Published in final edited form as:
Toxicol Sci. 2006 September ; 93(1): 213–222.

Phenotypic Anchoring of Acetaminophen-Induced Oxidative Stress with Gene Expression Profiles in Rat Liver

Christine L. Powell[*,†], **Oksana Kosyk**[†], **Pamela K. Ross**[†], **Robert Schoonhoven**[†], **Gunnar Boysen**[†], **James A. Swenberg**[*,†], **Alexandra N. Heinloth**[‡], **Gary A. Boorman**[§], **Michael L. Cunningham**[*,¶], **Richard S. Paules**[‡], and **Ivan Rusyn1**[*,†]

* *Curriculum in Toxicology, and*

†*Department of Environmental Sciences and Engineering, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599; and*

‡*National Center for Toxicogenomics,*

§*Environmental Toxicology Program, and*

¶*Laboratory of Pharmacology and Chemistry, National Institute of Environmental Health Sciences, Research Triangle Park, NC 27709*

Abstract

Toxicogenomics provides the ability to examine in greater detail the underlying molecular events that precede and accompany toxicity, thus allowing prediction of adverse events at much earlier times compared to classical toxicological endpoints. Acetaminophen (APAP) is a pharmaceutical that has similar metabolic and toxic responses in rodents and humans. Recent gene expression profiling studies with APAP found an oxidative stress signature at a sub-toxic dose that we hypothesized can be phenotypically anchored to conventional biomarkers of oxidative stress. Liver tissue was obtained from experimental animals used to generate microarray data where male rats were given APAP at sub-toxic (150 mg/kg), or overtly toxic (1500 and 2000 mg/kg) doses and sacrificed at 6, 24, or 48 hrs. Oxidative stress in liver was evaluated by a diverse panel of markers that included assessing expression of base excision repair (BER) genes, quantifying oxidative lesions in genomic DNA, and evaluating protein and lipid oxidation. A sub-toxic dose of APAP produced significant accumulation of nitrotyrosine protein adducts, while both sub-toxic and toxic doses caused a significant increase in 8-hydroxy-deoxyguanosine. Only toxic doses of APAP significantly induced expression levels of BER genes. None of the doses examined resulted in a significant increase in the number of abasic sites, or in the amount of lipid peroxidation. The accumulation of nitrotyrosine and 8-hydroxy-deoxyguanosine adducts phenotypically anchors the oxidative stress gene expression signature observed with a sub-toxic dose of APAP, lending support to the validity of gene expression studies as a sensitive and biologically-meaningful endpoint in toxicology.

Keywords

toxicogenomics; acetaminophen; nitrotyrosine; 8-hydroxy-deoxyguanosine; oxidative stress; lipid peroxidation

Corresponding Author: Ivan Rusyn, M.D., Ph.D., CB#7431, Department of Environmental Sciences and Engineering, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-7431, Phone/Fax: (919) 843-2596, Email: iir@unc.edu.

INTRODUCTION

Toxicogenomics is an area in toxicology that elucidates how the entire genome is involved in biological responses of organisms exposed to environmental toxicants. Expectations for this new field have been high with promises of obtaining in much greater detail the molecular events that precede and accompany toxicity, thus allowing prediction of a toxic insult at much earlier stages than classical measures. Initial studies have been encouraging with gene-specific signatures that predict and classify unknown hepatotoxicants based on a preliminary training set of chemicals (Hamadeh *et al.* 2002). This work has led to the hypothesis that it is possible to define signature patterns of altered gene expression that indicate specific adverse effects of chemicals, drugs, or environmental exposures.

In order for gene expression profiling to become a well recognized and valuable tool in toxicology, it should be characterized for its ability to reflect the results derived from classical toxicology assays (*e.g.*, histopathology, and clinical chemistry) in a dose- and time-dependent manner (Paules 2003). Such phenotypic anchoring removes subjectivity from interpretation of expression data by distinguishing between the toxicological effect signal from other gene expression changes that may be unrelated to toxicity, such as the therapeutic effects of a compound. Unfortunately, much of the available toxicogenomic data that has been published to date, with few exceptions, has been limited to a description of alterations in gene expression patterns.

Toxicology studies, in themselves, are quite complex with sources of variability resulting from the dose and delivery of the chemical under study, the choice of animal species, and the differences in biological and pathological responses of various tissues (Boorman *et al.* 2002). The combination of this with the known technical variability in genomic studies (Bammler *et al.* 2005) underscores the importance of careful validation of alterations in gene expression patterns. In most cases, expression data can be phenotypically anchored using classical toxicological methods; however, the apparent lack of sensitivity for most toxicity assays will make this difficult for altered expression patterns observed at sub-toxic doses. Thus, corroboration of such expression data sets will require the use of more sensitive, complex assays.

The results of gene expression profiling studies can serve as a guide in the search for specific genes and/or proteins that could be used as biomarkers of incipient toxicity, or can predict the pathological changes that are yet to be realized by morphological analysis. Recently, a proof of concept study was conducted whereby rats, the preferred model organism in toxicity testing, were administered the hepatotoxicant, acetaminophen (APAP), and gene expression profiling was performed (Heinloth *et al.* 2004). It was demonstrated that alterations in expression patterns at a low, sub-toxic dose (*i.e.*, no apparent toxicity was detected by histopathology or clinical chemistry) may reveal signs of subtle cellular injury that are exacerbated at higher doses. Specifically, it was found that altered gene expression patterns were suggestive of mitochondrial dysfunction and oxidative stress and with increasing dose there was concomitant increase in the magnitude of response and number of genes represented within the same vital cellular pathways. However, the specificity of these gene expression changes to the mechanism of APAP hepatotoxicity cannot be discerned without further research. Here, we have undertaken a study to substantiate these findings of a gene expression signature for oxidative stress by a sub-toxic of APAP in rat liver using a panel of sensitive biomarkers of oxidative stress and oxidative DNA damage. The results of our studies show that a reduction in glutathione (GSH) content in the liver alongside with the accumulation of nitrotyrosine protein adducts and 8-hydroxy-deoxyguanosine lesions in DNA, events known to be a part of the mode of action of APAP, provide good and early phenotypic anchors for gene expression signature of APAP-induced oxidative stress, even at a sub-toxic dose.

MATERIALS AND METHODS

Animals and treatments

The studies detailed herein were performed using liver tissues (stored at −80°C) from previously published report (Heinloth *et al.* 2004) where male Fisher 344 rats were administered a single acute dose of acetaminophen by gavage at sub-toxic (150 mg/kg) or overtly toxic (1500 and 2000 mg/kg) doses in 0.5% aqueous methyl cellulose. Animals were sacrificed 6, 24, or 48 hrs following dosing at which point frozen and formalin-fixed liver samples were collected.

Determination of Liver Tissue Glutathione Levels

Approximately 50 mg frozen liver tissue was homogenized in 5% sulfosalicyclic acid, centrifuged at 8000 g for 10 min, and the supernatant assayed for reduced glutathione content following manufacturer's protocol (BioVision, Mountain View, CA).

Immunohistochemistry

Formalin-fixed, paraffin-embedded sections (6 μm) were mounted on glass slides. Sections were deparaffinized in xylene, rehydrated in a series of graded alcohol concentrations, and placed in phosphate-buffered saline (PBS) with 1% Tween 20. Immunostaining was performed using DAKO EnVision System HRP (Dako Cytomation, Carpinteria, CA) with primary antibody [1:200 nitrotyrosine (Molecular Probes; Eugene, OR); 1:200 malondialdehyde (MDA; Alpha Diagnostic, San Antonio, TX); and 1:200 8-hydroxyguanosine (Research Diagnostics; Flanders, NJ)] diluted in PBS containing 1% bovine serum albumin and incubated overnight at 4°C. Slides were counterstained with hematoxylin. In order to ensure the quantitative measurement of each immunoreaction, all sections from each animal and group to be compared were processed in parallel. Antibody specificity was determined by incubating each antibody with its respective antigen before immunostaining. Quantitative analysis of immunostained liver sections was performed using BIOQUANT software (BIOQUANT Image Analysis, Nashville, TN) by averaging percent area stained to total area within pericentral regions at 200× with exception for 8-OH-dG where percentage of positively stained nuclei to total nuclei in pericentral regions was determined.

Isolation of DNA

DNA was extracted by a procedure slightly modified from the method reported previously (Nakamura *et al.* 2000). To minimize formation of oxidative artifacts during isolation, 2,2,6,6-tetramethylpiperidinoxyl (TEMPO, 20 mM final concentration) was added to all solutions and all procedures were performed on ice. Briefly, frozen tissues were thawed and homogenized in PBS with a Tehran homogenizer (Wheaton Instruments, Millville, NJ). After centrifugation at 2,000 x g for 10 min, the nuclear pellets were incubated in lysis buffer (Applied Biosystems, Foster City, CA) overnight at 4°C with proteinase K (500 mg/ml; Applied Biosystems). DNA was extracted twice with a mixture of phenol/chloroform/water followed by ethanol precipitation. The extracted DNA was incubated in PBS (pH 7.4) with RNase A followed by DNA precipitation with cold ethanol. Then, the DNA pellet was resuspended in sterilized double distilled water. The DNA solution was stored at −80°C until assayed. The DNA extraction method used in this study is unlikely to modify the original number of AP sites and single strand breaks in genomic DNA from intact tissues or cells, based on re-extraction data of DNA exposed to high concentrations of methylmethane sulfonate (Swenberg and Nakamura, unpublished).

