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Elucidation of the Role of Poly(ADP-Ribose) Polymerase in Drug-Induced Toxicity

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Elucidation of the Role of Poly(ADP-Ribose) Polymerase in Drug-Induced Toxicity

by

Kambria K. Haire

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
Department of Environmental and Occupational Health
with a concentration in Toxicology and Risk Assessment
College of Public Health
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DEDICATION

To my wonderful mother, sister, and my love Jordan, thank you for tireless love and support through everything.

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TABLE OF CONTENTS

List of Tables	iii
List of Figures	iv
List of Abbreviations	vii
Abstract	x
Chapter One: Introduction	1
Statement of the Problem	2
Purpose of the Study	3
Chapter Two: Literature Review	5
Cocaine Background	5
Acetaminophen Background	7
Hepatotoxicity	10
Apoptosis	12
Necrosis	12
Other Outcomes of Hepatotoxicity	13
Cocaine and Hepatotoxicity	14
Acetaminophen and Hepatotoxicity	16
Poly(ADP-ribose) Polymerase (PARP)	17
Cocaine and PARP	20
Acetaminophen and PARP	21
Poly(ADP-ribose) Polymerase (PARP) Inhibition	22
Objective	23
Research Questions	24
Hypotheses	24
Chapter Three: Methods	25
Animals	25
Treatments	25
Experimental Design	26
Serum Biochemistry	26
Dose-Response Analysis	27
Temporal Analysis	27

Poly(ADP-ribose) Polymerase (PARP) Activity	28
Poly(ADP-ribose) Polymerase (PARP) Inhibition	32
Glutathione.....	33
DNA Fragmentation.....	33
Western Blot	34
Gross Pathology	36
Spectrophotometry	36
Statistical Analysis.....	37
Chapter Four: Results	38
Dose-Response.....	38
Temporal Analysis	40
Poly(ADP-Ribose) Polymerase Activity	44
Poly(ADP-Ribose) Polymerase Inhibition.....	50
Glutathione.....	58
DNA Fragmentation.....	63
Western Blot	66
Chapter Five: Discussion	73
Chapter Six: Conclusion	82
References.....	84
Appendix A: IACUC Approval	94
About the Author	End Page

LIST OF TABLES

Table 1:	Dose Response for Cocaine	27
Table 2:	Dose Response for APAP	28
Table 3:	Temporal Analysis for Cocaine	28
Table 4:	Temporal Analysis for APAP	28
Table 5:	PARP Activity for Cocaine.....	30
Table 6:	PARP Activity for APAP	31
Table 7:	PARP Inhibition: Activity, ALT, GSH, DNA Fragmentation, and Western Blot for Cocaine + DIQ at the 6 hour Time Point	32
Table 8:	PARP Inhibition: Activity, ALT, GSH, DNA Fragmentation, and Western Blot for APAP + DIQ at the 18 hour Time Point.....	32

LIST OF FIGURES

Figure 1:	Cocaine Metabolites.....	7
Figure 2:	Acetaminophen Metabolites	10
Figure 3:	PARP Structure.....	19
Figure 4:	Cell-Death Model.....	20
Figure 5:	Protein Qualification.....	31
Figure 6:	PARP Activity	31
Figure 7:	DNA Fragmentation Electrophoresis gel.....	35
Figure 8:	Western Blot Protein Ladder	36
Figure 9:	µQuant Spectrophotometer	37
Figure 10:	Cocaine-Dose Response Analysis.....	39
Figure 11:	APAP-Dose Response Analysis	40
Figure 12:	Cocaine Temporal Analysis.....	42
Figure 13:	Gross Pathology: Control + 50 mg/kg Cocaine	43
Figure 14:	APAP Temporal Analysis.....	44
Figure 15:	Gross Pathology: Control + 300 mg/kg APAP	45
Figure 16:	Cocaine and APAP Temporal Analysis: Bar Chart	46
Figure 17:	Cocaine and APAP Temporal Analysis: Line Graph	47
Figure 18:	PARP Activity for Cocaine.....	48

Figure 19:	PARP Activity Fold Change for Cocaine	49
Figure 20:	PARP Activity for APAP	50
Figure 21:	PARP Activity for Fold Change for APAP	51
Figure 22:	Cocaine and APAP PARP Analysis: Bar Chart.....	52
Figure 23:	Cocaine and PARP Analysis.....	53
Figure 24:	PARP Inhibition for Cocaine: ALT	54
Figure 25:	PARP Activity Fold Change for PARP Inhibition for Cocaine.....	55
Figure 26:	PARP Inhibition for APAP: ALT	56
Figure 27:	PARP Activity Fold Change for PARP Inhibition for APAP.....	57
Figure 28:	Cocaine and APAP PARP Inhibition: ALT Bar Chart	58
Figure 29:	Cocaine and APAP PARP Inhibition: ALT Line Graph	59
Figure 30:	PARP Inhibition for Cocaine: PARP Activity.....	60
Figure 31:	PARP Inhibition for APAP: PARP Activity.....	61
Figure 32:	PARP Inhibition for Cocaine and APAP: PARP Activity Bar Chart	62
Figure 33:	PARP Inhibition for Cocaine and APAP: PARP Activity Line Graph	63
Figure 34:	Glutathione Analysis for Cocaine.....	64
Figure 35:	Glutathione Analysis for APAP.....	65
Figure 36:	Glutathione Analysis for Cocaine and APAP: Bar Chart	66
Figure 37:	Glutathione Analysis for Cocaine and APAP: Line Graph	67
Figure 38:	DNA Fragmentation: Cocaine	68
Figure 39:	DNA Fragmentation: APAP	69
Figure 40:	DNA Fragmentation: Cocaine and APAP	70
Figure 41:	Western Blot: Cocaine	71

Figure 42: Western Blot: APAP72

LIST OF ABBREVIATIONS

3-AB	3-aminobenzamide
μL	microliter
AIF	apoptotic-inducing factor
ANOVA	analysis of variance
APAP-SG	acetaminophen-glutathione conjugate
ATP	adenine triphosphate
B-NADPH	beta-nicotinamide adenine dinucleotide phosphate
b/w	body weight
BCA	bicinchoninic acid
BSA	bovine serum albumin
ddH₂O	double distilled water
DIQ	1,5-dihydroxyisoquinoline
DNA	deoxyribonucleic acids
DTNB	5,5'-dithiobis-(2-nitrobenzoic acid)
DTT	1, 4-dithiothreitol
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
g	gram

GSH	glutathione
GSSG	oxidized glutathione
HCl	hydrochloric acid
hr	hour
HRP	horseradish peroxidase
IACUC	Institutional Animal Care and Use Committee
IP	intraperitoneal
IU/L	international units per liter
K₂HPO₄	dipotassium phosphate
kDa	kilodalton
kg	kilogram
KH₂ PO₄	monopotassium phosphate
KPE	potassium phosphate buffer with disodium ethylenediaminetetraacetic acid
M	molar
mg	milligrams
mM	millimolar
MPA	metaphosphoric acid
N	normal
Na₂CO₃	sodium carbonate
NAD⁺	nicotinamide adenine dinucleotide
Na₂EDTA	disodium ethylenediaminetetraacetic acid
NaCl	sodium chloride
NADB	6-biotin-17-nicotinamide-adenine-dinucleotide

NaHCO₃	sodium bicarbonate
NaOH	sodium hydroxide
NIDA	National Institute on Drug Abuse
NLS	nuclear location signal
Orange G	6X Orange G DNA Loading Dye
PARP-HSA	PARP-High Specific Activity Enzyme
PBS	phosphate-buffered saline
rcf	relative centrifugal force
RIPA	radioummunoprecipitation assay buffer
RNAse	ribonuclease
SDS	sodium dodecyl sulfate
TBS-T	tris-buffered saline + 0.1% Tween-20
TMB	3,3',5,5'-tetramethylbenzidine
U	units

ABSTRACT

Drug toxicity may cause liver injury, resulting in damage to cells and tissues. This damage can lead to cytotoxic events that may result in an activation of poly(ADP-ribose) polymerase (PARP). A study was conducted to determine if cocaine and acetaminophen toxicity lead to DNA damage and to the activation of the repair protein, PARP in the liver using the hepatotoxicants: cocaine and acetaminophen (APAP).

A dose-response analysis for cocaine concluded that a dose as low as 20 mg/kg resulted in elevated ALT levels. A higher dose of 60 mg/kg was tested for analyses but resulted in severe hemorrhaging. The dose-response analyses for APAP resulted in no elevated liver enzyme levels for a 75 mg/kg and 150 mg/kg dose. A dose of 50 mg/kg for cocaine, and a dose of 300 mg/kg for APAP, were used to analyze temporal trends for both toxicants. Both cocaine and APAP produced incremental increases in ALT at the 2 hour, 6 hour, 18 hour, and 24 hour time points, respectively. PARP activity analysis for cocaine measured the highest activity at the 2 hour and 6 hour time points. PARP analysis for acetaminophen measured gradual increases until the 18 hour time point where the highest level of PARP activity was measured.

A PARP inhibition analysis was conducted with cocaine and (APAP) to understand the impact of a PARP inhibitor, 1,5-dihydroxyisoquinoline (DIQ), on PARP activity in the liver. A 50 mg/kg dose of cocaine or a 300 mg/kg dose of APAP was administered, followed by a 10 mg/kg dose of DIQ at 1) the time of initial toxicant dose (0 hour), or 2) 1 hour after initial

toxicant dose (1hr). The PARP inhibition analysis for cocaine and APAP was conducted at 6 and 18 hours post initial dose, respectively, when the highest levels of PARP were observed. Inhibition analyses determined that ALT declined significantly when DIQ was administered immediately following the initial toxicant dose for both toxicants. DIQ administered 1 hour after initial toxicant dose resulted in slightly higher ALT than the 0 hour time point. Decreases in PARP activity were observed at the 0 hour time point, with slightly higher PARP levels observed at the 1 hour time point. Decreased PARP activity was observed following DIQ treatment with both, a concurrent drug treatment and treatment following drug administration. Cocaine and APAP treatment did not cause DNA fragmentation. A liver glutathione (GSH) analysis conducted for cocaine and APAP did not correlate with DIQ alteration of PARP activity. The mechanism of DIQ effects on drug-induced hepatotoxicity appears to be GSH independent. DIQ was effective in reducing drug-induced hepatotoxicity and preserving organ function.

CHAPTER ONE:

INTRODUCTION

Recent data reported by the CDC states that drug overdose was the leading cause of death among adults aged 25-64 years old, causing more overall deaths than motor vehicle accidents (Centers for Disease Control and Prevention, 2015). While drug overdose is on the rise among the adult population within the United States, the use of prescription drugs has drove the recent statistics for both drug usage, as well as drug overdose to alarming heights. However, the debate continues to exist as to how much of a role does pharmaceutical and recreational drugs contribute to these alarming statistics.

A drug is defined as any chemical compound that may be used for treatment or diagnosis of a condition or disease, for pain relief, or for the feeling that it causes (CDC, 2015). It is the misuse of drugs that lead to a potential overdose. During an overdose, an excessive amount of a drug may be ingested, inhaled, or absorbed that may lead to body injury (CDC, 2015). Two major classes of drugs that may lead to a potential overdose are recreational and pharmaceutical drugs. Recreational drugs are those drugs that are used without medical justification for the effects that they elicit (Merriam-Webster, 2015). These drugs may be habit-forming or addictive. Some examples of recreational drugs include cocaine, amphetamines, methamphetamines, LSD, heroin, cannabis, and ecstasy. Pharmaceutical drugs are those drugs that are prescribed and are considered to be safe and effective (World Health Organization, 2015). Some examples of

pharmaceutical drugs include Acetaminophen, Ranitidine, Diazepam, Naproxen, and Furosemide. An overdose of either a recreational or pharmaceutical drug may lead to a number of adverse health effects, including acute liver injury (CDC, 2015; WHO, 2015).

As of 2007, the Centers for Disease Control and Prevention reported that as many as 120 people die per day as a result of drug overdose (CDC, 2015). This alarming statistic includes both prescription drug abuse as well as recreational drug use. In 2012, drug overdose was the leading cause of injury death. Overdosing of drugs led to more deaths than motor vehicle traffic crashes among individuals between the ages of 25 to 64 (CDC, 2015). With the risk for death from overdose increasing each year, it is imperative that research studies are conducted to determine any potential way that we may combat these alarming death statistics.

Statement of the Problem

With recent increases observed in the number of individuals overdosing on prescription drugs, there has also been an increase in the usage of recreational drugs as well (CDC, 2015). The age of drug usage for recreational drugs continues to decrease every year. While youth have the lowest death rates from drug overdose, drug abuse among all ages contributed to more than 2.5 million visits in the emergency room in 2012 (CDC, 2015). One of the major recreational drug's that is gaining popularity among youth in grades eight through twelve is cocaine. What is more alarming about the use of cocaine among the youth population is that the usage of crack cocaine far surpasses the usage of cocaine among young people (CDC, 2015; National Institute on Drug Abuse, 2015). As of 2014, there has been a recent increase in the usage of crack cocaine among youth aged 12 to 17 years of age (NIDA, 2015). While the physiological impacts from

cocaine are much more acute, extensive liver damage may result from consuming high doses of this recreational drug (McCluskey, Harbison, Sava, Johnson, Harbison, 2012; NIDA, 2015).

While prescription drug abuse may contribute the overwhelming majority of pharmaceutical drug abuse, over the counter drugs also contribute to the recent increase in the number of overdoses from pharmaceutical products. One of the major reasons for increase in abuse of pharmaceutical drugs is due to the idea that pharmaceutical drugs pose less of a health risk because they are prescribed medications (CDC, 2015; NIDA, 2015). Pharmaceutical drugs are seen as safe and present less addictive properties as opposed to recreational drugs (CDC, 2015). However, it is this false misconception that leads to the misuse and abuse of pharmaceutical drugs. Just as this misconception is prevalent among prescription drug abusers, this theory also resonates for abuse of over-the-counter drugs as well. Acetaminophen, also known as Tylenol, is commonly used as a prescribed medication, as well as an over-the-counter drug. A common analgesic or pain reliever, acetaminophen is one of the most commonly used pharmaceutical drugs on the market today (United States National Library of Medicine, 2015). Because acetaminophen is an over-the-counter drug, it is deemed safe if the recommended dose is consumed. However, acetaminophen is not recommended for large consumption with a single dose, or for long-term usage due to its potential to cause extensive damage to the liver (United States National Library of Medicine, 2015). It is this concern that leads to precaution of physicians and other health professionals for consumers that may misuse this pharmaceutical drug.

Purpose of the Study

The purpose of this study is to determine the impacts of a pharmaceutical drug and

recreational drug overdose on the role of poly(ADP-Ribose) polymerase (PARP) protein activity in the liver. The pharmaceutical drug acetaminophen and the recreational drug cocaine will be used to characterize the role of PARP in hepatotoxicity. A dose-response analysis will be conducted to determine the optimal dose to elicit cytotoxicity in the liver caused by an overdose of cocaine and acetaminophen. A temporal analysis will be conducted to understand the role of time in drug-induced hepatotoxicity for the two hepatotoxicants. PARP activity will be measured to characterize the role of PARP in cocaine and acetaminophen-induced hepatotoxicity and the onset of change. Lastly, an efficacy study inhibiting PARP activity with 1,5-dihydroxyisoquinoline (DIQ) will be conducted to determine if there is a measurable reduction in damage from blocking activation of the PARP protein.

The ability to inhibit the PARP activation mechanism will help to increase the amount of time an individual will have to seek medical attention following an overdose. This will help to potentially decrease mortality rates for drug overdose if an individual is able to seek medical attention soon after administration of the drug. Furthermore, this will also help to better understand the cytotoxic impacts of acetaminophen and cocaine in the liver, while also explaining PARP protein activity in the liver in the presence of these known hepatotoxicants.

CHAPTER TWO: LITERATURE REVIEW

Cocaine Background

Cocaine is one of the most commonly used recreational drugs, with only marijuana and heroin usage being more common among drug abusers of illicit drugs (NIDA, 2015). Some alternative names for cocaine include coke, blow, powder, and snow (NIDA, 2015). Common uses include the form of both cocaine and crack cocaine. Classified as a stimulant, cocaine produces a temporary increase in functionality due to its impact on the neurotransmitter dopamine (NIDA, 2015). The use of cocaine blocks the dopamine transporter and prevents the reuptake of dopamine (Siegel, 1991). Cocaine produces an overproduction of dopamine, but those dopamine molecules have nowhere to go so they bounce around between the pre- and post-synapses at the synaptic cleft (Siegel, 1991). In turn, this reaction contributes to the excitatory or stimulatory feelings observed after consumption of the drug.

Cocaine originates from the coca plant found mostly in South America. Historically, cocaine was used as a common anesthetic, particularly during nasal surgery (Kester, Vrana, & Karpa, 2012). Common routes of administration include chewing, insufflation (snorting), inhalation (smoking), dermal, and intravenous. Injecting cocaine or smoking cocaine will provide a quicker and stronger but short lasting high as opposed to insufflation or snorting cocaine (NIDA, 2015). A common treatment method for an overdose of cocaine is the

administration of Diazepam or other benzodiazepines. Administration of Diazepam is used to treat symptoms such as elevated heart rate and blood pressure but will not alter hepatotoxicity (NIDA, 2015).

While there are a number of potential routes of administration for cocaine, the end result is significant impacts on the central nervous and cardiovascular system (Mehanny, Abdel-Rahman, 1991; Kulberg, 1986; Thompson, Shuster, Shaw, 1979; Labib, Turkall, Abdel-Rahman, 2002; Ascenzi, Clementi, Polticelli, 2003). The binding of cocaine to dopamine receptors produces a stimulatory response due to the inhibition of the re-uptake of dopamine from the synaptic cleft. The lack of binding of 3dopamine to its receptors produces feelings of euphoria (Xu et al., 1994). The stimulatory effects of cocaine on the heart may severely impact heart rate and blood pressure, along with causing damage to heart cells by reactive oxygen species (Kovacic, 2005; Labib, Turkall, Abdel-Rahman, 2002).

Cocaine is primarily metabolized in the liver and produces two major metabolites of ecgonine methyl ester and benzoylecgonine (Labib, Turkall, Abdel-Rahman, 2002; Stewart, Inaba, Lucassen, Kalow, 1979; Ascenzi, Clementi, Polticelli, 2003; Stewart, Inaba, Lucassen, Kalow, 1979). (See Figure 1.) During hepatotoxic events minor metabolites such as norcocaine or cocaethylene are produced. Ecgonine methyl ester is metabolized via a deesterification metabolic pathway, while the more common metabolite benzoylecgonine is metabolized via a demethylation metabolic pathway. Benzoylecgonine is unlikely to produce any reactive species that may be detectable within the liver (Kovacic, 2005; Labib, Turkall, Abdel-Rahman, 2002; Freeman and Harbison, 1981; Boess, Ndikum-Moffor, Boelsterli, Roberts, 2003vira). One minor oxidative metabolic route producing norcocaine may produce a number of byproduce such as nococaine nitroxide, N-hydroxynorcocaine, norcocaine nitrosonium, cocaine iminium, N-

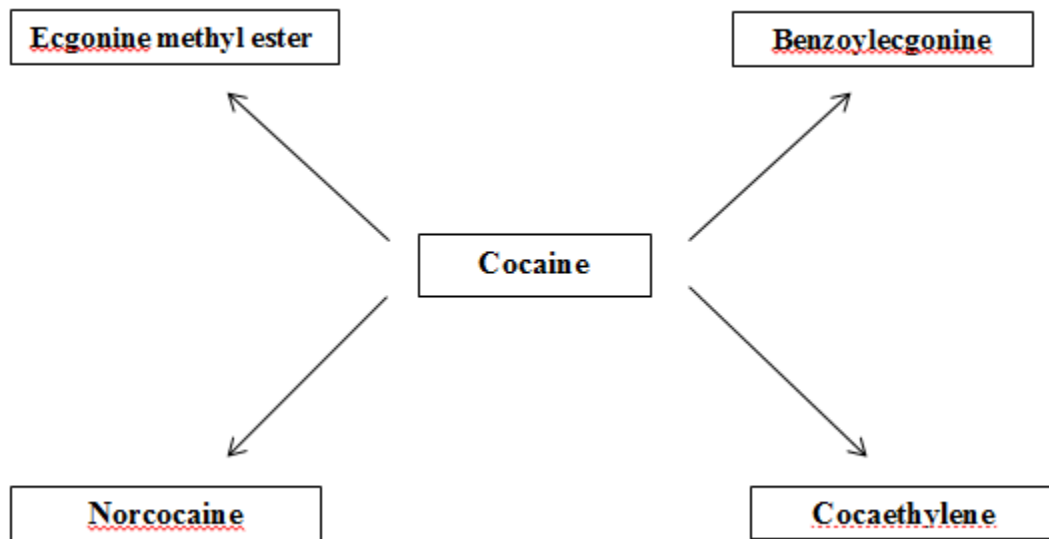


Figure 1. Cocaine Metabolites. Metabolism of cocaine produces the metabolites of ecgonine methyl ester, benzoyllecgonine, norcocaine, and cocaethylene (Stewart, Inaba, Lucassen, Kalow, 1979; Ascenzi, Clementi, Polticelli, 2003; Thompson, Shuster, Shaw, 1979; Roth, Harbison, James, Tobin, Roberts, 1992; Kovacic, 2004).

hydroxy, and formaldehyde, which have been shown to affect cocaine toxicity (Kovacic, 2005; Labib, Turkall, Abdel-Rahman, 2002; Thompson, Shuster, Shaw, 1979). The production of cocaethylene as a minor metabolite may result from cocaine metabolism in the presence of alcohol (Ascenzi, Clementi, Polticelli, 2003). These metabolites may react with a number of enzymes throughout the body to impact several different organ systems such as the central nervous system and the cardiovascular system (Kovacic, 2005; Labib, Turkall, Abdel-Rahman, 2002).

Acetaminophen Background

Acetaminophen is a common analgesic drug used safely at therapeutic doses, but may

cause liver injury with an overdose (James, Mayeux, Hinson, 2003; Dönmez, Uysal, Poyrazoglu, Er Öztas, Türker, Kaldirim, Korkmaz, 2015). A common over-the-counter drug suggested for women during pregnancy, acetaminophen is rarely recognized as a toxic drug. Common routes of administration include intravenously, orally, and rectally. Acetaminophen overdose is one of the leading causes of acute liver injury (McGill, Lebofsky, Norris, Slawson, Bajt, Xie, Williams, Wilkins, Rollins, Jaeschke, 2013; Lee, 2004; Yaman, Isbilir, Cakir, Uysal, 2011). While acetaminophen overdose may cause acute liver injury, it is unlikely to cause widespread liver damage that could result in a liver transplant. Due to the acute impacts of acetaminophen liver injury resulting from an overdose, it is important to use proper clinical diagnostic tests to identify acetaminophen hepatotoxicity (Dönmez, Uysal, Poyrazoglu, Er Öztas, Türker, Kaldirim, Korkmaz, 2015; Yaman, Isbilir, Cakir, Uysal, 2011). Delayed symptoms from an overdose include nausea and vomiting that may develop more than eight hours after ingestion (Rumack, 1983). Common treatments for an overdose include charcoal and N-acetylcysteine, the latter commonly used in Europe (Linden & Rumack, 1984; Rumack, 1983).

With origins dating back well over 100 years, acetaminophen has been a commonly used analgesic for a number of medical conditions (Brune, Renner, Tiegs, 2015; Spooner & Harvey, 1976). Originating in a laboratory from p-nitrophenol, paracetamol gained more popularity overseas than in the United States. It wasn't until its efficacy as an analgesic was recognized in the mid-1900s that acetaminophen gained widespread use in the United States (Spooner & Harvey, 1976; Black, 1984). A patent was granted for acetaminophen use in the United States during the 1980s (Black, 1984).

Because acetaminophen is a common analgesic, resultant target areas may vary throughout the body (Rumack, 1983; Linden & Rumack, 1984; James, Mayeux, Hinson, 2003).

When taken as a therapeutic drug, acetaminophen has little to no toxic impacts on the body as most of its metabolites are excreted in the urine (Miners, Osborne, Tonkin, Birkett, 1992). It is in the presence of an overdose when symptoms may be observed in an individual that may require medical attention (Budnitz, Lovegrove, Crosby, 2011). During an overdose, hepatotoxic events may produce widespread liver damage (Shi et al., 2012; James, Mayeux, Hinson, 2003; Jaeschke, McGill, Williams, Ramachandran, 2011; McGill, Sharpe, Williams, Taha, Curry, Jaeschke, 2012). While charcoal and N-acetylcysteine may be used for treatment of an acute overdose, often prolonged therapeutic use of acetaminophen may also result in widespread liver damage as well. Resultant liver damage from acute and chronic use may be irreversible in extreme cases (McGill et al., 2013; Williams, Koerner, Lampe, Farhood, Jaeschke, 2011; Jaeschke, McGill, Williams, Ramachandran, 2011; McGill, Sharpe, Williams, Taha, Curry, Jaeschke, 2012).

