The Effect of Aging on Acetaminophen Pharmacokinetics, Toxicity and Nrf2 in Fischer 344 Rats

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We investigated the effect of aging on hepatic pharmacokinetics and the degree of hepatotoxicity following a toxic dose of acetaminophen. Young and old male Fischer 344 rats were treated with 800 mg/kg acetaminophen (young n = 8, old n = 5) or saline (young n = 9, old n = 9). Serum measurements showed old rats treated with acetaminophen had significantly lower serum alanine aminotransferase and higher acetaminophen and acetaminophen glucuronide levels and creatinine, compared with acetaminophen treated young rats (p < .05). Immunoblotting and activity assays showed old saline-treated rats had twofold lower cytochrome P450 2E1 activity and threefold higher NAD(P)H quinone oxidoreductase 1 protein expression and activity than young saline-treated rats (p < .05), although Nrf2, glutathione cysteine ligase–modulatory subunit, glutathione cysteine ligase–catalytic subunit, and cytochrome P450 2E1 protein expressions were unchanged. Primary hepatocytes isolated from young rats treated with 10 mM acetaminophen had lower survival than those from old rats (52.4% ± 5.8%, young; 83.6% ± 1.7%, old, p < .05). The pharmacokinetic changes described may decrease susceptibility to acetaminophen-induced hepatotoxicity but may increase risk of nephrotoxicity in old age.

Key Words: Acetaminophen—Toxicity—Aging—Pharmacokinetics.

Received February 21, 2013; Accepted May 28, 2013

Decision Editor: Placido Navas, PhD

ACETAMINOPHEN (paracetamol) is an analgesic and antipyretic taken commonly by people of all ages. At therapeutic doses, it is considered safe. However, when taken excessively, acetaminophen can cause hepatotoxicity (1,2). Currently, acetaminophen is the most frequent cause of drug-induced liver injury in humans (3). Hepatotoxicity has been reported to occur predominantly in adolescents and young adults; however, the majority of acetaminophen associated deaths occur in old age (1,3). In older adults, acetaminophen toxicity is frequently related to accidental therapeutic overdoses. Age-related changes in the metabolism and toxicity of acetaminophen are poorly understood (4). A better understanding of acetaminophen toxicity and pharmacokinetics in old age is required to improve assessment and management of acetaminophen toxicity and to establish safe therapeutic dosing guidelines for older adults.

A therapeutic dose of acetaminophen is mainly metabolized (>90%) via Phase II metabolism, through glucuronidation and sulfation, resulting in nontoxic excretable conjugates (acetaminophen glucuronide and sulfate). Approximately 5%–10% of acetaminophen is metabolized via Phase I cytochrome P450 metabolism, particularly Cytochrome P450 2E1 (CYP2E1), into a hepatoxic intermediate, N-acetyl-p-benzoquinone imine (NAPQI) (2). NAPQI is metabolized by NAD(P)H quinone oxireductase 1 (NQO1) back to its parent acetaminophen compound (5) or detoxified by the antioxidant glutathione (6). In young and middle-aged adults, following an excessive dose of acetaminophen, Phase II metabolism is saturated and Phase I metabolism is enhanced resulting in increased levels of NAPQI (6). Eventually, as overwhelming concentrations of NAPQI form, glutathione stores are depleted and NAPQI covalently binds to cellular proteins. Binding of NAPQI to mitochondrial proteins leads to downstream oxidative stress resulting in irreversible damage (6), such as hepatic apoptosis and/or necrosis and leakage of alanine aminotransferase (ALT) into the blood. In severe cases, liver failure ensues (6).

The effects of old age on acetaminophen-induced hepatotoxicity has not been widely studied. Aging is associated with changes in both Phase I and II metabolism (7–9). In humans and rodents, a reduction or no changes
in glucuronidation and sulfation of acetaminophen have been reported in old age (10,11). Other investigators have documented decreased CYP2E1 activity in old age (12,13), which could result in reduced formation of toxic intermediates. The detoxification of NAPQI may also be altered as glutathione is decreased or unchanged in old age (14–16), whereas NQO1 has been found to increase (17,18). In addition, Nuclear factor (erythroid-derived 2)-like 2 (Nrf2) a transcription factor that regulates NQO1 expression and glutathione synthesis by acting on glutathione cysteine ligase (GCL) is decreased in aged rats (15,19–21). Together these results suggest hepatic acetaminophen pharmacokinetic changes do occur with old age and indicate a need to investigate these processes further to understand the risk of toxicity with acetaminophen in old age.