Apurinic/apyrimidinic (AP) sites

AP sites were measured following a procedure reported by Nakamura and Swenberg (Nakamura and Swenberg 1999). Briefly, 8 μg of DNA in 150 μl of phosphate-buffered saline was incubated with 1 mM aldehyde reactive probe at 37°C for 10 min. After precipitation using cold ethanol, DNA was resuspended in TE buffer (10 mM Tris-HCl, pH 7.4, containing 1 mM EDTA). DNA (250 ng) in TE buffer was heat-denatured and loaded on a nitrocellulose membrane (110 ng DNA/slot, Hybond-C Super, Amersham Pharmacia Biotech, Piscataway, NJ). The nitrocellulose membrane was soaked with 5×SSC and then baked in a vacuum oven for 30 min. The membrane was preincubated with 10 ml of Tris-HCl containing bovine serum albumin for 15 min and then incubated in the same solution containing streptavidin-conjugated horseradish peroxidase at room temperature for 45 min. After rinsing the nitrocellulose membrane, the enzymatic activity on the membrane was visualized by enhanced chemiluminescence reagents (Amersham). The nitrocellulose filter was exposed to x-ray film, and the developed film analyzed using a Kodak Image Station 440. Quantitation was based on comparisons to internal standard DNA containing a known amount of AP sites.

Detection and Quantification of 8-OH-dG by Capillary LC-MS/MS

The measurement of 8-OH-dG by LC-MS/MS was adapted from the method described by Liao (Liao 2003). To digest DNA into individual nucleosides, DNA (30 to 50 μg) was dissolved in 80 mM Tris-HCl buffer/20 mM MgCl2 (pH 7.0) with 2.75 pmoles [$^{15}N_5$]-8-OH-dG internal standard prior to digestion with 40U of DNase I for 10 min at 37°C. Next, 2.7 mU of phosphodiesterase I and 2U of alkaline phosphatase were added and incubated for an additional 1h. The released 8-OH-dG was purified by reverse phase HPLC using a Beckman Ultrasphere ODS C18 column (5 um, 4.6 x 250 mm, Beckman, Fullerton, CA). The isocratic mobile phase was 7% MeOH in 10 mM ammonium formate (pH 4.3) with a flow rate 1 mL/min. Fractions were collected 2 min preceding and following the elution of 8-OH-dG. The quantitative analysis of 8-OH-dG by capillary LC-electrospray-MS/MS was performed with an 1100 capillary high-performance liquid chromatograph (Agilent, Wilmington, DE) coupled to a TSQ-Quantum triple quad mass analyzer (ThermoFinnigan, San Jose, CA). A 3.5 μm Zorbax XDB-C18 column (0.3 mm x 150 mm; Agilent) was operated with a binary mobile phase of 2% 10 mM ammonium formate (pH 4.3) and 98% methanol followed by a linear gradient increase of methanol from 2% to 30% from 0 to 5 min, holding at 30% for 10 min, and immediate return to initial conditions that was held for 15 min. Both analyte and internal standard were detected by single reaction monitoring of the transition of nucleoside to base adduct *m/z* 284.2 to 168.2 and *m/z* 289.2 to 173.2, respectively. MS conditions were as follows: spray voltage, 2200 V; heated capillary temperature, 350°C. All reagents were purchased from Sigma-Aldrich (St. Louis, MO) and were ACS grade or higher.

Ribonuclease protection assays

Total RNA was isolated using RNeasy total RNA (Qiagen, Valencia, CA) and dissolved in RNase-free water. Samples were stored at −80°C until assayed. The quality of preparations was determined using an Agilent Bio-Analyzer® (Agilent Technologies, Palo Alto, CA). The RNase protection assays were performed on 20 μg of individual total RNA samples using a RiboQuant^{TM} multi-probe RNase protection assay kit (rBER, BD PharMingen, San Diego, CA) essentially as described elsewhere (Rusyn *et al.* 2000). Riboprobes were synthesized in the presence of [^{32}P]dUTP to yield labeled antisense RNA probes. Protected fragments were separated on 5% polyacrylamide nucleic acid separation gels, dried and exposed to x-ray film. The intensity of protected bands was quantified using Kodak 1D Image Analysis Software (New Haven, CT). Band intensity was normalized to the reference gene L32, which in our experience has shown to be stably expressed in the liver without undue expression modulation by various treatments.

Statistical analysis

Results are reported as means ± SD with n = 3 in each group. Treatment groups were compared using one-way ANOVA analysis followed by Tukey's multiple comparison post-hoc test, where appropriate. A $p<0.05$ was selected before the study to determine statistical differences between groups.

RESULTS

A previous toxicogenomic study of APAP-induced toxicity in rat liver revealed an oxidative stress signature with a sub-toxic dose at 6h that was also present after exposure to overtly toxic doses (Heinloth *et al.* 2004). Specifically, this gene signature included the induction of cAMP inducible gene 1, heterogenous nuclear ribonucleoproteins A1/B1, phospholipase C gamma1, metallothionein (MT1a), phytoene dehydrogenase, and H2AX histone family. To substantiate the link of this oxidative stress gene expression signature to incipient liver toxicity, we examined the liver tissue from this earlier study to measure a diverse panel of endpoints for oxidative stress and DNA damage. The study by Heinloth et al. (2004) also found that livers of animals treated with a sub-toxic (150 mg/kg) dose of APAP were histologically indistinguishable from controls. In contrast, rats treated with 1500 and 2000 mg/kg exhibited mild to moderate centrilobular necrosis and inflammatory lesions that was most prominent at 24h and coincided with a significant increase in serum alanine aminotransferase (ALT) activity, 2952 ± 261 and 5047 ± 728, respectively.

Glutathione depletion by the reactive metabolite of APAP, *N*-acetyl-*p*-benzoquinone imine (NAPQI), is thought to play an important causal role in APAP-induced hepatotoxicity. As such, liver tissue from rats given a single acute dose by gavage of vehicle or APAP at sub-toxic (150 mg/kg) or overtly toxic (1500 mg/kg) doses at 6 and 24 hrs post-dosing were analyzed for reduced GSH content. APAP treatment at sub-toxic and overtly toxic doses led to a 30 and 58% depletion of GSH content, respectively, compared to control animals at 6 hrs post-dosing (Table 1). The GSH content in the liver of these animals returned to control levels by 24 hrs.

Peroxynitrite, an oxidant and nitrating species, is formed from the reaction of superoxide and nitric oxide (NO·) which can lead to the formation of 3-nitrotyrosine protein adducts (Radi *et al.* 1991; Pryor and Squadrito 1995). Liver sections from rats given a single acute dose by gavage of vehicle or APAP at sub-toxic (150 mg/kg) or overtly toxic (1500 and 2000 mg/kg) doses were examined for the presence of nitrotyrosine protein adducts by immunohistochemistry. Control animals as well as those given an overtly toxic dose of APAP exhibited minimal to non-detectable levels of nitrotyrosine (Figure 1A, 1C-D); in contrast, rats given a sub-toxic dose of APAP exhibited extensive localized staining of hepatocytes within pericentral regions of the liver lobule (Figures 1B and E) which resolved to control levels by 48hrs. Microscopic examination showed the presence of nitrotyrosine adducts within both cellular and nuclear compartments of hepatocytes. Quantitative analysis of liver sections from control animals showed that nitrotyrosine comprised less than 5% of total cellular area in pericentral regions of the liver lobule as opposed to those given a sub-toxic dose of APAP where nitrotyrosine comprised 80% and 30% of total cellular area at 6 and 24 hrs post-dosing, respectively (Figure 1F).

The covalent binding of NAPQI to mitochondrial proteins can lead to an increased production of reactive oxygen species (ROS) that can react with DNA (Mitchell *et al.* 1973b). First, we examined 8-OH-dG, a widely-used marker of oxidative DNA damage, by immunohistochemical detection (Figures 2A-C). Microscopic examination of liver sections from control animals revealed sporadic distribution of positively stained nuclei but sections from animals given either sub-toxic or overtly toxic dose had a concentration of positively stained nuclei within the pericentral region (Figure 2D). Image analysis was performed to

determine the percentage of positively stained nuclei (Figure 2E). Both sub-toxic and overtly toxic doses of APAP led to a significant accumulation of 8-OH-dG adducts at 6 hrs post-dosing.