The complex mechanism for acetaminophen has been researched for a number of years due to the impacts of intermediate metabolites on various cells and tissues throughout the body (McGill, Williams, Xie, Ramachandran, Jaeschke, 2012; Gujral, Knight, Farhood, Bajt, Jaeschke, 2002; James, Mayeux, Hinson, 2003; McGill et al., 2013; Lawson, Fisher, Simmons, Farhood, Jaeschke, 1999). Acetaminophen metabolism occurs via three pathways. The two most common pathways include glucuronidation and sulfation (Kessler, Kessler, Auyeung, Ritter, 2002; McGill, Sharpe, Williams, C., Taha, M., Curry, S., Jaeschke, H., 2012; Koch-Weser, 1976; Miners, Penhall, Robson, Birkett, 1988). (See Figure 2.) The minor third metabolic pathway involves the oxidation of acetaminophen molecules that are produced by cytochrome -P450 enzymes to produce the reactive metabolite of N-acetyl-p-benzoquinone imine (NAPQI) (Manyike, Kharasch, Kalhorn, Slattery, 2000). Glutathione (GSH) detoxifies NAPQI molecule

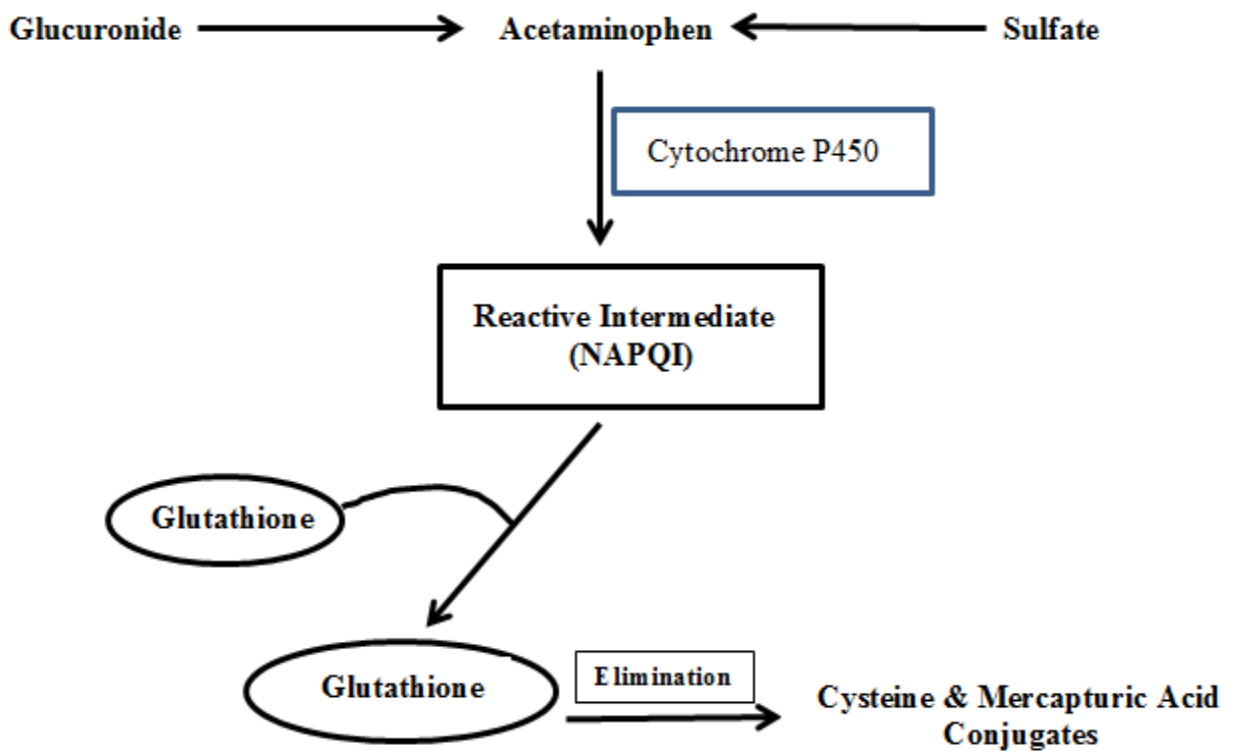


Figure 2. Acetaminophen Metabolism. Metabolism of acetaminophen produces metabolites via the processes of glucuronidation and sulfation, along with the formation of the reactive metabolite N-acetyl-p-benzoquinone imine (NAPQI) (Food and Drug Administration, 2002; James, Mayheux, Hinson, 2003; Jaeschke, McGill, Williams, Ramachandran, 2011).

through a conjugation reaction to reduce damage to liver cells and tissue from toxic metabolites (McGill, Sharpe, Williams, C., Taha, M., Curry, S., Jaeschke, H., 2012; Nelson, 1990; Williams, Koerner, Lampe, Farhood, Jaeschke, 2011; Cohen, Pumford, Khairallah, Boekelheide, Pohl, Amouzadeh, Hinson, 1997; Manyike, Kharasch, Kalhorn, Slattery, 2000; Mitchell, Jollow, Potter, Davis, Gillette, Brodie, 1973).

Hepatotoxicity

Hepatotoxic events may result from a number of acute and chronic drug use. While

chronic usage is a repeated use of drugs over a prolonged period of time, toxicity resulting from acute usage may occur from consumption of a single dose of a drug. Both chronic and acute drug usage may result in drug-induced liver injury (Begrache, Massart, Robin, Borgne-Sanchez, Fromenty, 2011; Björnsson, 2009). However, it is the metabolism of the drug to its toxic metabolites that may create widespread liver damage. Two common outcomes resulting from drug toxicity are apoptosis and necrosis. Both outcomes impact cell signaling and result in extreme damage to liver cells and tissues (Gujral, Knight, Farhood, Bajt, Jaeschke, 2002; Hong, Dawson, Dawson, 2004; El-Hassan et al., 2003; Adams et al., 2001).

Toxicity in the liver may be measured a number of different ways. The most common method for determining liver damage is through the testing of serum biomarkers. Abnormal levels of serum biomarkers may provide an indirect indication of potential liver damage that may exist (Ozer, Ratner, Shaw, Bailey, Schomaker, 2008; Amacher, 1998; Amacher, Alder, Hearth, Townsend, 2005; Boone et al., 2005; Ramaiah, 2007). Considered the gold standard for detection of liver injury, alanine aminotransferase (ALT) is the most frequently used serum biomarker for assessing hepatotoxic effects (Ozer, Ratner, Shaw, Bailey, Schomaker, 2008; Amacher, 1998; Amacher, 2002). Similar to ALT, aspartate aminotransferase (AST) is considered the second most common serum biomarker used for measuring hepatotoxicity (Ozer, Ratner, Shaw, Bailey, Schomaker, 2008). Although AST is less specific than ALT, abnormalities with both liver enzymes indicate potential liver damage that may exist. Some other common serum biomarkers used to determine hepatotoxicity include bilirubin, bile acids, gamma-glutamyl transferase, and glutamate dehydrogenase (Ozer, Ratner, Shaw, Bailey, Schomaker, 2008; Amacher, 2002). Most liver enzyme assays may be conducted with colorimetric detection methods, more in-depth

ELISA assays may be used to determine any abnormalities for any non-colorimetric biomarkers (Ozer, Ratner, Shaw, Bailey, Schomaker, 2008).

Apoptosis

A cellular pathway ending in apoptosis may result from a number of potential metabolic pathways. Common apoptotic pathways may result from minor cellular damage to improper cell signaling. An alternative result from apoptosis is the activation of initiator and effector caspase. Irrespective of the mechanistic pathway, the ending result for apoptotic signaling is cell death. Cells processing through apoptotic pathways result in cell shrinkage and cell phagocytosis while the cell membrane remains intact (Alison, Sarraf, 1992; Sen, 1992; Proskuryakov, Konoplyannikov, Gabai, 2003). Other metabolic processes impacted by apoptosis include DNA fragmentation and caspase (Jaeschke, H., Cover, C., Bajt, M., 2006; McGill, Sharpe, Williams, Taha, Curry, Jaeschke, 2012; Cohen, 1997; Sen, 1992). While cell death may be the ultimate outcome from apoptotic signaling, often the damage to tissues is not widespread (Sen, 1992).

Necrosis

An alternative cellular pathway ending is via necrosis. A necrotic pathway is more severe in that the cellular and tissue damage is widespread than for apoptosis. While cells may shrink and die with apoptosis, during necrosis cells swell and burst. This potential outcome leads to breaking of the cell membrane and cell contents are released from inside the cell causing massive widespread tissue damage (Hong, Dawson, Dawson, 2004; Walker, Harmon, Gobé, Kerr, 1988). Some outcomes that may result from a necrotic pathway may include ATP depletion, free radicals, and reactive oxygen species (Jaeschke, H., Cover, C., Bajt, M., 2006; McGill, Sharpe,

Williams, Taha, Curry, Jaeschke, 2012; Begriche, Massart, Robin, Borgne-Sanchez, Fromenty, 2011). A necrosis ending result is more detrimental to cells and tissues due to the widespread damage that is caused with the bursting of cells and the release of free radicals to potentially generate further damage to other nearby cells (Proskuryakov, Konoplyannikov, Gabai, 2003).

Other Outcomes of Hepatotoxicity

There are a number of outcomes that may result from potential hepatotoxic events. Each of the resultant outcomes result in either necrosis or apoptosis as damage to cells and tissues occur. Some common alternative outcomes that may result from hepatotoxic events include DNA fragmentation, activation of caspases, and ATP depletion. Activation of any of these processes will most likely result in cell death and tissue damage. Most damage that occurs as a result of these outcomes is irreversible.

DNA fragmentation expresses the cleaving of DNA that occurs during apoptotic signaling. Apoptotic signaling causes histones wrapped around DNA to unravel and expose DNA strands. Exposed strands are susceptible to degradation as other parts of the cell die during the apoptosis process. Another potential outcome leading to apoptosis that may arise from an apoptotic pathway is the activation of caspases (Hong, Dawson, Dawson, 2004; Malhi, Gores, Lemasters, 2006; Jiang, Wang, 2004). Activation of caspases may result from a number of potential mechanisms, but the end result of caspase activation is activation of apoptotic pathways (McGill, Sharpe, Williams, Taha, Curry, Jaeschke, 2012; Gujral, Knight, Farhood, Bajt, Jaeschke, 2002; Davidson and Eastham, 1966; Hong, Dawson, Dawson, 2004; Jaeschke, H., Cover, C., Bajt, M., 2006; Majno, Joris, 1995). ATP depletion results from a necrotic pathway. A depletion of ATP stores results in cell swelling, where cell membranes eventually burst leading

to cell death (Malhi, Gores, Lemasters, 2006; Majno, Joris, 1995; Goldblatt, Trump, Stowell, 1965; Nieminen, Gores, Wray, Tanaka, Herman, Lemasters, 1988; Herman, Nieminen, Gores, Lemasters, 1988). Significant reductions to glutathione may also result from hepatotoxic events such as the production of reactive oxygen species. Because glutathione works as an antioxidant to repair damaged cells, reductions in levels may result in more widespread damage due to cells' inability to prevent damage from reactive metabolites produced as a result of drug-induced toxicity (Nýdlova, Vrbová, Česla, Jankovičová, Ventura, Roušar, 2013; McCluskey, Harbison, Sava, Johnson, Harbison, 2012; Labib, Turkall, Abdel-Rahman, 2002; McCluskey, Sava, Harbison, Muro-Cacho, Johnson, Ping, Harbison, 2011; Dönmez, Uysal, Poyrazoglu, Er Öztas, Türker, Kaldirim, Korkmaz, 2015; Cover et al. 2005a; James, Mayeux, Hinson, 2003; McGill et al. 2013; McGill, Sharpe, Williams, Taha, Curry, Jaeschke, 2012).

Cocaine and Hepatotoxicity

The liver is a known target of cocaine toxicity. The hepatic injury that may result from cocaine overdose is likely to be extensive throughout many liver cells and tissue. Studies have shown that whether the dose is acute or chronic, measured liver enzymes indicate that some level of damage to the liver occurs from either frequency of administration of the drug (Mehanny, Abdel-Rahman, 1991; Evans, Dwivedi, Harbison, 1977; Shuster, Freeman, and Harbison, 1977; Freeman, Harbison, 1981; Devi, Chan, 1997). Hepatotoxicity resulting from acute cocaine doses has been shown to be dependent upon the induction cytochrome P450 oxidative enzymes (Mehanny, Abdel-Rahman, 1991; Thompson, Shuster, Shaw, 1979; Freeman, Harbison, 1978; Labib, Turkall, Abdel-Rahman, 2002; Freeman, Harbison, 1981; Devi, Chan, 1997). It is through this oxidative pathway that reactive oxygen species are produced (Labib, Turkall, Abdel-

Rahman, 2002; Aoki, Ohmori, Takimoto, Ota, Yoshida, 1997). The production of these reactive species causes damage to a number of organelles within the cell, such as damage to the cell membrane and malfunctioning of mitochondria (Labib, Turkall, Abdel-Rahman, 2002; Devi, Chan, 1997; Ndikum-Moffor, Roberts, 2003). As a result glutathione levels are expected to be reduced along with ATP stores within the cell. Depletion of ATP stores impact the cell's ability to withstand damage from ROS produced from cocaine toxicity (Labib, Turkall, Abdel-Rahman, 2002; Devi and Chan, 1996; Evans, 1983; Kovacic, 2004). These hepatotoxic events lead to a defective cell that swells and eventually bursts. This damage eventually causes what has been observed as necrotic damage to cells and tissues resulting from cocaine hepatotoxicity (Roth, Harbison, James, Tobin, Roberts, 1992).

Research has shown that pretreatments may help to reduce the extent of liver damage that results from cocaine hepatotoxic events. Studies evaluated the effectiveness of pretreatments on both acute and chronic cocaine drug treatments (Labib, Turkall, Abdel-Rahman, 2002; McCluskey, Harbison, Sava, Johnson, Harbison, 2012; McCluskey, Sava, Harbison, Muro-Cacho, Johnson, Ping, Harbison, 2011). The purpose is to reduce ALT levels, restore ATP, and increase the production of glutathione. Research has shown that improvements of these elements have significant impacts on reducing widespread liver damage, and in some cases, may also reverse observed damage from cocaine-induced hepatotoxicity (McCluskey, Harbison, Sava, Johnson, Harbison, 2012; Ozer, Ratner, Shaw, Bailey, Schomaker, 2007). However, the key to altering the toxicity produced by reactive oxygen species is to protect the cell membrane and mitochondria to maintain viable cells that can increase glutathione production that will aid in the reduction of toxic cocaine metabolites (Donnelly, Boyer, Petersen, Ross, 1988; McCluskey, Harbison, Sava, Johnson, Harbison, 2012).

Acetaminophen and Hepatotoxicity

Acetaminophen hepatotoxicity may result from drug-induced liver injury that may cause permanent damage to liver cells and tissue (Enomoto, Itoh, Nagayoshi, Haruta, Kimura, O'Connor, Harada, Yamamoto, 2001; Ingawale, Mandlik, Naik, 2014; Brodie, Reid, Cho, Sipes, Krishna, Gillette, 1971; Mitchell, Jollow, Potter, Davis, Gillette, Brodie, 1973; Reid, Krishna, Mitchell, Moskowitz, Brodie, 1971; Cover et al, 2005a; James, Mayeux, Hinson, 2003; Dönmez, Uysal, Poyrazoglu, Er Öztas, Türker, Kaldirim, Korkmaz, 2015). Hepatotoxic events develop from the production of the toxic metabolite, N-acetyl-p-benzoquinone imine (NAPQI), by cytochrome p450 enzymes. The production of NAPQI depletes glutathione and binds to the amino acid cysteine within the cell (McGill, Williams, Xie, Ramachandran, Jaeschke, 2012; Nelson, 1990; James, Mayeux, Hinson, 2003; Cohen, Pumford, Khairallah, Boekelheide, Pohl, Amouzadeh, Hinson, 1997; Nýdlova, Vrbová, Česla, Jankovičová, Ventura, Roušar, 2013). This process stimulates the toxic events of oxidative stress resulting from damage caused by reactive oxygen species to various parts of the cell (McGill, Williams, Xie, Ramachandran, Jaeschke, 2012; Kon, Kim, Jaeschke, Lemasters, 2004; Jaeschke, H., McGill, M., Ramachandran, A., 2012; Cohen, Pumford, Khairallah, Boekelheide, Pohl, Amouzadeh, Hinson, 1997; Cover et al., 2005b).

Exposures to toxic doses of acetaminophen may cause widespread mitochondrial damage (McGill, Sharpe, Williams, Taha, Curry, S., Jaeschke, 2012; Jaeschke, H., McGill, M., Ramachandran, A., 2012). This mitochondrial dysfunction impacts the cytochrome p450 protein mechanism. The mitochondrial damage to cytochrome p450 can lead to oxidative stress, which may result in a production of free radicals (Park, Smith, Combs, Kehrer, 1988; Jaeschke, Gujral, Bajt, 2004; Yoon, Kim, Lee, Chung, Kim, 2006; Jaeschke, Cover, Bajt, 2006; McGill, Williams,

Xie, Ramachandran, Jaeschke, 2012; Cover et al., 2005b). These free radicals cause DNA fragmentation that can serve as a precursor to necrotic cell death and injuries to liver tissue (Cover et al., 2005a; McGill, Sharpe, Williams, Taha, Curry, Jaeschke, 2012; Williams, Koerner, Lampe, Farhood, Jaeschke, 2011; James, Mayeux, Hinson, 2003; Kröger, Ehrlich, Klewer, Grätz, Dietrich, Miesel, 1996; Jaeschke, McGill, Williams, Ramachandran, 2011).

Pretreatments have also been shown to improve hepatotoxic events resulting from acetaminophen-induced toxicity (Dönmez, Uysal, Poyrazoglu, Er Öztas, Türker, Kaldirim, Korkmaz, 2015; Ray, Kamendulis, Gurule, Yorkin, Corcoran, 1993). Administered pretreatments aid in the reduction of elevations in ALT, and improve glutathione and ATP stores that result from acetaminophen-induced hepatotoxic events. Reducing the production of the toxic metabolite, NAPQI, will result in the improvement of glutathione stores that will improve cellular function (Shi et al., 2012; Fernandes et al., 2011; Purnell, Whish, 1980; Dönmez, Uysal, Poyrazoglu, Er Öztas, Türker, Kaldirim, Korkmaz, 2015). Improving the damage that results from hepatotoxic events may help to prevent widespread liver damage and reverse damage to liver cells and tissue (Shi et al., 2012; Dönmez, Uysal, Poyrazoglu, Er Öztas, Türker, Kaldirim, Korkmaz, 2015; Fernandes et al., 2011; Purnell, Whish, 1980).

Poly(ADP-ribose) Polymerase (PARP)

The role of cellular death in drug-induced toxicity has been studied for a number of decades. While it is understood that the metabolic production of toxic metabolites contributes to the damage in major cell organelles, the intermediate mechanistic pathways and contributors continues to be debated (Cover et al., 2005b; Wang, Dawson, Dawson, 2009; James, Mayeux, Hinson, 2003; Virág, Robaszkiewicz, Rodriguez-Vargas, Oliver, 2013). Damage to DNA is

considered to be the main cellular response that results from the toxic events (Hong, Dawson, Dawson, 2004). Resultant nicks and tears to single and double stranded DNA triggers a cellular response to activate the enzyme poly(ADP-ribose) polymerase (PARP) by catalyzing modified nuclear proteins via poly-ADP ribosylation (Wang, Dawson, Dawson. 2009; Cover et al., 2005b; Scobie et al., 2014; Bouchard, Rouleau, Poirier, 2003; Dönmez, Uysal, Poyrazoglu, Er Öztas, Türker, Kaldirim, Korkmaz, 2015; Virág, 2005). PARP is a 116 kDa protein containing 3 main domains: an N-terminal DNA-binding domain (42 kDa), a central automodification domain (16 kDa), and a C-terminal domain (55kDa). The N-terminal DNA-binding domain contains two zinc fingers that aid recognizing nicks and tears to DNA strands (Hong, Dawson, Dawson, 2004; Wang, Dawson, Dawson. 2009; Cover et al. 2005a; Bouchard, Rouleau, Poirier, 2003). (See Figure 3.)

In the presence of drug and chemical-induced toxicity, an overproduction of PARP is activated to compensate for the loss of cellular energy resulting from mitochondria dysfunction and rapid depletion of NAD^+ and ATP stores (McCluskey, Sava, Harbison, Muro-Cacho, Johnson, Ping, Harbsion, 2011; Cover et al., 2005a; Scobie et al., 2014; Dönmez, Uysal, Poyrazoglu, Er Öztas, Türker, Kaldirim, Korkmaz, 2015; Virág, Robaszkiewicz, Rodriguez-Vargas, Oliver, 2013; Aredia, Scovassi, 2014). Excessive activation of this PARP enzyme depletes nicotinamide adenine dinucleotide (NAD^+) within the cell. This depletion in NAD^+ triggers a rapid depletion of adenosine triphosphate (ATP) within the cell. Rapid catabolism of NAD^+ impacts energy metabolism by causing mitochondrial dysfunction and decreases function of the nucleus which eventually leads to cell death (Hong, Dawson, Dawson, 2004; McCluskey, Harbison, Sava, Johnson, Harbison, 2012; McCluskey, Sava, Harbison, Muro-Cacho, Johnson,

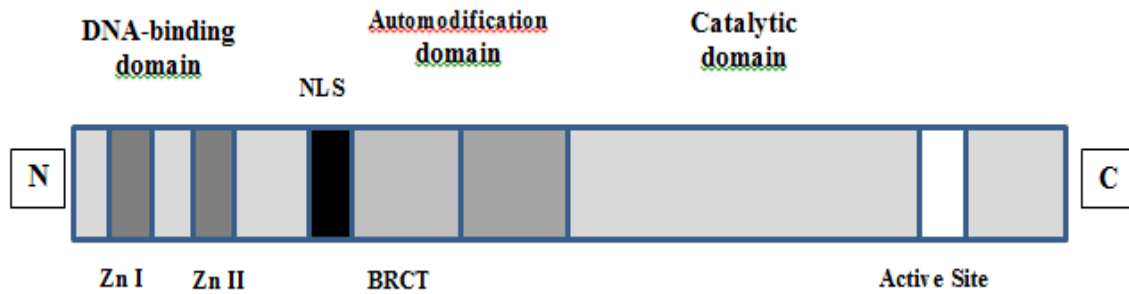


Figure 3. PARP Structure. A PARP structure containing the three main domains and two zinc fingers (Bouchard, Rouleau, Poirier, 2003; Hong, Dawson, Dawson, 2004).

Ping, Harbison, 2011; Bouchard, Rouleau, Poirier, 2003). The cell death that results is triggered from the activation of one of the two common cellular death pathways: apoptosis or necrosis (McCluskey, Harbison, Sava, Johnson, Harbison, 2012; Wang, Dawson, Dawson. 2009; Bouchard, Rouleau, Poirier, 2003; Ha & Snyder, 1999; Virág, Robaszkiewicz, Rodriguez-Vargas, Oliver, 2013). (See Figure 4.) Biochemical changes resulting from apoptosis include a cell's lack of ability to repair damage to DNA. Apoptosis results from the activation and cleavage of effector caspases: caspase 3, caspase 6, and caspase 7. Other cases where an apoptotic pathway may be activated include mitochondrial dysfunction and other proteases or profactors that may trigger activation of effector and effector caspase pathways. Activation results in the shrinking of cells where the cell eventually commits what is known as "cell suicide" or prograded cell death. The alternative common cellular death pathway of necrosis includes an inflammatory response. Resulting from an over-activation of PARP due to decreases in cellular function, cells activating this pathway produce a breakdown of the cell membrane due to swelling. Eventually cells burst resulting in widespread cell and tissue damage (Hong, Dawson, Dawson, 2004; Wang, Dawson, Dawson. 2009; Bouchard, Rouleau, Poirier, 2003; McCluskey, Harbison, Sava, Johnson, Harbison, 2012; Cover et al., 2005b; Dönmez, Uysal,

Poyrazoglu, Er Öztas, Türker, Kaldirim, Korkmaz, 2015; Ha & Snyder, 1999; Virág, Robaszkiewicz, Rodriguez-Vargas, Oliver, 2013).

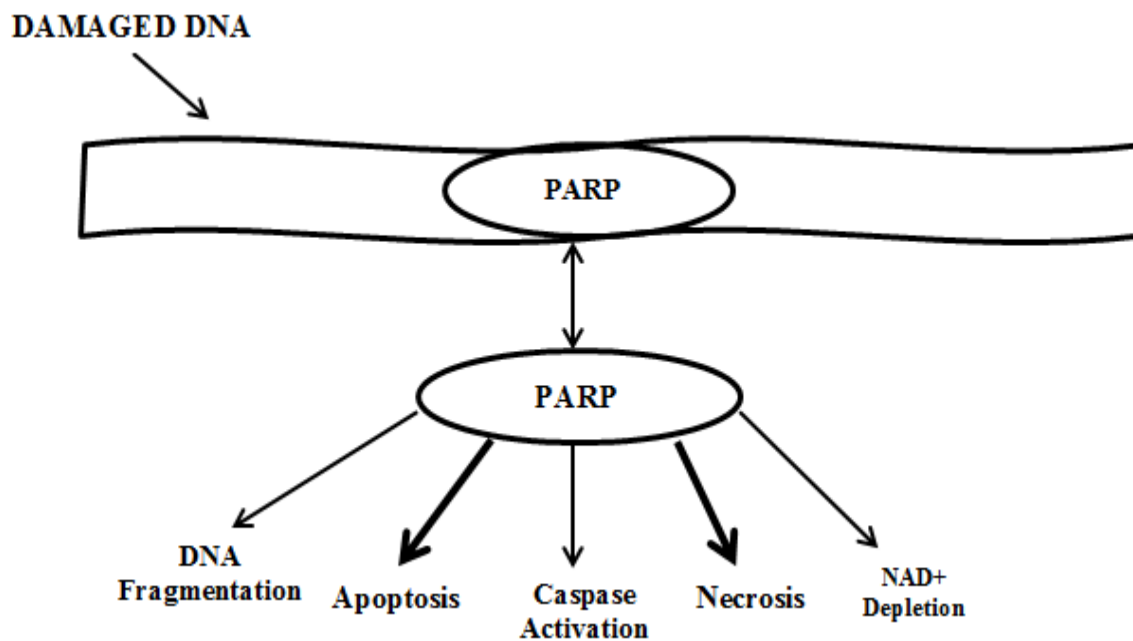


Figure 4. Cell Death Model. Model displaying the outcomes of cell death resulting from activation of PARP (Bouchard, Rouleau, Poirier, 2003; Hong, Dawson, Dawson, 2004; Wang, Dawson, Dawson, 2009).

Cocaine and PARP

Cocaine has been shown to be toxic to the liver and produce hepatotoxic events resulting from an acute or chronic dose (McCluskey, Harbison, Sava, Johnson, Harbison, 2012; Scobie et al., 2014; Price, Muro-Cacho, Harbison, 1999; Evans, 1983). While the mechanism for cocaine-induced hepatotoxicity is not fully understood, the production of the norcocaine metabolite from cocaine metabolism via cytochrome-P450 enzymes has been shown to elicit cytotoxic events causing damage to liver cells and tissues (McCluskey, Harbison, Sava, Johnson, Harbison, 2012; Roth, Harbison, James, Tobin, Roberts, 1992; Mehanny, Abdel-Rahman, 1991; Thompson, Shuster, Shaw, 1979; Stewart, Inaba, Lucassen, Kalow, 1979). What was once thought to

potentially be an apoptotic cell death pathway caused by cocaine has now been shown to be a necrotic cell death pathway. Toxic doses of cocaine has been shown to deplete ATP and NAD⁺ stores, along with causing mitochondrial dysfunction and damage to DNA (McCluskey, Harbison, Sava, Johnson, Harbison, 2012; Thompson, Shuster, Shaw, 1979; Roth, Harbison, James, Tobin, Roberts, 1992; Evans, 1983; Devi & Chan, 1997). These cellular events lead to the activation of PARP in order to attempt to replenish depleted ATP and NAD⁺ stores. However, in the presence of cocaine-induced hepatotoxicity, an overproduction of the PARP enzyme causes widespread damage to liver cells and tissues as cellular and mitochondrial function is impaired. Eventually, hepatocyte membranes are damaged by reactive oxygen species and the cell is no longer able to repair itself. As cellular function diminishes, cells swell and burst causing widespread necrotic cell death (McCluskey, Harbison, Sava, Johnson, Harbison, 2012; Kovacic, 2005; Aoki, Ohmori, Takimoto, Ota, Yoshida, 1997; Roth, Harbison, James, Tobin, Roberts, 1992; Devi & Chan, 1997; Evans, 1983).