To date, very few studies have investigated the effect of aging on acetaminophen toxicity. Some of these studies were confounded by higher drug exposure as a consequence of significant weight gains in their aging animal models and dosing per kg of total body weight. Such studies generally indicated that old animals were more susceptible to toxicity (22,23). In addition, obese animals are known to be at greater risk of acetaminophen-induced organ injury (24). In contrast, only one study by Rikans in 1988 (14) has investigated the effect of aging on acetaminophen toxicity in an aging model free from age-associated obesity, Fischer 344 rats. This study found an age-associated decrease in susceptibility based on ALT and glutathione concentrations. The authors also found a reduction of CYP450 expression with aging. However, due to the technological and knowledge limitations at the time, they were unable to draw definitive conclusions on the mechanism of decreased susceptibility with aging. Therefore, this study was undertaken to better understand and further investigate the influence of aging on the risk of toxicity with acetaminophen in old age.

**Experimental Procedures**

**Animals**

Young adult (6–7 mo, 373.5 ± 5.6 g) and old (26–28 mo, 418.4 ± 12.3 g) male Fischer 344 rats were obtained from Harlan Laboratories (Harlan, United States). A separate cohort of young adult male Fischer 344 rats (4–6 mo, 362.4 ± 11.5 g) were purchased from the Animal Research Centre (Perth, Australia) for primary cell culture experiments. Animals had access to water and food ad libitum. The study was approved by the Royal North Shore Hospital Animal Care and Ethics Committee and the University of Sydney Animal Ethics Committee.

**Materials**

Acetaminophen and reagents required for the enzyme activity assays were purchased from Sigma–Aldrich (Sydney, Australia). 4-20% and 12% mini-PROTEAN precast gels were purchased from Bio-Rad (Sydney, Australia). The Glutathione Assay kit was obtained from Cayman Chemicals (#703002, Sapphire Bioscience, Sydney Australia) The primary antibodies Nrf2 (sc-722), alpha tubulin (sc-5286), and NQO1 were purchased from Santa Cruz (ThermoFisher Scientific, Melbourne, Australia), whereas GCL-catalytic subunit (GCLC; ab80841), GCL-modulatory subunit (GCLM; ab81445), and CYP2E1 (ab19140) primary antibodies were sourced from Abcam (Sapphire Bioscience). Transcription factor II B (TFIIB; #4169) primary antibody was purchased from Cell Signaling Technology (Genesearch, Brisbane, Australia). Anti-rabbit (A9169) and Anti-mouse secondary (ab6728) antibodies were purchased from Sigma–Aldrich and Abcam (Sapphire Bioscience), respectively. Reagents for the primary cell culture experiments were obtained from Sigma–Aldrich and Invitrogen (Mulgrave, Australia).

**Acetaminophen Treatment of Animals and Tissue Collection**

Animals were fasted overnight for 16 hours before an intraperitoneal injection with 800 mg/kg of acetaminophen in saline or saline vehicle. Animals were injected between 10 am and 12 am to avoid temporal variation in acetaminophen metabolism (25). Four hours postinjection, animals were anaesthetized with an intraperitoneal injection of Ketamine (75 mg/kg; Parnell Laboratories Pty Ltd, Sydney, Australia) and Xylazine (10 mg/kg; Troy Laboratories Pty Ltd, Sydney, Australia). A midline laparotomy was performed. Blood was taken from the inferior vena cava and hepatic (300 U) was then injected into the same vessel. This was followed by cannulation of the portal vein with an 18G intravenous catheter (BD, Sydney, Australia) through which the liver was perfused in situ at 1–1.5 mL/min/g of liver with oxygenated Krebs–Henseleit Bicarbonate buffer (95% O₂, 5% CO₂, 37°C) to remove blood from the liver tissue.

The liver was excised and segments were placed into Cryovials (ProsciTech, Brisbane, Australia) and snap frozen in liquid nitrogen for subsequent enzyme activity assays and immunoblotting. A section of the main lobe was also fixed in 10% neutral formalin (ProSciTech) for histology.

