The assessment of an *in-vitro* model for evaluating the role of PARP in ethanol-mediated hepatotoxicity

Jayme P Coyle, A Mayo-Perez¹, M Bourgeois, G Johnson, S Morris², RD Harbison

ABSTRACT

This investigation aims to assess whether the hepatocellular carcinoma cell line, HepG2, is an appropriate model to assess the role of poly (ADP-ribose) polymerase (PARP) during acute ethanol toxicosis. HepG2 cells were dosed with graded concentrations of ethanol, ranging from 100 mM to 800 mM, for 6 hours to assess PARP activity induction, while another parallel experiment examined cellular damage via medium aspartate aminotransferase activity and cellular viability via MTT reduction. Aspartate aminotransferase activity was significantly elevated at 600 mM ethanol (FOLD; \(P < 0.01\), with further increases at the 800 mM dose (1.43 fold; \(P < 0.001\)), compared to controls. Cellular viability was not significantly decreased compared to controls among all dose groups. PARP activity measured in total cell lysates showed a significant decreasing trend with respect to ethanol dose, reaching statistical significance at the 100 mM dose group (\(P < 0.05\)). Paradoxically, exposure to 50 \(\mu\)M etoposide (Positive apoptosis-inducing control) did not demonstrate significant PARP activity ablation. When analyzing PARP activity observation temporally, a significant correlation (\(R^2 =0.5314\)) was observed between activity and assay sequence. Overall, a clear HepG2 insensitivity to ethanol was observed.

Key Words: CYP2E1, Ethanol, HepG2, Hepatotoxicity, *In vitro*, Poly (ADP-Ribose) Polymerase

INTRODUCTION

Ethanol is a ubiquitous recreational drug associated with severe chronic liver disorders such as cirrhosis. Several studies have attempted to elucidate acute mechanisms of ethanol hepatotoxicosis to apply intervention agents to attenuate hepatic injury using *in-vivo* and *in-vitro* models.[¹,²] Among the studies, a common factor has remained of interest, the role of reactive oxygen species (ROS) in mediating hepatotoxicity.[³]

Ethanol is predominantly metabolized in hepatocytes by alcohol dehydrogenase (EC 1.1.1.1.) to acetaldehyde under low-dose conditions, while cytochrome P450 2E1 (CYP2E1) essentially metabolizes the remaining portion yielding acetaldehyde and several ROS. Under high acute doses, transient transcriptional induction of CYP2E1 compensates for the comparatively low ethanol CYP2E1 binding affinity, thus promoting enhanced hepatocyte ethanol clearance.[⁴,⁵] Consequently, increased microsomal metabolism enhances ROS load of hepatocytes. ROS have been implicated in precipitating intracellular oxidative stress and cytotoxicity in several cell types, including hepatocytes, signifying a plausible unified physiological phenomenon.[⁶-⁸]

The HepG2 immortalized hepatocellular carcinoma cell line has received extensive utilization as toxicological and pharmacological models, especially for ethanol toxicosis, as described later. However, recent evidence has demonstrated a required constitutive CYP2E1 expression level for inducing ROS-generating microsomal ethanol metabolism; a phenotype rescued after stable transfection with the CYP2E1 gene.[⁹] The dependence of acute ethanol-mediated hepatic injury on ROS formation has been demonstrated repeatedly (Reviewed by.[¹⁰,¹¹] Conversely, CYP2E1-deficient cells do not exhibit significant oxidative damage at clinically relevant doses.[¹²] These results further strengthen observations of oxidant quenching in attenuating rat hepatotoxicity *in vivo*.¹³ Subsequent investigations using ethanol have revealed an unstable toxic susceptibility profile of...
Nevertheless, current reports continue to utilize the model in demonstrating ethanol sensitivity phenotype, followed subsequently by antioxidant rescue, albeit without designation of CYP2E1 transfection status.\(^{15-18}\)