Acetaminophen and PARP

Hepatotoxic events resulting from an acetaminophen overdose may result in a number impacts to hepatocyte function. The resultant hepatotoxic events may be caused by either an acute or chronic dose. Impacts to hepatocytes include depletion of ATP and glutathione (GSH), mitochondrial dysfunction, and inflammation. These events cause tearing of DNA strands that may become permanently damaged and lead to cell death (Dönmez, Uysal, Poyrazoglu, Er Öztas, Türker, Kaldirim, Korkmaz, 2015; Cover, C., et al., 2005a; McGill, Sharpe, Williams, Taha, Curry, Jaeschke, 2012; McGill et al., 2013; James, Mayeux, Hinson, 2003; Gujral, Knight, farhood, Bajt, Jaeschke, 2002; McGill, Williams, Xie, Ramachandran, Jaeschke, 2012; Mitchell,

Jollow, Potter, Davis, Gillette, Brodie, 1973). The damage to DNA may lead to activation of poly(ADP-ribose) polymerases (PARP) in the nucleus of a cell. However, too much production of PARP leads to bursting of cells and necrotic cell death (Ha, H., Snyder, S., 1999; Cover, C., et al., 2005a; Gujral, Knight, Farhood, Bajt, Jaeschke, 2002; McGill, Sharpe, Williams, Taha, Curry, Jaeschke, 2012; Williams, Koerner, Lampe, Farhood, Jaeschke, 2011; McGill et al., 2013; Mitchell, Jollow, Potter, Davis, Gillette, Brodie, 1973).

Poly(ADP-ribose) Polymerase (PARP) Inhibition

Due to the widespread damage resulting from the activation of PARP, inhibiting the activity of PARP via treatments with an inhibitor has been studied for a number of drugs and chemicals (McCluskey, Sava, Harbison, Muro-Cacho, Johnson, Ping, Harbison, 2011; McCluskey, Harbison, Sava, Johnson, Harbison, 2012; Virág, Szabó, 2002; Virág, 2005; Dönmez, Uysal, Poyrazoglu, Er Öztas, Türker, Kaldirim, Korkmaz, 2015; Southan, Szabó, 2003; Hall, Muro-Cacho, Abritis, Johnson, Harbison, 2009-2010; Banasik, Stedeford, Strosznajder, Takehashi, Tanaka, Ueda, 2011; Shi et al., 2011). While treatments have varied tremendously due to their impacts on the metabolism of the drug being studied, the efficacy of treatments has provided a number of potential options to decrease cell and tissue damage throughout the body (McCluskey, Sava, Harbison, Muro-Cacho, Johnson, Ping, Harbison, 2011; McCluskey, Harbison, Sava, Johnson, Harbison, 2012; Virág, Szabó, 2002; Virág, 2005; Southan, Szabó, 2003; de la Lastra, Villegas, Sánchez-Fidalgo, 2007; Shi et al., 2011). PARP inhibitors have been used to understand the role of inhibition on PARP activity, as well their role in cellular functions and signaling. Because PARP inhibition target DNA and mitochondria, the ability for an inhibitor to decrease the depletion of ATP and NAD⁺ while inhibiting necrosis or apoptosis can

prove to be a key component in its ability to prevent drug-induced hepatotoxicity (Virág, Szabó, 2002; Virág, 2005; Cover et al., 2005a; Dönmez, Uysal, Poyrazoglu, Er Öztas, Türker, Kaldirim, Korkmaz, 2015; Southan, Szabó, 2003; de la Lastra, Villegas, Sánchez-Fidalgo, 2007; Hall, Muro-Cacho, Abritis, Johnson, Harbison, 2009-2010).

The use of water-soluble PARP inhibitors can be used to determine the role on PARP activity without potential interferences from chemical reactions and impacts on results gathered from biochemical assays (McCluskey, Sava, Harbison, Muro-Cacho, Johnson, Ping, Harbison, 2011; Thiermann, 2002; Cover et al., 2005a; Dönmez, Uysal, Poyrazoglu, Er Öztas, Türker, Kaldirim, Korkmaz, 2015). A common water soluble PARP inhibitor, 1,5-dihydroxyisoquinoline (DIQ), has been found to decrease PARP activity with little to no effects on the metabolism of drug or chemicals used for inducing hepatotoxicity (McCluskey, Harbison, Sava, Johnson, Harbison, 2012). Along with their ability to cause less interference with enzymatic reactions, water soluble PARP inhibitors such as DIQ have been effective in preventing hepatotoxic events (McCluskey, Sava, Harbison, Muro-Cacho, Johnson, Ping, Harbison, 2011; McCluskey, Harbison, Sava, Johnson, Harbison, 2012; Cover et al., 2005a; Thiermann, 2002).

Objective

To determine if cocaine and acetaminophen-induced toxicity leads to the activation of the repair protein, poly(ADP-ribose) polymerase (PARP) in the liver. If activation of this PARP protein occurs, can this PARP activity be inhibited by the PARP inhibitor 1,5-dihydroxyisoquinoline (DIQ).

Research Questions

Research Question 1:

Are dosing and temporal factors correlated with increases in serum liver enzyme levels?

Research Questions 2:

Is there a correlation between temporal factors and PARP activity?

Research Question 3:

Is an increase in PARP activity indicative of hepatic biochemical changes relevant to the mechanism of drug-induced hepatotoxicity?

Research Question 4:

Does DIQ provide protection against PARP activity for drug-induced toxicity with cocaine and acetaminophen?

Hypotheses

Research Question 1:

Dosing and temporal factors are correlated with increases in serum liver enzyme levels.

Research Question 2:

There is a correlation between temporal factors and PARP activity.

Research Question 3:

An increase in PARP activity contributes to the mechanism of drug-induced hepatotoxicity.

Research Question 4:

DIQ decreases PARP activity and drug-induced toxicity caused by cocaine and acetaminophen.

CHAPTER THREE:

METHODS

Animals

Male, CD-1 mice (30 ± 5 g), age 8 weeks, were obtained from Charles River Laboratories (Wilmington, Massachusetts). Animals were housed under controlled conditions and exposed to a 12-hour light/dark cycle with free access to food and water. Animals were housed 5 animals per cage and were allowed 7 days to acclimate prior to testing. All of the experiments were conducted in accordance to the University of South Florida Institutional Animal Care and Use Committee (IACUC) guidelines.

Treatments

Drugs were administered through intraperitoneal (IP) injection at a volume of $50\mu\text{L}/10\text{g}$ of body weight. All solutions were prepared fresh prior to treatment. Cocaine hydrochloride (cocaine) was obtained from Sigma-Aldrich (St. Louis, MO) and 4-Acetamidophenol, 98%, (APAP) was obtained from Acros Organics (Morris Plains, NJ). The PARP inhibitor, 1,5-dihydroxyisoquinoline (DIQ), was obtained from AdipoGen (San Diego, CA). The PARP inhibitor was administered through intraperitoneal (IP) injection at a volume of $50\mu\text{L}/10\text{g}$ of body weight (b/w). Cocaine, APAP, and DIQ were dissolved in warm 0.9% saline prior to administration. Groups for comparison were treated with either (I) saline vehicle, (II) cocaine,

(III) APAP, (IV) cocaine and DIQ, or (V) APAP and DIQ. The combined administration of toxicant and inhibitor did not exceed the maximum allowed volume of 200 μ L/10g b/w. The injection sites for toxicants and PARP inhibitor were administered distant to one another to avoid chemical interaction.

Experimental Design

Animals were randomized into treatment groups as shown in Tables 1 through 8. Animals were randomly assigned to control groups where the number of animals in each treatment group was approximately double that of the number in the control groups. The number of animals in each treatment group ranged from 4 to 8 depending on the size of the experiment.

Serum Biochemistry

Alanine aminotransferase (ALT), a biomarker of hepatotoxicity, was quantified in triplicate using a colorimetric endpoint kit from TECO Diagnostics (Anaheim, CA) from the modified method by Reitman et al. (Reitman & Frankel, 1957). Whole blood was collected by cardiac puncture in microcentrifuge tubes and allowed to clot at room temperature for a minimum of 20 minutes. Serum was obtained from each individual sample by centrifugation at 600 rpm for 20 minutes. Serum samples were transferred to a new microcentrifuge tube and stored at -20°C until time of assay. All serum samples were analyzed within a 24 hour period. Serum samples were diluted with 0.9% saline for assay calculations. Standard curve was optimized for high concentrations using sodium pyruvate from Sigma-Aldrich (St. Louis, MO). Regents (ALT (SGPT) substrate, ALT (SGPT) color reagent, and ALT (SGPT) calibrator) were transferred to microplate with diluted samples at timed intervals, immediately followed by heat incubation at

37°C for specified time increments. Assay sample processing was modified in order to analyze samples using a 96-well assay plate. Assay plates were read by a spectrophotometer at 505 nm. Results are presented as total international units of ALT per serum sample. Modifications were made to calibration calculations using sodium pyruvate due to excessive levels of ALT measured among samples.

Dose-Response Analysis

To determine the optimal dose for cocaine and APAP-induced hepatotoxicity, a dose-response study was conducted. Animals were sacrificed 24 hours after treatment. Animals treated with cocaine were administered (1) Vehicle only, (2) 20 mg/kg dose, (3) 30 mg/kg dose, (4) 40 mg/kg dose, (5) 50 mg/kg dose, or (6) 60 mg/kg dose. (See Table 1.) Animals treated with APAP were administered (1) Vehicle only, (2) 75 mg/kg dose, (3) 150 mg/kg dose, (4) 200 mg/kg dose, or (5) 300 mg/kg dose. (See Table 2.) Blood samples were collected by cardiac puncture to determine alanine transaminase (ALT).

Table 1. Dose-Response for Cocaine.

Treatment Group	Chemicals	Dose
Group I	Vehicle Only	0 mg/kg
Group II	Cocaine	20 mg/kg
Group II	Cocaine	30 mg/kg
Group II	Cocaine	40 mg/kg
Group II	Cocaine	50 mg/kg
Group II	Cocaine	60 mg/kg

Temporal Analysis

To determine the optimal time for cocaine and APAP-induced hepatotoxicity, a time study was

conducted. Animals were administered a (II) 50 mg/kg dose of cocaine or a (III) 300 mg/kg dose of APAP. Animals were sacrificed at a 2 hour, 6 hour, 18 hour, or 24 hour time point following treatment (See Tables 3 & 4.) Blood samples were collected by cardiac puncture to determine alanine transaminase (ALT).

Table 2. Dose-Response for APAP.

Treatment Group	Chemicals	Dose
Group I	Vehicle Only	0 mg/kg
Group III	APAP	75 mg/kg
Group III	APAP	150 mg/kg
Group III	APAP	200 mg/kg
Group III	APAP	300 mg/kg

Table 3. Temporal Analysis for Cocaine.

Treatment Group	Chemicals	Dose	Time
Group I	Vehicle Only	0 mg/kg	2 hr, 6 hr, 18 hr, 24 hr
Group II	Cocaine	50 mg/kg	2 hr
Group II	Cocaine	50 mg/kg	6 hr
Group II	Cocaine	50 mg/kg	18 hr
Group II	Cocaine	50 mg/kg	24 hr

Table 4. Temporal Analysis for APAP.

Treatment Group	Chemicals	Dose	Time
Group I	Vehicle Only	0 mg/kg	2 hr, 6 hr, 18 hr, 24 hr
Group III	APAP	300 mg/kg	2 hr
Group III	APAP	300 mg/kg	6 hr
Group III	APAP	300 mg/kg	18 hr
Group III	APAP	300 mg/kg	24 hr

Poly(ADP-ribose) Polymerase (PARP) Activity

An indirect colorimetric method to measure poly(ADP-ribose) polymerase (PARP) was

used to measure the activity of PARP in liver tissue following cocaine or APAP treatments. (See Tables 5 & 6.) Livers were isolated and perfused with normal saline, and stored at -80°C until used for assay. PARP activity was measured using an 80 ± 10 mg sample of liver tissue homogenized with a mortar and pestle, sonicated at a medium setting, and centrifuged at 0.6 rcf for 5 minutes in a 1X lysis buffer (Triton X-100, D-1-1,4-dithiothreitol (DTT), double distilled water (ddH₂O), sodium chloride (NaCl), and 10X PARP Buffer (Tris base, magnesium chloride (MgCl₂•6H₂O), bovine serum albumin (BSA), 1.78M hydrochloric acid (HCl), and ddH₂O) to remove excess proteins. A transfer of supernatant to a new tube was centrifuged at 4.0 rcf for 10 minutes. After 10 minutes the supernatant is discarded and the nuclear fraction remains. The 1X PARP buffer was added to the nuclear fraction for each sample, with the addition of glycerol, and was stored at -80°C in order to prevent degradation of proteins. Protein concentration quantification was determined utilizing the biuret method for a bicinchoninic acid (BCA) assay prior to PARP activity measurements for sample loading. Samples were diluted with saline and working reagents A and B were combined and added to change protein samples from green to purple during a 30 minute incubation at 37°C . (See Figure 5.) Concentrations of protein were calculated for 20 μg of protein/well and diluted with 1X PARP buffer on Day 3 of PARP activity measurement. PARP activity measurement consisted of a three day process using an enzyme-linked immunosorbent assay (ELISA) microplate, (Day 1) plating of histones, (Day 2) blocking step, (Day 3) PARP activity detection. Day 1 consisted of preparing a histone coating solution consisting of sodium carbonate (Na₂CO₃), sodium bicarbonate (NaHCO₃), stock histone solution (Fraction V Histones), and ddH₂O, and coating each well of a 96-well plate with histone coating solution. Day 2 consisted of a blocking step that began with a washing step with phosphate-buffered saline (PBS) of the 96-well ELISA microplate with 1X PBS and 1X PBS + 0.05%

Tween-20. Washing steps were followed by the addition of a 3% BSA blocking solution to each well. After Day 1 and Day 2 procedure was conducted, microplate was stored at -20°C. Day 3 consisted of measurement of PARP activity where sample dilutions were calculated from protein quantification conducted prior to Day 3 assay. PARP-High Specific Activity Enzyme (HSA) was used to create calibration curve. Assay processing begins with a 1X PBS and 1X PBS + 0.05% Tween-20 washing step, followed by the addition of samples diluted with 1X PARP buffer to the 96-well ELISA microplate. Reagents used on Day 3 consists of 1X PARP substrate (6-biotin-17-nicotinamide-adenine-dinucleotide (NAD^B), sheared herring sperm DNA, 10X PARP buffer, and ddH₂O) (1 hour incubation at room temperature), 1:60,000 streptavidin-conjugated horseradish peroxidase (streptavidin-HRP)) (1 hour incubation at room temperature), 3,3',5,5'-tetramethylbenzidine (TMB) (20 minute incubation at room temperature), and 0.2M HCl (10 minute incubation at room temperature). After the addition of each reagent to the 96-well ELISA microplate a washing step is conducted with 1X PBS and 1X PBS + 0.05% Tween-20. The microplate was read at 450 nm using an ELISA microplate reader at the end of the Day 3 procedure. (See Figure 6.) A standard curve using a fitted regression model was used to quantify samples. PARP activity was reported as IU per 20 µg of protein per well.

Table 5. PARP Activity for Cocaine.

Treatment Group	Chemicals	Dose	Time
Group I	Vehicle Only	0 mg/kg	2 hr, 6 hr, 18 hr, 24 hr
Group II	Cocaine	50 mg/kg	2 hr
Group II	Cocaine	50 mg/kg	6 hr
Group II	Cocaine	50 mg/kg	18 hr
Group II	Cocaine	50 mg/kg	24 hr

Table 6. PARP Activity for APAP.

Treatment Group	Chemicals	Dose	Time
Group I	Vehicle Only	0 mg/kg	2 hr, 6 hr, 18 hr, 24 hr
Group III	APAP	300 mg/kg	2 hr
Group III	APAP	300 mg/kg	6 hr
Group III	APAP	300 mg/kg	18 hr
Group III	APAP	300 mg/kg	24 hr



Figure 5. Protein Quantification. A endpoint colorimetric bicinchoninic acid (BCA) assay used to quantify the concentration of protein used for PARP activity analysis.

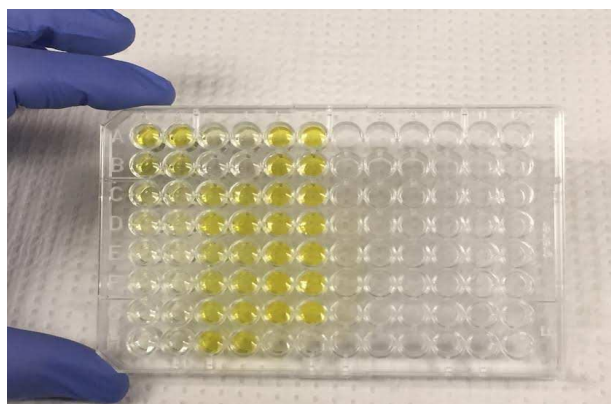


Figure 6. PARP Activity. A resultant PARP analysis assay with a 96-well microplate for an endpoint colorimetric assay measured by spectrophotometry.

Poly(ADP-ribose) Polymerase (PARP) Inhibition

An inhibitory efficacy study was conducted to determine if a PARP inhibitor, DIQ (AdipoGen), reduced ALT and PARP activity. A 10 mg/kg dose of DIQ was dissolved in warm saline and administered via IP injection at the time points of 0 hour, concurrent with treatment dose, or 1 hour after treatment dose. PARP inhibition analyses were conducted at time points where PARP activity was highest to measure any reductions in PARP activity that occurred following cocaine and APAP treatment. (See Tables 7 & 8.) Cocaine PARP inhibition analyses were conducted at the 6 hour time point. APAP PARP inhibition analyses were conducted at the 18 hour time point. The effect of DIQ on PARP inhibition was also analyzed along with glutathione (GSH), DNA fragmentation, and Western Blot.

Table 7. PARP Inhibition: Activity, ALT, GSH, DNA Fragmentation, and Western Blot for Cocaine + DIQ at the 6 hour Time Point.

Treatment Group	Chemicals	Dose	Time
Group I	Vehicle Only	0 mg/kg	0 hr, 1 hr
Group II	Cocaine Only	50 mg/kg	0 hr, 1 hr
Group IV	Cocaine + DIQ	50 mg/kg + 10 mg/kg	0 hr
Group IV	Cocaine + DIQ	50 mg/kg + 10 mg/kg	1 hr

Table 8. PARP Inhibition: Activity, ALT, GSH, DNA Fragmentation, and Western Blot for APAP + DIQ at the 18 hour Time Point.

Treatment Group	Chemicals	Dose	Time
Group I	Vehicle Only	0 mg/kg	0 hr, 1 hr
Group III	APAP Only	300 mg/kg	0 hr, 1 hr
Group V	APAP + DIQ	300 mg/kg + 10 mg/kg	0 hr
Group V	APAP + DIQ	300 mg/kg + 10 mg/kg	1 hr

Glutathione

An analysis was conducted to determine the role of glutathione (GSH) on PARP activity. A modified colorimetric kinetic glutathione assay measured the ratio of the reduced form of glutathione to its oxidized form by Griffith and Rahman et al (Griffith, 1980; Rahman, Kode, & Biswas, 2006). Livers were isolated and perfused with normal saline, and stored at -80°C until needed for assay. Prior to assay a 30 ± 10 mg sample of liver tissue was homogenized with a mortar and pestle on ice and centrifuged at 4.0 rcf for 10 minutes in a GSH extraction reagent (metaphosphoric acid (MPA), 5-sulfosalicylic acid, and double distilled water (ddH₂O)). The homogenized tissue for each sample was stored at -80°C in order to prevent degradation. Aliquot samples were transferred into two tubes for measurement of total glutathione and oxidized glutathione. Samples were analyzed utilizing a 96-well microplate. Standards and samples were diluted with KPE buffer (0.1 M KH₂PO₄, 97.6 mM K₂HPO₄, Na₂EDTA, and ddH₂O) prior to assay. Reagents for assay include 0.066% w/v Ellman's Reagent (DTNB), 0.066% w/v β-NADPH, and glutathione reductase (10 Units/mL), working substrate reagent (1:1 DTNB:glutathione reductase), 2-vinylpyridine working reagent, and triethanolamine working reagent. Total glutathione procedures were conducted first, followed by oxidized glutathione. The microplate was read five times at 412 nm using a microplate reader at the end of each procedure. Oxidized glutathione is calculated from total glutathione to compute a reduced to oxidized glutathione ratio.

DNA Fragmentation

A DNA fragmentation analysis using a modified Herrmann et al. method was conducted to determine if fragmented DNA results from hepatotoxic events with cocaine and APAP

overdose (Hermann, Lorenz, Voll, Grünke, Woith, and Kalden, 1994). DNA fragmentation analyses were conducted with cocaine, APAP, cocaine + DIQ, and APAP + DIQ samples with 1.6% agarose gel. (See Figure 7.) The two day assay procedure consisted of tissue preparation (Day 1) and DNA analysis (Day 2). Livers were isolated and perfused with normal saline, and stored at -80°C until needed for assay. Day 1 consisted of tissue preparation of a 20 ± 10 mg sample of liver tissue saturated in a LB_{9.0} buffer (1M Tris base, 500mM ethylenediaminetetraacetic acid (EDTA), 4M sodium chloride (NaCl), 10% sodium dodecyl sulfate (SDS), 1M sodium hydroxide (NaOH), 10 mg/mL proteinase K, autoclaved ddH₂O) and placed on a heating block/dry bath to dissolve for at least 12 hours. Day 2 of assay included addition of the reagents RNase, 4M sodium acetate, and 70% ethanol. Samples were placed in a centrifuge for 10 minutes at 14 rcf. Samples were incubated for 1 hour at -20°C. After 1 hour, a small volume of each sample was transferred to a new microcentrifuge tube and XCog (10 mM Tris base, 12 mM EDTA, 50% v/v Glycerol, 0.03% w/v Xylene Cyanol, 0.15% w/v Orange G, and HCl) was added to each sample before samples were added to wells. A resuspension buffer (3 mM Tris base, 0.2 mM EDTA, and HCl) was used as a loading control. A 1.6% agarose gel with ethidium bromide was used to run DNA samples in a 1X TAE buffer solution (Sigma-Aldrich) at 75V for 2 hours. After 2 hours, gel was placed on UV light and photos were taken.

Western Blot

A western blot analysis was conducted to identify PARP protein sequences in liver tissues for a 50 mg/kg dose of cocaine at the 6 hour time point and a 300 mg/kg dose of APAP at the 18 hour time point. Livers were isolated and perfused with normal saline, and stored at -80°C

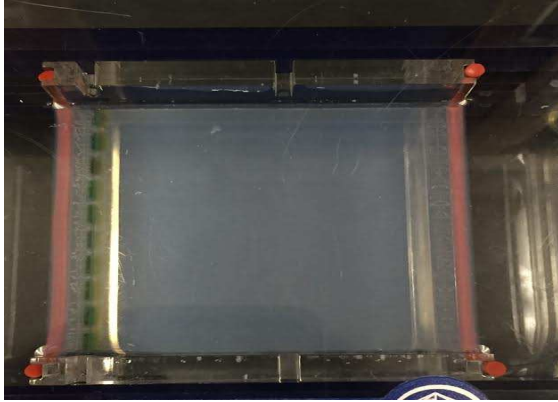


Figure 7. DNA Fragmentation Electrophoresis Gel. An electrophoresis gel used to analyze treatment samples for fragmented DNA.

until needed for assay. Prior to assay a 10 ± 10 mg sample of liver tissue was homogenized with a mortar and pestle on ice and centrifuged at 14.0 rcf for 5 minutes in a radioimmunoprecipitation assay (RIPA) buffer (1M Tris base, 500mM ethylenediaminetetraacetic acid (EDTA), 4M sodium chloride (NaCl), 10% sodium dodecyl sulfate (SDS), Triton X-100, 1.78M HCl, deoxycholic acid, and autoclaved ddH₂O). After centrifugation, sample lysate was stored at -80°C until time of assay to prevent degradation of proteins. Protein concentration quantification was determined utilizing the biuret method for a BCA assay prior to western blot analysis. Samples were diluted with saline and working reagents A and B were combined and added to change protein samples from green to purple during a 30 minute incubation at 37°C. Concentrations of protein were calculated for 10-15U of protein/well and diluted with saline and Laemmli sample buffer prior to assay. Diluted samples + Laemmli sample buffer were loaded on a 4-10.5% Tris-glycine polyacrylamide gel for electrophoresis at 12V. Proteins were transferred to nitrocellulose membranes, which were incubated in 3% BSA blocking buffer (30 minute incubation at room temperature), 1:10,000 primary antibodies (one hour incubation at room temperature), 1:50,000 secondary antibodies (1 hour incubation at room

temperature), and streptavidin-HRP (25 minutes at room temperature). After the addition of each reagent, membranes were washed with Tris-buffered saline + 0.1% Tween-20 (TBS-T).

Resultant nitrocellulose and gels were quantified using a Thermo Scientific PageRuler Unstained Protein Ladder (10 to 200 kDa). (See Figure 8.) The molecular weight for PARP measures at 116 kDa (Boulares et al. 1999; Hong, Dawson, Dawson, 2004).

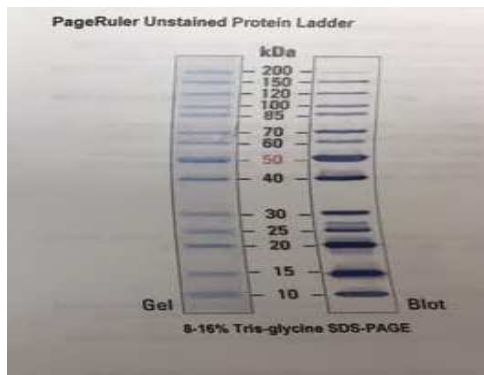


Figure 8. Western Blot Protein Ladder. A protein ladder display used to measure the molecular weight for PARP at 116 kDa.