**Serum Biochemistry**

The collected blood was allowed to clot at room temperature before separating the serum by centrifugation at 10,000 rpm (7.378g) for 10 minutes (Sigma centrifuge 1–14, John Morris Scientific Pty Ltd, Australia). Sera were then stored in aliquots at −80°C. Serum Liver Function Tests were carried out to assess liver injury and serum creatinine concentration was measured to assess kidney damage. These tests were conducted by a National Association of
Testing Authorities accredited hospital laboratory, Pacific Laboratory Medicine Services at Royal North Shore Hospital (Sydney, Australia).

Histology

Fixed liver tissue was embedded in paraffin and 5 µm sections were mounted on slides. Slides were processed for Haematoxylin and Eosin staining in the National Association of Testing Authorities accredited hospital laboratory of the Pathology department of Royal Prince Alfred Hospital, Sydney, Australia. Histopathology was described by an anatomical pathologist (C.M.), who was blinded to the age and treatment groups of the samples. Animals with histopathologic evidence of age-related hepatic malignancy, lymphoproliferative disease, or other liver diseases were excluded (n = 4 old saline treated and n = 3 old acetaminophen treated).

Acetaminophen and Acetaminophen Metabolite Levels

Concentrations of acetaminophen and its sulfate and glucuronide metabolites were measured in serum by ultra violet detection method in association with high-performance liquid chromatography with a Shimadzu 10A VP instrument using the method of Jensen and coworkers (26).

Primary Hepatocyte Isolation and Acetaminophen Treatment

Primary hepatocytes from young and old male F344 rats were isolated by a two-step collagenase perfusion in situ (27). The viability of isolated hepatocytes was determined by trypan blue staining, and it was 86.2% ± 1.1% for the young rats and 61.5% ± 4.2% for old rats. The hepatocytes were seeded on 24- or 48-well collagen I-coated plates (BD) at a density of 6.25 × 10^4/cm^2 in Dulbecco’s modified eagle medium (Invitrogen) supplemented with 4% fetal calf serum (Sigma–Aldrich), and the cells were fractionated.

Western Analysis

Liver tissue was homogenized with a Dounce homogeniser (Sigma–Aldrich), and the cells were fractionated into cytosolic, nuclear, and mitochondrial fractions using the method of Ishigami and coworkers (29) and Kislinger and coworkers (30). For NQO1 protein expression levels, livers were homogenized in sucrose–Tris buffer (0.25 M sucrose, 10 mM Tris–HCl, pH 7.4) and centrifuged (Optima XL–100K Ultracentrifuge, Beckman Coulter, Australia) at 50,000 rpm for 60 min at 4°C, following the method used by Aleksunes and coworkers (31), and the supernatants were collected.

The fractions were purified using the ReadyPrep 2-D cleanup Bio-Rad Kit. Protein levels were measured with the Bio-Rad RC DC protein kit (#500-0122). Samples were made up with 3x blue loading buffer (Cell Signalling Technology, Sydney, Australia) and run on Bio-Rad Mini-PROTEAN TGX precast gel. This was followed by semidry transfer (Hoefer #Te77xp, Hoefer, Holliston, United States) onto 0.45 µm nitrocellulose membrane (Bio-Rad). Proteins were probed with primary antibodies specific to CYP2E1, GCLC, and GCLM in the cytosolic fractions and Nrf2 in the nuclear fractions followed by the secondary antibody. Bands were detected by exposure to Super Signal West Pico (ThermoFisher Scientific) and subsequent chemiluminescence detection with ImageQuant LAS 4000 biomolecular imager (GE Healthcare Life Sciences, NSW, Australia), followed by digital quantification using Multi Gauge version 3.0 software. Target proteins were identified based on molecular size and verified with positive controls and blocking peptides specific to the antibody. Target proteins were normalized to cytosolic and nuclear loading controls using alpha tubulin and transcription factor II B, respectively. Results were displayed as arbitrary densitometric values relative to young control as 1.

CYP2E1 Activity

Liver microsomes were prepared by the method of Shoaf and coworkers (32). CYP2E1 activity was measured as described by Roberts (1995) (33), via aniline hydroxylase activity, as conversion of aniline to p-aminophenol occurs. P-aminophenol absorption was measured at the wavelength of 620 nm.