Then, we determined the amounts of 8-OH-dG after APAP treatment using a recently developed capillary LC-MS/MS method as described in Materials and Methods. A calibration curve was obtained by using 275.5 fmoles internal standard and variable amounts of 8-OH-dG ranging from 0.5 to 228 fmoles/μl. The ratio of the peak areas of 8-OH-dG versus internal standard was plotted against the known amounts of 8-OH-dG yielding a linear calibration curve with a correlation coefficient of 1.0 (Figure 3A). Endogenous levels of 8-OH-dG in control rat liver were ~ 1 adduct per 10^6 dG (Figure 3B), in agreement with recent consensus reports from European Standards Committee on Oxidative DNA Damage (ESCODD 2003). Analysis of liver tissues from rats treated with a sub-toxic and overtly toxic dose of APAP found a 3 to 4-fold increase in 8-OH-dG adducts, respectively, over control (Figure 3B), confirming our results with immunohistochemical detection.

It is believed that the predominant pathway used for removal of oxidized bases from DNA is the base excision repair (BER) pathway. A multi-probe RNase protection assay for BER enzymes was used, since this approach distinguishes the presence of multiple expressed DNA repair genes simultaneously from a single sample, thus allowing for sensitive comparative analysis of different mRNA products both within and between samples. Sub-toxic dose −150 mg/kg (data not shown), had no effect on expression of BER genes as compared to controls; however, a time- and dose-dependent increase (1.5 to 3-fold) in mRNA for proliferating–cell nuclear antigen (*Pcna*), poly ADP-ribose polymerase (*Parp*), AP endonuclease 1, 8-oxoguanine DNA glycoslyase 1 (*Ogg1*), and polymerases β and δ was observed for 1500 mg/kg dose of APAP (Table 2). In addition, expression of *Mgmt*, an enzyme involved in the direct repair of alkylated guanine residues and not involved in repair of oxidative DNA lesions, was unaffected in all treatments examined, conferring specificity to BER pathway.

Next, to determine whether the number of mutagenic and clastogenic apurinic/apyrimidinic (AP) sites was increased following exposure to APAP, AP sites were measured using a slot blot assay. AP sites can be generated spontaneously by chemical depurination of labile bases and enzymatically by DNA glycosylases in a process of BER. In addition, ROS may induce sugar lesions directly by hydrogen abstraction of deoxyribose, frequently resulting in oxidized AP sites (Pogozelski and Tullius 1998). Although APAP at overtly toxic doses induced BER genes as shown above, there were no significant increases in the number of AP sites generated for any of the treatments (Figure 4).

Through the metabolic activation of APAP, both superoxide and peroxynitrite are generated that subsequently may initiate lipid peroxidation by Fenton chemistry (Radi *et al.* 1991). Lipid peroxidation was evaluated by immunostaining for malondialdehyde (Khan *et al.* 2002) in liver sections from rats given vehicle, or a single acute dose by gavage of APAP at sub-toxic (150 mg/kg) and overtly toxic (1500 mg/kg) doses at 6, 24, or 48 hrs (Figures 5A–C). Quantitative image analysis (Figure 5D) found no statistically significant difference (one-way ANOVA, $p<0.05$) between treatment groups and controls.

DISCUSSION

In the present study, we investigated whether altered gene expression patterns that are suggestive of oxidative stress at a sub-toxic dose of APAP, when no apparent toxicity was detected using routine histopathological and clinical chemistry measurements, could be phenotypically anchored by using a panel of sensitive biomarkers for oxidative stress and oxidative DNA damage. Our results substantiate the previously reported gene expression profiling data (Heinloth *et al.* 2004) demonstrating that the sub-toxic dose of APAP (150 mg/

kg) does induce oxidative stress as demonstrated by the significant accumulation of nitrotyrosine protein adducts, 8-OH-dG DNA lesions, and the reduction in GSH content 6 hrs after treatment. This confirms that gene expression signatures can potentially serve as useful sensitive indicators of toxicity. Furthermore, these data support the utility of gene expression profiling as a sensitive and biologically-relevant endpoint in toxicology.

APAP is a common over-the-counter medication used for its analgesic and antipyretic properties; however, it is also one of the leading causes of drug-induced liver failure (Ostapowicz *et al.* 2002). At pharmacological doses, APAP is metabolized by sulfation and glucuronidation, and to a lesser extent, by cytochrome CYP2E1 that produces a reactive metabolite, NAPQI, which is detoxified by conjugation with GSH (Dahlin *et al.* 1984; Mitchell *et al.* 1973a). APAP-induced hepatotoxicity occurs when GSH reserves are exhausted allowing covalent binding of NAPQI to critical cellular proteins as APAP-cysteine adducts (Cohen *et al.* 1997), ultimately disrupting their cellular function, see Scheme 1. Many of these covalently bound proteins are within the mitochondria (Qiu *et al.* 1998) resulting in reduced respiration (Donnelly *et al.* 1994) and increased superoxide production (Jaeschke 1990). Superoxide either reacts with nitric oxide to produce peroxynitrite, which is responsible for protein nitration (Hinson *et al.* 1998), or dismutates to hydrogen peroxide whereby it can oxidize cellular macromolecules. The presence of nitric oxide, which is induced by APAP (Gardner *et al.* 1998), is thought to block propagation of lipid peroxidation (O'Donnell *et al.* 1997). It has been postulated that loss of mitochondrial function and concomitant generation of oxidative stress are central to APAP-induced hepatotoxicity (James *et al.* 2003a).

It is well recognized that the metabolic activation of APAP leading to GSH depletion is an important step in APAP-induced liver toxicity. As expected, an overtly toxic dose of APAP given to rats significantly reduced GSH content to 60% less than control at 6 hrs post-dosing. Most surprisingly, a sub-toxic dose also significantly reduced GSH, albeit to a lesser extent. This data suggests that depletion of GSH must reach a certain threshold for toxicity to ensue and may explain why there is no apparent clinical signs of toxicity with a sub-toxic dose of APAP.

A number of studies have reported elevated levels of nitrotyrosine protein adducts that precede and accompany APAP-induced hepatotoxicity in mice (Hinson *et al.* 1998; James *et al.* 2003b). In this study, only rats given a sub-toxic dose of APAP, not overtly toxic doses, had significantly elevated levels of nitrotyrosine protein adducts in liver compared to controls at 6 and 24 hrs post-dosing. The detection of these adducts was limited to the pericentral regions of the liver where metabolic activation of APAP would occur and toxicity would develop at higher doses. Also, an inverse relationship appears to exist between the levels of nitrotyrosine protein adducts and GSH content in the liver. Between 6 and 24 hrs, the levels of nitrotyrosine decreased from 17- to 8-fold over controls. The reduction in nitrotyrosine protein adducts during this time may be due to several contributing factors such as enzymatic reversal of protein nitration (Kamisaki *et al.* 1998), increased rate of protein degradation (Gow *et al.* 1996), as well as decreased ROS production with concomitant recovery of GSH content in the liver.

The detection of nitrated protein adducts with a sub-toxic dose of APAP support the presence of oxidant stress as indicated by earlier gene expression studies. However, the inability to detect these adducts with overtly toxic doses demonstrates that the oxidant species formed in the presence of sub-toxic and overtly toxic doses of APAP in the rat are not identical. It has been shown that nitration of tyrosine residues is not limited to peroxynitrite exposure but can occur via peroxidase enzymes such as glutathione peroxidase (Gaut *et al.* 2002) which is impaired during APAP toxicity. In addition, it has been demonstrated that APAP is highly effective at preventing tyrosine nitration by peroxynitrite (Whiteman *et al.* 1996; Lakshmi *et al.* 2000). Thus, in this study overtly toxic doses of APAP, unlike sub-toxic doses, may be able to compete

with tyrosine for peroxynitrite which may explain the differences observed with nitrotyrosine levels between these two dosing groups. Moreover, this data would suggest that nitrotyrosine is not associated with APAP-induced hepatotoxicity in the rat; an observation that is in direct opposition to what has been observed in numerous studies with mice. This may well reflect a mechanistic difference in APAP metabolism between these two rodent species.

The generation of ROS by either APAP metabolism or resulting mitochondrial damage can lead to direct or indirect oxidative DNA damage. Immunohistochemical and mass spectrometry methods found a significant accumulation of the potentially mutagenic DNA lesion, 8-OH-dG, at sub-toxic and overtly toxic doses of APAP. Accumulation of 8-OH-dG lesions preceded the onset of hepatic injury as reported by ALT and histopathology. The formation of 8-OH-dG from APAP exposure potentially results from mitochondrial oxidant stress where both superoxide and peroxynitrite are produced and can either directly or indirectly oxidize guanines in DNA (Douki and Cadet 1996; Steenken 1989).