Gross Pathology

Livers were isolated and partially perfused with normal saline following necropsy of each animal. Visual findings were recorded to correlate with ALT and PARP activity. Photographs were taken at the 24 hour time point for a 50 mg/kg dose of cocaine, a 300 mg/kg dose of APAP, and controls.

Spectrophotometry

Assays requiring colorimetric endpoint measurements (ALT, PARP, PARP Inhibition, and GSH) were quantified using the μ Quant Spectrophotometer (BioTek, Winooski, VT) with

the KC-Junior Analytical software (BioTek). (See Figure 9.) Each colorimetric endpoint was assessed at the wavelength stated within the assay section above.



Figure 9. μQuant Spectrophotometer. The spectrophotometer used to read microplates and quantify biochemical medium as the amount of absorbed light that is proportionate to the concentration of the measured solution.

Statistical Analysis

Statistical analysis was performed utilizing Microsoft Excel and the software program, SAS version 9.3. A one-way analysis of variance (ANOVA) test was performed to test comparisons among groups. Comparisons between treatment groups were conducted with independent sample t-tests. Statistical differences were considered significant with $p \leq 0.05$. Adjustments for multiple comparisons were conducted utilizing a Tukey test.

CHAPTER FOUR:

RESULTS

Dose-Response

A dose-response analysis was conducted for cocaine and acetaminophen (APAP) to determine the optimal dose that produces hepatotoxic events. A single dose of cocaine or APAP was administered via intraperitoneal (IP) injections (n=6) and evaluated animals 24 hours after treatment. A measurement of the alanine aminotransferase (ALT) was chosen to analyze the severity of liver damage that resulted from drug toxicity. The determined optimal dose for both toxicants was used to analyze poly(ADP-ribose) polymerase (PARP) activity.

Serum ALT (IU/L) measurements were conducted for a 20 mg/kg, 30 mg/kg, 40 mg/kg, and 50 mg/kg, and 60 mg/kg dosages of cocaine at 24 hours following treatment. (See Figure 10.) Elevated serum ALT levels were measured for each dose. ALT increased incrementally for the 20, 30, 40, and 50 mg/kg dosages. ALT for the control group ranged from 30-40 IU/L. The highest ALT level of 1749 IU/L was observed at the 50 mg/kg dose group. A decreased level of 1362 IU/L was observed with the 60 mg/kg dose. The 60 mg/kg dose resulted in severe hemorrhaging that caused a decrease in the amount of drug metabolized by the liver. This resulted in a decrease in ALT levels observed from the 50 to 60 mg/kg dose groups. Each dose group was statistically significantly increased at a ($p < 0.05$) significance level when compared to controls.

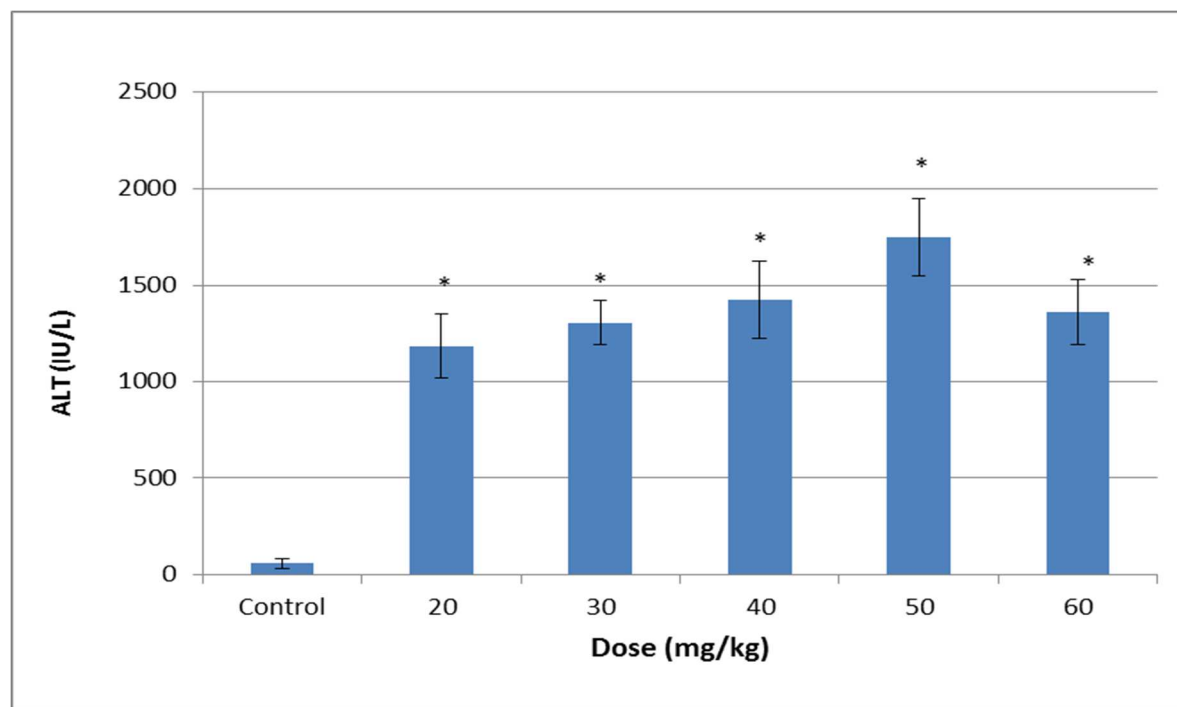


Figure 10. Cocaine Dose-Response Analysis. A dose response analysis was conducted for a 20 mg/kg, 30 mg/kg, 40 mg/kg, 50 mg/kg dose, and 60 mg/kg dose of cocaine at 24 hours following treatment (n=6). Mean serum ALT levels with standard deviations are denoted with error bars. An (*) denotes statistical significance at ($p < 0.05$) when compared to controls.

Serum ALT measurements were also conducted for 75 mg/kg, 150 mg/kg, 200 mg/kg, and 300 mg/kg doses of acetaminophen (APAP) at 24 hours following treatment (See Figure 11.) ALT for the control group ranged from 30-40 IU/L. Each dose group resulted in elevations in ALT. Serum ALT levels for the 75 mg/kg (45 IU/L) and 150 mg/kg (316 IU/L) dose groups did not result in significant elevations of ALT. A 200 mg/kg dose of APAP resulted in a significant increase in serum ALT of 2252 IU/L. Higher serum measurements in ALT were observed with the 200 mg/kg and 300 mg/kg dose groups. The highest observed serum ALT was measured with the 300 mg/kg dose group at 4311 IU/L. Each dose group was statistically significant at a ($p < 0.05$) significance level when compared to controls.

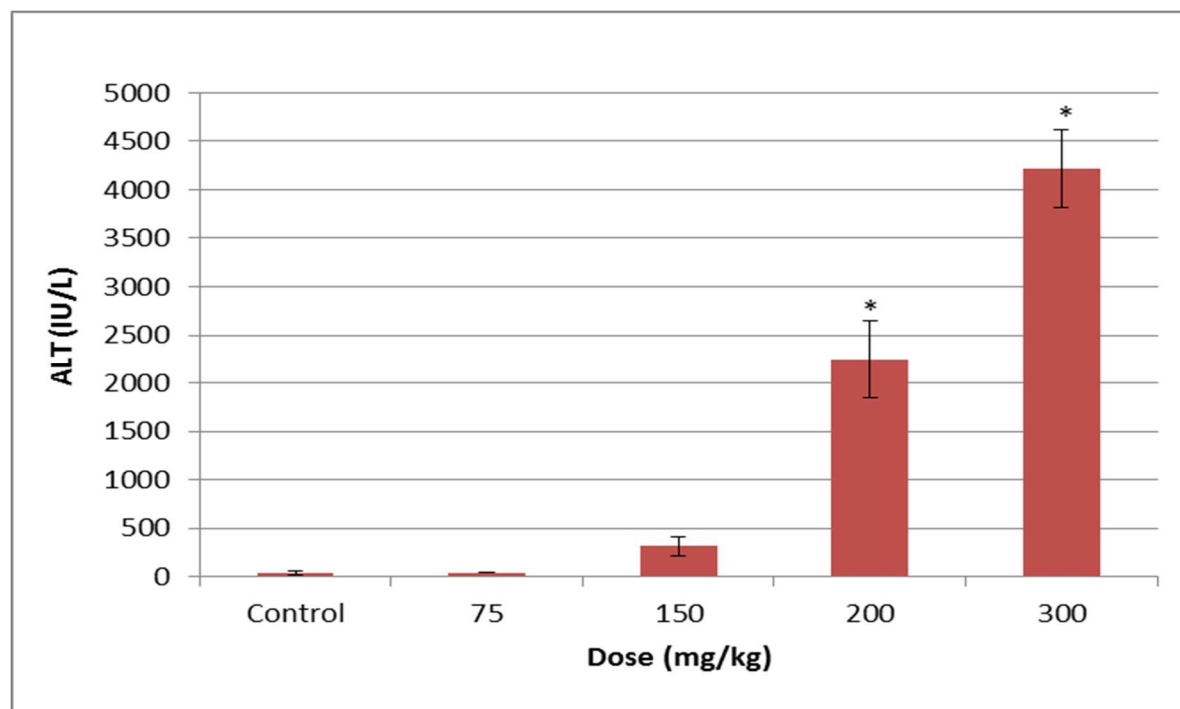


Figure 11. APAP Dose-Response Analysis. A dose-response analysis comparing serum ALT was conducted for a 75 mg/kg, 150 mg/kg, 200 mg/kg, and 300 mg/kg dose of acetaminophen (APAP) at 24 hours following treatment (n=6). Standard deviations are denoted with error bars. An (*) denotes statistical significance at ($p < 0.05$) when compared to controls.

Temporal Analysis

A temporal analysis was conducted for cocaine and acetaminophen (APAP) to determine the optimal time to measure PARP activity. A single dose of cocaine or APAP was administered via intraperitoneal (IP) injection (n=6) where serum and livers were harvested at 2 hour, 6 hour, 18 hour, and 24 hour time points. A measurement of serum ALT (IU/L) was chosen to analyze the severity of liver damage that resulted from drug-induced toxicity at each time point. The optimal time point for both toxicants was based on the observed serum ALT levels following drug treatment to analyze poly(ADP-ribose) polymerase (PARP) activity.

A 50 mg/kg dose of cocaine was determined to be the optimal dose that produced hepatotoxicity but did not cause death. This 50/kg dose of cocaine was used to conduct a

temporal analysis of serum ALT levels over a 24 hour period. ALT levels were conducted at a 2 hour, 6 hour, 18 hour, and 24 hour time point following treatment. (See Figure 12.) Serum ALT for the control group ranged from 30-40 IU/L. Serum ALT levels were measured at each time point. An initial increase in ALT at the 2 hour following treatment time point was measured at 450 IU/L. A significant increase in serum ALT was observed from the 2 to 6 hour time point following treatment with a measurement of 1847 IU/L observed at the 6 hour time point. Serum ALT increased incrementally during the 6, 18, and 24 hour time points following treatment. The highest observed serum ALT level of 3231 IU/L was observed at the 24 hour time point. Serum ALT measurements at the 6 hour, 18 hour, and 24 hour time points were statistically significant at a ($p < 0.05$) significance level when compared to controls.

Liver samples from a saline control and a 50 mg/kg dose of cocaine were collected at a 24 hour time point following treatment. (See Figure 13.) When compared to the control liver sample, the cocaine liver appears smaller in size, including abnormal shapes of the lobes of the liver sample. Some hemorrhaging and necrosis appears on the surface of the liver. Mottling on the surface of the liver was also observed at the 24 hour time point when compared to controls.

Serum ALT measurements were conducted at a 2 hour, 6 hour, 18 hour, and 24 hour time point following APAP treatments. (See Figure 14.) Serum ALT for the control group ranged from 30-40 IU/L. Elevated ALT levels were measured for each time point. A significant increase in ALT was observed at the 2 hour time point with a measurement of 1507 IU/L. ALT increased incrementally at each time point. The highest observed serum ALT of 4994 IU/L was observed at the 24 hour time point. ALT measurements at the 2 hour, 6 hour, 18 hour, and 24 hour time points were statistically significant at a ($p < 0.05$) significance level when compared to controls.

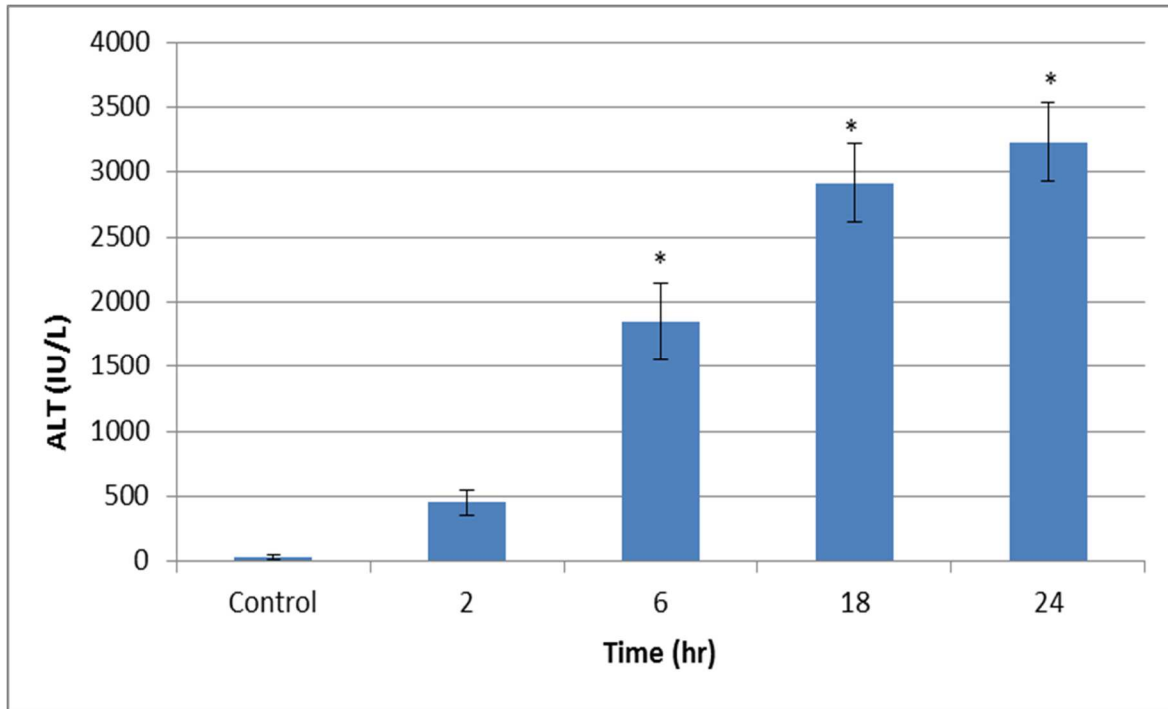


Figure 12. Cocaine Temporal Analysis. A temporal analysis for a 50 mg/kg dose of cocaine was conducted at a 2 hour, 6 hour, 18 hour, and 24 hour time points following treatment (n=6). Standard deviations are denoted with error bars. An (*) denotes statistical significance at ($p < 0.05$) when compared to controls.

Liver samples from a saline control and a 300 mg/kg dose of APAP treated animal were collected at 24 hours following treatment. (See Figure 15.) When compared to the control liver sample, lobes of the liver appear smaller in size when compared to controls. Significant hemorrhaging and necrosis appears throughout the liver. Slight discoloration is observed on the outer edges of the APAP liver sample.

A comparison of serum ALT for a 50 mg/kg dose of cocaine and a 300 mg/kg dose of APAP at a 2 hour, 6 hour, 18 hour, and 24 hour time points following treatment. (See Figure 16.)



Figure 13. Gross Pathology: Control + 50 mg/kg Cocaine. Liver samples were harvested for a saline control (left) and a 50 mg/kg dose of cocaine (right) at 24 hours following treatment.

ALT increased at a much faster rate following APAP treatment compared to cocaine. A significant increase in serum ALT following cocaine treatment was observed at the 2 and 6 hour time point. A significant increase was observed at the 18 hour and 24 hour time points for both toxicants following treatments.

The line graph comparison for cocaine and APAP-induced serum ALT changes at a 2 hour, 6 hour, 18 hour, and 24 hour time points following treatments shows a steady increase in ALT for APAP from the 2 hour to 24 hour time point when compared to controls. (See Figure 17.) The graph also shows a steady increase from the 2 hour to 18 hour time point for cocaine. A much smaller increase was observed for cocaine from the 18 hour to 24 hour time points when compared to controls.

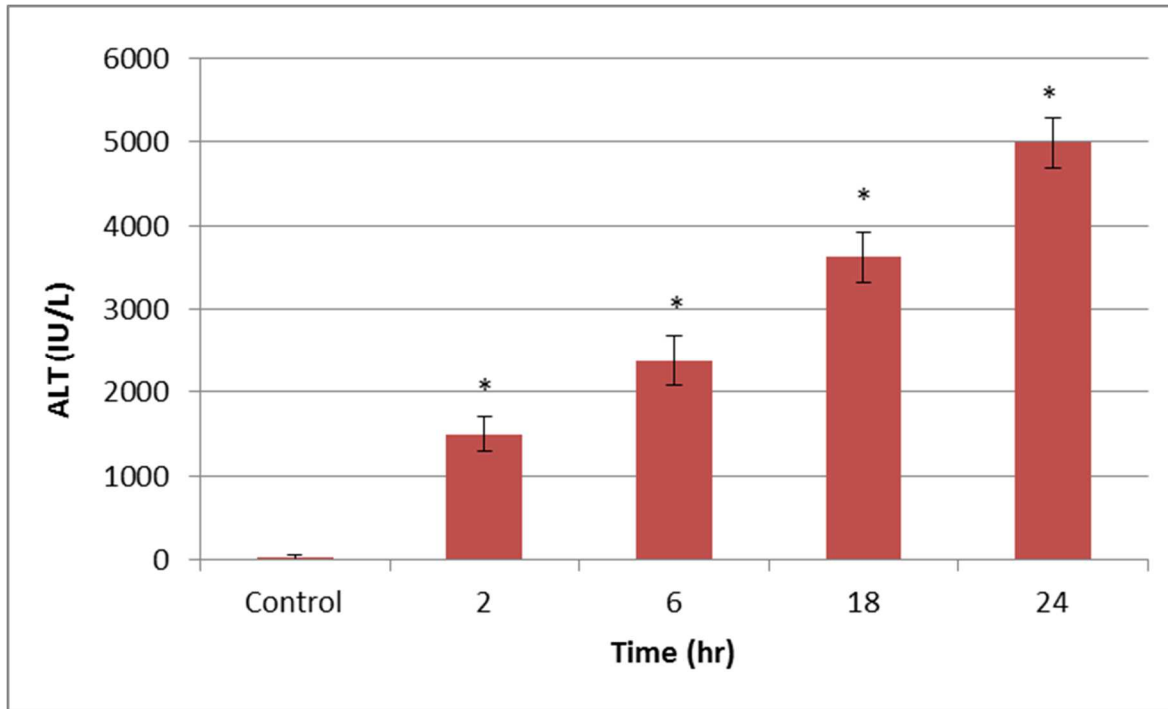


Figure 14. APAP Temporal Analysis. A temporal analysis for a 300 mg/kg dose of APAP was conducted at a 2 hour, 6 hour, 18 hour, and 24 hour time points following treatment (n=6). Standard deviations are denoted with error bars. An (*) denotes statistical significance at ($p < 0.05$) when compared to controls.

Poly(ADP-Ribose) Polymerase (PARP) Activity

A 50 mg/kg dose of cocaine and a 300 mg/kg dose of acetaminophen (APAP) were determined to be the optimal dose to analyze PARP activity. This optimal dose for cocaine and APAP was used to conduct a PARP activity analysis over a 24 hour period. PARP activity (IU/20 μ g of protein) was measured at a 2 hour, 6 hour, 18 hour, and 24 hour time point following cocaine and APAP treatments. An ALT and PARP activity analysis was conducted for both cocaine and APAP-induced hepatotoxicity.



Figure 15. Gross Pathology: Liver samples were harvested for a saline control (left) and a 300 mg/kg APAP dose (right) at 24 hours following treatment.

PARP activity was measured at a 2 hour, 6 hour, 18 hour, and 24 hour time point following treatment with a 50 mg/kg dose of cocaine. (See Figure 18.) Activation of PARP activity was measured at each time point. Elevations in PARP activity were measured for each time point. PARP activity for the control measured at 0.2 IU/20 μ g of protein. Cocaine increased PARP activity at the 2 hour time point following treatment. The highest activity was 1.43 IU/20 μ g of protein was measured at the 6 hour time point. An increase in activity was also measured from the 2 to 6 hour time point. A decrease in PARP activity was also observed from the 6 to 18 hour time point but remained significantly increased above controls. The lowest amount of PARP activity was measured at the 24 hour time point at 1.04 IU/20 μ g of protein. PARP activity measurements at the 2 hour, 6 hour, 18 hour, and 24 hour time points were statistically significantly elevated at a ($p < 0.05$) significance level when compared to controls.

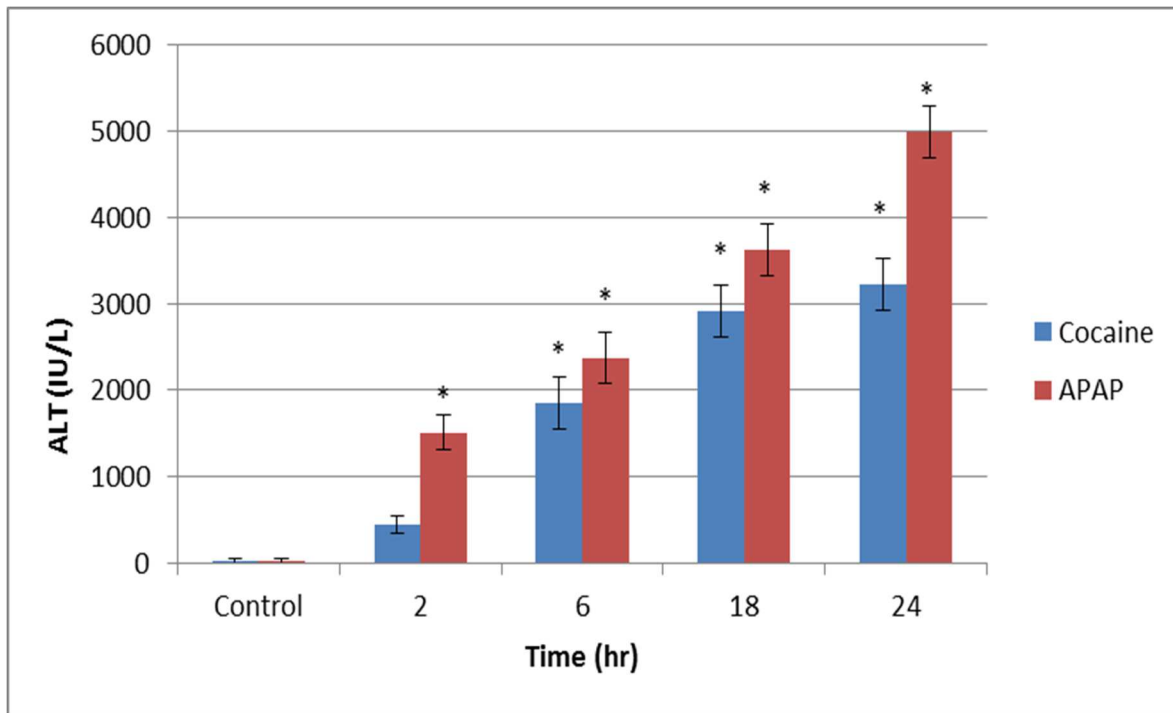


Figure 16. Cocaine and APAP Temporal Analysis: Bar Chart. A bar chart comparison of the serum ALT temporal differences between a 50 mg/kg dose of cocaine and a 300 mg/kg dose of APAP at a 2 hour, 6 hour, 18 hour, and 24 hour time points following treatments (n=6). Standard deviations are denoted with error bars. An (*) denotes statistical significance at ($p < 0.05$) when compared to controls.

PARP activity increased in liver tissue at a 2 hour, 6 hour, 18 hour, and 24 hour time point following a 50 mg/kg dose of cocaine. (See Figure 19) A seven fold increase for PARP activity shows that the largest increase in PARP activity was observed at the 6 hour time point when compared to controls. Lower increases were observed at the 18 and 24 hour time points. The smallest increase was observed at the 24 hour time point with a 5.60 increase measured.

PARP activity was measured at 2 hour, 6 hour, 18 hour, and 24 hour time point for a 300 mg/kg dose of APAP. (See Figure 20.) An activation of PARP activity was measured at each time point. Elevations in PARP activity were measured for each time point. PARP activity for

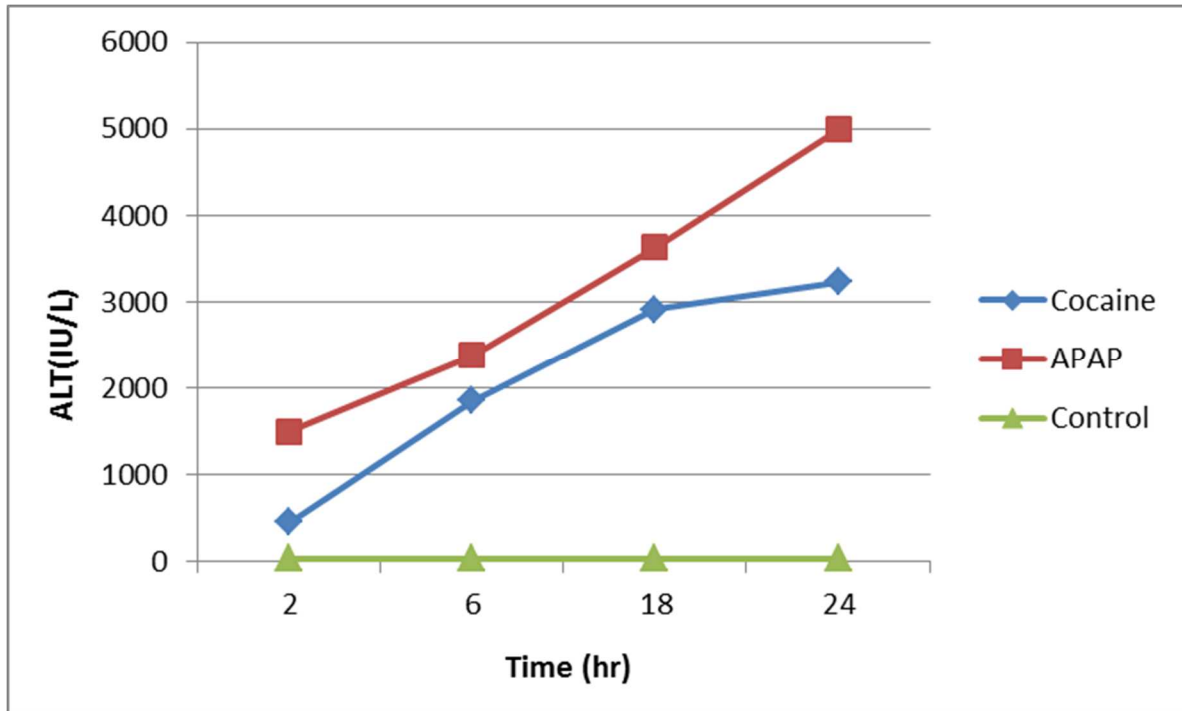


Figure 17. Cocaine and Temporal Analysis: Line Graph. A line graph comparison of the temporal analysis for serum ALT following a 50 mg/kg dose of cocaine and a 300 mg/kg dose of APAP was conducted at a 2 hour, 6 hour, 18 hour, and 24 hour time points following treatment (n=6).

the control was 0.23 IU/20 μ g of protein. APAP increased PARP activity at the 2 hour and 6 hour time points. The highest activity of 1.55 IU/20 μ g of protein was measured at the 18 hour time point. A significant increase in activity was also measured at the 6 and 18 hour time points. A decrease in PARP activity was observed at the 18 and 24 hour time points but remained above the controls. The lowest amount of PARP activity was measured at the 24 hour time point at 1.23 IU/20 μ g of protein. Increases in PARP activity at the 2 hour, 6 hour, 18 hour, and 24 hour time points were statistically significant at a ($p < 0.05$) significance level when compared to controls.