NQO1 Activity

Following the method used by Aleksunes and coworkers (31), NQO1 enzymatic activity was measured over 1 minute using the reduction of dichlorophenolindophenol (DCPIP) at 595 nm with NADPH as the reducing cofactor. Briefly NQO1 activity was determined at 27°C in a reaction mixture containing liver cytosol, 200 µmol/L NADPH, 40 µmol/L DCPIP, 25 mmol/L Tris–HCl buffer pH 7.4, and 0.7 mg/mL bovine serum albumin. Parallel reactions were conducted with 20 µmol/L of selective NQO1 inhibitor dicumarol. The rates of dicumarol-sensitive NQO1 activity were determined by the difference between uninhibited and
dicumarol-inhibited rates. This was further normalized to the total cytosolic protein as previously described (34).

**Liver Glutathione Concentrations**

Hepatic concentrations of oxidized and reduced glutathione were determined with a commercial Glutathione Assay kit from Cayman Chemicals.

**Statistical Analysis**

Statistical analysis was conducted using IBM SPSS statistics version 20. Data were analyzed using analysis of variance with posthoc Tukey test, a Kruskal Wallis test or student t test where appropriate. p values less than 0.05 were considered significant. Data are presented as mean ± SEM.

**RESULTS**

**Animal Details**

Ages, body weights (pre and post fasting) and liver weights of animals are shown in Table 1.

**Evidence of Toxicity**

**Serum Biochemistry**.—There was no difference in serum ALT concentration (Table 1) in the aged animal groups with acetaminophen treatment, whereas ALT was significantly increased in the young animal group treated with acetaminophen (55 ± 3 U/L, young saline; 109 ± 14 U/L, young acetaminophen, p < .05). This same pattern was observed in serum aspartate aminotransferase levels (Table 1).

Serum creatinine concentration was significantly elevated only in old animals treated with acetaminophen (41 ± 2.2 μmol/L, young acetaminophen; 65 ± 8 μmol/L, old acetaminophen, p < .05; Table 1).

**Histology**.—On hematoxylin and eosin staining, zone 3 necrosis was observed in one young rat treated with acetaminophen, and early zone 3 necrosis was observed in one old rat treated with acetaminophen. Mild hepatic steatosis (5%–10%) was observed in one young control (11.1% of group), one young acetaminophen-treated rat (12.5% of group), four old controls (44.4% of group), and two old acetaminophen-treated rats (40.0% of group).

**Serum Acetaminophen and Acetaminophen-Conjugated Metabolites**

Serum acetaminophen (334.4 ± 11.0 μg/mL, young; 571.75 ± 159.0 μg/mL, old) and acetaminophen glucuronide metabolite levels (84.3 ± 3.5 μg/mL, young; 397.6 ± 8.3 μg/mL, old; Figure 1) were significantly higher in the old acetaminophen-treated group than the young acetaminophen-treated group (p < .05). Acetaminophen sulfate was not significantly higher in old than in young acetaminophen-treated rats.

**Acetaminophen Treatment of Primary Hepatocytes**

Following acetaminophen treatment, the viability of primary hepatocytes from old rats was significantly higher

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**Table 1. The Body and Liver Weights and Serum Biochemistry of Young and Old Animals Treated With Saline or Acetaminophen**