Recently, the quantified expression of base excision DNA repair (BER) genes was shown to be a sensitive *in vivo* biomarker of chemical-induced oxidative stress (Rusyn *et al.* 2000). Moreover, because this pathway encompasses broad specificity and multiple routes of repair, it allows greater sensitivity in the ability to detect oxidative DNA damage. The measurement of multiple genes involved in the BER pathway by an RNase protection assay was able to detect up-regulation of gene expression that correlated with the onset of centrilobular hepatic necrosis in addition to the rise and fall of ALT. However, the assay was unable to detect significant increases of BER genes at a sub-toxic dose where genomic profiling generated an oxidative stress signature that consisted primarily of genes that are involved in protecting the cell from oxidative stress. It is known that the redox state of the cell is one of many mechanisms involved in activating transcription factors involved in regulating the expression of DNA repair genes (Fritz *et al.* 2003). Thus, it may be that at sub-toxic doses of APAP the apparent increase in expression of anti-oxidants, such as metallothioneins, may be sufficient in maintaining redox equilibrium.

The accumulation of apurinic/apyrimidinic (AP) sites can result from oxidative DNA damage through an intermediary step of BER pathway, enzymatic cleavage, and chemical depurination. The induction of BER pathway and, in particular, AP endonuclease gene by APAP was not, however, corroborated by the accumulation of AP sites by any of the APAP treatments examined. A lack of evidence for an increase in AP sites may be manifested in the limited dose and time regimens examined in this study. Since the development of APAP toxicity typically occurs within the first 6h of exposure, the detection of abasic sites would be limited if repair occurred rapidly. Alternatively, the repair pathway could involve another route whereby generation of an abasic site is obsolete. The dissociation between expression of BER genes and accumulation of AP sites is not an unusual phenomenon and has been observed with other chemical hepatotoxicants (Rusyn *et al.* 2000).

The role of lipid peroxidation in APAP-induced hepatotoxicity has been controversial (Wendel *et al.* 1979; Knight *et al.* 2003; Hinson *et al.* 2002). Mitochondrial dysfunction leads to both increased production of superoxide and formation of peroxynitrite that are both capable of initiating lipid peroxidation; however, biochemical studies have shown that nitric oxide can prevent the propagation of lipid peroxidation reactions (O'Donnell *et al.* 1997). This is supported by the fact that inhibiting nitric oxide production during APAP exposure leads to enhanced lipid peroxidation (Hinson *et al.* 2002). Our studies revealed that lipid peroxidation, as measured by the presence of malondialdehyde, was not observed to be significant for any doses or times examined. Despite the generation of ROS/RNS by APAP, our work does not support the role of lipid peroxidation as a mediator of APAP-induced hepatotoxicity.

It should be considered that alterations in gene expression that are potentially indicative of cellular injury with an adverse outcome but are unsubstantiated by classical measures of toxicity may be a mere reflection of the tissue's capacity to cope. As demonstrated in this study, an APAP-induced oxidant signature generated by a sub-toxic dose was corroborated using sensitive biomarkers for oxidative stress and DNA damage. However, the presence of genes having an anti-oxidant role within this signature may explain the lack of observable toxicity by classical measures. In order to discern whether gene expression alterations in critical cellular pathways represent benign homeostatic adjustments, indications of the potential for adverse effects or in fact represent adverse effects, especially at doses and times with no observable toxicity, will require the application of more sophisticated and sensitive tools that provide a mechanistic link between a chemical and the observed toxic effect.

In general, the acceptance of microarray expression data as a relevant endpoint in toxicological studies requires careful interpretation and validation. It has been suggested that this should be achieved using classical toxicological endpoints such as histopathology and clinical chemistry (Henry *et al.* 2002). One of the great promises of toxicogenomics is that it will be able to detect and predict toxicity at much earlier stages compared to existing methods; however, restricting validation of expression data to only classical endpoints, with their inherent lack of sensitivity, would bring the advancement of toxicogenomics as well as toxicology to an impasse. Most importantly, the enhanced sensitivity of microarray studies to detect subtle, early alterations in vital cellular pathways that may be indicative of adverse effects, but display no observable toxicity by conventional measures, can have serious ramifications in policy and regulatory decision making.

In summary, we show that incipient signs of oxidative stress can be observed with a sub-toxic dose of APAP based on the significant accumulation of both nitrotyrosine protein adducts and 8-OH-dG DNA lesions, markers anchored on the mechanism of APAP-induced liver toxicity. The use of sensitive biomarkers of oxidative stress and oxidative DNA damage revealed not only that mechanistic differences may exist in APAP metabolism between sub-toxic and overtly toxic doses in rats but also among rodent species. Gene expression profiling is a sensitive tool capable of detecting subtle cellular disturbances at doses and times unobtainable by classical toxicological measures. Thus, it has the potential to serve an essential role in predicative toxicology by generating gene signatures as biomarkers of incipient toxicity.

Acknowledgements

Financial support for these studies was provided, in part, by grants from the National Institutes of Health: U19-ES11391, P42-ES05948, R42-ES11746, P30-ES10126, and NIEHS Intramural Research Program. I.R. was a recipient of a Transition to Independent Position award K22-ES11660. C.L.P. was supported, in part, by a National Institute of Environmental Health Sciences Training Grant T32-ES07126. The authors thank Dr. David Malarkey (NIEHS) and Ms. Julie Foley (NIEHS) for careful review of the manuscript.

References

1. Bammler T, Beyer RP, Bhattacharya S, Boorman GA, Boyles A, Bradford BU, Bumgarner RE, Bushel PR, Chaturvedi K, Choi D, Cunningham ML, Deng S, Dressman HK, Fannin RD, Farin FM, Freedman JF, Fry RC, Harper A, Humble MC, Hurban P, Kavanagh TJ, Kaufmann WK, Kerr KF, Jing L, Lapidus JA, Lasarev MR, Li J, Li YJ, Lobenhofer EK, Lu X, Malek RL, Milton S, Nagalla SR, O'Malley JP, Palmer VS, Pattee P, Paules RS, Perou CM, Phillips K, Qin L, Qiu Y, Quigley SD, Rodland M, Rusyn I, Samson LD, Schwartz DA, Shi Y, Shin JL, Sieber SO, Slifer S, Speer MC, Spencer PS, Sproles DI, Swenberg JA, Suk WA, Sullivan RC, Tian R, Tennant RW, Todd SA, Tucker CJ, Houten BV, Weis BK, Xuan S, Zarbl H. Members of the Toxicogenomics Research Consortium. Standardizing global gene expression analysis between laboratories and across platforms. Nat Methods 2005;2(5):351–356. [PubMed: 15846362]

2. Boorman GA, Haseman JK, Waters MD, Hardisty JF, Sills RC. Quality review procedures necessary for rodent pathology databases and toxicogenomic studies: the National Toxicology Program experience. Toxicol Pathol 2002;30(1):88–92. [PubMed: 11890481]

3. Cohen SD, Pumford NR, Khairallah EA, Boekelheide K, Pohl LR, Amouzadeh HR, Hinson JA. Selective protein covalent binding and target organ toxicity. Toxicol Appl Pharmacol 1997;143(1):1–12. [PubMed: 9073586]

4. Dahlin DC, Miwa GT, Lu AY, Nelson SD. N-acetyl-p-benzoquinone imine: a cytochrome P-450-mediated oxidation product of acetaminophen. Proc Natl Acad Sci U S A 1984;81(5):1327–1331. [PubMed: 6424115]

5. Donnelly PJ, Walker RM, Racz WJ. Inhibition of mitochondrial respiration in vivo is an early event in acetaminophen-induced hepatotoxicity. Arch Toxicol 1994;68 (2):110–118. [PubMed: 8179480]

6. Douki T, Cadet J. Peroxynitrite mediated oxidation of purine bases of nucleosides and isolated DNA. Free Radic Res 1996;24(5):369–380. [PubMed: 8733941]

7. Measurement of DNA oxidation in human cells by chromatographic and enzymic methods. Free Radic Biol Med 2003;34(8):1089–1099. [PubMed: 12684094]ESCODD

8. Fritz G, Grosch S, Tomicic M, Kaina B. APE/Ref-1 and the mammalian response to genotoxic stress. Toxicology 2003;193(1–2):67–78. [PubMed: 14599768]

9. Gardner CR, Heck DE, Yang CS, Thomas PE, Zhang XJ, DeGeorge GL, Laskin JD, Laskin DL. Role of nitric oxide in acetaminophen-induced hepatotoxicity in the rat. Hepatology 1998;27(3):748–754. [PubMed: 9500703]

10. Gaut JP, Byun J, Tran HD, Lauber WM, Carroll JA, Hotchkiss RS, Belaaouaj A, Heinecke JW. Myeloperoxidase produces nitrating oxidants in vivo. J Clin Invest 2002;109(10):1311–1319. [PubMed: 12021246]

11. Gow AJ, Duran D, Malcolm S, Ischiropoulos H. Effects of peroxynitrite-induced protein modifications on tyrosine phosphorylation and degradation. FEBS Lett 1996;385(1–2):63–66. [PubMed: 8641468]

12. Hamadeh HK, Bushel PR, Jayadev S, Martin K, DiSorbo O, Sieber S, Bennett L, Tennant R, Stoll R, Barrett JC, Blanchard K, Paules RS, Afshari CA. Gene expression analysis reveals chemical-specific profiles. Toxicol Sci 2002;67(2):219–231. [PubMed: 12011481]