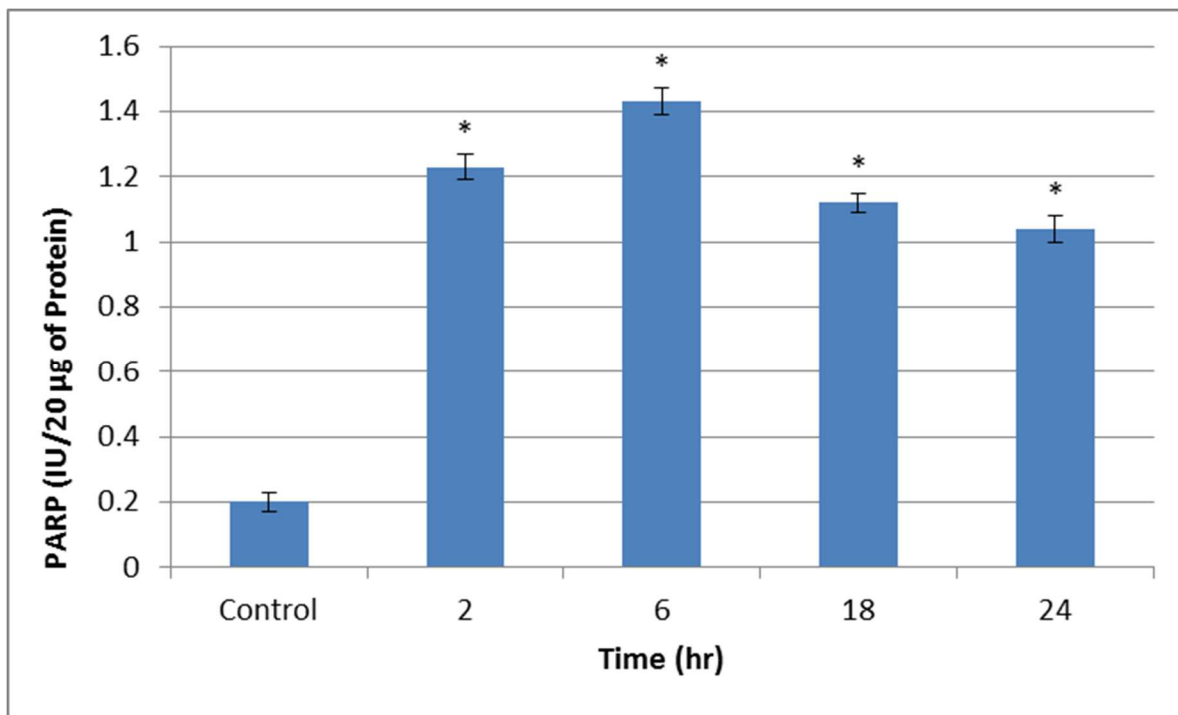


Figure 18. PARP Activity for Cocaine. A PARP analysis of liver tissue following 50 mg/kg dose of cocaine at 2 hour, 6 hour, 18 hour, and 24 hour time points following an initial dose (n=6). Standard deviations are denoted with error bars. An (*) denotes statistical significance at ($p < 0.05$) when compared to controls.

Increases in PARP activity were measured at the 2 hour, 6 hour, 18 hour, and 24 hour time points following a 300 mg/kg dose of APAP. (See Figure 21.) The largest increase was observed at the 18 hour time point with a measurement of 7.75. A lower level was observed at the 24 hour time point. The smallest increase was observed at the 2 hour time point with a measurement of a 2.50 fold increase.

A comparison of liver PARP activity following a 50 mg/kg dose of cocaine and a 300 mg/kg dose of APAP at a 2 hour, 6 hour, 18 hour, and 24 hour time points following treatments. (See Figure 22.) A delayed elevation of PARP activity was observed following APAP treatment. Cocaine-induced PARP activity showed significant increases at the earlier time points. PARP

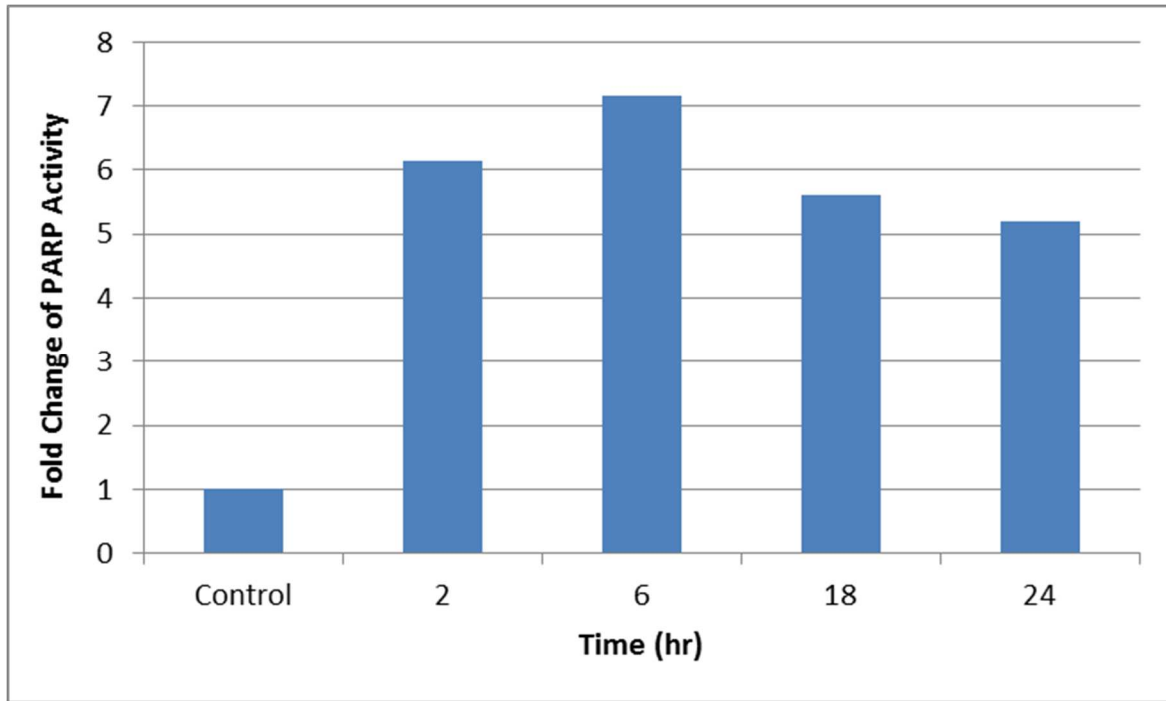


Figure 19. PARP Activity Fold Change for Cocaine. Increases in PARP activity following a 50 mg/kg dose of cocaine at 2 hour, 6 hour, 18 hour, and 24 hour time points following treatment (n=6).

activity levels are higher at the 2 and 6 hour time points for cocaine when compared to APAP. APAP-induced PARP activity levels are higher at the 18 and 24 hour time points. Cocaine and APAP showed a decrease in PARP activity from the 18 to the 24 hour time point.

The line graph comparison of increase in PARP activity following a 50 mg/kg dose of cocaine and a 300 mg/kg dose of APAP at 2 hour, 6 hour, 18 hour, and 24 hour time points following treatment shows a significant difference in PARP activity over the observed 24 hour period. (See Figure 23.) PARP activity was higher at early time points within the 24 hour observation period for cocaine treatments. PARP activity had a slower rate of increase for APAP treatments during the 24 hour observation period. APAP-induced PARP activity was higher following cocaine treatments during the 24 hour observation period.

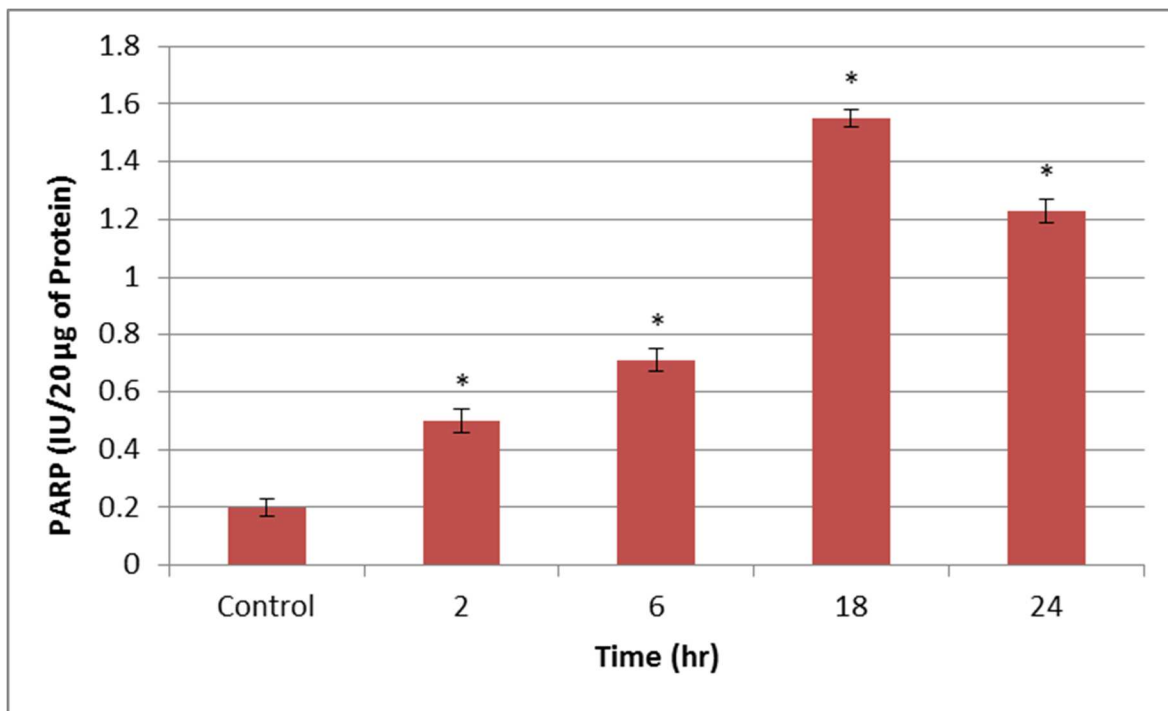


Figure 20. PARP Activity for APAP. A PARP activity for liver samples for a 300 mg/kg dose of APAP at a 2 hour, 6 hour, 18 hour, and 24 hour time points following treatment (n=6). Standard deviations are denoted with error bars. An (*) denotes statistical significance at ($p < 0.05$) when compared to controls.

Poly(ADP-Ribose) Polymerase (PARP) Inhibition

A 50 mg/kg dose of cocaine at a 6 hour time point and a 300 mg/kg dose of acetaminophen (APAP) at an 18 hour time point were determined to be the optimal doses and time to analyze PARP inhibition. These optimal doses for cocaine and APAP were used to evaluate PARP activity over a 24 hour period. These time points were chosen because the highest levels of PARP activity were observed for the optimal dose at the observed time points following treatment. A 10 mg/kg dose of 1,5-dihydroxyisoquinoline (DIQ), a PARP inhibitor, was used to evaluate the effect of PARP inhibition on cocaine and APAP-induced hepatotoxicity. An ALT and PARP activity analysis was conducted for both toxicants.

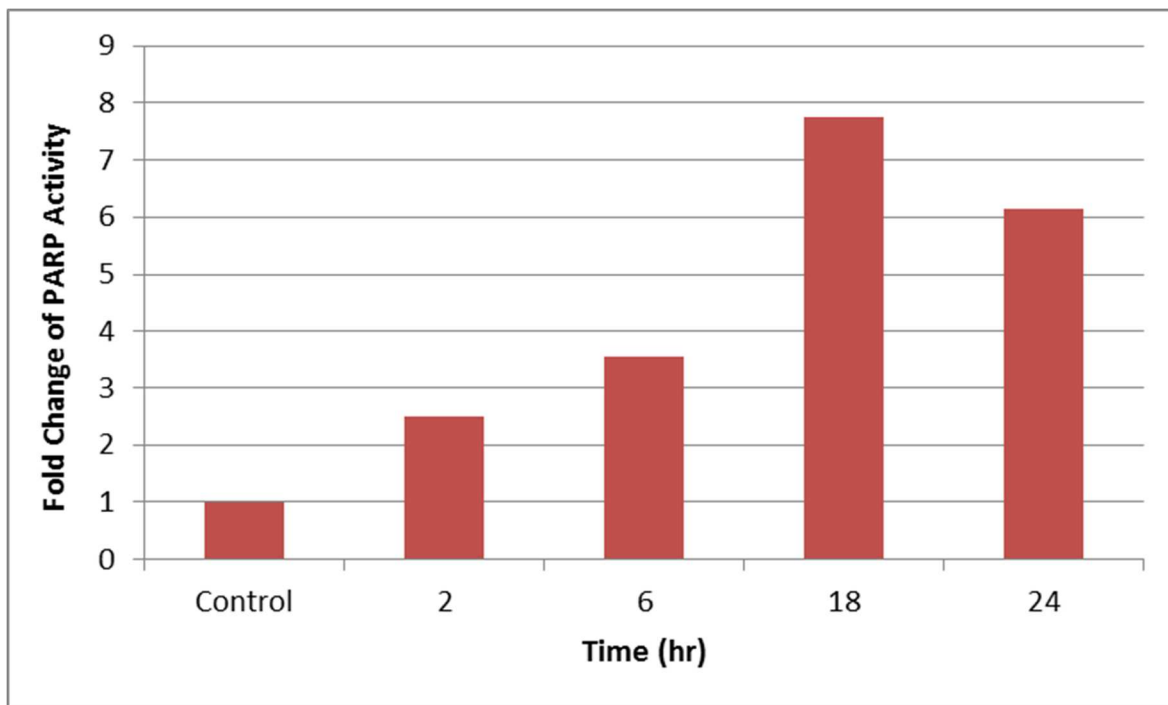


Figure 21. PARP Activity Fold Change for APAP. Increases in PARP activity following a 300 mg/kg dose of APAP at 2 hour, 6 hour, 18 hour, and 24 hour time points following an initial dose (n=6).

A PARP inhibition ALT analysis for a 50 mg/kg dose of cocaine at the 6 hour time point shows an elevation in serum ALT of 1677 IU/L following treatment. (See Figure 24.) A DIQ injection administered concurrently with cocaine significantly reduced serum ALT to 201 IU/L. DIQ administration at the 1 hour post cocaine treatment time point was not as effective in reducing elevations in serum ALT.

DIQ treatment reduced cocaine-induced increased PARP activity by two fold. (See Figure 25.) DIQ treatment administered one hour following cocaine was not as effective as concurrent treatment. A larger reduction in PARP activity resulted from concurrent treatment of DIQ when compared to the cocaine only dose group.

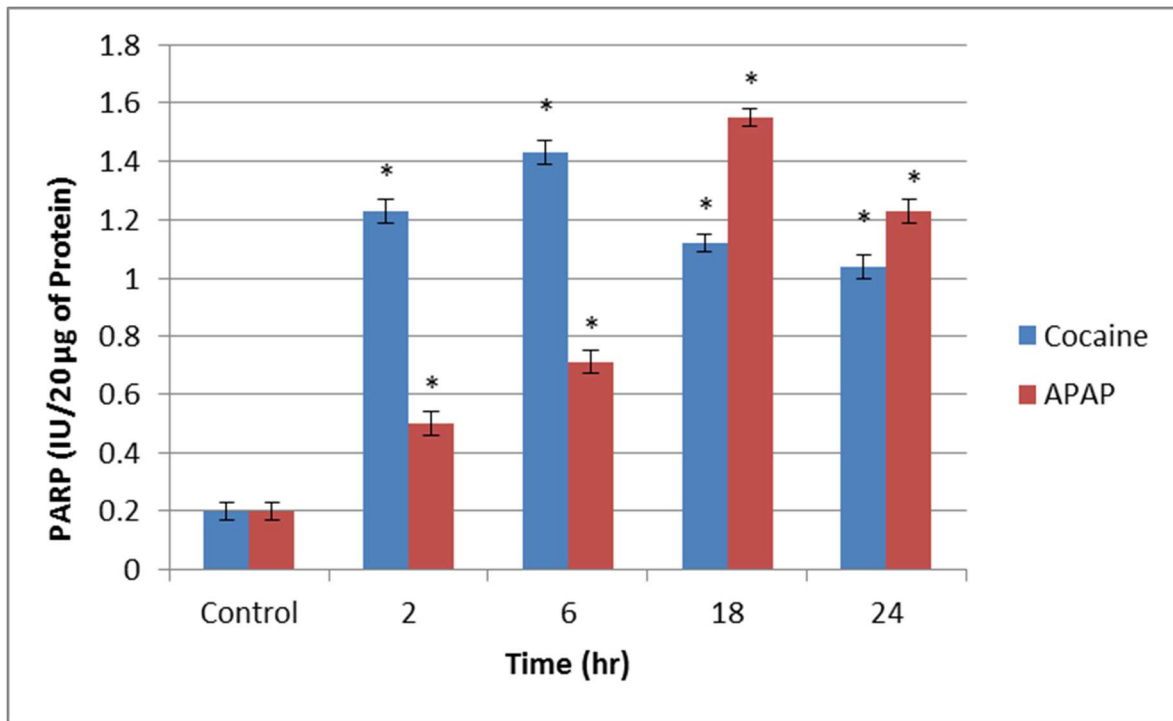


Figure 22. Cocaine and APAP PARP Analysis: Bar Chart. A bar chart comparison of liver PARP activity following a 50 mg/kg dose of cocaine and a 300 mg/kg dose of APAP was conducted at 2 hour, 6 hour, 18 hour, and 24 hour time points following treatments (n=6). Standard deviations are denoted with error bars. An (*) denotes statistical significance at (p < 0.05) when compared to controls.

A 300 mg/kg dose of APAP significantly increased ALT 18 hours following treatment with an observed measurement of 4003 IU/L. (See Figure 26). Concurrent treatment with DIQ decreased that elevation to 856 IU/L. An APAP-induced increase in ALT was observed when DIQ was administered 1 hour following APAP treatment. The elevation of ALT was also reduced by DIQ treatment. The effect was not as robust when compared to concurrent DIQ administration with cocaine treatment.

DIQ treatment decreased APAP-induced elevation of PARP activity by more than 4 fold at 18 hours following treatment. (See Figure 27.) DIQ decreased PARP activity with concurrent

treatment and treatment 1 hour following APAP dose. DIQ treatment 1 hour following APAP was less effective than concurrent treatment.

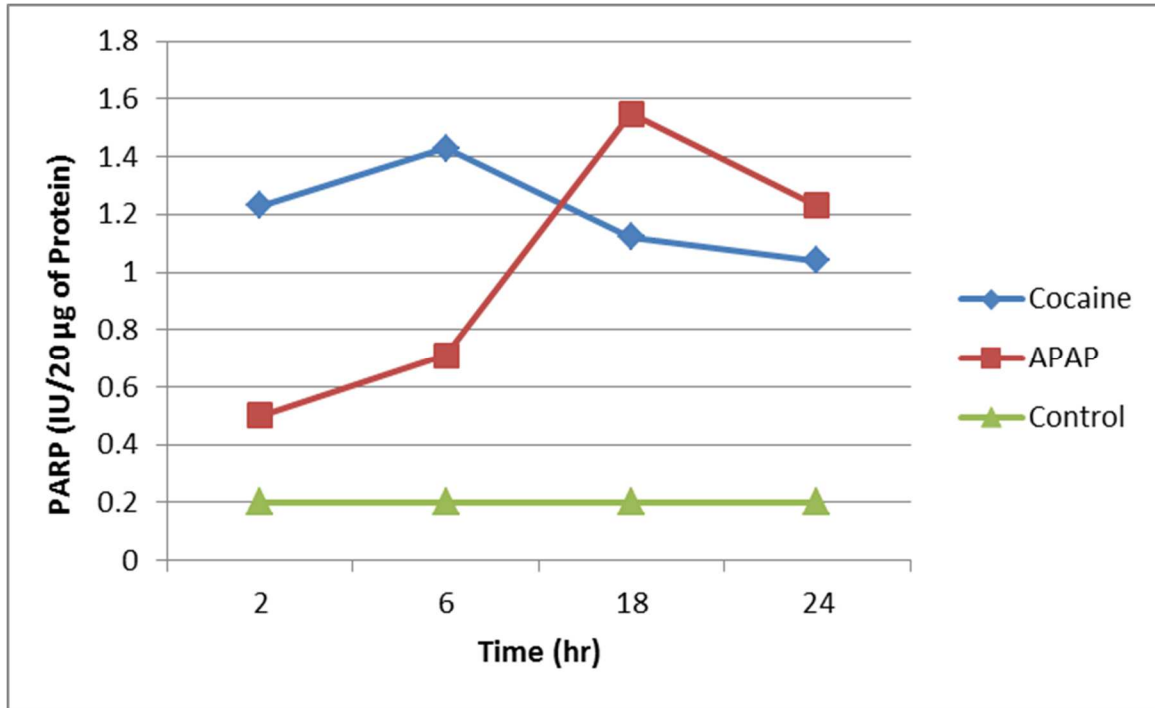


Figure 23. Cocaine and PARP Analysis: Line Graph. A line graph comparison of PARP activity for a 50 mg/kg dose of cocaine and a 300 mg/kg dose of APAP was conducted at 2 hour, 6 hour, 18 hour, and 24 hour time points following treatment (n=6).

A comparison of the effect of PARP inhibition on a 50 mg/kg dose of cocaine at a 6 hour time point and a 300 mg/kg dose of APAP at an 18 hour time point was measured at the stated time points. A DIQ dose was administered concurrently or 1 hour following drug treatment. (See Figure 28.) Higher serum ALT was measured for APAP treatments when compared to cocaine. The measured serum ALT for the both toxicants produced a significant decrease to elevations with DIQ administration at the 0 hour post treatment time point. A larger decrease in serum ALT was observed for APAP treatments with a concurrent DIQ administration. Increases for cocaine

and APAP-induced samples with DIQ administration were observed at the 1 hour post initial dose time point when compared to controls.

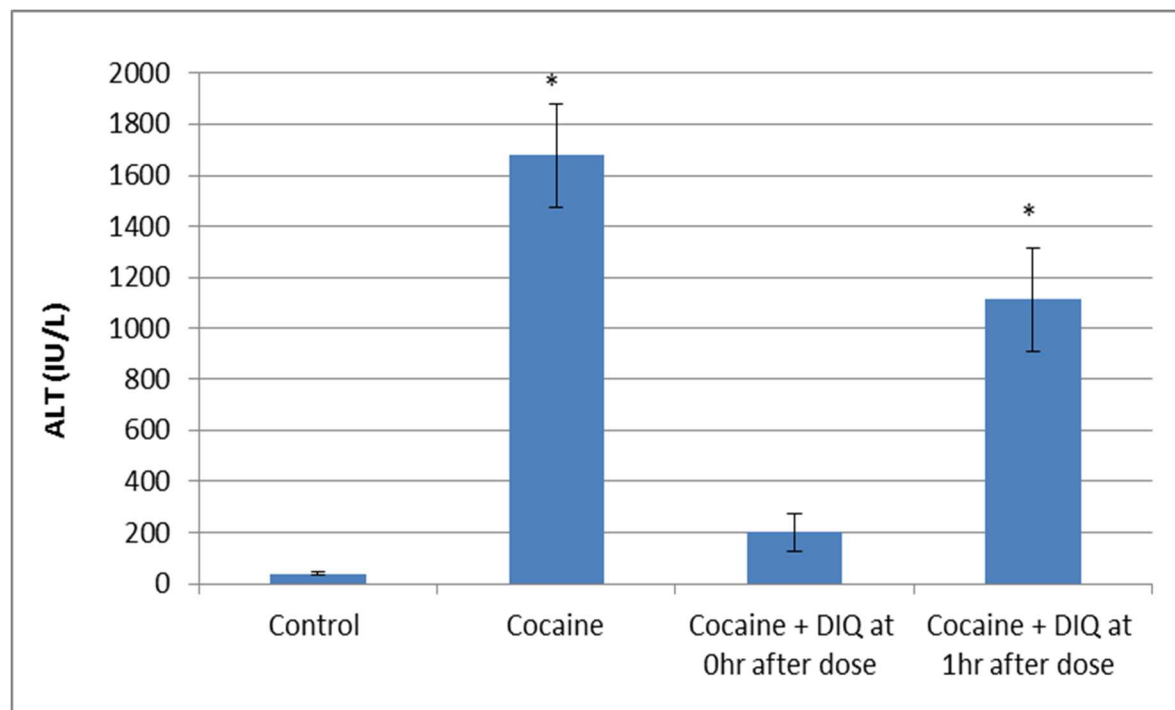


Figure 24. PARP Inhibition for Cocaine: ALT. A serum ALT PARP inhibition analysis following a 50 mg/kg dose of cocaine at the 6 hour time point with the administration of a PARP inhibitor, DIQ (n=6). Standard deviations are denoted with error bars. An (*) denotes statistical significance at ($p < 0.05$) when compared to controls.

