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The Assessment of an In-vitro Model for Evaluating the Role of PARP in Ethanol-mediated Hepatotoxicity

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The Assessment of an In-vitro Model for Evaluating the Role of PARP in Ethanol-mediated Hepatotoxicity

by

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ABSTRACT

This investigation assesses the role of poly(ADP-ribose) polymerase in ethanol-mediated hepatotoxicity using the untransfected HepG2 hepatocellular carcinoma line, an established, well-characterized toxicological model. HepG2 cells were treated with ethanol at concentrations between 100 mM and 800 mM, and assessed for markers of cytotoxicity. PARP-1 activity in total cell protein lysates was quantified as a proxy of apoptotic induction at six hours. Our results demonstrated a 1.43-fold AST activity increase in culture medium isolates of cells exposed to 800 mM without significant effect on cellular viability. PARP-1 activity varied greatly and results for enzyme activity remained inconclusive. The results suggest a high degree of insensitivity to ethanol toxicity and nuclear enzyme activity, demonstrating the metabolic irrelevance of untransfected HepG2 in ethanol toxicosis. There is a need to characterize phase 1 metabolic enzyme expression profiles relevant to ethanol for CYP2E1 and ADH pathways to facilitate comparisons across toxicological models using transfected, as well as the untransfected HepG2 model.

CHAPTER ONE:

INTRODUCTION

Ethanol

Ethanol (Ethyl alcohol; CAS No. 64-17-5) is a widely consumed neurologic and hepatic toxicant. According to a Substance Abuse and Mental Health Services Administration report (2012), approximately 23.6% of adults admitted engaging in at least one binge drinking event within the past year. At high doses, the requirement of hepatic detoxification increases substantially, as does the risk of neurotoxicity and hepatotoxicity acetaldehyde and reactive by-products (Reviewed in Albano et al., 2006). Pathological features of hepatic intoxication range from acute, transient steatosis (Lipid accumulation in hepatocytes), steatohepatitis (Lipid accumulation with cytokine-mediated inflammation), and cellular death, to more chronic, permanent manifestations, such as fibrosis and cirrhosis; such entities are aggregated into the pathological category called alcohol-induced liver disease (Goldin et al., 1993; Reviewed in Lucey et al., 2009; Massey & Arteel, 2012). An accepted theory in alcohol-induced liver injury pathogenesis involves a two-stage mechanism consisting of sensitization, e.g., reduction in endogenous antioxidant or suppression repair mechanisms, and subsequent priming resulting in a macrophage-mediated inflammatory response; such responses can lead to cellular damage or death (Takeyama et al., 1996; Tsukamoto et al., 2001). Attention on ethanol-mediated hepatotoxicosis has focused on mediators of oxidative stress originating from two general pathways: the indirect formation of reactive oxygen and nitrogen species as a byproduct of phase I metabolism, and direct

metabolism of ethanol to acetaldehyde–an electrophilic species which forms biomolecule adducts when detoxification does not occur (Tsukamoto et al., 2001). The role of reactive oxidative species in ethanol toxicosis has been demonstrated by the work of Di Luzio (1963). In attempting to further elucidate the mechanisms and biological consequences of ethanol-mediated hepatotoxicity *in vivo* and *in vitro*, models have facilitated characterization of clinically-relevant pathogenesis (Ambadath et al., 2010; Carter & Wands, 1988; Di Luzio, 1958, 1963; Garcia-Ruiz et al., 1994; Navasumrit et al., 2000; Rodeiro et al., 2008; Susin et al., 1999; Reviewed in Higuchi et al., 1996; Hoek & Pastorino, 2002). One of the models utilized as an *in vitro* model for ethanol-induced hepatotoxicity is the HepG2-an immortalized hepatocellular carcinoma cell line derived from a 15 year old Caucasian male with variable retention of putative biophysical functions of the originating parenchyma (Knowles et al., 1980).

The HepG2 Model

Since the establishment of the HepG2 cell line, the HepG2 model continues to appreciate utilization in toxicological testing. Early reports by Neuman and colleagues (1993) using this model demonstrated detectable toxicosis at clinically relevant serum concentrations, e.g., above 40 mM, in accordance with other studies (Guitierrez-Ruiz et al., 1999; Kurose et al., 1997), while others reported similar toxicosis as low as 1 mM (Castaneda & Kinne, 2000, 2001; Castaneda & Rosin-Steiner, 2006). Simultaneously, the differential expression of constitutive phase I metabolic enzymes was recognized, notably with some batches demonstrating significantly decreased expression levels of alcohol dehydrogenase (ADH; E.C. 1.1.1.1) and the ethanol-inducible cytochrome P450 2E1 (CYP2E1; E.C. 1.14.14.1) isozyme (Coon & Koop, 1987; Hasumura et al., 1975; Rodriguez-Antona et al., 2002; Tam, 1992). In compensation,

Cederbaum's group established a stable transfected HepG2 subtype with an attenuated CYP2E1 mRNA transcription profile (Dai et al., 1993). Ethanol-inducible, dose-dependent CYP2E1 mRNA transcriptional up-regulation was confirmed in accordance with physiologically-relevant MEOS levels and restored ethanol sensitivity to their HepG2 model, thus, implying sufficient contribution of CYP2E1 in oxidative stress-mediated hepatotoxicosis (Carroccio et al., 1994; Cederbaum, 2011; Ingelman-Sundberg et al., 1993). Direct comparisons of the CYP2E1 transfected and untransfected HepG2 models have attributed the untransfected HepG2 model as insensitive to ethanol-mediated cytotoxicity (Wu & Cederbaum, 1996, 1999), despite data suggesting a significant contribution of alcohol dehydrogenase to ethanol metabolism (≥50%). Under high doses, CYP2E1 becomes transcriptionally up-regulated and metabolizes the majority of the remaining ethanol fraction, albeit with an ethanol-specific K_m approximately 10-fold lower than that of ADH; catalase contributes an almost negligible fraction but does indeed participate. All three metabolic pathways produce acetaldehyde as well as reactive byproducts implicated in oxidative stress (Teschke et al., 1976).

ADH and CYP2E1 are responsible for reactive oxygen species (ROS) generation dose-dependently (Szuster-Ciesielska et al., 2008; Wu & Cederbaum, 1996) with resultant reductions in endogenous antioxidant pools, such as reduced glutathione (GSH), and formation of lipid peroxidation (Devi et al., 1993; Gutierrez-Ruiz et al., 1999; Kang et al., 2011; Khanal et al., 2009; Kurose et al., 1997). Further *in vivo* evidence demonstrates significant ethanol dose-dependent metabolically-derived inhibition of catalase and CuZn-superoxide dismutase (SOD) by superoxide and hydrogen peroxide, respectively, as well as acetaldehyde-mediated SOD inhibition in hepatocytes (Balasubramaniyan et al., 2007; Das et al., 2010; Ingelman-Sundberg & Johansson, 1984; Kono & Fridovich, 1982; Yang et al., 2008). One report did not confirm this observation

(Peng et al., 2010). The data imply ROS generation occurs irrespective of hepatocyte model source so long as ROS generation occurs, and, thus, an inherent physiological phenomenon reproducible both *in vivo* and *in vitro*. Together, these results suggest firstly, ethanol administration can result in ROS generation and hepatotoxicosis under physiological conditions, and, secondly, that the integrity of constitutive phase I enzyme metabolic profile dictates the extent to which damage occurs along a constant administered dose. Therefore, the potential for ROS-mediated oxidative stress shall depend on the basal metabolic profile of the HepG2 model tested.

The work of Hewitt and Hewitt (2004) has illustrated phenotypic variations across batched HepG2 cells which can account for some variability in between group toxicological endpoint heterogeneity. Collectively, although CYP2E1 mRNA expression tends to be low, basal expression may vary significantly between batches of cells and dependent upon culturing conditions, e.g., time from isolation to exposure, plating time, passage number, etc.; problems shared similarly by primary hepatocyte isolates (Richert et al., 2006). Thus, unwarranted assumptions regarding the metabolic phenotype between batched HepG2 cells can be erroneous, and, as such, the degree of separation from primary hepatocyte isolates and *in vivo* models cannot be denounced unless either genotypic expression profiles or phenotypic proxies of pertinent metabolic enzymes have been demonstrated (Hart et al., 2010; Hewitt & Hewitt, 2004).