13. Heinloth AN, Irwin RD, Boorman GA, Nettesheim P, Fannin RD, Sieber SO, Snell ML, Tucker CJ, Li L, Travlos GS, Vansant G, Blackshear PE, Tennant RW, Cunningham ML, Paules RS. Gene expression profiling of rat livers reveals indicators of potential adverse effects. Toxicol Sci 2004;80 (1):193–202. [PubMed: 15084756]

14. Henry CJ, Phillips R, Carpanini F, Corton JC, Craig K, Igarashi K, Leboeuf R, Marchant G, Osborn K, Pennie WD, Smith LL, Teta MJ, Vu V. Use of genomics in toxicology and epidemiology: findings and recommendations of a workshop. Environ Health Perspect 2002;110(10):1047–1050. [PubMed: 12361931]

15. Hinson JA, Bucci TJ, Irwin LK, Michael SL, Mayeux PR. Effect of inhibitors of nitric oxide synthase on acetaminophen-induced hepatotoxicity in mice. Nitric Oxide 2002;6(2):160–167. [PubMed: 11890740]

16. Hinson JA, Pike SL, Pumford NR, Mayeux PR. Nitrotyrosine-protein adducts in hepatic centrilobular areas following toxic doses of acetaminophen in mice. Chem Res Toxicol 1998;11(6):604–607. [PubMed: 9625727]

17. Jaeschke H. Glutathione disulfide formation and oxidant stress during acetaminophen-induced hepatotoxicity in mice in vivo: the protective effect of allopurinol. J Pharmacol Exp Ther 1990;255 (3):935–941. [PubMed: 2262912]

18. James LP, Mayeux PR, Hinson JA. Acetaminophen-induced hepatotoxicity. Drug Metab Dispos 2003a;31(12):1499–1506. [PubMed: 14625346]

19. James LP, McCullough SS, Lamps LW, Hinson JA. Effect of N-acetylcysteine on acetaminophen toxicity in mice: relationship to reactive nitrogen and cytokine formation. Toxicol Sci 2003b;75(2): 458–467. [PubMed: 12883092]

20. Kamisaki Y, Wada K, Bian K, Balabanli B, Davis K, Martin E, Behbod F, Lee YC, Murad F. An activity in rat tissues that modifies nitrotyrosine-containing proteins. Proc Natl Acad Sci U S A 1998;95(20):11584–11589. [PubMed: 9751709]

21. Khan MF, Wu X, Tipnis UR, Ansari GA, Boor PJ. Protein adducts of malondialdehyde and 4-hydroxynonenal in livers of iron loaded rats: quantitation and localization. Toxicology 2002;173(3): 193–201. [PubMed: 11960672]
22. Knight TR, Fariss MW, Farhood A, Jaeschke H. Role of lipid peroxidation as a mechanism of liver injury after acetaminophen overdose in mice. Toxicol Sci 2003;76(1):229–236. [PubMed: 12944590]
23. Lakshmi VM, Hsu FF, Davis BB, Zenser TV. Nitrating reactive nitric oxygen species transform acetaminophen to 3-nitroacetaminophen. Chem Res Toxicol 2000;13 (9):891–899. [PubMed: 10995262]
24. Liao, S. Quantification of 8-hydroxy-2′-deoxyguanosine in DNA by capillary liquid chromatography-electrospray tandem mass spectrometry. 2003. University of North Carolina at Chapel Hill. (Thesis/Dissertation).
25. Mitchell JR, Jollow DJ, Potter WZ, Davis DC, Gillette JR, Brodie BB. Acetaminophen-induced hepatic necrosis. I Role of drug metabolism. J Pharmacol Exp Ther 1973a;187(1):185–194. [PubMed: 4746326]
26. Mitchell JR, Jollow DJ, Potter WZ, Davis DC, Gillette JR, Brodie BB. Acetaminophen-induced hepatic necrosis. I Role of drug metabolism. J Pharmacol Exp Ther 1973b;187(1):185–194. [PubMed: 4746326]
27. Nakamura J, La DK, Swenberg JA. 5′-nicked apurinic/apyrimidinic sites are resistant to b-elimination by b-polymerase and are persistent in human cultured cells after oxidative stress. J Biol Chem 2000;275(8):5323–5328. [PubMed: 10681505]
28. Nakamura J, Swenberg JA. Endogenous apurinic/apyrimidinic sites in genomic DNA of mammalian tissues. Cancer Res 1999;59(11):2522–2526. [PubMed: 10363965]
29. O'Donnell VB, Chumley PH, Hogg N, Bloodsworth A, Darley-Usmar VM, Freeman BA. Nitric oxide inhibition of lipid peroxidation: kinetics of reaction with lipid peroxyl radicals and comparison with alpha-tocopherol. Biochemistry 1997;36(49):15216–15223. [PubMed: 9398249]
30. Ostapowicz G, Fontana RJ, Schiodt FV, Larson A, Davern TJ, Han SH, McCashland TM, Shakil AO, Hay JE, Hynan L, Crippin JS, Blei AT, Samuel G, Reisch J, Lee WM. Results of a prospective study of acute liver failure at 17 tertiary care centers in the United States. Ann Intern Med 2002;137(12): 947–954. [PubMed: 12484709]
31. Paules R. Phenotypic Anchoring: Linking Cause and Effect. Environ Health Perspect 2003;111 (6):A338–A339. [PubMed: 12760838]
32. Pogozelski WK, Tullius TD. Oxidative strand scission of nucleic acids: Routes initiated by hydrogen abstraction from the sugar moiety. Chemical Review 1998;98:1089–1107.
33. Pryor WA, Squadrito GL. The chemistry of peroxynitrite: a product from the reaction of nitric oxide with superoxide. Am J Physiol 1995;268(5 Pt 1):L699–L722. [PubMed: 7762673]
34. Qiu Y, Benet LZ, Burlingame AL. Identification of the hepatic protein targets of reactive metabolites of acetaminophen in vivo in mice using two-dimensional gel electrophoresis and mass spectrometry. J Biol Chem 1998;273(28):17940–17953. [PubMed: 9651401]
35. Radi R, Beckman JS, Bush KM, Freeman BA. Peroxynitrite-induced membrane lipid peroxidation: the cytotoxic potential of superoxide and nitric oxide. Arch Biochem Biophys 1991;288(2):481–487. [PubMed: 1654835]
36. Rusyn I, Denissenko MF, Wong VA, Butterworth BE, Cunningham ML, Upton PB, Thurman RG, Swenberg JA. Expression of base excision repair enzymes in rat and mouse liver is induced by peroxisome proliferators and is dependent upon carcinogenic potency. Carcinogenesis 2000;21(12): 2141–2145. [PubMed: 11133801]
37. Steenken S. Structure, acid/base properties and transformation reactions of purine radicals. Free Rad Res Comm 1989;6(2–3):117–120.
38. Wendel A, Feuerstein S, Konz KH. Acute paracetamol intoxication of starved mice leads to lipid peroxidation in vivo. Biochem Pharmacol 1979;28(13):2051–2055. [PubMed: 475847]
39. Whiteman M, Kaur H, Halliwell B. Protection against peroxynitrite dependent tyrosine nitration and alpha 1-antiproteinase inactivation by some anti-inflammatory drugs and by the antibiotic tetracycline. Ann Rheum Dis 1996;55(6):383–387. [PubMed: 8694578]

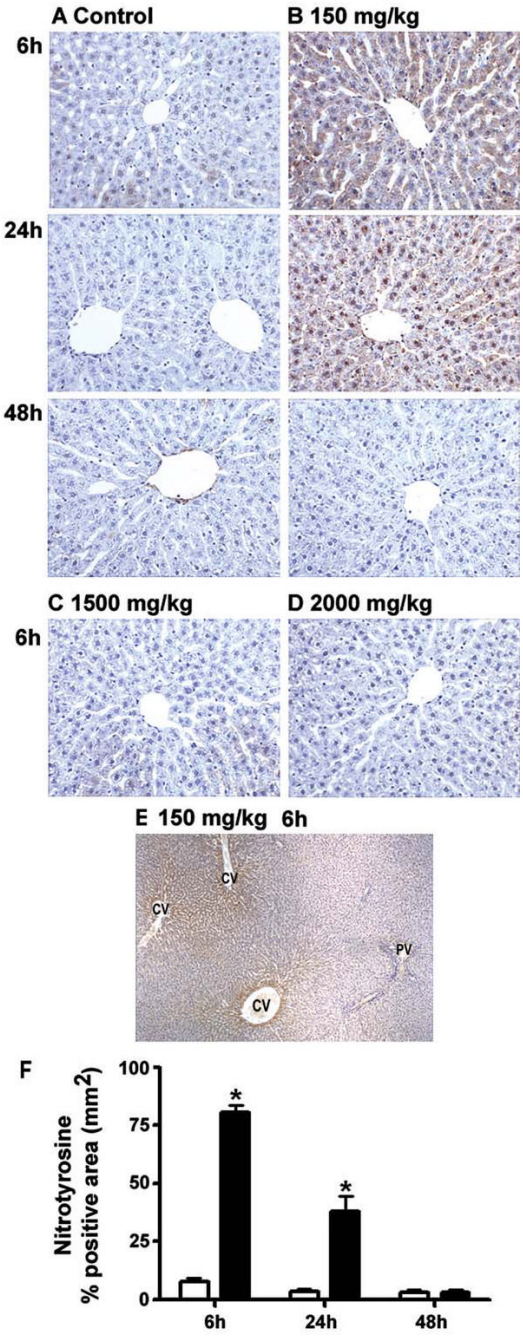


Figure 1. A Sub-toxic dose of acetaminophen significantly increases nitro-tyrosine protein adducts in rat liver