A line graph comparison of serum ALT levels for PARP inhibition following a 50 mg/kg dose of cocaine at a 6 hour time point and a 300 mg/kg dose of APAP at an 18 hour time point was measured at the stated time points. Serum ALT levels were measured at a concurrent DIQ dose and 1 hour post initial dose time points. (See Figure 29.) The line graph shows a very similar pattern for both toxicants following DIQ-induced PARP inhibition when compared to controls. Steeper declines and increases were observed for APAP samples. A large decrease in serum ALT was observed with the concurrent administration of DIQ. A slight elevation was

observed when DIQ was administered at the 1 hour time point for both toxicants when compared to controls. A measurement for toxicant + DIQ at the 1 hour time point increases to serum ALT levels observed for the toxicant only treatment group for both drugs.

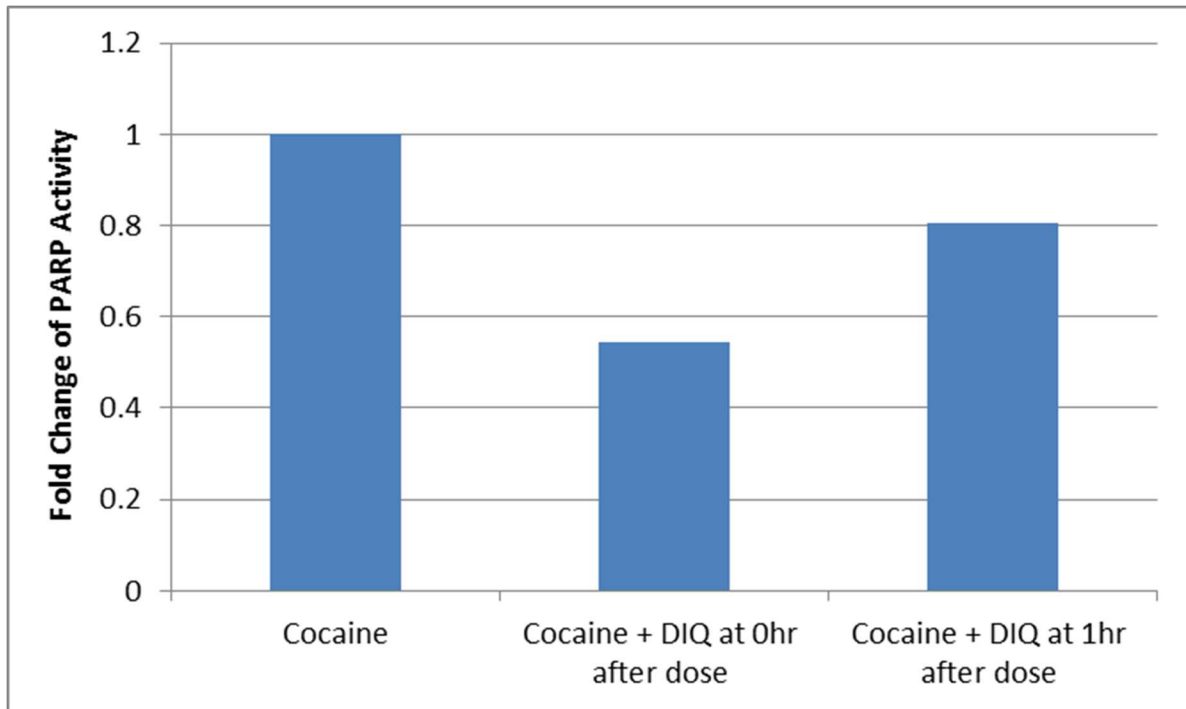


Figure 25. PARP Activity Fold Change for PARP Inhibition for Cocaine. Decreases in PARP activity following a 50 mg/kg dose of cocaine at a 6 hour time point of an initial dose with a concurrent 10 mg/kg DIQ treatment and a DIQ treatment 1 hour following drug treatment. (n=6).

A PARP activity analysis for PARP inhibition was conducted with a 50 mg/kg dose of cocaine at the 6 hour time point. (See Figure 30.) The cocaine treatment caused an activation of PARP and a large increase in PARP activity of 1.36 IU/20 μ g of protein when compared to controls. A decrease in PARP activity was observed for a concurrent dose of DIQ at 0.74 IU/20 μ g protein. DIQ administered 1 hour following cocaine treatment was not as effective with a measurement of 1.09 IU/20 μ g protein.

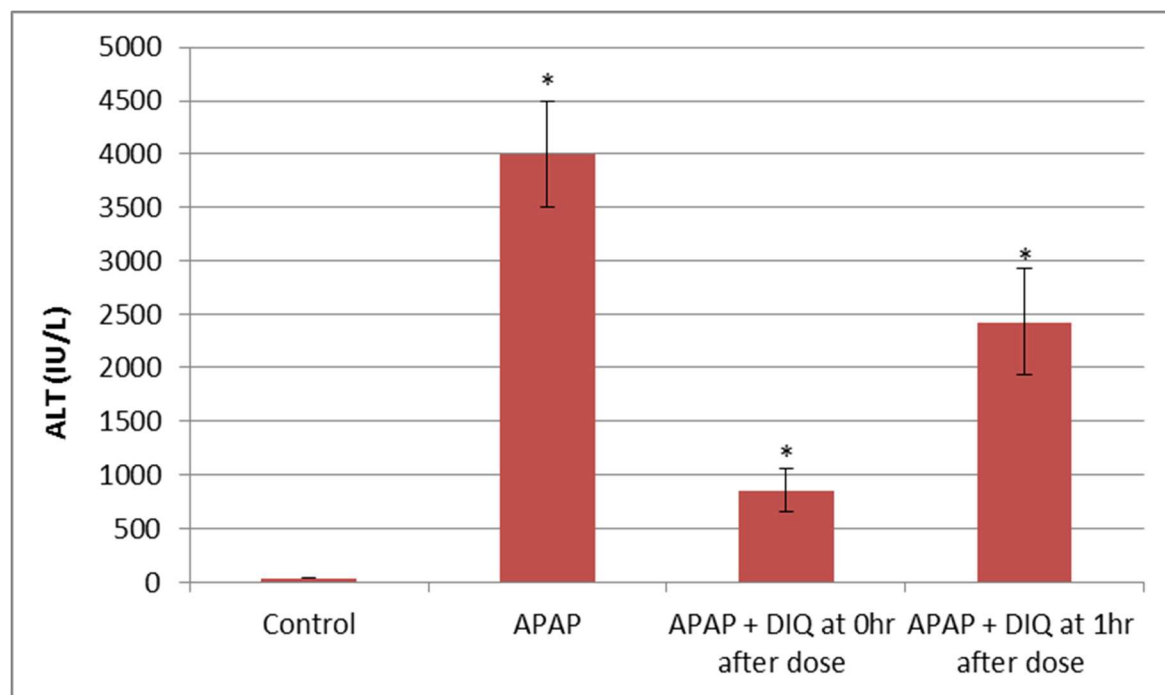


Figure 26. PARP Inhibition for APAP: ALT. A serum ALT PARP inhibition analysis following a 300 mg/kg dose of APAP at the 18 hour time point with the administration of a PARP inhibitor, DIQ (n=6). Standard deviations are denoted with error bars. An (*) denotes statistical significance at ($p < 0.05$) when compared to controls.

PARP activity was measured following a 300 mg/kg dose of APAP at the 18 hour time point following a concurrent DIQ treatment and a 1 hour following APAP treatment. (See Figure 31.) The APAP treatment shows an activation of PARP and a large increase in PARP activity at 1.52 IU/20 μg protein when compared to controls. A concurrent DIQ treatment decreased PARP activity at 0.51 IU/20 μg protein. A slight increase in PARP activity was observed when DIQ was administered 1 hour following APAP treatment time point at 1.10 IU/20 μg protein.

A combined analysis of PARP activity with PARP inhibition shows a relatively similar trend for both cocaine and APAP. (See Figure 32.) PARP activity following APAP treatment

resulted in a lower decrease in PARP activity with a concurrent dose of DIQ when compared to cocaine. A larger increase was measured at 1 hour following APAP treatment time point than cocaine. Both drugs resulted in a decrease in PARP activity with concurrent administration of DIQ following treatments. Measurements observed at the 1 hour time point show that DIQ was less effective in decreasing PARP activity for cocaine and APAP-induced hepatotoxicity.

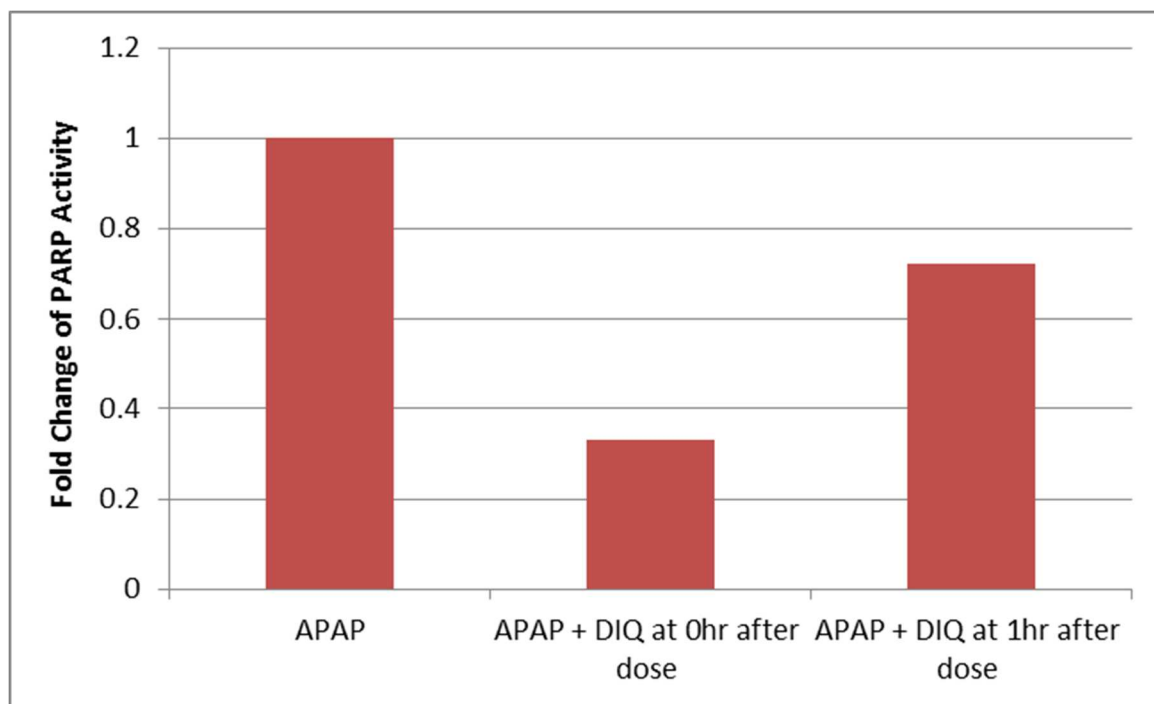


Figure 27. PARP Activity Fold Change for PARP Inhibition for APAP. DIQ treatment resulted in a fold decrease in PARP activity following a 300 mg/kg dose of APAP at the 18 hour time point for a concurrent treatment and a 1 hour following drug treatment time points (n=6).

A line graph comparison of PARP activity with PARP inhibition shows a relatively similar trend for both cocaine and APAP. (See Figure 33.) PARP activity following APAP treatment resulted in a lower decrease in PARP activity with a concurrent dose of DIQ when compared to cocaine. The line graph shows a similar pattern for cocaine and APAP treatments

with DIQ when compared to controls. A significant decrease was observed with a concurrent dose of DIQ for both toxicants. A slight elevation was observed 1 hour following treatment for cocaine and acetaminophen treatments when compared to controls.

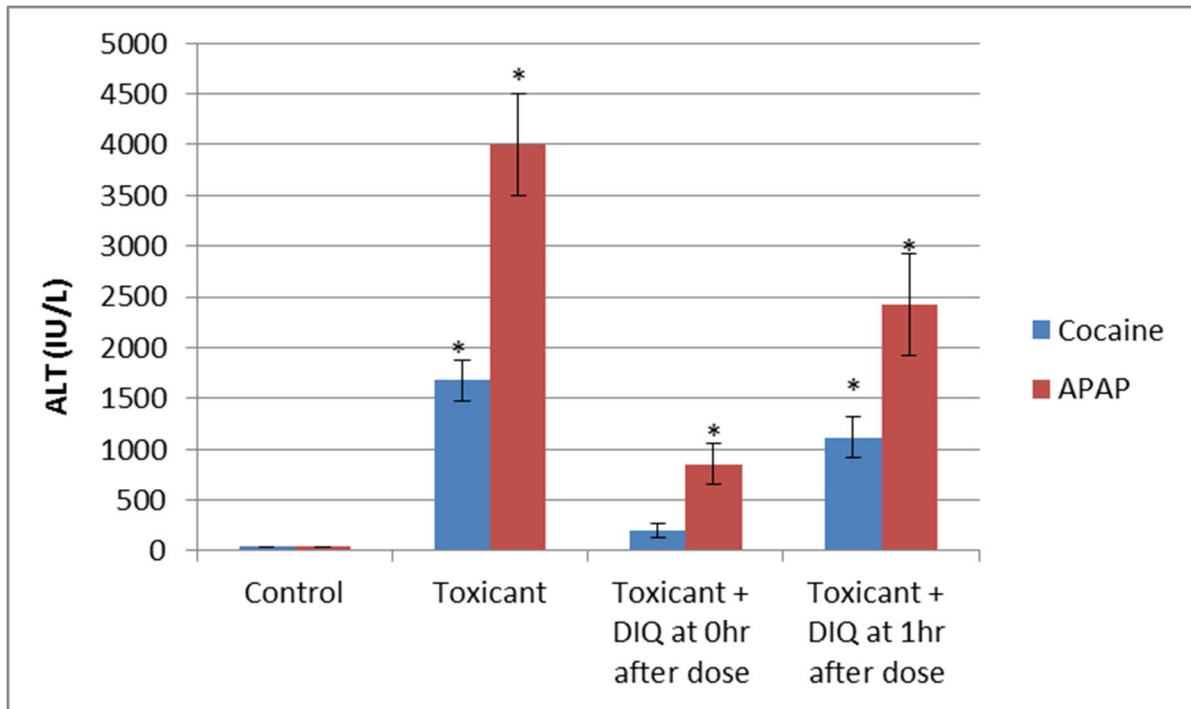


Figure 28. Cocaine and APAP PARP Inhibition: ALT Bar Chart. A bar chart comparison of a 50 mg/kg dose of cocaine at the 6 hour time point with the administration of DIQ concurrently or 1 hour following cocaine treatment (n=6) and a 300 mg/kg dose of cocaine at the 18 hour time point with the administration of DIQ concurrently or 1 hour following APAP treatment (n=6). Standard deviations are denoted with error bars. An (*) denotes statistical significance at (p < 0.05) when compared to controls.

Glutathione

A glutathione (GSH) analysis was conducted for a 50 mg/kg dose of cocaine at the 6 hour time point and a 300 mg/kg dose of acetaminophen (APAP) at the 18 hour time point to

understand the role of GSH in PARP activity following the administration of a PARP inhibitor. An analysis was conducted following cocaine and APAP treatments with a 10 mg/kg dose of DIQ as a concurrent dose or 1 hour after following treatment. Glutathione was measured as a ratio of GSH (reduced glutathione) to GSSG (oxidized glutathione) for each toxicant at the above time points.

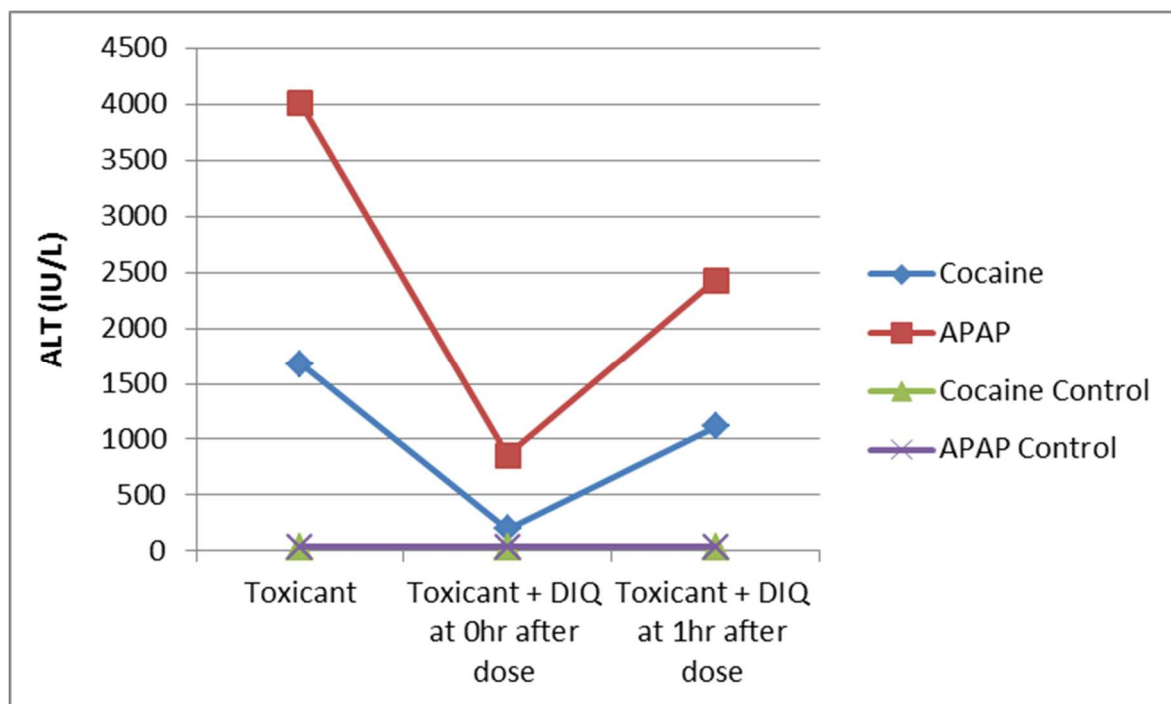


Figure 29. Cocaine and APAP PARP Inhibition: ALT Line Graph. A line graph comparison of a 50 mg/kg dose of cocaine at the 6 hour time point with the concurrent administration of DIQ or 1 hour following cocaine treatment (n=6) and a 300 mg/kg dose of APAP at the 18 hour time point with the concurrent administration of DIQ or 1 hour time point following APAP treatment (n=6).

The glutathione ratio for 50 mg/kg dose of cocaine at the 6 hour time point decreased by more than half when compared to controls to 9.10. (See Figure 34.) The ratio decreased for a concurrent dose of DIQ. A concurrent administration of DIQ with cocaine treatment reduced the

ratio to 1.96 when compared to animals treated with only cocaine. An increase in the oxidized to reduced ratio of glutathione was observed for DIQ 1 hour following treatment at 4.17.

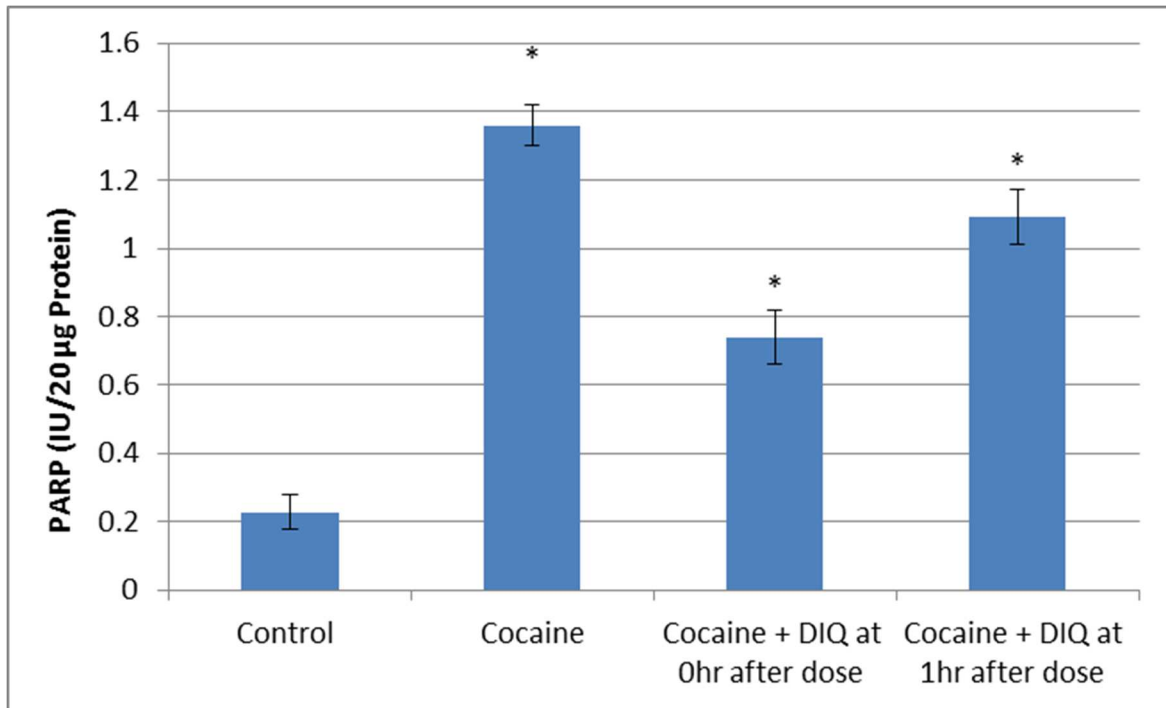


Figure 30. PARP Inhibition for Cocaine: PARP Activity. PARP activity for a 50 mg/kg dose of cocaine at the 6 hour time point with the concurrent administration of DIQ or administration of DIQ 1 hour following cocaine treatment (n=6). Standard deviations are denoted with error bars. An (*) denotes statistical significance at ($p < 0.05$) when compared to controls.

A glutathione analysis was conducted with a GSH to GSSG ratio for a 300mg/kg dose of APAP at an 18 hour time point to determine the role of glutathione in PARP activity. (See Figure 35.) An increase in the ratio was measured following APAP treatment at 3.29 and at 5.40 for a concurrent dose of DIQ when compared to 1 hour following treatment. A decrease in the GSH/GSSG ratio was observed with a DIQ dose administered 1 hour following treatment.

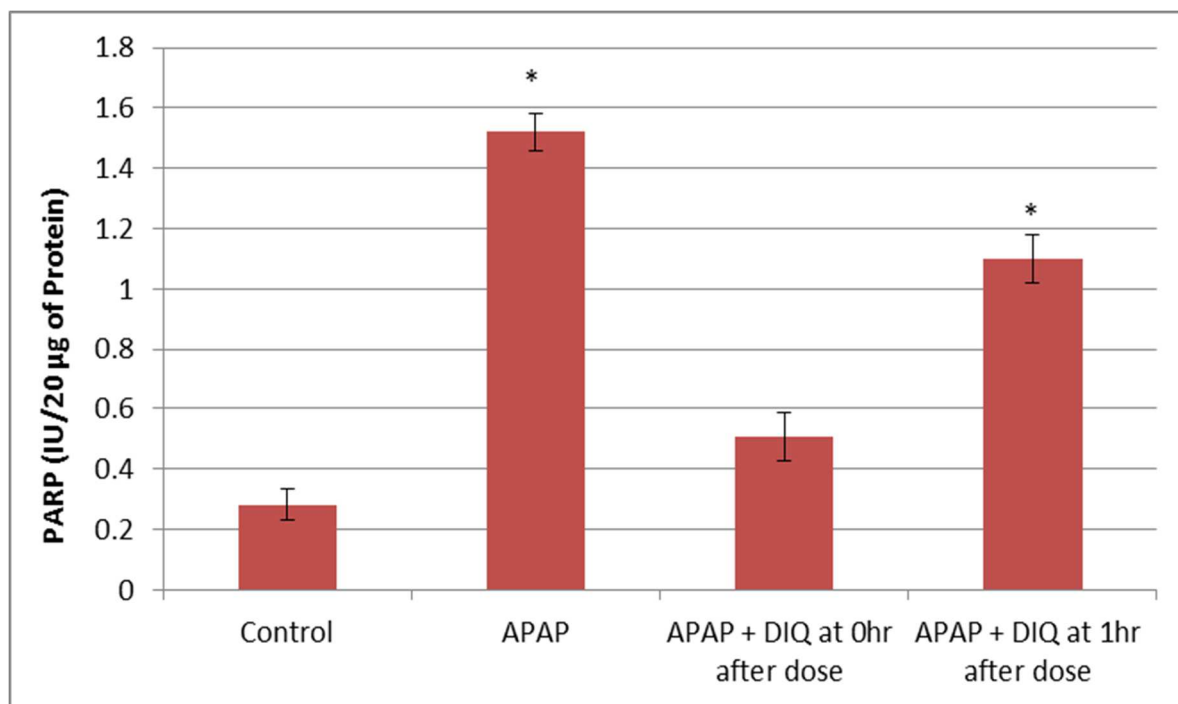


Figure 31. PARP Inhibition for APAP: PARP Activity. PARP activity for a 300 mg/kg dose of APAP at the 18 hour time point with the concurrent administration of DIQ or administration of DIQ 1 hour following cocaine treatment (n=6). Standard deviations are denoted with error bars. An (*) denotes statistical significance at ($p < 0.05$) when compared to controls.