Recent analyses investigating HepG2 expression profiles by differential microarray and qualitative real-time polymerase chain reaction have indeed confirmed numerous samples with significantly reduced CYP2E1 and ADH mRNA expression profiles compared to hepatocyte isolates, fresh hepatocellular carcinoma isolates, and the immortalized HepaRG cell line (Costantini et al., 2013; Guo et al., 2011; Hart et al., 2010). Two profiles displayed significant reduction in both phase I enzyme mRNA (Guo et al., 2011; Hart et al., 2010), while Costantini and col-

leagues (2013) did not directly report ethanol-dependent metabolic enzyme characterization; HepG2 mRNA expression among several genes involving cell cycle, signaling, and DNA response collectively exhibited 2646 significantly down-regulated and 3586 significantly up-regulated genes compared to hepatocytes. Of note, however, the precision genetic variation, and, thus, the absolute ratio of up-regulated or down-regulated genes, shall depend on the genotypic profile of the underlying control hepatocytes (Ponsoda et al., 2001; Rogue et al., 2012). Contemporary studies have not fully characterized phenotypic variability within the HepG2 model, and whether attenuation of CYP2E1 and ADH expression profiles through transfected, or co-transfected, cells may be appropriate for a particular application (Hewitt & Hewitt, 2004). Despite suggested variation, several studies continue to utilize the HepG2 model both transfected with CYP2E1 and/or ADH, as well as those not designated as transfected, with variable metabolic success.

Several recent studies utilizing HepG2 model not designated as transfected (Herein: non-designate HepG2) with either CYP2E1 or ADH have contributed evidence suggesting a therapeutic role of antioxidants or oxidative species-quenching mechanisms in abrogating ethanol-mediated toxicosis (Farshori et al., 2013; Kang et al., 2011; Kumar et al., 2011; Reddy et al., 2008; Senthil Kumar et al., 2012), while other have focused on elucidating subcellular interactions among apoptotic and necrotic machinery in dictating ethanol-induced cellular fate (Balasubramaniyan et al., 2007; Gutierrez-Ruiz et al., 1999, 2001; Szuster-Ciesielska et al., 2008; Yang et al., 2008). Studies have reported detectable toxicosis at concentrations ranging between 100 mM (Senthil Kumar et al., 2012) and 300mM (Farshori et al., 2013) to greater than 500 mM for one study (Kang et al., 2011), demonstrating highly variable toxic thresholds. Across the aforementioned studies, proxies of toxicosis have remained relatively consistent in characterizing

cytotoxicity markers including, but not limited to: quantification of endogenous antioxidants utilizing total GSH or GSH/GSSG ratio; cellular viability via trypan blue exclusion, Annexin V, propidium iodide, and Hoechst staining; plasma membrane integrity via quantification of aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamyltranferase (GGT); mitochondrial membrane electrochemical gradient potential via JC-1, rhodamine 123, and Ca^{2+} quantification; and oxidative stress biomarkers via malondialdehyde formation, thiobarbituric acid reactive substances assessment, and NO production. Notably, these methods are also consistent with those assessing transfected HepG2 cells, conferring a degree of comparability across studies. With respect to AST leakage, all studies mentioned utilizing the non-designate HepG2 model, with the exception of Gutierrez-Ruiz and colleagues (1999), demonstrated significant dose-dependent elevations of AST activity at 24 hours post exposure to ethanol. Though the magnitude of AST activity increases depend on treatment conditions and individual variation, studies *in vivo* have confirmed hepatic pathogenesis of high acute ethanol doses via AST liberation from serum samples (Ambadath et al., 2010; Cederbaum, 2011; Das et al., 2010). Together with ALT and lipid peroxidation products, AST has been noted as a stable and sensitive marker of hepatotoxicity *in vivo* and *in vitro* (Cohen & Kaplan, 1979; Meagher et al., 1999). Conclusively, some non-designate HepG2 cells have exhibited cytotoxicity generally homologous to those of transfected HepG2 and animal models.

An early report by Di Luzio (1963) exhibited the therapeutic role of antioxidants in attenuating acute ethanol-induced hepatotoxicosis *in vivo*, implicating oxidative stress as an etiological factor in the pathogenesis of steatosis and hepatocyte injury; results from this study assisted in focusing the therapeutic paradigm towards oxidative species quenching. Other markers and cytotoxic mechanisms of ethanol toxicosis focus on inflammatory mediators and allow diversifi-

cation of ethanol-mediated mechanistic hepatotoxicity, especially *in vivo*; nevertheless, this aspect shall not be further addressed in this study. As such, a fundamental link between unquenched ROS-mediated oxidative stress and DNA lesions, e.g., single strand breaks, *in vivo* and *in vitro* demonstrated the requirement of repair machinery induction following high acute ethanol dosing. As general repair enzymes respond to DNA lesions, one enzyme remains of particular concern in contemporary repair signaling and nucleosome modification (Navasumrit et al., 2000).

Poly(ADP-Ribose) Polymerase

The poly(ADP-ribose) polymerase (PARP) family includes 18 known members, exhibiting immense variation in molecular weight, domain composition, cellular function, and localization, are phylogenetically linked by a conserved ADP-ribosylating catalytic domain, (Reviewed in Ame et al., 2004; Hassa & Hottiger, 2008). PARP-1 [E.C. 2.4.2.30], the most abundant isoform found within the nucleus, has received considerable attention for its role in transcriptional regulation (Frizzell et al., 2009; Ju et al., 2004; Krishnakumar et al., 2008, 2010b; Wacker et al., 2007), chromosome structure modification (Huletsky et al., 1989; Lonskaya et al., 2005; Tulin & Spadling, 2003), metabolism and homeostasis (Bai et al., 2011; Luo & Kraus, 2012), stalled replication fork reinitiation (Bryant et al., 2009; de Murcia et al., 1983; Simbulan-Rosenthal et al, 1998), and cellular signaling and cycling (Monaco et al., 2005). In addition to the catalytic domain, PARP-1 contains putative domains exemplifying its role in regulated DNA repair. Two zinc finger domains have been implicated in localizing PARP-1 to DNA lesions, while simultaneously delineating DNA damage type (single- versus double-strand breaks) for initiating lesion-specific repair pathways (Ahel et al., 2008; Ali et al., 2012; Eustermann et al., 2011; Ikejima et

al., 2009; Langelier et al, 2011; Pion et al, 2003). A third zinc domain has a distinct function with possible interdomain interactions for modulating catalysis, DNA binding, transactivation (Langelier et al., 2008, 2010; Lilyestrom et al., 2010; Tao et al., 2008). A BRCA-like automodification domain allows self-post-translational ADP-ribosylation for regulating hetero-ADP-ribosylation (D'Amours et al, 1999), while a WGR [tryptophan-, glycine-, and arginine-rich] domain, of unknown specific function, is imperative for catalysis (Altmeyer et al., 2009). Together, the WGR and ART (ADP-ribose transferase) domains compose the catalytic domain necessary for core interaction with DNA; this composite of domains phylogenetically distinguishes PARP-1 from the other isozymes of the poly(ADP-ribose) polymerase family (Ruf et al, 1996, 1998).

The initial report by Chambon and colleagues (1963) describes the necessity for both DNA and oxidized nicotinamide-adenine dinucleotide (NAD^+) in PARP-1 activation, resulting in the formation of polymerized ribose residues-a process called poly ADP-ribosylation. Later studies revealed the necessity for a nucleic store of adenine triphosphate (ATP) in cell sustainability during ADP-ribosylation, especially after widespread DNA lesion formation (Benjamin et al., 1980a, 1980b; Berger, 1985; Durkacz et al, 1980; Juarez-Salinas et al., 1979; Kameshita et al., 1984; Satoh et al., 1994). Under basal levels of endogenous DNA damage, PARP-1 acts as a DNA nick sensor generally through recognition of non-B-type DNA conformations to initiate transient repair (Lonskaya et al., 2005). Translocalization to nicks has been attributed to the inherently flexibility of nicked strands, otherwise stereochemically prevented by unstressed, B-type DNA, and affords a mechanistic explanation of PARP-1's sensitivity in localization to damaged nucleotides or nucleosome constituents (Le Cam et al., 1994). Consequently, transient histone ADP-ribosylation electrochemically promotes an open DNA conformation, enabling access

by repair machinery, such as XRCC1 for base excision repair, MRE11 for nucleotide excision repair, and Ku70 for non-homologous end-joining (Bouchard et al., 2003; Caldecott, 2003; El-Khamisy et al., 2003; Mathis & Althaus, 1987; Pleschke et al., 2000; Schreiber et al., 2002; Veuger et al., 2004). To limit aberrant overactivation, the highly ubiquitous PARP-1 remains predominantly latent until activation, which, when activated by post-translational modification, can reach levels 500-fold of the basal level and effectively precluded the requirement of transcriptional up-regulation during cytotoxic stress (David et al., 2009; Haince et al, 2008; Hassa & Hottiger, 2008; Ju et al., 2004; Ludwig et al., 1988; Tulin & Spradling, 2003; Zaremba et al., 2009). Therefore, negative regulation of PARP is necessary to discourage over-activation and energy depletion.