Representative micrographs (200×) of liver tissue from rats immunostained for nitrotyrosine after treatment with (A) methyl cellulose control or (B) 150 mg/kg acetaminophen at 6, 24, and 48hrs post-dosing and (C) 1500 mg/kg and (D) 2000 mg/kg acetaminophen at 6 hrs post-dosing*. Representative micrograph (40×) of liver tissue from rats after treatment with 150 mg/kg acetaminophen demonstrating centralobular localization of nitrotyrosine staining (D). CV, central vein; PV, portal vein. Immunostained liver sections for control (□) and 150 mg/kg (■) APAP were quantified by averaging percent area stained to total area at 200× in pericentral regions (F). Data are presented as mean ± SD, n = 3 biological replicates per group.

Data significantly different from control, $p < 0.01$, is denoted by asterisk. *Analysis of overtly toxic doses of APAP at 24 and 48 hrs post-dosing was not performed due to the extensive presence of necrotic tissue that often stains non-specifically.

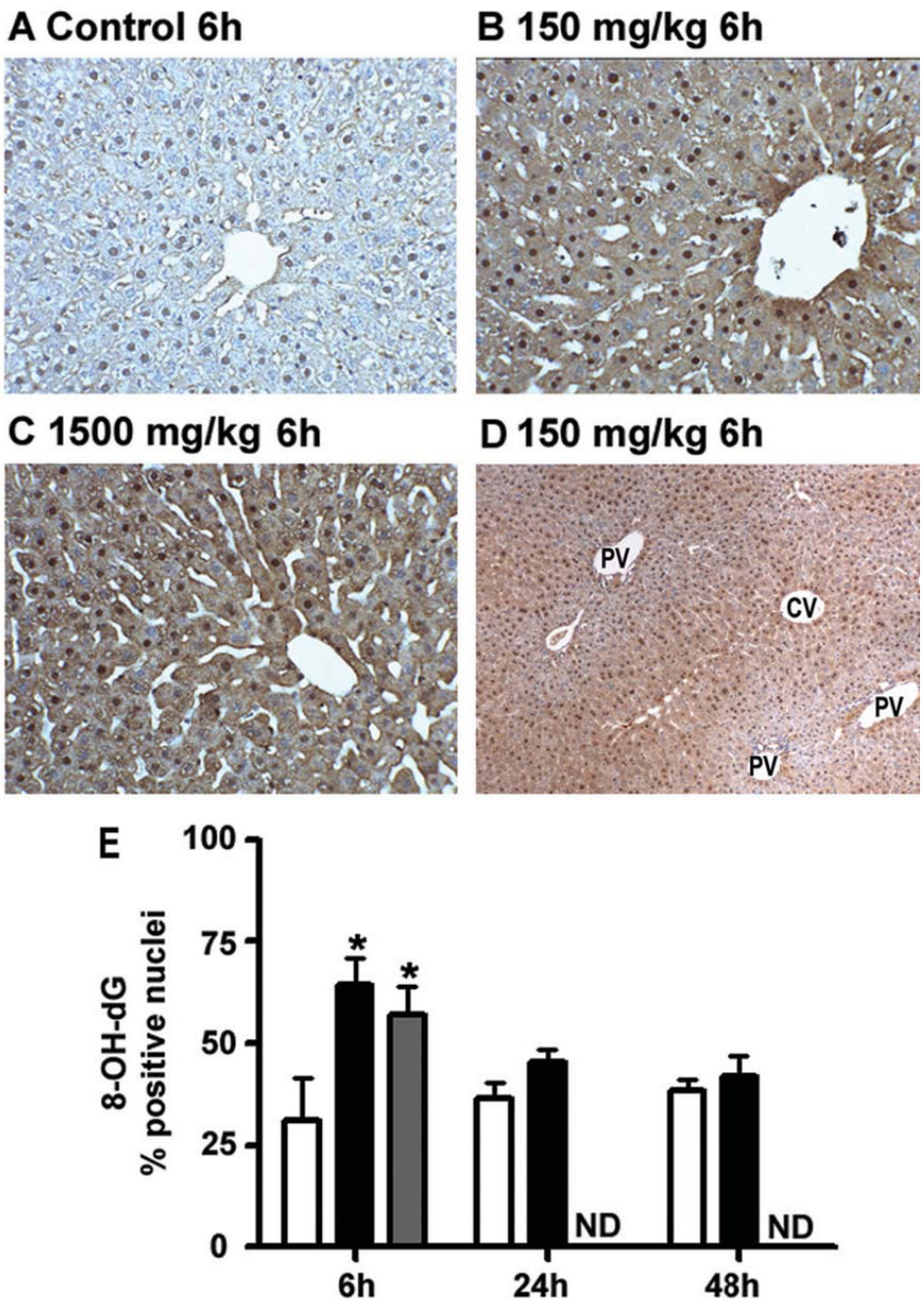


Figure 2. Rat liver genomic DNA significantly accumulates 8-OH-dG adducts after 6 hrs treatment with sub-toxic and overtly toxic doses of acetaminophen

Representative micrographs (200×) of liver tissue immunostained for 8-OH-dG from rats treated with (A) methyl cellulose control, (B) 150 mg/kg, or (C) 1500 mg/kg acetaminophen 6 hrs post-dosing. Representative micrograph (40×) of liver tissue from rats after treatment with 150 mg/kg acetaminophen demonstrating centralobular localization of 8-OH-dG adducts (D). . CV, central vein; PV, portal vein. Immuno-stained liver sections for control (□), 150 mg/kg (■), or 1500 mg/kg (▩) APAP were quantified by averaging percent nuclei stained to total nuclei within pericentral regions at 200× (E). Data are presented as mean ± SD, n = 3

biological replicates per group. Data significantly different from control, $p < 0.05$, is denoted by asterisk. ND = not determined