Chromatid oxidative stress has been extensively demonstrated to promote induction of poly (ADP-ribose) polymerase (PARP) activity and chromatin modification.\(^{19,20}\) In linking ethanol and PARP activation, Cherian and colleagues\(^{21}\) demonstrated transient poly (ADP-ribose)ylation (PARylation) in fetal cortical neurons after ethanol exposure. PAR levels spiked between six to eight hours post exposure, supplying evidence of a link between ethanol toxicosis and PARP activity. Ha and Snyder\(^{22}\) described a necrotic-like death resulting from intracellular oxidized nicotinamide adenine dinucleotide (NAD\(^+\)) reservoir depletion as a direct result of PARP overactivation in accordance with several previous reports\(^{23-25}\) an endpoint that can be therapeutically attenuated by PARP inhibition or through endogenous pathways involving caspase-3 catalytic cleavage.\(^{26}\)

The PARP family, first described by Chambon and colleagues\(^{27}\) includes 16 members classified based on the putative ADP-ribose catalytic domain. Only one nuclear isoform, PARP-1, has the signature chromatid-interacting zinc fingers allowing histone ribosylation.\(^{28}\) Under periods of nuclear oxidative stress, PARP-1 acts as a DNA break and nick sensor resulting in transient ribosylation using endogenous NAD\(^+\) as the ribose monomer substrate donor. Electrostatic interactions between ribose moieties and the negatively charged chromatid induce an open chromatin conformation while concomitantly rescuing undamaged histones from proteasomal degradation.\(^{19,20,29,30}\)

Other DNA repair machinery, such as XRCC1, become recruited by local PARylation, indicating that PARP plays a role in orchestrating chromatid repair mechanisms.\(^{31,32}\) In averting energy crisis by ATP, NAD\(^+\) depletion, and cellular necrosis, nicotinamide, the by-product of ribosylation, provides a competitive negative-feedback loop resulting in local PARP activity regulation.\(^{33}\) Transient PARP auto-PARylation of the automodification domain promotes decreased affinity for chromatin and PAR catalysis. Initial activity increases arise from PAR hydrolysis by poly (ADP-ribose) glycohydrolase (PARG). Interestingly, PARG also plays an antagonistic modulatory role in PARP activity by hydrolyzing covalent PARylation of chromatid, after which PAR chains become solubilized. These solubilized PAR chains are thought to be associated with apoptosis inducible factor translocation to initiate parthanatos.\(^{26,34,35}\)

Several lines of evidence have suggested attenuation of toxic insult associated through therapeutic inhibition of PARP inhibition with resultant rescue of intracellular energy stores.\(^{36-38}\)

Nevertheless, the role of PARP in acute ethanol toxicity remains incompletely understood, especially in hepatocytes. This report is an initial investigation in ascertaining the feasibility of using the untransfected HepG2 cell line as a toxicological model for assessing the role of PARP \textit{in vitro}, which may later serve to elucidate PARP’s role and the efficacy of its inhibition in ethanol hepatotoxicosis.

**MATERIALS AND METHODS**

**Cell culture, passage, and seeding**

HepG2 hepatocellular carcinoma cell line was purchased from the American Tissue Culture Collection (ATCC; Catalog no. HB-8065; Manassas, VA). HepG2 cells were cultured in Dulbecco’s modified Eagle Medium containing phenol red (DMEM; CellGro, Manassas, VA) supplemented with 10 mM HEPE buffer (Sigma Aldrich, St. Louis, MO), 100 IU/mL penicillin and 100 μg/mL streptomycin (ATCC), and 10% fetal bovine serum (ATCC) at 37°C under a 5% carbon dioxide humidified atmosphere. Growth medium was exchanged every three to four days when confluent. When reaching 80% confluence, cells were washed with calcium- and magnesium-free phosphate buffered saline solution (CellGro), disaggregated by light 0.05% w/v trypsinization with EDTA (CellGro), collected by centrifugation (250 ×g), and resuspended in fresh growth medium. Cells were either plated for continued propagation at a ratio of 1:3 or 1:6 or counted via hemacytometer using trypan blue (CellGro) for seeding experimental plates. All experimental samples were seeded overnight prior to sample preparation in order to allow proper plate adherence.