Internuclear NAD^+ levels are replenished partially by endogenous nicotinamide cycling and utilization competition, while regulatory mechanism buffer against instances of depletion; for example, endogenous intranuclear production of NAD^+ is constrained by NMNAT-1, while a few nuclear enzymes compete for free NAD^+, e.g., SIRT1 and PARP-1 (Bai et al., 2011; Berger et al., 2007; Kolthur-Seetharam et al., 2006; Revollo et al., 2004; Zhang et al., 2009). When DNA damage occurs, PARP-1 and DNA-PK competitively bind DNA lesions, limiting PARP-1 lesion localization and ADP-ribosylation activation (D'Silva et al., 1999; Masson et al., 1998; Veuger et al., 2004). Post-translation modification by protein kinase C and through the IGF-I associated ERK cascade results in decreases in PARP activity (Bauer et al. 1992; Beckert et al., 2006; Tanaka et al., 1987). Nicotinamide, the catalysis by-product of ADP-ribosylation, acts as a strong negative-feedback by transient, reversible inhibition of PARP (Hageman & Stierum, 2001; Zhang & Kraus, 2009). Interestingly, an important ADP-ribose acceptor is the PARP-1 BRCA domain which ablates ADP-ribosylation, limiting excessive enzyme activation and, sub-

sequently, leading to a negative regulatory quiescent PARP-1 state (Adamietz, 1987; D'Amours et al., 1999; de Murcia et al., 1983; Fontan-Lozano et al., 2010; Happel & Doenecke, 2009; Kawaichi et al. 1981; Messner et al., 2010; Ogata et al., 1981; Virag & Szabo, 2002). Under circumstances favoring ADP-ribosylation, poly(ADP-ribose) glycohydrolase (PARG; E.C. 3.2.1.143) hydrolyzes ADP-ribose from acceptor proteins, such as PARP-1, thus, functionally linking PARG in PARP-1 activation (Erdelyi et al., 2009; Frizzell et al., 2009). While both PARG and ADP-ribose hydrolase (ARH3) have been shown to hydrolyze ADP-ribose polymers within the nucleus and cytoplasm, polymer hydrolysis remains imperative for restoration of an unstressed ante-lesion, B-type conformation (de Murcia et al., 1986; Niere et al., 2012; Oka et al., 2006). New evidence suggest an ever-increasing role of PARP-1 not only in general repair enzyme recruitment, but in selective orchestration of base excision repair (BER), non-homologous end joining (NHEJ), or homologous recombination (HR), depending on nick characteristics (Audebert et al, 2004; Frank-Vaillant & Marcand, 2002; Hochegger et al., 2006; Mao et al., 2011; McCabe et al., 2006; Saberi et al, 2007; Wang et al, 2006). Also, maintaining integrity of nucleosomes has been suggested as histones, which are targets of oxidizing species, have been shown to exhibit preferential resistance to ADP-ribosylation by PARP-1 unless oxidatively damaged; histone ADP-ribosylation increases their affinity for proteolytic destruction by 20S (Catalgol et al., 2010; Mayer-Kuckuk et al., 1999; Ullrich et al. 1999a, 1999b, 2000; Ullrich & Grune, 2001). Oxidative stress is a natural occurrence with sufficient reserve capacity to protect the cell under low level oxidative stressor situations; however, under heavy oxidation, the effects can overwhelm repair mechanism reserves and lead to deleterious effects.

While oxidative stress-mediated cytotoxicity has been demonstrated to activate PARP-1, overactivation of by extensive oxidative stress may precipitate depletion of nuclear NAD^+ and

ATP (Berger et al., 1986; Carson et al, 1988; Filipovic et al. 1999; Ha & Snyder, 1999; Schraufstatter et al., 1986; Sims et al., 1981, 1983; Virag et al. 1998a, 1998b; Yamamoto et al., 1981; Zhang et al., 1994). Nitric oxide, produced from inducible nitric oxide synthase, can follow oxidative stress events resulting in the evolution of peroxynitrite-a known oxidative stress mediator shown also to contribute to DNA damage and PARP-1 overactivation (Cuzzocrea et al., 1998; Koh et al., 2005). While cellular death from PARP-mediated energy depletion has been an attractive mechanism (Benjamin et al., 1980a; Berger, 1985), research has offered evidence suggesting that energy depletion is not sufficient in causing PARP-mediated death, but rather nuclear translocation of mitochondrial apoptosis-inducing factor (AIF), an inner mitochondrial membrane embedded flavoprotein, remains paramount in completing the parthanatos pathway-a recently recognized cellular death subtype (Andrabi et al., 2006, 2008; David et al., 2009; Galluzzi et al., 2012; Pospisilik et al., 2007; Wang et al., 2011). Previous reports have attributed cellular death via necrosis (Filipovic et al., 1999; Ha & Snyder, 1999; Simbulan-Rosenthal et al., 1998). It is now known that parthanatos has subtle, but distinct, morphological manifestations delineating it from apoptosis and necrosis; however, mechanistic cross-talk exists, including a late inflammatory response with apoptotic machinery induction. For example, parthanatos requires AIF translocation for causing DNA fragmentation and condensation, but late caspase-3 activation occurs, and is a suggestive biomarker of apoptosis (Robaszkiewicz et al., 2012; Yu et al., 2002, 2006). Mechanistically, AIF translocation must commence only after a specific course of events. First and foremost, the formation of the mitochondrial permeability transition pore (MPT)-a complex of proteins forming a transmembrane conductance pore-assembles after ablation of the mitochondrial transmembrane potential ($\Delta\Psi_m$), calcium influx, and energy production secession (Bernardi, 1992; Bernardi et al., 1994; Crompton, 1999). AIF was shown to constrain a putative,

high affinity ADP-ribose binding domain, functionally linking PARP-1 activation, AIF, and parthanatos progression (Alano et al., 2004; Hong et al., 2004; Ye et al., 2002). Andrabi and colleagues (2006) demonstrated that ADP-ribose polymers of considerable length have been shown to directly induce ADP-ribose-bound AIF-dependent cellular death. Preceding translocation, the hydrophobic AIF anchor must be cleaved, though source of cleavage is not known exactly, and the role of calpain in AIF solubilization during parthanatos has been precarious (Otera et al., 2005; Susin et al., 1999; Vosler et al., 2009; Wang et al., 2009). Negative regulation of PARP-1-mediated AIF translocation by PARG was demonstrated using PARG knockout mice, which were rescued by the PARG wild-type phenotype (Zhou et al., 2011). Alternately, administration of PARP inhibitors have been shown to shunt the parthanatos pathway while simultaneously conserving nucleic energy stores, especially when ADP-ribosylation activity abrogation reaches 90% or above (Satoh et al., 1994).

HepG2, the Role of PARP, and Ethanol-Mediated Toxicity

In correlating ethanol toxicosis, oxidative stress, the HepG2 hepatocellular carcinoma model, and PARP-1 activity attenuation, Cherian and colleagues (2008) have demonstrated PARP activation with subsequent ADP-ribosylation in cultured fetal cortical neurons upon administration with ethanol. Late induction of caspase-3 did not inhibit PARP-1 activation below a threshold precluding significant histone ADP-ribosylation six hours post dose. A previous report in isolated macrophages demonstrated a dose-dependent cleavage of PARP-1. Conferring late apoptotic machinery induction; however, ADP-ribosylation, a proxy of PARP-1 activity, was not measured (Brown et al., 2007). Since hepatic mitochondrially-generated reactive species and acetaldehyde can definitively elicit DNA damage *in vivo* and in the HepG2 model resultant of

ethanol administration (Cederbaum et al., 2011; Navasumrit et al., 2000), the activation and characterization of PARP-1 in the HepG2 model has not been elucidated completely. Inhibition of PARP has been shown to rescue cells from death under circumstances of ischemic-reperfusion injury and excitotoxicity affording evidence for the role of PARP inhibition of attenuating acute oxidative damage (Eliasson et al., 1997; Endres et al., 1997; Zhang et al., 1994). The current investigation aims to assess the feasibility of utilizing the non-transfected HepG2 model as a model of ethanol-mediated hepatotoxicosis and the role of PARP-1 therein. Additionally, more evidence regarding the metabolic relevance of the HepG2 model shall be generated to exemplify the reproducibility of ethanol-mediated toxicosis as demonstrated by contemporary literature.