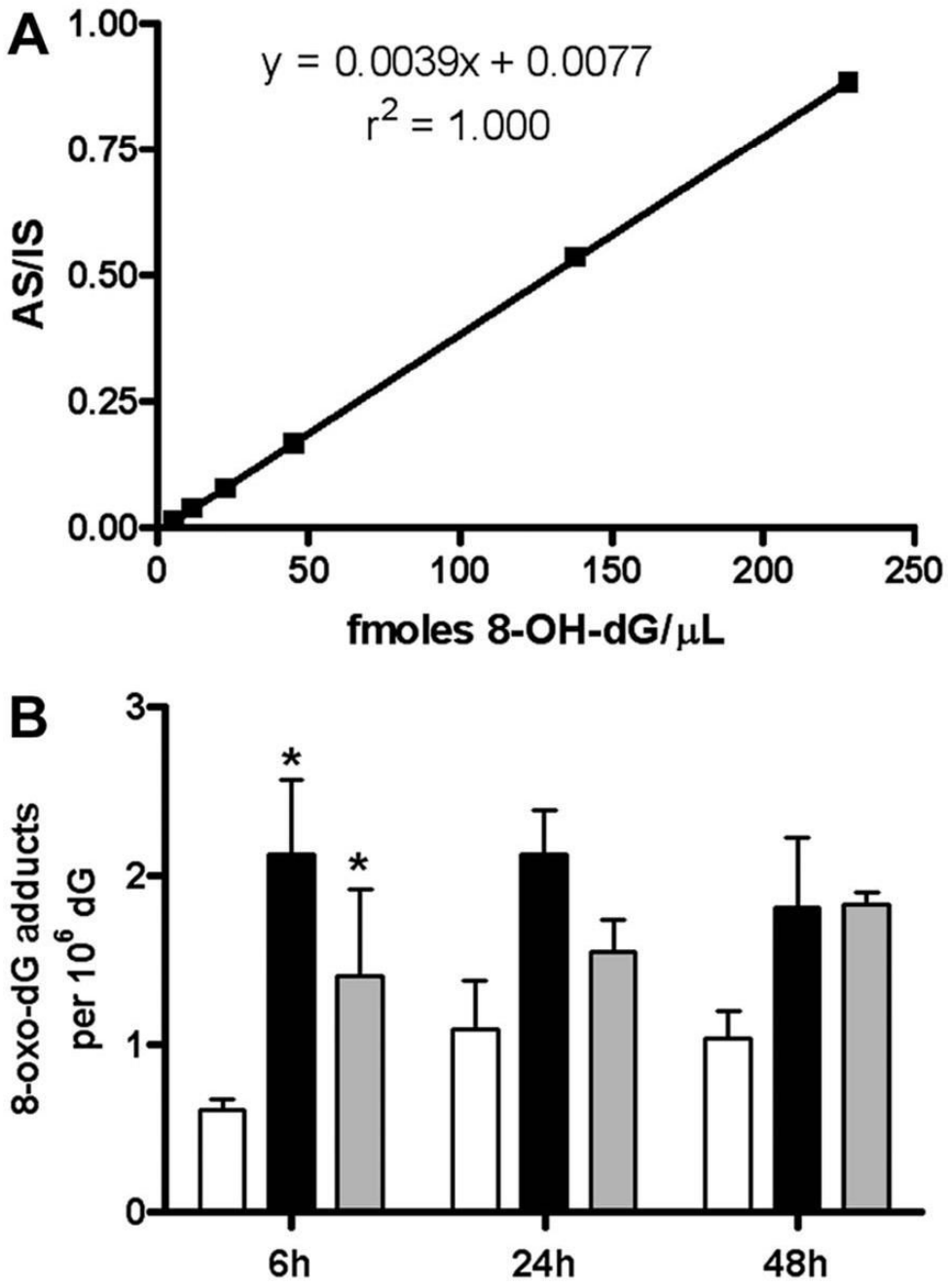


Figure 3. A sub-toxic dose of APAP significantly accumulates 8-OH-dG DNA adducts in rat liver as measured by capillary LC-MS/MS

(A) Standard calibration curve for 8-OH-dG by capillary LC-MS/MS. The ratio of the peak areas of 8-OH-dG (AS) to 275.5 fmoles [$^{15}N_5$]-8-OH-dG (AI) as the internal standard plotted against the amount of 8-OH-dG ranging from 5.5 to 228 fmoles/μl. (B) Quantitative measure of 8-OH-dG DNA adducts in rat liver from control (□), 150 mg/kg (■), or 1500 mg/kg (▩) APAP. Data are presented as mean ± SD from 3 animals per group. *Statistical difference ($p < 0.05$) from control group using one-way ANOVA followed by Tukey's multiple comparison post-hoc test.

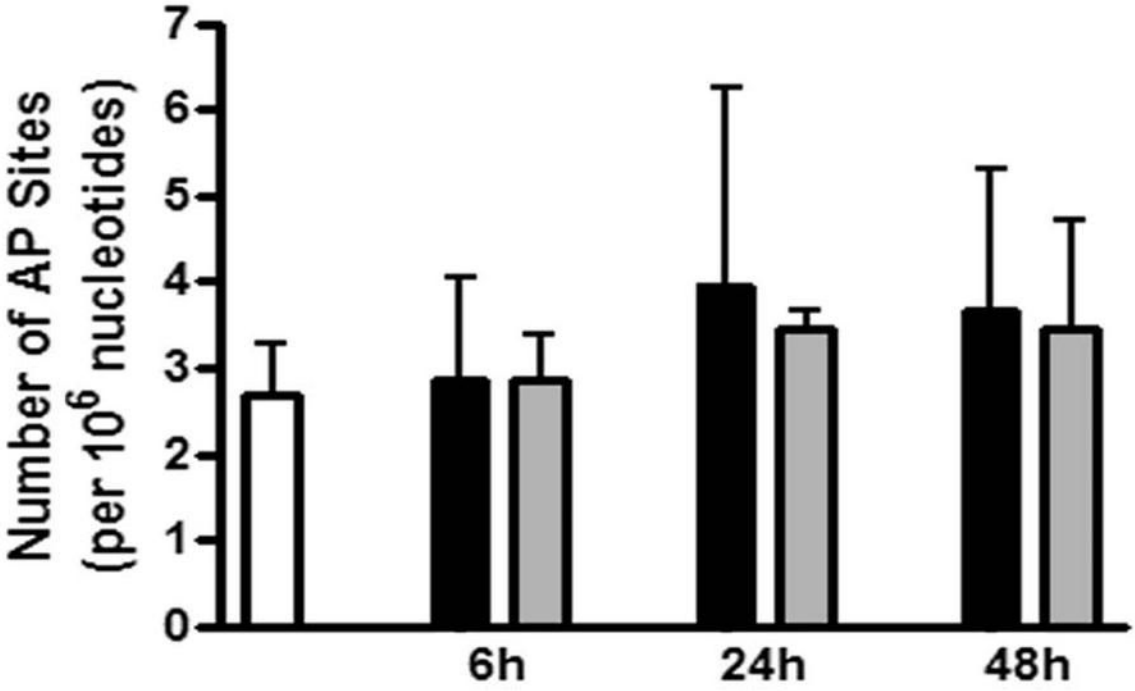


Figure 4. Acetaminophen has no effect on the accumulation of apurinic/apyrimidinic (AP) sites in rat liver

The number of AP sites in genomic DNA isolated from livers of control (□) and 150 mg/kg (■), or 1500 mg/kg (▩) APAP at 6, 24, and 48h. The control is pooled RNA from three biological replicates and then 6, 24, and 48h time points averaged together. Data is given as mean ± SD, n = 3. Statistical analysis by one-factor ANOVA ($p < 0.05$) found no significant difference between acetaminophen treated groups and control.