A single 96-well microplate was seeded with 2,500 cells per well in replicates of five in preparation for aspartate aminotransferase (AST) and cellular viability measurements; AST and cellular viability data were ascertained from the same experimental pool. The medium extraction required for AST quantification left the underlying cell layer intact, thus permitting viability assay without complication. A six-point standard calibration curve ranging from zero cells per well (Growth medium only) to 10,000 cells per well was plated on the same 96-well plate to calculate cellular viability; standards for each dose group were plated in triplicate. A second plate was plated at 2,500 cells per well in replicates of five for each dose group in order to prepare total cell lysates for quantifying PARP activity.

**Reagent preparation and exposure solutions**

A stock 800 mM solution was prepared from USP grade 200 proof ethanol (supplied by the University of South Florida Medical College), from which serial dilutions were formulated to yield the final concentrations:
100 µM, 300 µM, 600 µM, and 800 µM; complete growth medium served as the negative control vehicle. Etoposide, a positive apoptosis control, was diluted to 50 µM in complete growth medium from a stock 10 mM solution supplied by Trevigen (Gaithersburg, MD). All samples were incubated in 200 µL of exposure medium or vehicle for the incubation periods designated below. In order to minimize ethanol volatilization from the exposure medium, all overnight exposures were conducted under an ethanol-saturated atmosphere at 37°C and 5% CO₂ in accordance with previous methods.

The protein lysate solution required to perform PARP activity quantification was formulated as per manufacturer’s recommendation, and is as follows: 20X I-PAR buffer, 0.4M NaCl (Sigma Aldrich), 0.1% Triton X-100 (Sigma Aldrich), protease inhibitor cocktail (Sigma Aldrich), and double distilled water.

Cytological endpoint assays

Three different endpoints were assessed via colorimetric spectrophotometry using the µQuant universal microplate spectrophotometer (BioTek, Winooski, VT). Standard calibration curves were established for each endpoint based on the requirements for each respective endpoint per manufacturer’s instructions. Protein quantification was also performed using a standard BCA kit (Pierce, Rockford, IL) per manufacturer’s instructions and quantified by spectrophotometry via colorimetric endpoint. All plates assayed were 96-well flat-bottom tissue-treated microplates (CellTreat, Shirley, MA), except for the PARP activity assay for which proprietary 8-well strips were supplied. The µQuant spectrophotometric data was integrated using the KCjunior™ interface software.

Aspartate aminotransferase activity

Aspartate aminotransferase (AST), a specific in-vitro biomarker, was used as a proxy of hepatotoxicity. HepG2 cells were plated in a 96-well microplate at 2,500 cells per well in replicates of five per dose group. Each dose group was incubated with 200 µL of the respective medium incubated in ethanol (100, 300, 600, or 800 mM), etoposide (positive control), or vehicle (negative control) for 24 hours. After incubation, 100 µL of exposure medium or vehicle was extracted to a pre-chilled Eppendorf centrifuge tube and stored at −20°C until assaying. All samples were processed within 72 hours of medium extraction.

AST quantification was performed for each sample in triplicate using a standard diagnostic assay kit (Teco Diagnostics, Anaheim, CA) per manufacturer’s instructions, with minor modification. The kit was scaled down stoichiometrically to accommodate assay execution in a 96-well plate required by the spectrophotometer, rather than in a test tube suggested in the instructions.

Concomitantly, a six-point standard curve was constructed of serial dilutions from a stock calibration standard supplied by the manufacturer; each standard was plated in duplicate.

Cellular viability

Indirect cellular viability was quantified using the mitochondrial 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay described by Mosmann. The samples utilized for AST medium activity were also used for MTT, as the cells themselves were not compromised during half well medium extraction. In post analysis, one sample was excluded as an outlier result, which reduced the 600 mM group to n = 4. The outlier sample, however, did not affect the AST data; thus, it was not excluded from the respective cytotoxicity analysis. All remaining dose groups as well as positive control received data from five replicates. MTT was performed using a manufactured assay kit (Cayman Chemical, Ann Arbor, MI) per manufacturer’s instruction with minor modification described below.