CHAPTER TWO:

METHODS

Cell Culture

HepG2 human hepatocellular carcinoma cell line was obtained from the American Type Culture Collect (ATCC) (Manassa, VA) and cultured at 37° C and 5% CO_2 under humidified conditions. Complete supplemented medium consisted of Debulcco's Modified Essential Medium (Corning, Manassa, VA) containing 10% fetal bovine serum (ATCC), 10 mM HEPES buffer (Sigma, St. Louis, MO) and 100U/mL and100mg/mL of penicillin and streptomycin, respectively (ATCC). Supplemented medium was changed every two to three days. At 80-90% confluence, cells were subcultured by light trypsinization [0.1% Trypsin EDTA in PBS (Corning)] for 4 minutes at 37 °C after brief washing with phosphate buffered saline without calcium or magnesium (Corning). Trypsinization was stopped with one to two volumes of supplemented medium and separated by centrifugation at 250 x g for four minutes; thereafter, the supernatant was aspirated and the cells reconstituted in supplemented medium for subsequent plating in tissue-culture treated plates for either propagation or experimentation at known concentrations. Subculturing occurred every four to six days-the time necessary to reach confluence. HepG2 cells used for experimentation fell within the 6^{th} and 12^{th} passage in order to ensure consistency and to resist time-dependent genotype variation. Exposure periods to either vehicle or ethanol commenced only after allowing the cells to reattach overnight.

Treatment

HepG2 cells were plated in either 6-well flat-bottom plates at 100,000 cells (Corning) or in 96-well flat-bottom plates (Celltreat, Shirley, MA) at 2,500 cells per well and allowed to attach overnight in both circumstances. In preparing for experimentation, the supplemented medium was aspirated which was followed by subsequent addition of vehicle (Supplemented medium) or supplemented medium containing a predetermined concentration of USP grade 200 proof ethanol (Concentrations: 50-800µM, as specified). Both controls and exposed cells were incubated for 6 hours prior to preparation of PARP-1 lysates and 24 hours for assessing MTT and AST. The exposure chamber was preloaded with 200-proof ethanol to restrict ethanol volatilization from experimental medium in accordance with previous reports (Devi et al. 1993; Heitman et al. 1987; Maffi et al., 2008). Doses for ethanol exposure, unless otherwise specified, include: 0mM, 100mM, 300mM, 600mM, and 800mM. Etoposide, supplied by Trevigen (Gaithersburg, MD), was used as positive apoptosis control and was added to supplemented medium at a concentration of (50 µM). HepG2 cells were assessed for establishing ethanol-mediated dose-responses according to cellular viability, aspartate aminotransferase (Cytotoxicity marker), and PARP activation.

Cytotoxicity Assessment

Direct cytotoxicity of cells post ethanol exposure was assessed by trypan blue exclusion or MTT cell proliferation assay (Cayman Chemical, Ann Arbor, MI) per (Devi et al., 1993; Mosmann, 1983). After exposure to ethanol in 6-well or 96-well plates, the vehicle or exposure medium was aspirated and snap frozen for quantifying AST activity. For trypan blue exclusion, HepG2 cells were disaggregated using soft trypsinization and reconstituted in 1000µL supple-

mented medium. A 50 µL aliquot was combined in a 1:1 ratio with trypan blue (0.1% trypan blue in PBS; Corning) for dye exclusion viability assessment via hemacytometer. Except for two samples, a minimum of 500 cells were counted (Exceptions: 488 cells [50 mM] and 252 cells [100 mM]). Alternatively, HepG2 viability was performed by adding 10µL of 10 mg/L 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) solution in 90µL supplemented medium (A 10% solution) via manufacturer's instructions (Santa Cruz Biotechnology, Santa Cruz, CA). After incubation for two hours, the medium was aspirated via pipette, and the insoluble formazin was solubilized in 200 µL of dimethyl sulfoxide (Sigma) and read at 570 nm. Viability, in both cases, was normalized against the control and presented as percent of cells live. A six-point standard curve was utilized in order to quantify samples. Results of the MTT were not significantly impacted by varying concentrations of ethanol in media.

AST (Aspartate aminotransferase), a known biomarker for cytotoxicity, was measured as a proxy of hepatotoxicity as a result of ethanol exposure. Post exposure, vehicle and exposure media was collected and stored at -20° C until analysis which typically occurred within 24 hours. Quantification was performed in replicates of five using an AST colorimetric endpoint kit (TECO Diagnostics, Anaheim, CA) per manufacturer's instructions according to the method described by Reitman and Frankel (1957). However, the assay was down-sized stoichiometrically in order to facilitate assay performance in 96-well assay plates and read via spectrophotometer at 530 nm. The results were represented as total international units of AST per cells plated based on an established standard curve from a calibrator supplied by the manufacturer. Addition of ethanol to media did not significantly impact results of AST quantification in absence of HepG2 cells (Data not shown).

ALT (Alanine-aminotransferase) is also a biomarker of hepatotoxicity, is widely used in analytical chemistry. The medium was prepared similar to the procedure outlined for AST. Post exposure, vehicle and exposure media were collected and centrifuged to isolate pure medium from the insoluble fraction. The media supernatant were then extracted and stored at -20° C until analysis which occurred within 24 hours. Quantification was performed in triplicate using an ALT colorimetric endpoint kit (TECO Diagnostics, Anaheim, CA) per manufacturer's instructions; however, the assay conditions were downsized in order to process samples in 96-well assay plates and read via spectrophotometer at 505 nm. The results were represented total international units of ALT per cells plated based on a calibrator provided by the manufacturer. Addition of ethanol to media did not significantly impact results of ALT quantification in absence of HepG2 cells (Data not shown). This biomarker was ultimately cancelled due to confounding from medium containing 36 millimoles sodium pyruvate, a constituent necessary for indirectly measuring ALT via the prescribed commercial assay kit.

Poly(ADP-ribose) Polymerase Activity

Poly(ADP-ribose) polymerase-1 activity was measured from cell lysate protein using a PARP-1 colorimetric assay kit (Trevigen) per manufacturer's instructions. Vehicle and exposure medium were removed, and HepG2 cells were washed twice briefly with ice-cold DPBS to eliminate any residual medium. 100 µL of cold cell lysate solution containing diluted (1X) PARP buffer (Trevigen), Triton-X100 (Sigma), 0.4M NaCl, and a protease inhibitor cocktail (Sigma) was added and incubated on ice for 30 minutes with a brief, 10 second mixing period via tapping at 5 minute intervals. Recovery of cellular lysates from the 96-well tissue treated plates was complete with little remaining residue. As per manufacturer's instructions (Trevigen), the sam-

ples were homogenized via vortexing without centrifugation to relieve the insoluble fraction. Sample protein concentration was determined using a Pierce BCA colorimetric kit (Pierce, Rockford, IL) with aliquoted samples diluted 1:10 in deionized water via manufacturer's instructions. Samples were performed in duplicate and read via spectrophotometer at 562 nm. Cell lysate stock was stored at -80° C until performance of PARP apoptosis assay according to manufacturer's instructions. Relative PARP-1 activity per sample was standardized by using 20 µg protein lysate per well after subtracting the calculated bovine serum albumin concentration, a lysis blank during BCA quantification provided a concentration per cell lysate solution; each sample quantified PARP activity in duplicate and read via spectrophotometer at 450 nm. Relative PARP activity was quantified against known concentration of PARP enzyme activity based on an established standard curve using PARP enzyme provided by the manufacture, and reported as total PARP activity (miliunits [mU] per 20 ng protein). 10 µM Etoposide served as positive control of PARP-1 cleavage and inactivation.

Spectrophotometric Quantification

Assays requiring assessment of colorimetric endpoints (AST, ALT, BCA, MTT, and PARP) were quantified using the µQuant Spectrophotometer (BioTek, Winooski, VT) with the KC-Junior Analytical software (BioTek). Each colorimetric endpoint was assessed at the wavelength suggested by the manufacturer as mentioned above.

Statistical Analysis

Statistical analysis was performed utilizing the software program SAS version 9.3 by performing an ANOVA test. Individual comparisons between treatments were made via independ-

ent samples Student's T-test. Statistical significance was determined when the probability of attaining the measured statistic fell below 5.0 % ($p \leq 0.05$). Adjustment for multiple comparisons was made via Tukey's adjustment.

CHAPTER THREE:

RESULTS

Cytotoxicity: A Summary

Considering the assays utilized in assessing cytotoxicity, a general trend can be discerned: the insensitivity of the HepG2 model towards ethanol toxicosis. Two distinct biological markers of toxicity were employed in order to elucidate the extent of cellular death. Firstly, and most importantly, cellular viability was assessed using two methods of analysis including trypan blue exclusion and the mitochondrial succinate dehydrogenase-dependent MTT reduction assay. Neither of these two assays demonstrated significant dose-dependent loss in cellular viability, though weak trend associations are implied. Secondly, the quantitative assessment of hepatic cytotoxicity biomarkers liberated in the vehicle and treatment medium was utilized in providing proxy evidence of cellular damage and membrane degradation. Alanine aminotransferase provides a sensitive measure of hepatotoxicity; however, logistical issues precluded continued utilization of this enzyme in accurately assessing a toxicological endpoint. Aspartate aminotransferase activity quantification demonstrated consistent exhibition of a relevant toxic endpoint. A dose-response relationship was observed with AST activity levels in sample culture medium with respect to ethanol concentration, albeit under high, clinically-irrelevant doses. Lastly, in assessing the activity of poly(ADP-ribose) polymerase under the aforementioned challenge, an inverse dose-dependent relationship was demonstrated which was credited to systematic error,

thus, weakening the observed association between PARP-1 activity modulation and ethanol concentration.