A comparison of the ratio of GSH to GSSG for a 50 mg/kg dose of cocaine and a 300 mg/kg dose of APAP with a concurrent administration of DIQ and 1 hour following treatment for both drugs reveals significant differences in the measured ratio. (See Figure 36.) A higher ratio was measured for control liver samples following cocaine treatments than for APAP treatments. The GSH to GSSG ratio decreased in liver samples following cocaine treatment. A concurrent dose of DIQ showed a decrease for the cocaine samples while an increase in the ratio was observed for the APAP samples. A similar inverse relationship was observed for DIQ

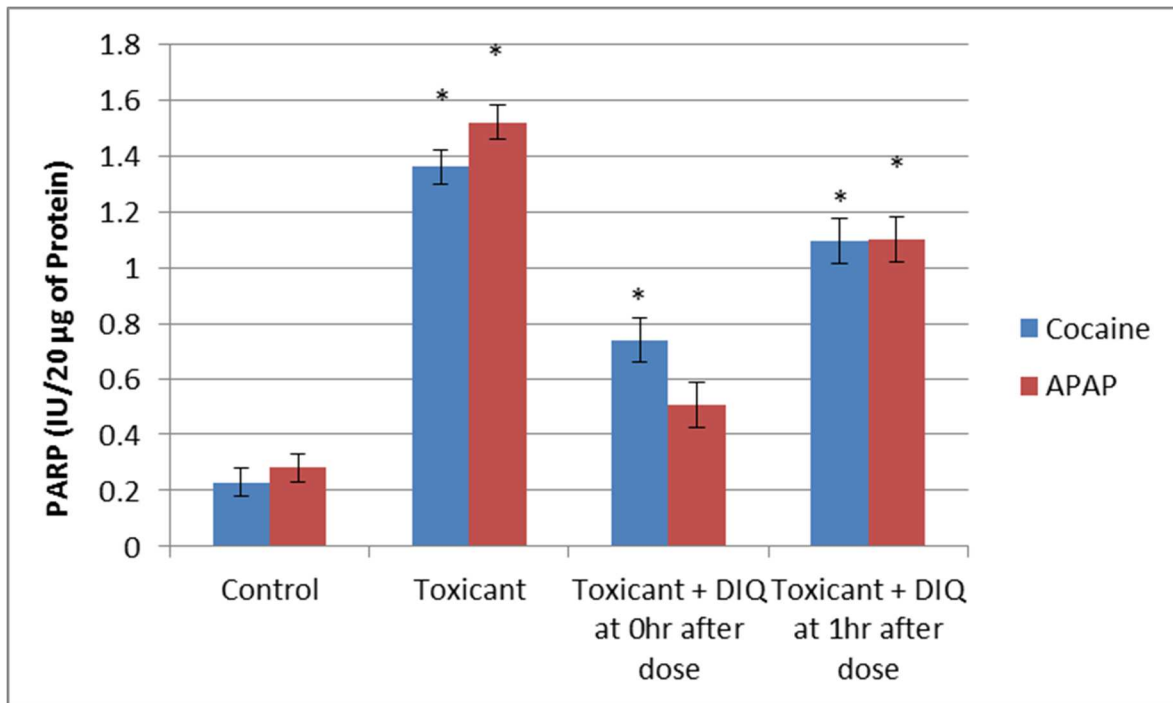


Figure 32. PARP Inhibition for Cocaine and APAP: PARP Activity Bar Chart. A bar chart comparison of PARP activity for a 50 mg/kg dose of cocaine at the 6 hour time point and a 300 mg/kg dose of APAP at the 18 hour time point with the concurrent administration of DIQ or administration of DIQ 1 hour following cocaine treatment. Standard deviations are denoted with error bars. An (*) denotes statistical significance at ($p < 0.05$) when compared to controls.

administration 1 hour following cocaine and APAP treatments. The inverse relationship observed for cocaine and APAP may express an independent relationship with glutathione and PARP activity in the presence of a PARP inhibitor.

The line graph for a glutathione ratio comparison following cocaine and APAP treatments shows an inverse relationship for both drugs with a concurrent administration of DIQ or 1 hour following treatment. (See Figure 37.) There is no clear linear pattern for the glutathione ratio following cocaine or APAP treatments with administration of DIQ. The glutathione ratio following cocaine and APAP treatments show individual linear trends that may suggest an independent relationship for PARP activity with glutathione for cocaine and APAP.

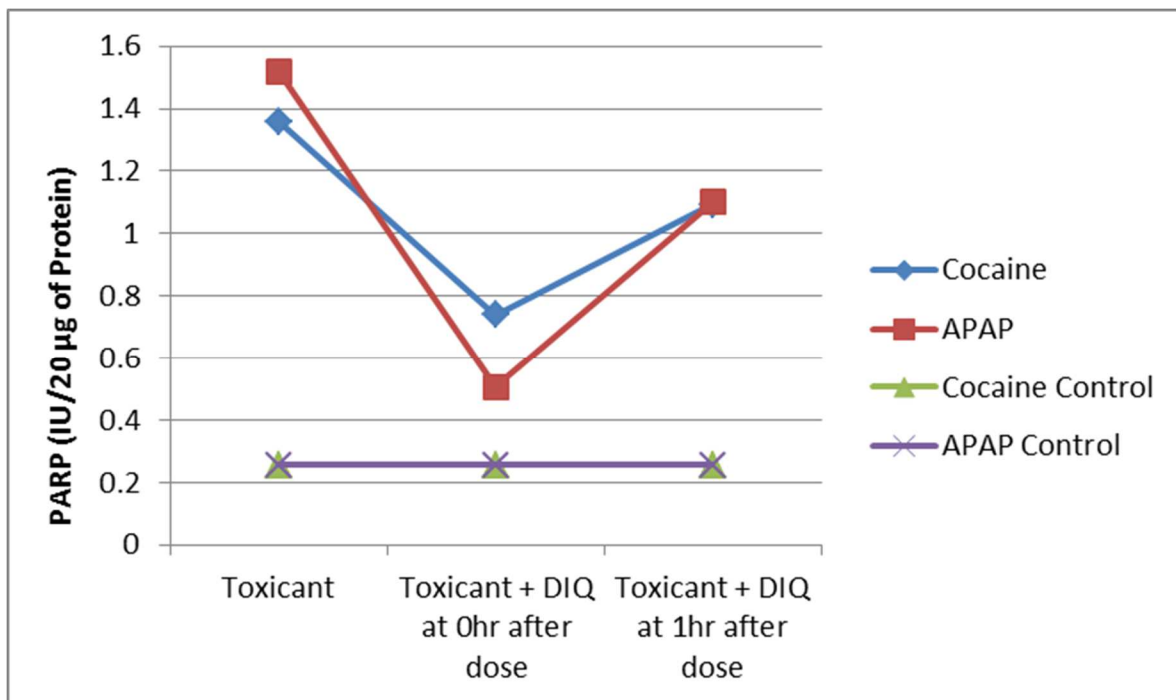


Figure 33. PARP Inhibition for Cocaine and APAP: PARP Activity Line Graph. A line graph comparison of PARP activity for a 50 mg/kg dose of cocaine at the 6 hour time point and a 300 mg/kg dose of APAP at the 18 hour time point with the concurrent administration of DIQ or administration of DIQ 1 hour following cocaine treatment. Standard deviations are denoted with error bars. An (*) denotes statistical significance at ($p < 0.05$) when compared to controls.

DNA Fragmentation

A 1.6% agarose gel was used to conduct a DNA fragmentation analysis for a 50 mg/kg cocaine dose at a 6 hour time point and a 300 mg/kg dose of APAP at an 18 hour time point. A 10 mg/kg dose of the PARP Inhibitor of DIQ was administered concurrently with cocaine and APAP treatments or at 1 hour following cocaine and APAP treatments. DNA was analyzed from collected liver samples for any fragments, tears, or shredding of DNA in each of the treatment samples. Herring sperm DNA was used as a positive control to show the presence of DNA with no fragments. An expression of fragmented DNA may suggest evidence of apoptosis as the resultant outcome of drug toxicity as opposed to necrosis.

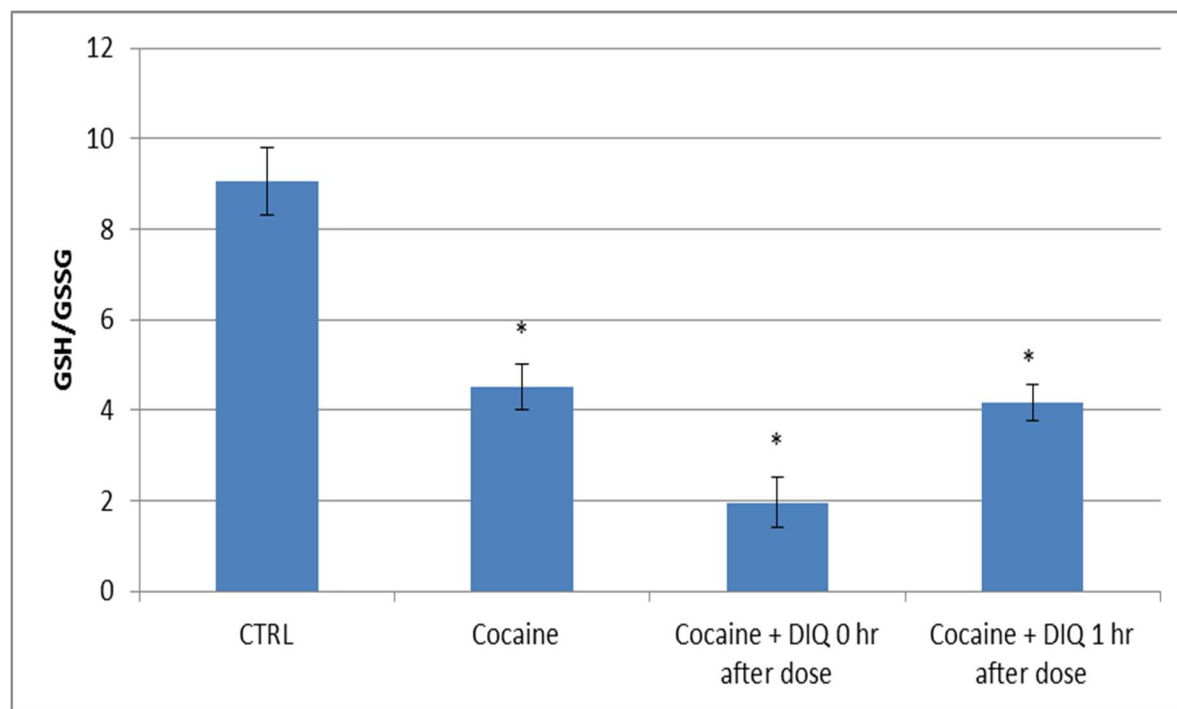


Figure 34. Glutathione Analysis for Cocaine. A 50 mg/kg dose of cocaine at a 6 hour time point measured a ratio of reduced glutathione to its oxidized form of GSSG. Measurements were conducted for concurrent administration of DIQ and 1 hour following treatment (n=6). Standard deviations are denoted with error bars. An (*) denotes statistical significance at ($p < 0.05$) when compared to controls.

A DNA fragmentation analyses was conducted to determine the impact of a cocaine treatment with a PARP inhibitor on DNA. (See Figure 38.) The resultant DNA fragmentation results show no cocaine treatment samples contained fragmented DNA. The results suggest that apoptosis is most likely not an outcome for cocaine-induced hepatotoxicity for a 50 mg/kg dose. Results also suggest that DIQ administered concurrently or 1 hour following cocaine treatment did not result in DNA fragmentation.

Fragmented DNA was analyzed for a 300 mg/kg APAP dose with a 10 mg/kg DIQ PARP inhibitor dose. (See Figure 39.) The resultant DNA fragmentation results shows no samples for APAP only treatments, APAP administered concurrently with DIQ, or 1 hour following

treatment resulted in fragmented DNA. The results suggest that apoptosis is likely not an outcome for APAP-induced hepatotoxicity. This also suggests that DIQ administered concurrently or 1 hour following APAP treatment did not result in DNA fragmentation.

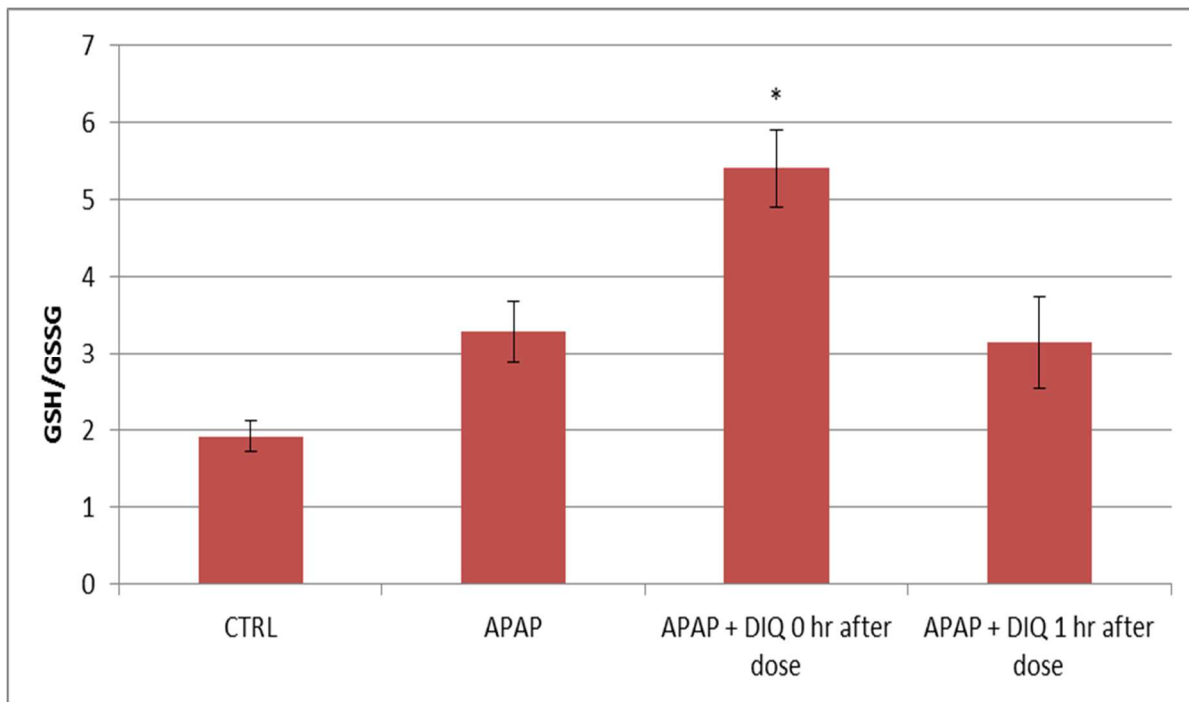


Figure 35. Glutathione Analysis for APAP. A 300 mg/kg dose of APAP at an 18 hour time point measured a ratio of glutathione to its oxidized form of GSSG. Measurements were conducted for concurrent administration of DIQ and 1 hour following treatment (n=6). Standard deviations are denoted with error bars. An (*) denotes statistical significance at ($p < 0.05$) when compared to controls.

A comparison of analysis for DNA fragmentation was conducted to express any resultant fragmentation for a 50 mg/kg dose of cocaine or a 300 mg/kg dose of APAP with a 10 mg/kg dose of DIQ at a 0 hour or 1 hour time point. (See Figure 40.) DNA fragmentation results show no fragmented DNA was observed following cocaine and APAP treatment with a concurrent DIQ dose or a DIQ dose 1 hour following treatment. These results also conclude that apoptosis is

not likely to be the ending result for cell damage resulting from PARP activation following cocaine and APAP treatments.

Western Blot

A western blot analysis was conducted for a 50 mg/kg cocaine or a 300 mg/kg dose of APAP with a 10 mg/kg concurrent dose of DIQ and 1 hour following treatment to analyze the expression of PARP in cocaine and APAP-induced liver samples. A protein ladder was used to measure the molecular weight of PARP at 116 kDa. PARP- High Specific Activity Enzyme (PARP-HSA) was used as a positive control to show the presence of PARP.

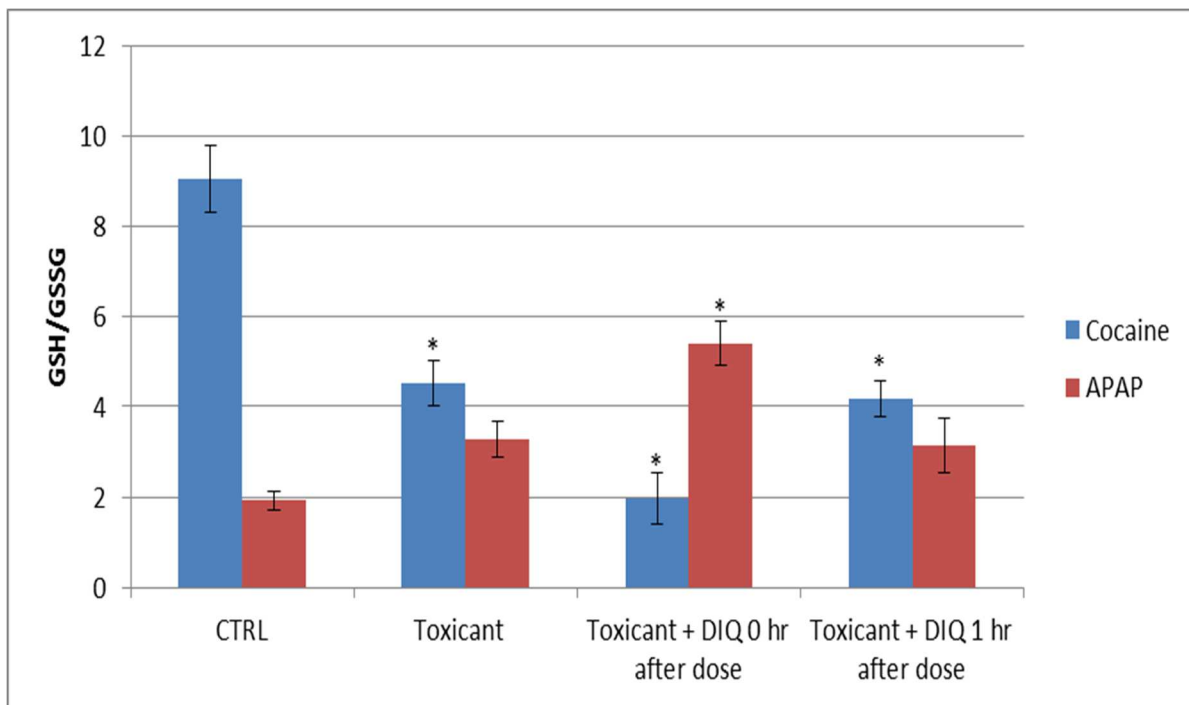


Figure 36. Glutathione for Cocaine and APAP: Bar Chart. A comparison of the glutathione ratio of GSH to GSSG for a 50 mg/kg dose of cocaine and a 300 mg/kg dose of APAP with the PARP inhibitor DIQ administered concurrently or 1 hour following drug treatment (n=6). Standard deviations are denoted with error bars. An (*) denotes statistical significance at ($p < 0.05$) when compared to controls.

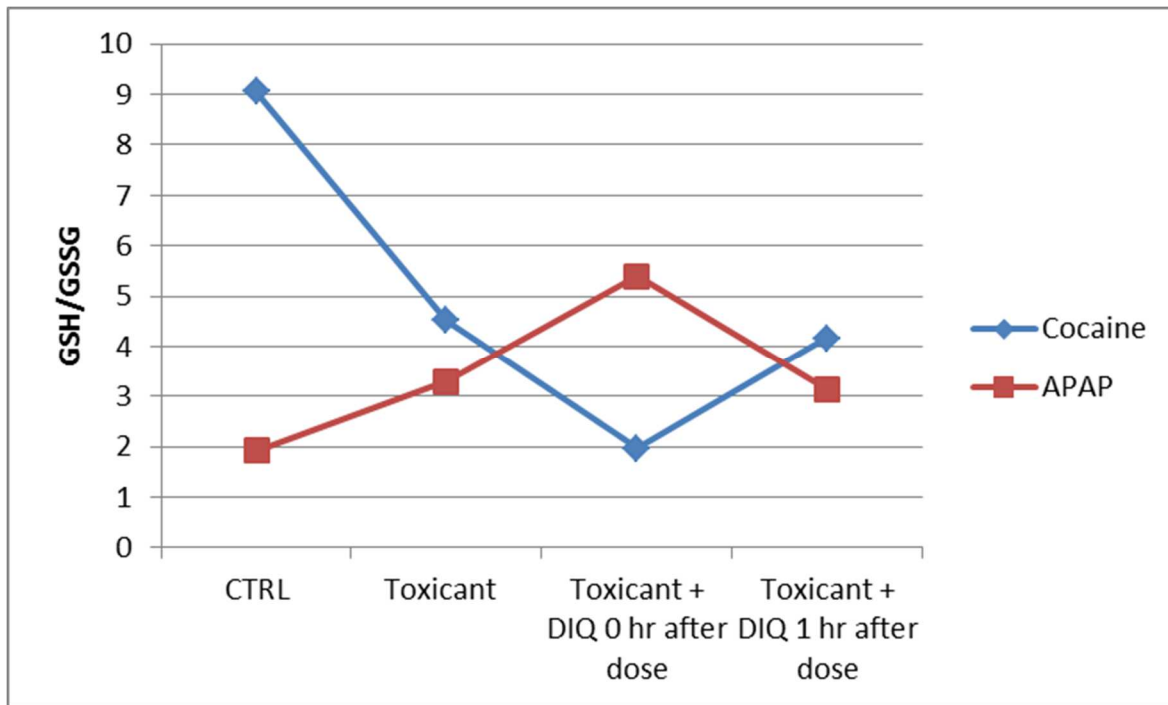


Figure 37. Glutathione for Cocaine and APAP: Line Graph. A comparison of the glutathione ratio of GSH to GSSG following a 50 mg/kg dose of cocaine and a 300 mg/kg dose of APAP with a concurrent dose of PARP inhibitor DIQ or a DIQ dose 1 hour following treatments (n=6). Standard deviations are denoted with error bars. An (*) denotes statistical significance at ($p < 0.05$) when compared to controls.

A western blot analysis was conducted for a 50 mg/kg cocaine with a concurrent 10 mg/kg dose of DIQ and 1 hour following treatment. (See Figure 41.) Lane 1 was a positive control of PARP-HSA with a molecular weight measuring at 116 kDa. Lane 2 was a protein ladder used to determine the molecular weight of PARP protein. Lane 3 (cocaine + DIQ administered 1 hour following treatment), lane 4 (cocaine + DIQ administered 1 hour following treatment), and lane 8 (cocaine treatment only) show light signals of PARP protein at 116 kDa, likely due to a weak transfer to nitrocellulose. Lane 5 (cocaine + a concurrent DIQ dose) and lane 7 (cocaine treatment only) shows strong signals of PARP protein at 116 kDa. Lane 6 (cocaine + a concurrent DIQ dose) does not show any strong PARP protein signals, likely due to

a bad transfer. This is likely due to low signaling or weak transfer. The cocaine saline control in lane 9 and the negative control (RIPA buffer) in lane 10 do not express any PARP signaling at 116 kDa. Lanes expressing PARP protein signaling express activation and presence of PARP activity observed from cocaine and APAP treatment with DIQ liver samples.

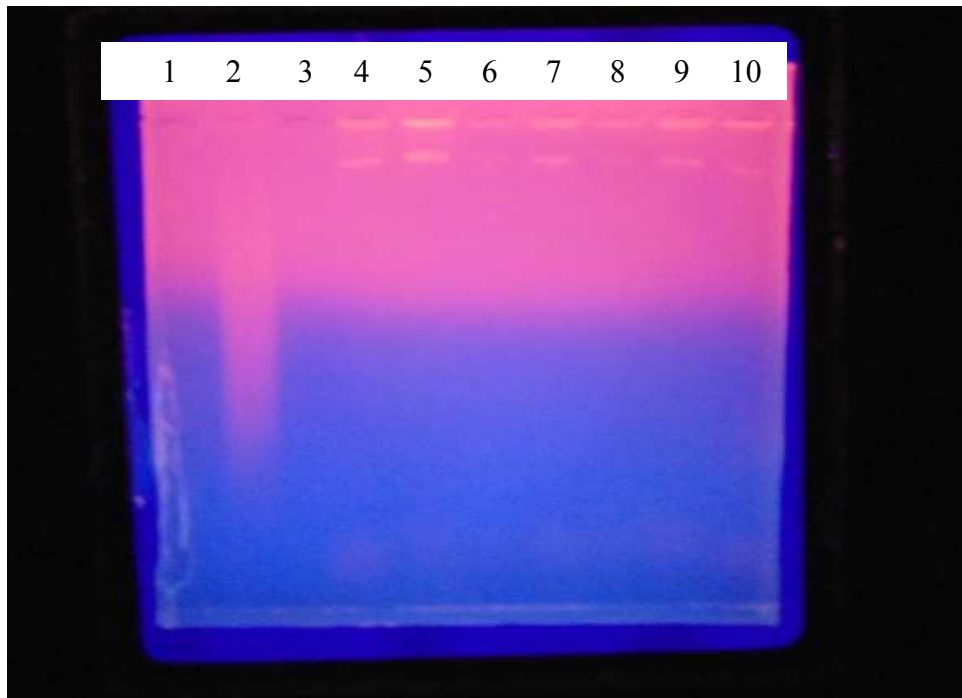


Figure 38. DNA Fragmentation: Cocaine. A 50 mg/kg dose of cocaine + 10 mg/kg DIQ dose concurrently or 1 hour following treatment in a 1.6% agarose gel. (1) (-) control (H₂O), (2) (+) control (Herring Sperm DNA), (3) loading control (resuspension buffer), (4) saline control, (5)-(6) cocaine dose only, (7)-(8) cocaine + concurrent DIQ dose, (9)-(10) cocaine + DIQ dose 1 hour following treatment.

A western blot analysis was conducted for a 300 mg/kg APAP and a 10 mg/kg concurrent DIQ dose and a DIQ dose 1 hour after treatment. (See Figure 42.) Lane 1 was a positive control of PARP-HSA expressing a low signal with a molecular weight measuring at 116 kDa. This is

likely due to low concentration of protein transferred during the assay. Lane 2 shows a protein ladder used for measuring molecular weight of PARP protein at 116 kDa. Lanes 3 & 4 (APAP + DIQ 1 hour following treatment), lanes 5 & 6 (APAP + a concurrent DIQ dose, and lanes 7 & 8 (APAP treatment only) express strong signals of PARP protein at 116 kDa. Lane 9 (APAP saline control) and lane 10 (negative control) show no signals of PARP protein at 116 kDa. Lanes expressing PARP protein signaling express activation and presence of PARP activity expressed with liver samples used for analysis.