<table>
<thead>
<tr>
<th></th>
<th>Young Saline</th>
<th>Young Acetaminophen</th>
<th>Old Saline</th>
<th>Old Acetaminophen</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>n</strong></td>
<td>9</td>
<td>8</td>
<td>9</td>
<td>5</td>
</tr>
<tr>
<td><strong>Age (mo)</strong></td>
<td>6.6 ± 2.2</td>
<td>6.5 ± 2.4</td>
<td>25.4 ± 6.1</td>
<td>26.6 ± 2.8</td>
</tr>
<tr>
<td><strong>Prefast weight (g)</strong></td>
<td>381.2 ± 9.4</td>
<td>379.3 ± 9.2</td>
<td>424.8 ± 14.5</td>
<td>429.1 ± 30</td>
</tr>
<tr>
<td><strong>Postfast weight (g)</strong></td>
<td>375.7 ± 8.1</td>
<td>371.3 ± 8.3</td>
<td>419.9 ± 12.2**</td>
<td>415.7 ± 28.9</td>
</tr>
<tr>
<td><strong>Liver weight (g)</strong></td>
<td>10.9 ± 0.4</td>
<td>10 ± 0.4</td>
<td>11.7 ± 0.4</td>
<td>11.8 ± 0.9</td>
</tr>
<tr>
<td><strong>Liver function test</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td><strong>Alanine transaminase (U/L)</strong></td>
<td>55 ± 3.4</td>
<td>109.3 ± 14.3***</td>
<td>5.4 ± 6.4</td>
<td>51 ± 8</td>
</tr>
<tr>
<td><strong>Aspartate transaminase (U/L)</strong></td>
<td>112.4 ± 11.3</td>
<td>349.6 ± 34.3***</td>
<td>120.8 ± 12.9</td>
<td>194.6 ± 29.1</td>
</tr>
<tr>
<td><strong>Albumin (g/L)</strong></td>
<td>13.6 ± 0.3</td>
<td>12.6 ± 0.4</td>
<td>10.4 ± 1*</td>
<td>10.8 ± 0.9</td>
</tr>
<tr>
<td><strong>Bilirubin (μmol/L)</strong></td>
<td>1 ± 0.2</td>
<td>1.3 ± 0.2</td>
<td>2.5 ± 0.2</td>
<td>1.6 ± 0.2</td>
</tr>
<tr>
<td><strong>Alkaline phosphatase (U/L)</strong></td>
<td>98.8 ± 5.3</td>
<td>85.3 ± 9.9</td>
<td>71.6 ± 9.4</td>
<td>48.2 ± 5.8***</td>
</tr>
<tr>
<td><strong>Gamma-glutamyl transpeptidase (U/L)</strong></td>
<td>1.2 ± 0.1</td>
<td>1.6 ± 1</td>
<td>2.1 ± 0.9</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td><strong>Protein (g/L)</strong></td>
<td>62.3 ± 0.8</td>
<td>57.9 ± 1.4</td>
<td>54.8 ± 5.1</td>
<td>59.2 ± 1.3</td>
</tr>
<tr>
<td><strong>Kidney function</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td><strong>Creatinine (μmol/L)</strong></td>
<td>33.5 ± 0.4</td>
<td>41.2 ± 2.2</td>
<td>33 ± 4.5</td>
<td>65.4 ± 8***</td>
</tr>
</tbody>
</table>

*Notes: Data are presented in mean ± SEM of n = 5–9 animals per group.
* p < .05 compared with young saline group.
** p < .05 compared with young acetaminophen group.
*** p < .05 compared with all other groups.
†Where n = 9, 7, 6, and 5 for young saline, young acetaminophen, old saline, and old acetaminophen, respectively.
‡Where n = 9, 7, 6, and 5 for young saline, young acetaminophen, old saline, and old acetaminophen, respectively.
Metabolic Enzyme Activity and Expression

CYP2E1 activity.—CYP2E1 activity was significantly higher in young than old saline-treated animals (4.1 ± 0.8 nmol/min/mg, young; 1.5 ± 0.4 nmol/min/mg, old, \( p < .05 \), Figure 3A). In acetaminophen-treated animals, there was a nonsignificant trend toward lower activity in old age.

NQO1 activity.—Figure 3B shows a threefold increase in NQO1 activity in old rats compared with young, with both saline (212.9 ± 30.8 µmol DCPIP/min/mg, young; 711.1 ± 67.6 µmol DCPIP/min/mg, old, \( p < .05 \), Figure 3A) and acetaminophen treatment (188.5 ± 16.3 µmol DCPIP/min/mg, young; 738.4 ± 135.0 µmol DCPIP/min/mg, old, \( p < .05 \)).

CYP2E1 and NQO1 protein expression.—CYP2E1 protein expression did not differ with age or treatment group (Figure 4A, \( p > .05 \)). As shown in Figure 4B, NQO1 protein expression increased fourfold in old rats compared with young, with both saline (1 ± 0.27, young; 4.4 ± 1.34, old, relative to young control = 1, \( p < .05 \)) and acetaminophen treatment (1.1 ± 0.45, young; 4.79 ± 1.14, old, relative to young control = 1, \( p < .05 \)).