Toxicological Endpoints

Cellular Viability

Preliminary trypan blue exclusion assessment showed an insignificant decreasing trend in viability over an increasing ethanol dose. The subtle decreasing trend was observed over ethanol doses ranging between 50 mM and 200 mM, such that at 200 mM, a 3% decrease in viability comparative to the control group was demonstrated (Data not shown). Therefore, the dose was extended to 800 mM in order to encompass higher orders of dose magnitude with a shift from trypan blue exclusion to MTT. Implementation of cytotoxic quantification via MTT reduction facilitated increased statistical power for committing erroneous false negatives regarding viability. Each sample was normalized against the control by expressing viability as a percent of the control whose viability was arbitrarily set to 100% as was performed for trypan blue exclusion.

Similar to the trypan blue exclusion assessment, MTT reduction demonstrated a non-significant trend of decreased viability over ethanol doses. However, the highest dose (800 mM) was required to achieve 95% viability. No statistical decreases or increases in cytoviability were observed, despite observation of slight increases in MTT reduction at the dose groups 300 mM and 600 mM, corresponding to 108.7% and 108.1%, respectively. An initial insignificant viability drop at 100 mM (97.7% of control) was also observed. Only the etoposide-treated group exhibited significant cellular death reaching 71.0% viability as normalized against the control ($p < 0.01$) [*Figure 1*]. These results suggest that, even under high doses, those far above clinical rele-

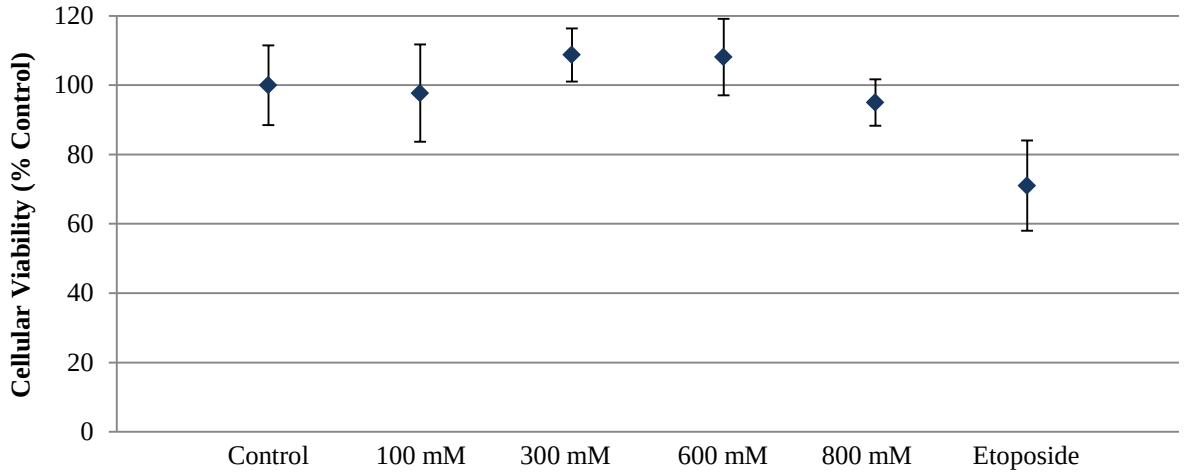

Figure 1. Cellular Viability. Cellular viability was assessed for HepG2 cells 24 hours after dosing with ethanol or etoposide (Positive apoptosis control). The mean viability was plotted with respective standard deviation (Error bars). No statistical differences were observed between ethanol groups in comparison to the control, though a slight negative trend was noted beginning at 800mM. The etoposide group was statistically lower than control ($p < 0.01$).

vance, insignificant effects on HepG2 viability were noted, implying a substantial insensitivity to ethanol relative to cellular viability during experimentation.

Alanine Aminotransferase (ALT)

Exposure of HepG2 to increasing doses of ethanol resulted in an insignificant dose-dependent decrease in alanine aminotransferase activity (Data not shown). As ALT serves as a specific proxy of hepatotoxicity, these results suggest decreased cytotoxicity with incremental increases in ethanol dose; these results were indeed remarkable. Consideration of the analytical chemical technique of the ALT assay kit allows elucidation of the unexpected observed results, which have been deemed erroneous for the following reason.

The ALT colorimetric endpoint assay kit utilizes an indirect method of assessing ALT activity quantification by assuming minimal contribution of both L-alanine and α-ketoglutarate in serum, or in this case medium. Liberation of ALT from hepatocytes into the medium can be quantified by the catalytic formation of pyruvate from the two aforementioned substrates by en-

dogenously-derived ALT. In order to quantify ALT activity, the catalysis product pyruvate undergoes acid-catalysis with 2,4-dinitrophenylhydrazine to form 2-4-dinotrophenyl-hydrozone, which is not produced endogenously, and absorbs as the colorimetric reporter wavelength of 505 nm recommended by the manufacturer. Since the basal Dulbecco's Modification of Eagle's Medium (DMEM) is supplemented with 4.5 g/L sodium pyruvate which, when formulated to a complete supplemented medium, approximately equates to 36 mM pyruvate. Pyruvate in the medium may lead to erroneously elevated ALT values for all samples, even among the highest dose concentration which was, during this was assay, 200 mM. With a decreasing viability, or reduced metabolic capacity, the pyruvate within the supplemented medium will not be consumed at the same rate as the controls (0 mM). As pyruvate concentration in the medium is high (36 mM), detection of differences of endogenously-derived, ALT-mediated pyruvate generation may not kinetically be independent from the initial concentration, especially since kinetic rate constants are dependent upon initial concentration of pyruvate. Therefore, the initial rate of pyruvate production under timed conditions may lead to an erroneous bias of results. This may account for the remarkable results observed, especially since neither dose group exhibited significant ALT elevations. With such high initial concentrations of pyruvate, and natural variability in L-alanine and α-ketoglutarate catalysis by ALT, the results can be biased towards the null. Therefore, the accuracy of estimating ALT activity in medium cannot be attributed to ALT activity alone, and utilization of the ALT colorimetric endpoint assay as a cytotoxic biomarker was not retained for further analysis.

Aspartate Aminotransferase (AST)

Aspartate aminotransferase activity serves as a biomarker of cytotoxicity and was assessed also using an indirect method according to the methods ascribed. Quantification of AST using medium isolates from samples after incubation of 24 hours was reported in international Units per treatment sample cell count (2,500). Each sample was performed in replicates of four (n = 4). To confirm the relative AST activity irrespective of plating density, two dose groups composed of three controls (0 mM) and three treatments (800 mM) of ethanol were plated in 6-well plates at 100,000 cells per well; each dose was plated in triplicate (n = 3). A four-point standard curve served as assurance of reagent condition, sample precision, and optical density linearity across the inclusive AST activity range. However, the calibration standard corresponding to zero AST IU/2,500 cells was subsequently dropped due to a systematic depression in AST activity for the remaining three calibration standards. These results suggest the possibility of AST calibration standard degradation as the recommended utilization of calibration standards should commence within five days post reconstitution-the assay was performed within one week after the ascribed expiration date. In any case, degradation occurred systematically, thus, unaffecting relative effect, e.g., percent or fold versus control. In order to quantify the relative precision of calibration standards, a three-point linear curve was established excluding the zero calibration point. The high coefficient of determination ($R^2 = 1.0$) demonstrated high precision and reagent stability over calibration standards suggesting similar stability may be afforded to the samples. Conclusively, the systematic depression observed among the calibration standards demonstrates a systematic depression in total AST activity within the entire sample pool without deleteriously affecting inter-sample relative AST comparisons; therefore, comparisons among groups can be made in relative AST effect without the presence of deleterious confounding. It

must be noted that the overall AST activity was depressed when quantified against the supplied standard. However, this does not present deleterious effects on data interpretation as relative percentage changes are not affected, nor comparisons against normal/abnormal clinical value ranges are to be made. Comparative analyses in relating AST activity per treatment group illuminate relative effects of ethanol and etoposide against the control group.