After the 24-hour exposure period, the remaining cellular exposure medium or vehicle was aspirated after initial AST medium sample draw and replaced with 100 µL growth medium supplemented with 10% MTT solution. Each sample was incubated for three hours at 37°C under a humidified 5% carbon dioxide atmosphere. Thereafter, the medium was aspirated carefully to avoid stirring the insoluble formazin crystals. Formazin crystals were resuspended in 100 µL of dimethyl sulfoxide (Sigma Aldrich) for 10 minutes with intermittent stirring and read via spectrophotometry at 570 nm.

PARP activity

HepG2 cells were plated at 2,500 cells per well in replicates of five and incubated in exposure medium or vehicle for six hours. The time point was chosen based on a report by Cherian and colleagues (2008) using fetal cortical neurons. After exposure, each sample was aspirated and gently washed twice with 200 µL ice-cold Dulbecco’s Phosphate Buffered Saline (D-PBS) without calcium or magnesium. To each well, 100 µL of cell lysate solution was added, followed by a 30-minute incubation on ice interceded by brief 10-second mixing periods via tapping at 5-minute intervals. Each sample lysate was then extracted from each well to a pre-chilled Eppendorf centrifuge tube and homogenized by vortexing for 10 seconds. A small aliquot was diluted 1:10 in deionized water for protein and quantified in duplicate using the Pierce BCA colorimetric protein quantification kit (Pierce) per manufacturer’s instruction while performing a lysate blank to adjust for lysate solution protein concentration (Approximately 100 ng/µL). The concentrated total cell lysate was immediately stored at −80°C until PARP activity quantification.
PARP activity was measured using a standard assay kit per manufacturer’s instructions (Treven, Gaithersburg, MD). Cell lysate samples were standardized at 20 ng of protein and performed in duplicate. A standard curve was established using a calibration standard provided by the assay kit. PARP activity was reported as total PARP activity in milliunits [mU] per 20 ng protein.

**Statistical analysis**
Statistical analysis was carried out using SAS 9.3 software for all colorimetric endpoints. Additionally, statistical adjustment for multiple comparisons was performed using Tukey’s method. Statistically significant comparisons against the control group were performed and followed conventional designations: * P < 0.05, ** P < 0.01, and *** P < 0.001.

**RESULTS**

**Aspartate aminotransferase activity**
AST liberation in medium was quantified in order to serve as a proxy of cytotoxicity in the HepG2 cell model and normalized against 2,500 cells per well. Though AST remains an insensitive biomarker of hepatotoxicity in vitro, its use in in vitro modeling precludes non-specific endogenous sources other than hepatocytes.

Under high doses of ethanol for 24 hours, insignificant levels of cytotoxicity were observed at, and below, 600 mM (P > 0.05) reaching statistically significant increases at 800 mM (P < 0.001) [Figure 1]. A slight, but insignificant, decreasing trend of AST at incrementally higher doses was observed up to 600 mM, above which a positive correlative trend to 800 mM was observed.

Over incremental increases in ethanol among the exposure groups, no significant difference in viability was observed (P > 0.05). The 100 mM demonstrated a slight decrease in viability at 97.7% of the control, while among the 300- and 600-mM dose groups, slight increases in viability (108.6% and 108.1% of control, respectively) were observed. Standard deviations for each of these two groups indicated that these values were within the range of normal variability. Among all ethanol exposure groups, the 800-mM dose group resulted in the greatest viability decrease, but remained high at 95.5% of the control group where a toxic threshold for viability was not demonstrated. The positive control group demonstrated a significant 36.4% decrease (P < 0.001) in cellular viability as expected, and in accordance with apoptotic induction.

**PARP activity**
PARP activity was quantified from total cell protein lysates after protein quantification and standardization to 20 ng of protein per reaction. Each dose group contained five experiments, except the control group with four replicates and positive control, which contained three experiments (Total n = 27); each sample was performed in duplicate when performing PARP activity quantification [Figure 2c].