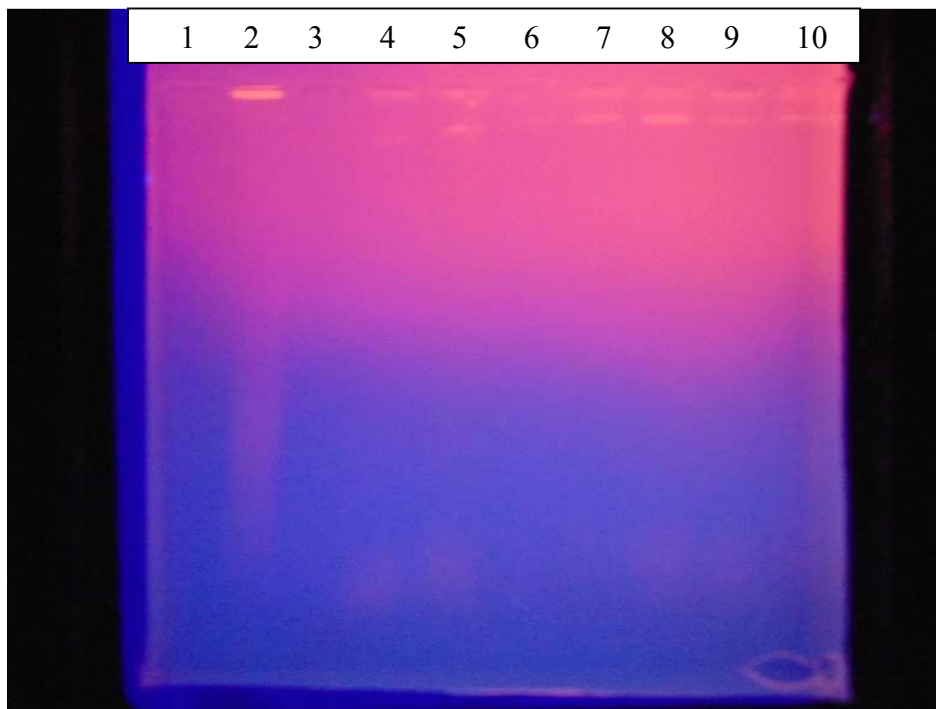


Figure 39. DNA Fragmentation: APAP. A 300 mg/kg dose of APAP + 10 mg/kg DIQ dose concurrently or 1 hour following treatment in a 1.6% agarose gel. (1) (-) control (H₂O), (2) (+) control (Herring Sperm DNA), (3) loading control (resuspension buffer), (4) saline control, (5)-(6) APAP dose only, (7)-(8) APAP + concurrent DIQ dose, (9)-(10) APAP + DIQ dose 1 hour following treatment .

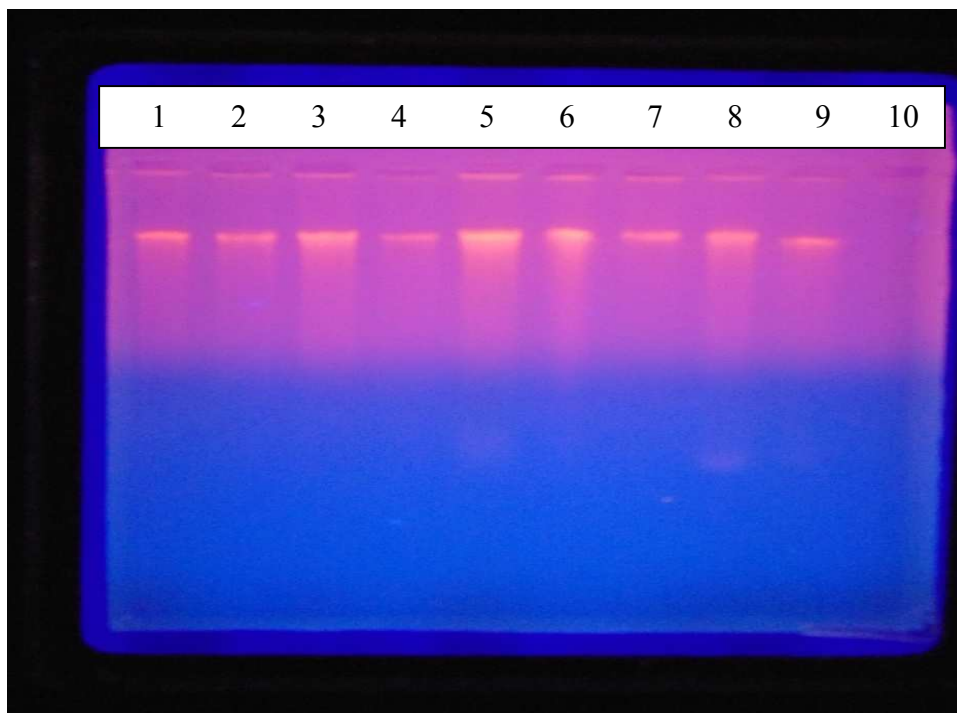


Figure 40. DNA Fragmentation: Cocaine & APAP. A 50 mg/kg dose of cocaine and a 300 mg/kg dose of APAP + 10 mg/kg concurrent DIQ dose and 1 hour following drug treatment in 1.6% agarose gel. (1) APAP + DIQ 1 hour after treatment, (2) cocaine + DIQ 1 hour after treatment, (3) APAP + a concurrent DIQ dose, (4) cocaine + a concurrent DIQ dose, (5) APAP treatment only, (6) cocaine treatment only, (7) APAP saline control, (8) cocaine saline control, (9) (+) control (Herring Sperm DNA), (10) (-) control (H₂O).



Figure 41. Western Blot: Cocaine. A 50 mg/kg dose of cocaine at the 6 hour time point + a concurrent 10 mg/kg DIQ dose and a DIQ dose 1 hour following treatment in 4-10.5% Tris-glycine polyacrylamide gel. (1) (+) control, PARP-HSA (2) protein ladder, (3)-(4) cocaine + DIQ 1 hour following treatment (5)-(6) cocaine + a concurrent DIQ dose, (7)-(8) cocaine treatment only, (9) cocaine saline control, (10) (-) control, RIPA buffer.

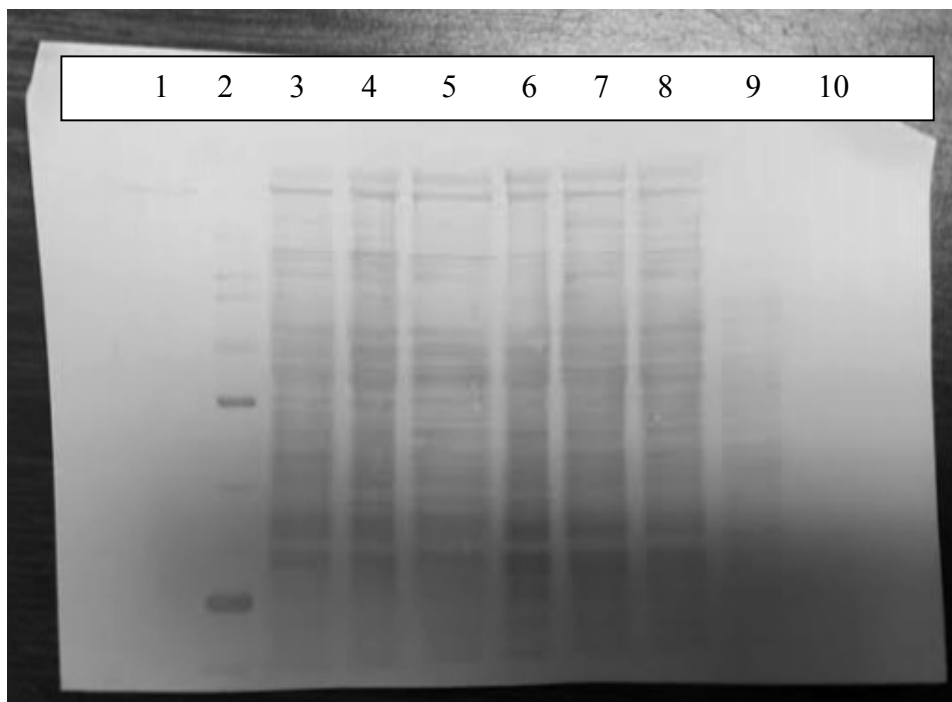


Figure 42. Western Blot: APAP. A 300 mg/kg dose of APAP at the 18 hour time point + a concurrent 10 mg/kg DIQ dose and a DIQ dose 1 hour following treatment in 4-10.5% Tris-glycine polyacrylamide gel. (1) (+) control, PARP-HSA (2) protein ladder, (3)-(4) APAP + DIQ 1 hour following treatment, (5)-(6) APAP + a concurrent DIQ dose, (7)-(8) APAP treatment only, (9) APAP saline control, (10) (-) control, RIPA buffer.

CHAPTER FIVE:

DISCUSSION

This experiment has shown that cocaine and acetaminophen-induced hepatotoxicity can activate poly(ADP-ribose) polymerase (PARP) activity in the liver. In the presence of an overdose, cocaine and acetaminophen (APAP) can produce elevations in PARP activity. The use of a PARP inhibitor following cocaine and APAP treatment, 1,5-dihydroxyisoquinoline (DIQ), was shown to reduce PARP activity in the liver. Cocaine and APAP-induced hepatotoxicity produced from cocaine and APAP treatments resulted in widespread liver damage producing significant elevations in serum ALT and increases in PARP activity in liver tissues.

A dose-response analysis conducted following a cocaine treatment produced an optimal dose of 50 mg/kg to evaluate cocaine-induced hepatotoxicity. While doses lower than 50 mg/kg produced significantly elevated serum alanine aminotransferase (ALT) levels, a 60 mg/kg dose caused severe hemorrhaging and resulted in a decrease in measured serum ALT that was likely due to the inability of the metabolism of cocaine following treatment. The measured ALT for the 60 mg/kg dose is inconsistent with Roth et al. and McCluskey et al. that chose a 60 mg/kg dose to analyze for experiments (McCluskey, Harbison, Sava, Johnson, Harbison, 2012). No studies have conducted a temporal analysis to determine the optimal time when PARP activity is highest for cocaine-induced hepatotoxicity. A comparative temporal analysis was conducted for PARP activity over a 24 hour period at a 2 hour, 6 hour, 18 hour, and 24 hour time point were serum

ALT was analyzed for any indications of cocaine-induced hepatotoxicity. While serum ALT levels increased incrementally at each time point, the highest ALT levels were observed at the 24 hour time point. Results from PARP analysis following cocaine treatment showed the most activity was observed at the 6 hours following treatment. It is expected for biochemical results to be delayed due to the time it takes for the drug to be metabolized by the liver. Results suggest that time may play a role in the difference between cocaine metabolism in the liver and the time for activation of PARP activity following treatment. These differences may exist with an acute and chronic cocaine dose. Scobie et al. measured elevations in PARP activity following a repeated cocaine dose from a single endpoint sacrifice time point in the brain. This may suggest that while elevations to PARP activity may be observed at a number of time points, the time point for peak PARP activity may exist during a specific period of time following cocaine treatments (Scobie, et al., 2014).

A dose-response analysis conducted for acetaminophen showed small elevations in serum ALT observed at the lower dose groups of 75 mg/kg and 150 mg/kg. Significant elevations in serum ALT were measured with a 200 mg/kg and 300 mg/kg dose group. A 300 mg/kg dose was chosen as the optimal dose to conduct a PARP analysis due to significant elevations in serum ALT observed and low mortality. A temporal analysis conducted by Cover et al. and Gujral et al. produced similar results for a 300 mg/kg dose of APAP of an increase in serum ALT over time an observed time period (Gujral, Knight, Farhood, Bajt, Jaeschke, 2002; Cover et al., 2005a). An analysis of a 12 hour observation time by Cover et al. and a gap between hours 6 and 24 for Gujral et al. suggest that while increases to serum ALT were observed as time progressed, some differences in serum ALT may have been observed at time points not observed during the study. These gaps in measurements may potentially impact analyzing PARP activity, as there may be

elevations in activity outside of the measured parameters that could suggest other periods of peak PARP activity. Results gathered from this study suggest that PARP activity and serum ALT may be independent of one another in reference to time. While increases in PARP activity following treatment may be observed, the importance of determining peak PARP activity may provide answers to more accurate administration of PARP inhibitors in the future.

A PARP inhibitor efficacy analysis was conducted with DIQ for acetaminophen and cocaine. Although DIQ is not a potent PARP inhibitor, a reduction in PARP activity was observed with a concurrent DIQ administration and a DIQ administration one hour following drug treatment. A serum ALT analysis for both toxicants testing the efficacy of the DIQ in reducing liver damage shows that a significant reduction in serum ALT was observed with a concurrent administration of DIQ, followed by a small increase observed one hour following treatment. Significant increases observed at the DIQ one hour following treatment time point shows that time does play a role in the reduction of potential liver damage that may result from drug-induced toxicity. While a delay in biochemical samples is expected with drug metabolism of the liver, serum ALT results alone may not prove effectiveness of a PARP inhibitor. A similar trend was observed with PARP activity following an acetaminophen and cocaine treatment to suggest that DIQ is effective at reducing PARP activity.

McCluskey et al. analyzed the effectiveness of DIQ with a 60 mg/kg cocaine dose on the liver. Reductions in serum ALT and PARP activity were measured in liver tissue samples. Study results also measured an increase in serum ALT at the one hour post initial dose time point. These results suggest that the effectiveness of a PARP inhibitor is impacted by time of administration. DIQ is most effective at inhibiting PARP activity and decreasing serum ALT levels when administered concurrently with a treatment dose (McCluskey, Harbison, Sava,

Johnson, Harbison, 2012). The significant differences in serum ALT for the measured PARP inhibitor time points suggest that while there is a window of time to reduce drug-induced liver injury, that window of time is very small. Results from this study for a 50 mg/kg cocaine dose produced similar results measuring a large increase in serum ALT from the DIQ administration concurrently with cocaine treatment to the one hour following treatment time point. No temporal analysis was conducted for PARP activity to determine the role of time on peak PARP activity.

A similar analysis was conducted for a 300 mg/kg dose of APAP with a concurrent 3-aminobenzamide (3-AB) dose by Cover et al. and Dönmez et al. (Dönmez, Uysal, Poyrazoglu, Er Öztas, Türker, Kaldirim, Korkmaz, 2015; Cover et al., 2005a). Both studies revealed a decrease in serum ALT levels. An increase in survival, glutathione, and PARP activity were observed for animals treated with 3-AB when compared to APAP treatment only animals. Results from this study produced similar results for PARP inhibition with DIQ administration for a 300 mg/kg APAP dose.

The results gathered from this study for the role of cocaine and acetaminophen on PARP inhibition with administration of DIQ on PARP activity supports studies that show DIQ protects against hepatotoxic events resulting from drug-induced toxicity (Cover et al., 2005a; McCluskey, Harbison, Sava, Johnson, Harbison, 2012; Dönmez, Uysal, Poyrazoglu, Er Öztas, Türker, Kaldirim, Korkmaz, 2015; McCluskey, Sava, Harbison, Muro-Cacho, Johnson, Ping, Harbison, 2011; Shi et al., 2012; Banasik, Stedeford, Strosznajder, Takehashi, Tanaka, Ueda, 2011). Cocaine and acetaminophen treatment produced an activation of PARP where elevated levels of PARP activity were also observed. Elevated levels of PARP activity and serum ALT suggest that significant damage to the liver resulted from toxic doses of acetaminophen and cocaine. Measurements for acetaminophen produced significantly higher measurements for ALT and

PARP activity when compared to cocaine. These were not expected results for acetaminophen when compared to cocaine, however, this may also be due to the route of administration for the toxicant dose. Administration of the 300 mg/kg dose of acetaminophen via the intraperitoneal route may have elicited a more extreme response due to metabolism of the drug and its ability to enter into the blood stream where toxic metabolites are produced in the liver.

Western blot analysis produced similar evidence with measurements of PARP weighing at 116 kDa was expressed for cocaine and acetaminophen. This supports elevations in serum ALT and PARP activity measured that would suggest activations of PARP activity following cocaine and acetaminophen treatment. Results also revealed the presence of non-specific binding among various proteins at various molecular weights. An analysis determining the cleavage of PARP may have provided a more in-depth understanding of the role of PARP and how it may differ following cocaine and acetaminophen treatment. A caspase-3 detection analysis would confirm whether apoptosis or necrosis was the hepatotoxic cell death pathway resulting in the observed cytotoxic damage.

A temporal analysis was conducted to determine the optimal time when PARP activity was highest following cocaine and acetaminophen treatments. A serum ALT analysis over a 24 hour period revealed that while elevations were observed for both drugs during the observed time period, significant differences were observed at each measured time point. The temporal analysis for PARP activity was also found to produce significantly different measurements at each observed time point. Cover et al. analyzed the role of time on a 300 mg/kg dose of acetaminophen over a 12 hour period (Cover et al., 2005a). Results to ALT and PARP activity revealed similar results in showing increases to activity observed over time. PARP activity measured for acetaminophen produced a delayed reaction over the observed period which were

also observed through results gathered from this study. Results for PARP activity following acetaminophen and cocaine treatments differed over the observed 24 hour time period. While acetaminophen produced delayed elevations in PARP activity occurring at the later time points within the observed 24 hour period. However, PARP activity results for cocaine produced varying results in activity at the earlier time points with decreases in activity observed at the later time points within the 24 hour observation period.

A temporal analysis was also conducted for the concurrent administration of the DIQ PARP inhibitor and 1 hour following treatment for both toxicants. A similar analysis was conducted by McCluskey et al. for a 60 mg/kg dose with cocaine with DIQ at a 0 hour, 1 hour, and 2 hours following treatment time point (McCluskey, Harbison, Sava, Johnson, Harbison, 2012). Similar results were measured for DIQ and its ability to provide protection against cocaine-induced hepatotoxicity events following cocaine treatment. The largest reduction in serum ALT was observed with a concurrent administration of DIQ, followed by large increases observed at the 1 hour following treatment time point. Similar results were gathered for cocaine and acetaminophen with a similar analysis for DIQ observed with a concurrent dose and a dose 1 hour following treatment time points. Reductions in PARP activity were observed with a concurrent DIQ administration and 1 hour following treatment for cocaine and acetaminophen. While a larger reduction in PARP activity was observed with concurrent administration of DIQ, results for both toxicants measured a much larger reduction for a concurrent administration of DIQ with acetaminophen treatment than cocaine treatment.

A glutathione analysis produced the ratio of reduced to oxidized glutathione. Ratios were gathered for a 50 mg/kg dose of cocaine at a 6 hour time point or a 300 mg/kg dose of acetaminophen at an 18 hour time point with a concurrent administration of DIQ and 1 hour

following treatment. Results from this analysis revealed a potential independent relationship for glutathione following cocaine and acetaminophen with DIQ administration. Alternatively, glutathione ratio measurements for the control group for acetaminophen were significantly lower than the toxicant dose group. Glutathione ratios measured for cocaine treated animals express that there may be a decrease in glutathione caused by cocaine and that DIQ may provide a delayed protection against hepatotoxic events at the 1 hour following treatment time point. Results gathered following acetaminophen treatment may suggest that the reactive metabolite, acetaminophen-glutathione conjugate (APAP-SG), produced during oxidation may produce toxic effects that may inhibit glutathione reductase activity. The inhibition of glutathione reductase by APAP-SG may suggest a competitive enzyme mechanism that could contribute to false elevations observed with acetaminophen treatments (Nýdlova, Vrbová, Česla, Jankovičová, Ventura, Roušar, 2013).

Gross pathology tissue samples following cocaine and APAP treatment at the 24 hour time point. The cocaine treatment samples display mottling on the surface with hemorrhaging observed on the surface of the liver. Resultant observations for the cocaine sample suggest that necrosis cell death is the pathway that results from cocaine-induced hepatotoxicity (Evans, 1983; Roth, Harbison, James, Tobin, Roberts, 1992; Thompson, Shuster, Shaw, 1979). A similar analysis was conducted for acetaminophen with significantly damaging impacts on the liver. Observations revealed a substantial amount of hemorrhaging on the surface of the liver. The results gathered from observing samples following acetaminophen treatment also suggest that necrosis is the cell death pathway for acetaminophen induced toxicity (Cover et al., 2005a; Dönmez, Uysal, Poyrazoglu, Er Öztas, Türker, Kaldirim, Korkmaz, 2015; Mitchell, Jollow,

Potter, Davis, Gillette, Brodie, 1973; Williams, Koerner, Lampe, Farhood, Jaeschke, 2011; Gujral, Knight, Farhood, Bajt, Jaeschke, 2002).

A DNA fragmentation analysis following cocaine and acetaminophen treatment evaluated the presence of fragmented DNA with PARP inhibited liver samples. Results from cocaine and cocaine + DIQ samples revealed no fragments to DNA in observed samples. Similar results were observed for APAP and APAP + DIQ samples. These results differ from Cover et al. that found the presence of fragmented DNA along with increases observed in fragmentation as time from initial dose increased (Cover et al., 2005a). While DNA fragmentation is considered to be a precursor for apoptotic signaling, presence of fragmented DNA has also been shown to produce necrotic cell death in liver cells. Some apoptotic cell death has been observed in the presence of acetaminophen induced drug toxicity (Cover et al., 2005a; Ray, Kamendulis, Gurule, Yorkin, Corcoran, 1993). However, recent studies analyzing acetaminophen hepatotoxicity have ruled out apoptosis as a cell death pathway (Cover et al., 2005a; Gujral, Knight, Farhood, Bajt, Jaeschke, 2002; Mitchell, Jollow, Potter, Davis, Gillette, Brodie, 1973).

Some possible limitations to this study include analyses used to determine PARP activity and cell death resulting from activation of PARP activity. The detection of glutathione and its role in PARP activity can be detected to determine the impacts of drug toxicity on cellular activity. While results may vary depending upon the toxicant, glutathione is expected to be depleted within the cell during toxic exposures to drugs and chemicals. The independent results gathered from this analysis with cocaine and acetaminophen presented alternative results that may distort the true role of glutathione on PARP activity. A western blot analysis analyzing the cleavage of PARP may have helped to better define the role of PARP activity following cocaine

and APAP treatments. More in-depth analyses for PARP activity may detect alternatives to cell death with drug-induced toxicity with administration PARP inhibitors.

There are a number of analyses that can be conducted to better understand the role of PARP activity in the presence of drug toxicity. Comparisons of drugs and chemicals help to better understand the metabolism of toxic metabolites activating PARP activity in the liver. Assays such as lipid peroxidation and caspase-3 can help to determine cell death pathways of necrosis or apoptosis for chemicals and drugs. Conducting analyses to understand that activity of PARP proteins during various stages of toxicity may help to understand better methods at inhibiting PARP activity. Evaluating the role of time with PARP activity is important to better understand the optimal time to supply PARP inhibitors in order to help elicit maximum potential when responding to hepatotoxic events.

CHAPTER SIX:

CONCLUSION

This study helped to prove the efficacy of the PARP inhibitor, DIQ, with cocaine and APAP-induced hepatotoxicity. Conducting a dose-response analysis following cocaine and acetaminophen treatment helped to determine the optimal dose that would elicit hepatotoxicity but not cause mortality. Results from this study helped to show that while there may be multiple doses that may contribute to significant elevations in serum ALT, determining the maximum dose to elicit PARP activity is key to determining the efficacy of potential PARP inhibitor treatment. Incorporating the element of time into an analysis will help to better understand PARP activity in order to produce the maximum effects from administration of PARP inhibitors. Time will also help to better understand the role of PARP activity for acute and chronic doses of drugs during drug-induced hepatotoxicity. Results from this study shows that time plays a major role in drug-induced hepatotoxicity following cocaine and acetaminophen treatment and the efficacy of PARP inhibitors.

The debate with apoptosis and necrosis cell death pathways helps to provide areas for future research between a number of drugs and chemicals. Determining intermediate impairments to cell function may lead to prevention of widespread damage resulting from these cell death pathways. As research for the efficacy of PARP inhibitors is studied to better understand the role of the production of toxic metabolites on PARP activity, new methods to

evaluate and understand metabolic differences that may exist for drugs and chemicals will help to propel research with PARP activity into new directions. Although drug-induced toxicity has been studied for a number of decades, creating new methods to evaluate cellular function as PARP is activated helps to allow for comparisons with various drugs and chemicals that will help with determining new methods to inhibit PARP activity in the future. A better understanding of the role of PARP activity in drug-induced hepatotoxicity may help to reduce widespread damage to the liver and may help to potentially save life by increasing the window of time for which an individual may be able to seek medical assistance.

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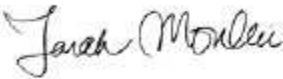
APPENDIX A:
IACUC APPROVAL



DIVISION OF RESEARCH INTEGRITY AND COMPLIANCE
INSTITUTIONAL ANIMAL CARE & USE COMMITTEE

MEMORANDUM

TO: Raymond Harbison, PhD

FROM: 
Farah Moulvi, MSPH, IACUC Coordinator
Institutional Animal Care & Use Committee
Research Integrity & Compliance

DATE: 7/3/2014

PROJECT TITLE: Pharmacological Modulation of Chemical-Induced Hepatotoxicity
by Poly(ADP-ribose)Polymerase (PARP) Inhibitors

FUNDING SOURCE: USF department, institute, center, etc.

IACUC PROTOCOL #: R IS00000608
PROTOCOL STATUS: APPROVED

The Institutional Animal Care and Use Committee (IACUC) reviewed your application requesting the use of animals in research for the above-entitled study. The IACUC APPROVED your request to use the following animals in your protocol for a one-year period beginning 7/2/2014:

Mouse: Charles Rivers/CD-1 (8-10 2848
weeks/25-30 g/ M)

Please take note of the following:

- IACUC approval is granted for a one-year period at the end of which, an annual renewal form must be submitted for years two (2) and three (3) of the protocol through the eIACUC system. After three years all continuing studies must be completely re-described in a new electronic application and submitted to IACUC for review.
- All Comparative Medicine pre-performance safety and logistic meetings must occur prior to implementation of this protocol. Please contact the program

coordinator at compmed@research.usf.edu to schedule a pre-performance meeting.

• **All modifications to the IACUC-Approved Protocol must be approved by the IACUC prior to initiating the modification.** Modifications can be submitted to the IACUC for review and approval as an Amendment or Procedural Change through the eIACUC system. These changes must be within the scope of the original research hypothesis, involve the original species and justified in writing. Any change in the IACUC-approved protocol that does not meet the latter definition is considered a major protocol change and requires the submission of a new application.

• **All costs invoiced to a grant account must be allocable to the purpose of the grant.** Costs allocable to one protocol may not be shifted to another in order to meet deficiencies caused by overruns, or for other reasons convenience. Rotation of charges among protocols by month without establishing that the rotation schedule credibly reflects the relative benefit to each protocol is unacceptable.

OFFICE OF RESEARCH & INNOVATION • DIVISION OF RESEARCH INTEGRITY AND COMPLIANCE
INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE
PHS No. A4100-01, AAALAC No. 58-15, USDA No. 58-15
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ABOUT THE AUTHOR

Kambria Haire is a graduate of Florida State University with a Bachelor's of Science degree in Exercise Science with a minor in Psychology (2010). She received a Master's in Public Health from Florida A&M University with concentrations in Environmental Health and Epidemiology (2012). Ms. Haire entered the PhD program in Toxicology and Risk Assessment in the Fall of 2012 at the University of South Florida within the Environmental and Occupational Health Department in the College of Public Health. Her research focused on the role of poly(ADP-ribose) polymerase in drug-induced toxicity. During her tenure at the University of South Florida she was a Graduate Assistant within the College of Nursing assisting with pre-requisite courses for the nursing program.