Comparison across dose groups resulted in a statistically significant dose-related increase in AST activity with respect to ethanol concentration ($p < 0.01$) [*Figure 2*]. An insignificant inverse dose-related trend was observed for the mean AST level between the 100 mM and 300 mM dose groups, credited to natural inter-sample variable. The AST activity trend began to become positively correlated with ethanol beginning with the 600 mM dose group, though comparison of the 600 mM group against the control group did not reach significance. Among the 800 mM dose group, a statistically significant 43.5% increase in AST activity was detected above control (Student's t-test; $p < 0.001$). Etoposide, the positive apoptotic control, exhibited a higher cytotoxicity biomarker level with an increase of 67.3% above the control, equally as significant as the 800 mM group. A significant increase in AST was observed between 600 mM and 800 mM, signifying a threshold of toxicity above 600 mM. These results indicate the necessity for a large dose in order to induce significant cytotoxicity in the tested HepG2 cells. Even at the 800 mM dose, decreases in cellular viability were not demonstrated by MTT reduction, suggesting that apical signs of ethanol-mediated toxicosis had indeed begun without significant morphological disruption affecting cellular or mitochondrial integrity.

The magnitude of relative affect (AST activity percent increase or decrease relative to the controls for the 800 mM dose group) between the two plating schemes was relatively homologous: 43.5% increase for AST IU/2,500 cells versus 51.1% increase for AST IU/100,000 cells.

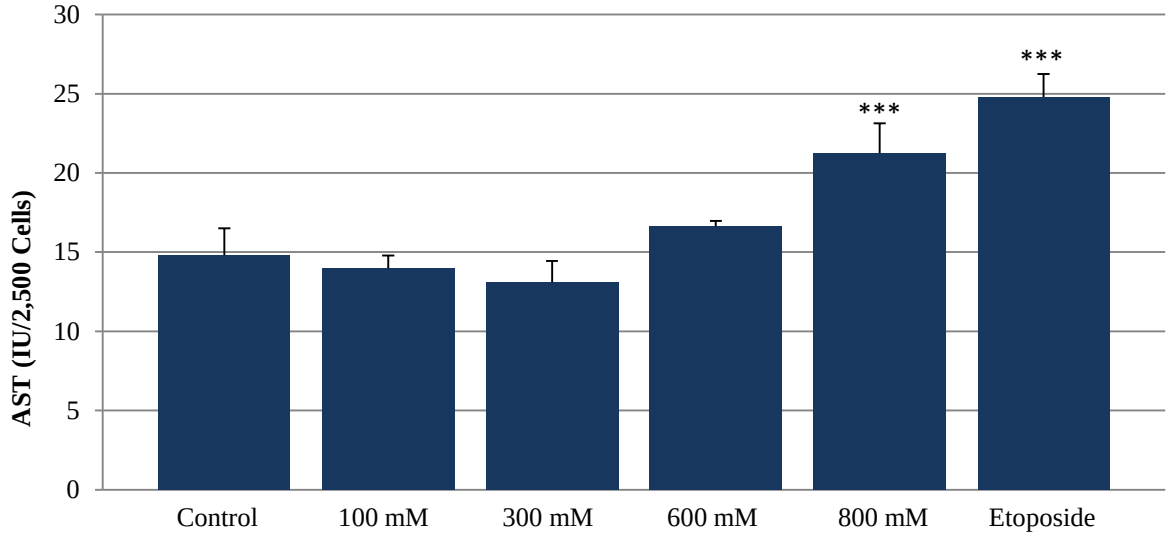

Figure 2. Aspartate Aminotransferase Activity. AST activity was quantified in medium isolates as a proxy of cytotoxicity dependent upon the dose of ethanol and etoposide. The mean of five samples was plotted with respective standard deviations (Error bars). An insignificant decreasing dose was observed up to 300 mM ethanol; at 600 mM, AST activity returned to control levels which continued to statistical significance at 800 mM ($p < 0.001$). Etoposide treatment was equally significant ($p < 0.001$) and exceeded the 800 mM ethanol dose AST level.

These results suggest that, though the absolute AST IU/cell activity may vary depending on plating density, though the relative effect of ethanol did not vary substantially under the aforementioned conditions.

Poly(ADP-ribose) Polymerase Activity

Protein isolates were prepared from 2,500 cells plated in a 96-well plate after the six hour ethanol exposure. After exposure, the cells were briefly washed twice in ice-cold working PBS and lysed with a prepared lysis buffer supplemented with Triton X-100. Protein lysates were then quantified using the methods described above whereby the amount of purified cellular protein required for conducting the PARP activity assay was standardized at 20 nanograms per reaction. A four-point PARP activity standard curve was established in order to cover the range of optical densities exhibited by the complete set of samples. As the calibration standard deviated from

linearity considerably, the line of best fit assumed the natural log transformation of along the x-axis (PARP activity in mU/20 ng), while transformation was not performed along the y-axis (Optical density). The natural log transformed fitted line resulted in a high coefficient of determination ($R^2 = 0.9981$) and conferring a more precise quantification of PARP activity as a function of optical density.

Mean active PARP activity, expressed as mU/20 ng protein, decreased with increasing dose [*Figure 3*]. The decrease in activity initially became significant at the 300 mM ethanol dose group with more pronounced decreases to 46.5% of control at the 800 mM dose group ($p < 0.01$). Dose groups 300 mM and 600 mM each equally exhibited activity approximately 61.7% of the control ($p < 0.05$). Interestingly, the application of etoposide, a known AIF-mediated apoptosis inducer, insignificantly reduced the active PARP activity to 76.1% of the control ($p > 0.05$) which was approximately equivalent to the 100 mM dose group. These results are perplexing as etoposide characteristically results in apoptosis initiation and subsequent caspase-3-dependent PARP-1 cleavage to the inactive 24 kDa and 89 kDa fragments. Of note, however, neither lysis cocktail nor assay buffer blank exhibited substantial optical density readings, thus demonstrating that the presence of albumin was an insignificant source of non-specific binding. Since the contribution of protein from the cell lysate was controlled at 20 nanograms of protein per assay well, and the activity of PARP-1 in the excess of NAD^+ and activated DNA is substantial under physiological conditions, these results may confirm isolation of a conformationally-active PARP-1 enzyme as reported by the high colorimetric chromophore optical density. Unfortunately, these observations cannot be confirmed without sensitive qualitative proteomic testing procedures.

In order to characterize one possible source of explaining the variation in PARP activity quantification, the most pertinent factor potentially affecting the data was determined to be order

of sample addition to the reaction well during the initial stages of conducting the PARP activity assay. ADP-ribosylation occurs at initial onset when adding the assay substrate reagent, the second constituent of the ADP-ribosylation step, to the ELISA plate, and is sensitive to time and procedural diversions. As such, plotting the sequence of reagent addition resulted in a significant time-dependent linear correlation with PARP-1 activity (R^2 = 0.5314) [*Figure 4*]. These data explain that 53.1% of the variation in the linear model can be explained by sequential addition of the reagents during assay execution, corresponding to significant contribution of the model variation by confounding. Therefore, it is highly likely that associations between PARP-1 activity and ethanol-concentration cannot be separated from confounding arising from the aforementioned source. Thus, statistical associations linking ethanol concentration and PARP-1 activity cannot be determined with certainty.

Taken together, the insensitivity of the HepG2 model in ethanol-mediated toxicosis does not represent a clinically-relevant toxicological endpoint, thus, conclusions regarding ethanol-mediated PARP-1 activity cannot be drawn.

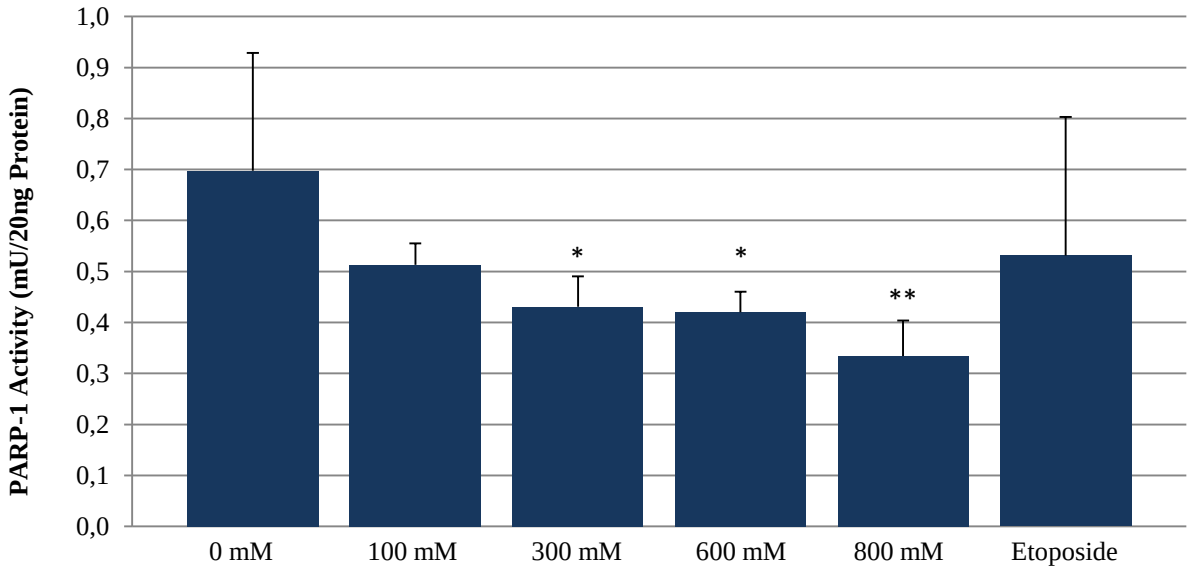

Figure 3. Poly(ADP-ribose) Polymerase Activity. Protein isolates were tested in order to quantify intact PARP-1 activity in screening for apoptosis. PARP activity is expressed as micro units PARP-1 per 20 nanograms protein (mU/20 ng protein); standard deviations are denoted with error bars. A dose-dependent relationship in PARP-1 activity was observed related to ethanol, but this this association was later credited to systematic error (See discussion). This becomes apparent with the medial position of etoposide within the range of activity. Statistical designations are as follows: * ($p < 0.05$); ** ($p < 0.01$).