The control group demonstrated approximately 0.698 miliUnits of PARP activity per 20 ng protein [Figure 2c]. With increasing doses of ethanol, PARP activity decreased, reaching statistical significance at 300 mM (0.431 mU/20 ng protein) and 600 mM (0.420 mU/20 ng protein; P < 0.05), respectively. At 800 mM, PARP activity was further suppressed reaching approximately 0.334 mU per 20 ng protein lysate (P < 0.01). The etoposide group, however, failed to result in a significant decrease in PARP activity compared to the control group and was measured
at 0.531 mU/20 ng protein ($P > 0.05$). The data was later reanalyzed in a time-dependent manner to elucidate variance among the obtained PARP activity results.

Evaluating PARP activity in relation to order of sample assay, a clear confounding measure was observed [Figure 2d]. In adding reagents according to the manufacturer’s protocol, the color reagent began development during assaying, which could not be rectified due to subsequent washing steps. As such, total evacuation of assay reagent occurred simultaneously while addition was sequential, leading to differential incubation residence times across all samples. The sequence revealed in Figure 2d began with the control group, followed sequentially by the etoposide-treated positive control group and the ethanol-treated experimental groups in ascending order of concentration (Total $n = 27$). When a simple fitted regression line was superimposed upon the data, the linear coefficient of determination was determined as 0.5314. These results demonstrate sufficient linear correlation to conclude a reagent sequence-dependent trend in PARP activity, especially since etoposide treatment did not result in significant activity ablation. Therefore, the data regarding PARP activity in relation to ethanol dose may be subject to variation arising from methodological variables.

**DISCUSSION**

**Alcohol insensitivity and PARP**

The current investigation demonstrated profound ethanol sensitivity in the HepG2 model. The cytotoxic threshold as measured by AST was between 600 mM and 800 mM; neither dose resulted in significant cytoplasmin losses. Conversely, the positive control did demonstrate significant cytotoxicity as measured by AST activity quantification with concomitant significant decreases in viability as expected. A negative volume–displacement control demonstrated that at the 800 mM ethanol dose level, pure volumetric dilution of the complete growth medium did not result in the obtained AST and viability changes above the control (Data not shown). Therefore, it may be assumed that toxicological endpoints were the result of the toxicant and not of dilution-dependent nutrient starvation.

Ethanol sensitivity has been shown in previous reports, the most relevant of which the authors demonstrated a toxic...
threshold between 500 mM and 1000 mM. The former dose demonstrated produced negligible (<5%) decrease in viability, where the latter 1000 mM dose resulted in cellular viability approaching 50% of controls. In the present study, dosing regimens up to 1000 mM were not attempted as the clinical relevance above even 60 mM becomes suspect at this level which may produce alcohol-induced coma or death if severe respiratory depression remains untreated. Nonetheless, Kang and others did not designate the HepG2 model as transfected. Their results were similar to those of the current investigation, which utilized a non-transfected HepG2 model. Early reports comparing ethanol sensitivity between the CYP2E1 transfected and untransfected model have demonstrated rescue of the ethanol sensitivity phenotype by stable transfection.

A wide body of evidence has suggested the precipitation of apoptosis in response to ethanol toxicosis particularly through a caspase-3-dependent pathway; this has been demonstrated even in the HepG2 model. Under high ROS loadings, oxidative stress precipitates chromatid oxidation, which requires cell cycle arrest and repair or, if severely damaged, apoptosis induction. As intracellular ATP stores have been implicated in orchestration of necrotic/apoptotic progression, PARP overactivation during high period of oxidative stress may promote the necrotic-like cellular death pathway parthanatos. Irrespective of the cellular death pathway, a detectable difference of PARP activity could be expected compared to controls. If the HepG2 cells were sufficiently insensitive to ethanol toxicosis, as suggestive of the AST and cellular viability assays, we can expect that PARP activity may be similar to that of the controls. Should this be the case, a dynamic equilibrium between oxidative recapitulation and ROS- or electrophile-mediated DNA damage would be reached, albeit at slightly elevated levels in accordance with the post-translational modification required to meet the demands of repair mechanisms during chromatin damage response. Otherwise, a dose-dependent relationship between ethanol and PARP activity would possibly be observed. In contrast, activity ablation would suggest a caspase-3-dependent cellular death pathway during apoptosis, such as induced by etoposide or in the later phases of parthanatos. Irrespective, the fate of PARP has been shown to be phenotypically constant: Catalytic cleavage inactivation. Administration of 68 µM etoposide to HL-60 cells, a leukemia cell line, demonstrated profound PARP activity ablation in accordance with a cascade-dependent apoptotic cascade. Alternately, selective induction of necrosis demonstrated a characteristically distinct PARP cleavage pattern presumably during the later stages of parthanatos, suggesting a caspase-3-independent pathway.