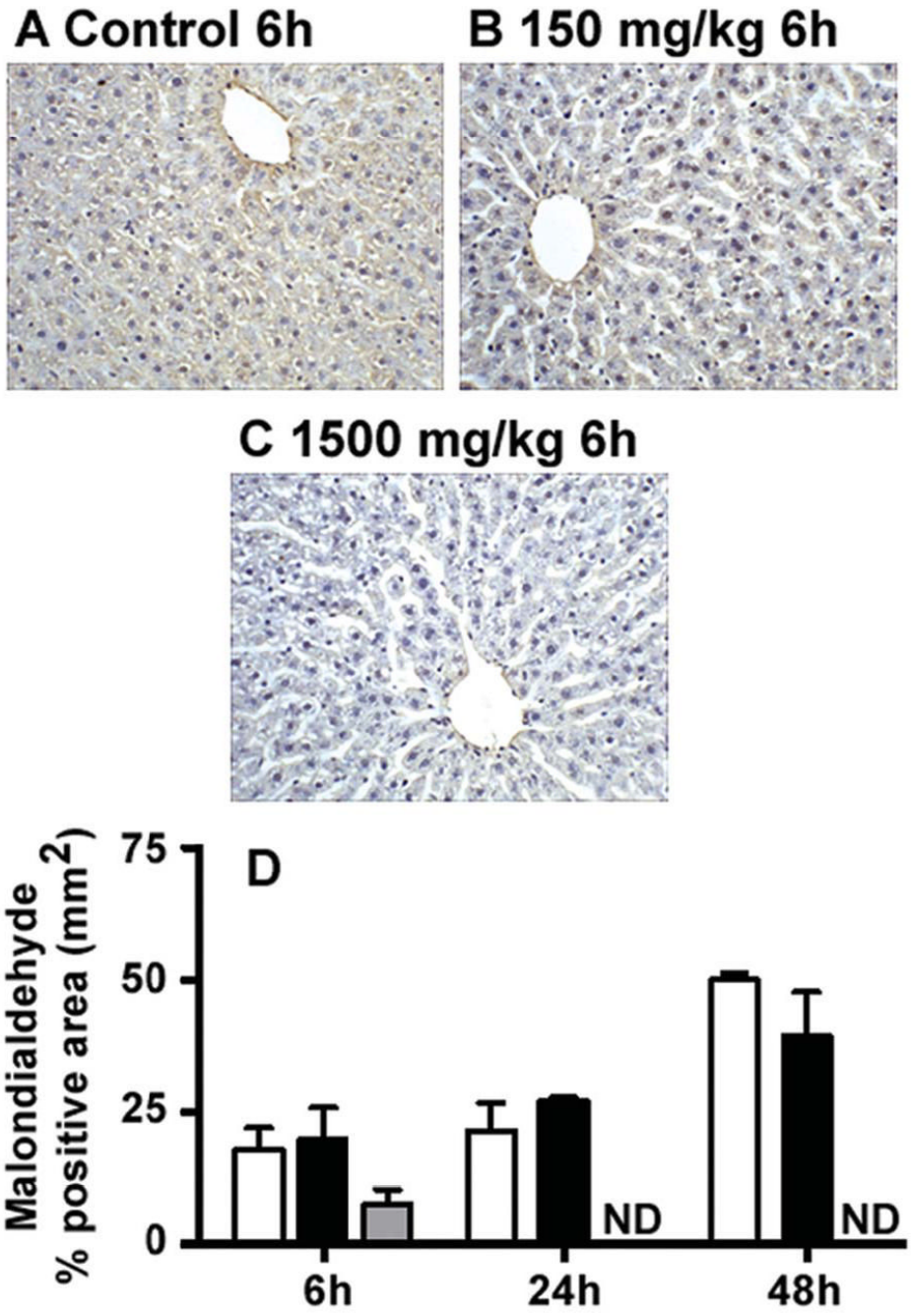
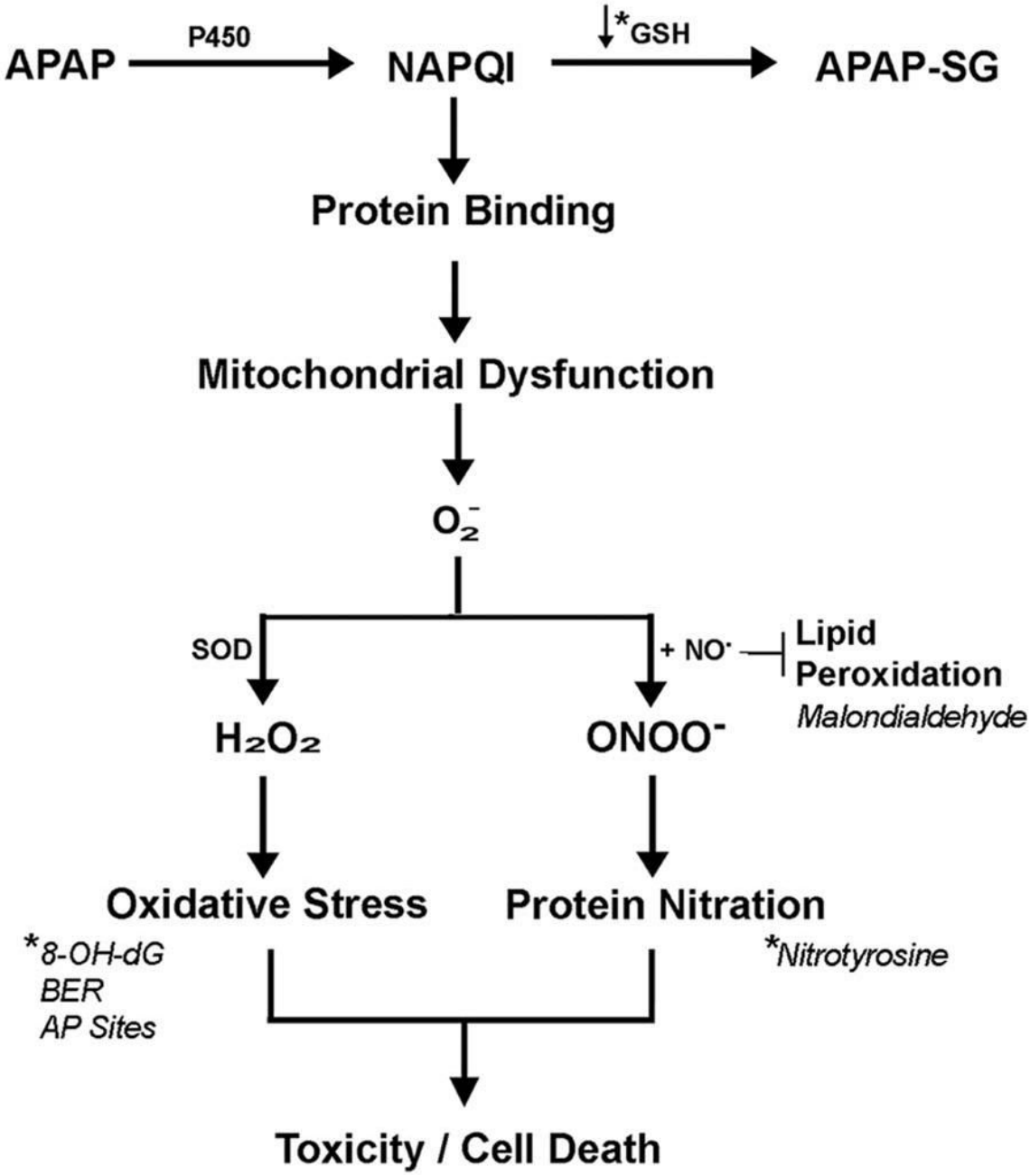


Figure 5. Acetaminophen does not promote lipid peroxidation in rat liver

Representative micrographs (200×) of liver tissue immunostained for malondialdehyde from rats treated with (A) methyl cellulose control, (B) 150 mg/kg, or (C) 1500 mg/kg acetaminophen 6 hrs post-dosing. Immunostained liver sections for control (☐), 150 mg/kg (■), or 1500 mg/kg (▩) APAP were quantified by averaging percent area stained to total area at 200× in pericentral regions (D). Data are presented as mean ± SD, n = 3 biological replicates per group. Statistical analysis by one-factor ANOVA ($p < 0.05$) found no significant difference between acetaminophen treated groups and control. ND = not determined.



Scheme 1.

Phenotypic anchors of gene expression profiling for oxidative stress are a reflection of the proposed mechanism of APAP-induced hepatotoxicity. APAP is metabolized by cytochrome P450s to a reactive metabolite, *N*-acetyl-*p*-benzoquinone imine (NAPQI), which depletes glutathione (GSH) and covalently binds to cellular proteins as APAP-cysteine adducts. Mitochondrial injury leads to increased production and release of reactive oxygen and nitrogen species that promotes oxidative stress and DNA damage. The depletion of GSH and coupling reaction of superoxide ($O_2.^-$) and nitric oxide (NO·) leads to formation of peroxynitrite ($ONOO^-$) that reacts with protein tyrosine residues. It has been proposed that the production of NO·, induced by APAP, terminates lipid peroxidation propagation. The concomitant loss of

mitochondrial function and generation of oxidative stress are postulated to have a central role in APAP-induced hepatoxicity. The sequence of events involved in APAP-induced hepatatoxicity are shown in *bold*, whereas the markers of oxidative stress and oxidative DNA damage measured in this study are shown in *italics*. *, markers that anchored gene expression signature suggestive of oxidative stress with a sub-toxic dose of APAP. The scheme is a summation of previously published reports on APAP-induced hepatotoxicity, see Discussion.

Table 1

Reduced glutathione concentration in rat liver following acetaminophen treatment.

Time	μmoles rGSH/g liver		
	Control	150 mg/kg	1500 mg/kg
6h	5.5 ± 0.43	3.8 ± 0.13*	2.3 ± 0.15*
24h	5.2 ± 0.68	5.9 ± 0.02	4.6 ± 0.75

Rats were administered a single acute dose of acetaminophen by gavage at sub-toxic (150 mg/kg) or overtly toxic (1500 mg/kg) doses in 0.5% aqueous methyl cellulose (vehicle control). Liver tissue collected at 6 or 24h post-dosing was analyzed for reduced glutathione (rGSH) content as described in Materials and Methods. Hepatic rGSH concentration is expressed as μmoles/g liver ± standard deviation from 3 animals per group.

* Statistical difference ($p < 0.01$) from control group using one-way ANOVA followed by Tukey's multiple comparison post-hoc test.

Table 2

Expression of DNA repair genes in rat liver after treatment with an overtly toxic dose (1500 mg/kg) of acetaminophen.

DNA Repair Gene	Control	6h	24h	48h
Ogg1, 8-oxoguanine DNA glycosylase 1	3.8 ± 0.8	3.9 ± 0.8	**8.2 ± 2.8***	**10.2 ± 1.5***
Mpg, N-methylpurine DNA glycosylase	6.0 ± 0.4	5.2 ± 1.3	4.8 ± 2.7	4.0 ± 0.8
Ape, purinic/apyrimidinic endonuclease 1	23.5 ± 1.4	21.6 ± 5.2	**34.8 ± 2.8***	30.8 ± 1.7
Pol β, polymerase (DNA directed) β	13.2 ± 0.9	11.9 ± 1.3	**18.7 ± 1.8***	15.8 ± 1.2
Pol δ, polymerase (DNA directed) δ	1.5 ± 0.3	1.7 ± 1.7	**6.0 ± 1.6***	**8.8 ± 2.8***
Pcna, proliferating cell nuclear antigen	20.7 ± 2.1	22.5 ± 2.6	33.6 ± 7.9	**50.9 ± 7.6***
Parp, poly (ADP-ribose) polymerase	15.3 ± 0.5	**21.4 ± 1.3***	**27.6 ± 3.8***	**29.1 ± 1.6***
Mgmt, O^6-methylguanine DNA ethyltransferase	29.8 ± 1.3	24.1 ± 5.7	32.2 ± 6.6	32.1 ± 0.1

Total RNA was isolated from liver samples and analyzed by RNase protection assay. The results are mean ± standard deviation from 3 animals per group. The relative expression of each gene was normalized to the expression of the housekeeping gene L32. The control is pooled RNA from three biological replicates and then 6, 24, and 48h time points averaged together. The results from animals given a sub-toxic dose of APAP (150 mg/kg) are not presented since they were not significantly different from controls.

* Statistical difference ($p < 0.05$) from control group using one-way ANOVA followed by Tukey's multiple comparison post-hoc test.