CHAPTER FOUR:

DISCUSSION

Poly(ADP-ribose) polymerase has been demonstrated to activate and ribosylate ethanol challenged DNA to isolated neuronal cells *in vitro* at 15 minutes and peaking around four hours with significant ADP-ribose residues detected at six hours; the time-point utilized for the current investigation (Cherian et al., 2008). The extra two hours allowed for increased time for metabolism and reactive species and DNA damage occurrence. Numerous reports have demonstrated reactive species formation during hepatic ethanol metabolism, especially considering by-products of the microsomal oxidative pathway member CYP2E1, though alcohol dehydrogenase can also result in the formation of oxidative species (Balasubramaniyan et al., 2007; Cederbaum, 2011; Di Luzio, 1963; Kurose et al., 1997; Yang et al., 2008). With increasing dose, the induction of CYP2E1 leads to the formation metabolically-derived hydroxyl radicals, further promoting hydrogen peroxide and superoxide formation and oxidative DNA lesion accumulation. Endogenous production of nitric oxide, necessary in biosignaling and vasodilation, can interact with superoxide to generate the strong oxidative species peroxynitrite; this phenomenon has been demonstrated to induce PARP activity (Cuzzocrea et al., 1998). Incidentally, acetaldehyde formation exacerbates oxidative stress potential through the inhibition of catalase, thus decreasing endogenous superoxide quenching capability. Taken together, increased production, with simultaneous inhibition of superoxide quenching, potentiates the probability of oxidative stress-generated mitochondrial dysfunction, DNA damage, and lipid peroxidation, while causing dis-

ruption of cellar processes and activation of DNA repair mechanisms, e.g., PARP activation (Kono & Fridovich, 1982; Koh et al., 2005).

Extensive xenobiotic insult to DNA has been an attractive model of elucidating the mechanism by which PARP is both characterized and exploited for therapeutic attenuation of certain diseases. For example, treatment of cells with hydrogen peroxide over-activates PARP-1 leading to endogenous NAD^+ depletion and energy crisis resulting in cellular death (Eliasson et al., 1997; Schraufstatter et al., 1986; Ullrich et al., 1999; Yu et al., 2006). Though the status of ADH and CYP2E1 was uncharacterized in the HepG2 in the current investigation, significant decreases in cellular viability at moderate to high ethanol doses were not observed suggesting low ROS generation activity from either metabolic enzyme. Nevertheless, the results were in accordance with previous observations. Kang and colleagues (2011) demonstrated that insignificant cellular death occurred among dose groups at or below 500 mM, and that the threshold of toxicity was assumed to fall between 500 mM and 1000 mM for their HepG2 batch. Our observations extended the mid-range dosage to include 600 mM and 800 mM, of which neither detected significant viability losses as measured by MTT. A trend towards such a decrease was initially observed with trypan blue exclusion, though these preliminary results may require confirmation to rebuke or confirm the negative results obtained by MTT reduction.

Morphological analysis was not included within the data set, but ethanol doses resulted in overt cellular membrane shrinkage whose extent was qualitatively dose-dependent. The 800 mM group appeared similar, though not as extensively damaged, as the etoposide-treated group. Since apical morphology alone cannot estimate underlying molecular pathogenesis, these remarks are left excluded from the results but remain worthy of consideration for further experi-

mentation. Other methods of subcellular morphological assessment, e.g., hematoxylin and eosin or periodic acid-Schiff staining, may help elucidate a qualitative assessment of gross pathology.

Consistent with several other reports utilizing the HepG2 model, ethanol did elicit leakage of aspartate aminotransferase dose-dependently. AST and ALT have been implied in assessing clinical ethanol toxicosis, particularly for chronic cirrhosis (Cohen & Kaplan, 1979; Nalpas et al., 1984). Of note, however, is the emphasis in Cohen and Kaplan's mention of biomarkers in post-necrotic cirrhosis. Xenobiotic-induced necrosis can follow extensive damage, particularly when repair mechanisms cannot adequately compensate for DNA damage or fill the energy depletion gap (Eguchi et al., 1997; Leist et al., 1997). Since studies related to PARP overactivation have displayed depletion of intracellular NAD^+ and subsequent depletion of ATP stores, the underlying energy crisis precludes the induction of active cellular death programs and may shift the balance to a necrotic-like death (Filipovic et al., 1999; Tentori et al., 2001). Energy depletion and cellular death attributed to PARP-1 overactivation have been associated with detectable quantitative increases in AST activity. This observation was confirmed in the current investigation, and is congruence with previous studies, albeit at orders of magnitude lower than those previously reported at lower ethanol doses (Kumar et al., 2011). For previous studies, levels above 80 mM did elicit significant AST activity in culture medium compared to controls, suggesting a clinically-relevant toxic threshold (Gutierrez-Ruiz et al., 1999; Neuman et al., 1993). Previous trials in the current investigation began with a dose range between 50 mM and 200 mM, within which significant increases in biomarkers of cytotoxicity were not detected. Other studies observed 2-fold to 6-fold increases in AST activity at modest ethanol doses above levels attained in the current investigation at considerably higher doses (Kumar et al., 2011; Senthil Kumar et al., 2012). Interestingly, all of the aforementioned studies did utilize HepG2

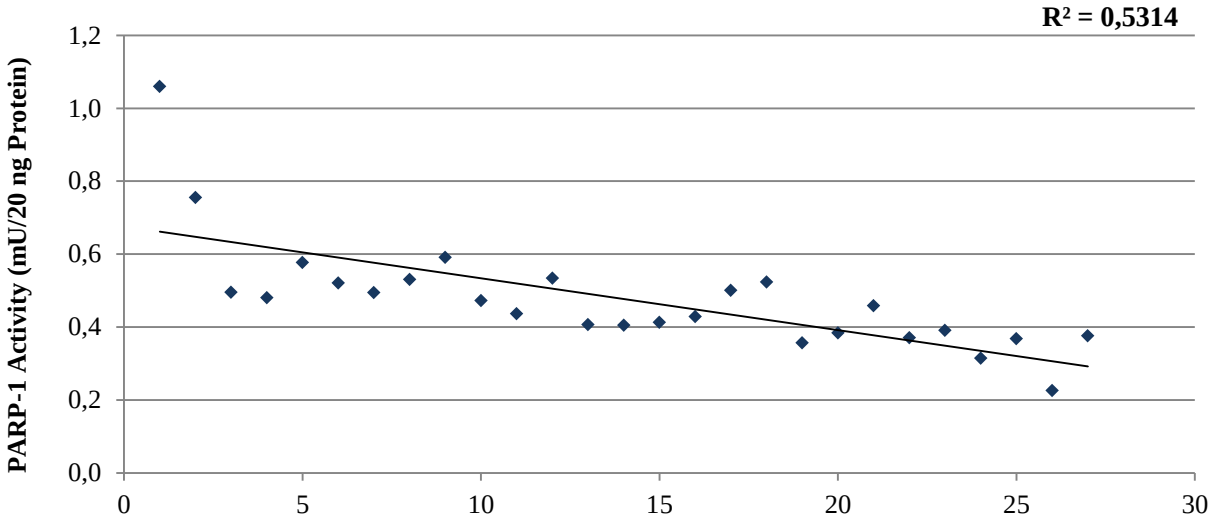

Figure 4. Poly(ADP-ribose) Polymerase Activity as a Function of Sequence. PARP-1 activity assessed by ELISA was analyzed previously to show an association due to dose. Subsequently, a sequential-association was observed in observed. Therefore, the PARP-1 activity was plotted relative to reagent addition revealing a high correlation (Coefficient of determination = 0.5314). Therefore, the variability of PARP-1 activity in the linear model measured could be explained by sequence alone, thus limiting strength of association with ethanol dose. The trend was significant ($p < 0.001$).

cells that were neither designated as transfected nor characterized for CYP2E1 or ADH mRNA transcription status. Consequently, these cells cannot be assumed to be a subtype of the parent HepG2 lineage with attenuated metabolism. In mentioning this, work by Wu & Cederbaum (1996) compared the untransfected line against the transfected line, noting ethanol insensitivity exhibited by the untransfected cells. Results obtained in the current study were place within this spectrum of complete ethanol insensitivity and sensitive, though nearer complete insensitivity, especially at doses below 800 mM. In any case, the AST biomarker of cytotoxicity was dose-dependent and may be attributed to the effective dose of ethanol.