Model phenotype variation: HepG2
Cultured cells, in particular the immortalized HepG2 cell line, demonstrate profound phenotypic changes between HepG2 batches—a phenomenon also confirmed in stable primary cultures, such as fresh tissue isolates and the HepaRG cell line with passage of time in culture and culture conditions. Interestingly, the work by Hewitt and Hewitt has demonstrated wide variability in putative phase I metabolic enzyme expression profiles even among HepG2 batches, suggesting severe potential to affect reproducibility among research groups. Genetic profiling of HepG2 cells compared to standard hepatocytes revealed 2646 significantly down-regulated and 3586 significantly up-regulated genes, offering insight into heterogeneous sensitivity in the literature, especially compared to hepatocyte models. For example, Castaneda and coworkers demonstrated significant ethanol-mediated apoptosis via the Fas receptor in HepG2 cells at low millimolar doses (e.g. 1 mM), far below those typically observed even among the CYP2E1 transfected model. Therefore, an unstable ethanol-mediated toxic threshold for the HepG2 cell line undermines the existence of a common mechanism to explain such phenotypic heterogeneity. Work by Wu and Cederbaum have credited HepG2 ethanol insensitivity to a lack of putative expression of CYP2E1, which is generally accepted as factor of ROS-mediated damage under acute high-dose ethanol doses in vivo and in vitro. Even CYP2E1-containing HepG2 cells have the capacity to mimic physiologically-relevant ethanol-mediated CYP2E1 transcriptional up-regulation. Thus, a lack of CYP2E1, especially with regard to transcriptional deficiency, would likely result in profound insensitivity. However, the current investigation was unable to reproduce reported HepG2 apoptotic sensitivity at concentrations (Up to 800 mM) compared to demonstrated effective concentrations, which themselves are far above the exposure range of clinical relevance (<1 mM to 80 mM, or higher). Therefore, reproducing a similar toxic outcome at low millimolar concentrations was precluded; an endpoint which the authors themselves report as yet unobserved in normal hepatocytes. Such heterogeneities undermine the extrapolatability of the untransfected.

CONCLUSION

This investigation attempted to characterize this phenomenon in the HepG2 cell line. Given the marginal increase in AST activity after large acute doses of ethanol, 1.43-fold at 800 mM compared to vehicle controls, and insignificant changes in cellular viability even at the highest dose, the clinical and toxicological relevance of the untransfected HepG2 model becomes a consideration. However, it is that the obtained data did not reflect relevant PARP response to etoposide treatment currently described within the literature. As parthanatos resembles characteristics of both necrosis and apoptosis, PARP activity would be expected to increase, decrease, or resist change during cellular degeneration, depending upon the time point chosen.
Several recent studies achieved reproducibility of previous results investigating ethanol-mediated toxicity with HepG2 cell line; many of these have focused on hepatoprotective antioxidant compounds. Paradoxically, others have challenged the relevance of the model arising from profound ethanol insensitivity; a phenotype partially rescued by stable transfection with the CYP2E1 gene. Although, even stable transfection seems to rescue the hepatocyte metabolic phenotype only partially, faintly reproducing results obtained in tissue isolates. Thus, the pathway leading to cytotoxic insult, i.e. CYP2E1-mediated ROS generation with subsequent oxidative stress, becomes essentially shunted. As a result, this model may not be metabolically representative in assessing hepatotoxic or hepatoprotective mechanisms in vitro without characterizing a proxy of CYP2E1 activity.

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