CONCLUSION

While acetaminophen is an effective analgesic-antipyretic when taken in large doses, it becomes toxic to the liver. NACA protected HepaRG cells against damage induced by acetaminophen toxicity and may, therefore, be a more useful antidote than NAC (the only approved antidote). However an *in vivo* study is needed and will be conducted in the near future.

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