An *in vivo* study by Cederbaum (2011) characterizing the role of CYP2E1 in ethanol hepatotoxicity demonstrated as serum AST activity increase between 50% and 100%, similar to those obtained in the current study, albeit at a substantially lower dose. Pathological lesions in the *in vivo* study exemplified focal necrosis, rather than a caspase-dependent apoptotic pathway. Under these conditions, oxidative stress was implied as the inducer of cellular death. Should the

HepG2 cells utilized in the current study lack physiologically-relevant ADH and CYP2E1 phenotypic expression, an almost complete insensitivity to ethanol-mediated hepatotoxicity would result. Although the obtained results generally signify low activity of constitutive phase I ethanol metabolism, assumptions of CYP2E1 and ADH phenotype across other batches cannot be made without proper characterization. Simply assuming a lack of CYP2E1 activity in the HepG2 model has proven erroneous due to several positive ethanol-sensitive antioxidant studies. Consequently, our observations are not congruent with the baseline HepG2 ethanol-sensitivity exemplified the current literature utilizing antioxidants as protective agents in ethanol-mediated hepatotoxicity using the HepG2 model (Farshori et al., 2013; Kumar et al., 2011; Reddy et al., 2008; Senthil Kumar et al., 2012). Should the previous positive studies utilized a subtype of the HepG2 model with an attenuated phenotypic profile, comparison would be unwarranted. On such subjects, the current study can only afford more evidence that suggests the lack of sensitivity to ethanol and that the characterization of underlying CYP2E1 and ADH activities should be performed prior to experimentation. Since the expression of these two metabolic enzymes have been demonstrated to critically affect oxidative-stress mediated cellular death pathways during ethanol toxicosis, their status is important for comparing past and current literature. Furthermore, as the activation of poly(ADP-ribose) polymerase depends upon the generation of oxidative species and DNA lesions, functionally linking ADH and CYP2E1-dependent ethanol metabolism with PARP-1 overactivation and consequential NAD^+/ATP depletion requires further investigation for possibly clinical relevance and biochemical mechanism elucidation.

In quantifying the active PARP in cellular lysates, the decreasing trend over ethanol dose groups was concluded as dependent upon sequence of reagent addition, rather than ethanol dose. This observation has implications for the integrity of the HepG2 hepatocellular carcinoma line as

a model for ethanol-mediated toxicosis. Assuming a low degree of non-specific binding, the linear correlation of ADP-ribosylation may be indicative of similar PARP activity across dose groups and etoposide as a function of time. After adjustment for protein concentration, even the etoposide-treated cells did not exhibit significant ADP-ribosylation ablation, despite significant etoposide-mediated decreases in cellular viability and increases in AST activity. Under apoptotic conditions, caspase-3 cleaves PARP into the inactive 89 kDa and 24 kDa fragments (Chaitanya et al., 2010; Kaufman et al., 1993; Lazebenik et al., 1994; Los et al., 1997; Nicholson et al., 1995; Simbulan-Rosenthal et al., 1998), thus, exemplifying that etoposide-treated cells could theoretically exhibit very low or negligible PARP activity following PARP-1 inactivation but halts after cleavage. As counter evidence, etoposide treatment did not ablate PARP-1 activity at 15 hours in one study suggesting late apoptosis may not result in significant cleavage of PARP *in vitro*, though this seems unlikely given the well-characterized toxicodynamics of etoposide (Soldani et al., 2001). The obtained results may possibly supply evidence that AIF-induced cellular death may not be suppressed at other points along the apoptotic pathway, especially at the initial onset. This observation would be in congruence with observations made by Cherian and colleagues (2008) in their investigation of PARP-1 induction by ethanol in neuronal cells. Early cellular death *in vitro* may differ from *in vivo* by residual cytokine- or chemokine-mediated effects of the overlaying parenchymal and stromal cells. In any case, treatment groups may not accurately be assessed for PARP cleavage status due to confounding demonstrated, and resulting in insufficient evidence for drawing conclusions on cellular death pathways during this investigation.

In the cases of extensive oxidative stress and subsequent energy depletion, PARP largely avoids caspase-mediated cleavage resulting in transient stabilization *in vitro* (Yu et al., 2002,

2006). This can lead to overactive PARP-1 enzyme signaled by extensive ADP-ribose polymer formation, ADP-ribose polymer translocation to the mitochondria, AIF nuclear translocation, and the initiation of parthanatos. Energy depletion alone has not been demonstrated sufficient in initiating pathogenesis leading to cellular death, though this has been credited rather to the depolarization of the mitochondrial membrane voltage potential ($\Delta\Psi_m$) and subsequent mitochondrial permeability transition pore formation (Andrabi et al., 2006; Hong et al., 2004). Significant AST activity signifies mitochondrial dysfunction and cytotoxicity. Mitochondrial injury was not confirmed as per the results of the MTT viability assay during investigation. As MTT reduction to formazin relies on the mitochondrial succinate dehydrogenase enzyme, the cellular membrane may have been disrupted to a limited extent by oxidation; however, succinate dehydrogenase activity was not significantly suppressed to restrict MTT reduction in ethanol-treated cells. Thus the damage induced by ethanol treatment in our investigation may not have been enough to dissipate the $\Delta\Psi_m$ and induce parthanatos and subsequent viability losses. The progression of the parthanatos pathway cannot be inferred by the given data, especially the extent of radical formation from ethanol-dependent metabolism, and shall require more sensitive methods to fully characterize subcellular interactions.

While characterization of PARP in the HepG2 model is required to properly place ethanol-mediated hepatotoxicosis and PARP activity within a framework of clinical relevance, the high degree of insensitivity of the HepG2 cells under investigation may preclude its utilization in elucidating the PARP's role in hepatotoxicity from some oxidative-stress-generating toxicants, especially considering differential involvement of constitutive phase I metabolic enzymes, such as ADH and CYP2E1. Characterization of basal metabolic activity would supply the most pertinent assurance of phase I oxidative stress generation, but may not assure physiologically-relevant

intoxication response cascade initiation. For example, effects of ethanol in the co-transfected HepG2 model did not inhibit the activation of the JAK-STAT pathway, a phenomenon confirmed in fresh rat hepatocyte isolates (Chen et al., 2001). Interestingly, cultured hepatocytes showed similar insensitivity to ethanol-induced STAT3 inhibition similar to the co-transfected HepG2 model. STAT3 inhibition was restored with administration of acetaldehyde, suggesting reductions in metabolism as a function of duration of time in culture irrespective of underlying metabolic capacity. A model assumed to retain germinal metabolic activity may indeed depreciate such metabolic capacity over time. Quantitatively addressing uncertainty in the model metabolic profile can increase comparability among studies investigating ethanol-mediated hepatotoxicity *in vitro*.

CHAPTER FIVE:

CONCLUSION

This investigation attempted to assess the role of poly(ADP-ribose) polymerase in ethanol-mediated hepatotoxicity using the HepG2 model, an established, well-characterized toxicological model. Several recent studies have utilized this model for therapeutic application of antioxidants in attenuating ethanol-mediated hepatotoxicosis. The assessment of the model in clinical relevance cannot be debated. Additionally, the characterization of PARP's role in ethanol-induced hepatotoxicity was undertaken. A significant trend in one cytotoxicity biomarker, aspartate aminotransferase, was observed, albeit only at ethanol concentrations far above clinical relevance. Assessment of viability did not reveal significant cellular viability losses compared to controls. Finally, PARP activity in isolated cell lysates did not yield information affording characterization for PARP's role in ethanol-mediated hepatotoxicosis. High variability in ethanol sensitivity in the HepG2 model may mask potential physiological phenomena, thus, precluding its utilization in toxicological studies. Transfection may only partially restore some metabolic pathways of physiological systems, but may lack representation of the underlying cytotoxic response to ethanol given an unknown degree of separation from hepatocytes *in vivo*. Therefore, genotypic or phenotypic characterization of metabolic enzymes may increase comparability across ethanol toxicological testing using the HepG2 hepatocellular carcinoma line, but the model may still be inappropriate for characterizing the role of PARP in ethanol-mediated hepatotoxicity.

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