Hepatic Glutathione Production and Concentration

GCL protein expression.—Protein expression of the GCLC and GCLM subunits of the glutathione rate-limiting enzymes were similar between age and matched treatment groups (Figure 4C, D).

Hepatic glutathione concentration.—Acetaminophen treatment significantly decreased total hepatic glutathione by 66.2% ± 6.6% and 50.1% ± 8.8% in young and old rats, respectively (Figure 5, 393 ± 39.66 mmol/g, young saline; 459.3 ± 33.4 mmol/g, old saline; 132.7 ± 253.97 mM/g, young acetaminophen; 229.4 ± 10.2 mM/g, old acetaminophen, \( p < .05 \)). No significant difference was observed between the two age groups when matched treatment groups were compared.

Nrf2 Expression

As shown in Figure 4E, nuclear expression of Nrf2 did not differ with age or treatment group.

Discussion

Old male Fischer 344 rats were less susceptible to acetaminophen-induced hepatotoxicity than younger rats despite higher levels of serum acetaminophen. Older rats displayed less CYP2E1 activity and enhanced NQO1 activity.
levels are consistent with previous studies. Serum ALT, which is a validated marker of hepatotoxicity (37), was only elevated in young acetaminophen-treated rats. Rikans (14) also observed a greater increase in serum ALT in young adult than in older rats 24 hours after a toxic dose of acetaminophen. Interestingly, like Rikans, our histopathologic analysis only showed minor signs of hepatotoxicity, which may be due to resistance in the rat species or, in our case, to tissue collection only 4 hours after administration of acetaminophen. Importantly, studies in humans receiving therapeutic acetaminophen have also observed higher serum acetaminophen levels in old age (38), with one case-control study finding no greater susceptibility to acetaminophen-induced hepatotoxicity despite higher serum acetaminophen levels in old age (39).

The higher acetaminophen levels and reduced hepatotoxicity observed in old age may be partly explained by our previous report of age-induced pseudocapillarization of liver sinusoids resulting in reduced hepatic uptake of acetaminophen (40). The reduced hepatocyte death that we observed in primary hepatocytes cultured from old animals compared with those from young adult animals in response to equivalent toxic concentrations of acetaminophen confirms that there are age-related differences in hepatocytes that contribute to decreased hepatotoxicity in old age (Figure 2). The age-induced metabolic changes reported in this study may explain these findings. The decrease in activity of CYP2E1 and increase in activity of NQO1 in our in vivo experiments in old rats compared with young rats are consistent with higher serum acetaminophen levels and decreased exposure to NAPQI in old age. The activity of CYP2E1 was significantly decreased in old age in saline-treated animals, with a trend toward reduced activity in old age in acetaminophen-treated animals, but no change was observed in protein expression between treatment or age groups, suggesting negative posttranslational regulation, as previously reported (12,13). This finding of decreased CYP2E1 activity and hepatotoxicity in old age is consistent with CYP2E1-deficient models, where susceptibility to acetaminophen-induced hepatotoxicity is significantly reduced (41). It is also consistent with models of increased CYP2E1 expression, where acetaminophen toxicity is increased, not only due to generation of toxic intermediates but also due to TNF-alpha toxicity mediated by c-Jun and oxidative stress (42).

Furthermore, NQO1 activity and protein expression were consistently increased threefold in old age, which has previously been reported with aging rats (17,18) but not previously assessed with acetaminophen toxicity in old age. Interestingly, the induction of recombinant human NQO1 enzyme has been shown to reduce NAPQI levels, whereas its inhibition enhances injury from acetaminophen in mice (5,43). The role of NQO1 in acetaminophen hepatotoxicity could be assessed with further studies using NQO1 inhibitors that are currently in development for cancer therapeutics.
Figure 4. Protein expression of Cytochrome P450 2E1 (CYP2E1), NAD(P)H quinone oxireductase 1 (NQO1) and glutathione synthesis regulating proteins of young (6 mo) and old (26 mo) rats 4 h after saline (solid bar) or acetaminophen (APAP; open bars) intraperitoneal injection. (A) CYP2E1, (B) NQO1, (C) Glutathione cysteine ligase–Catalytic subunit (GCLC), (D) Glutathione cysteine ligase–Modulatory subunit (GCLM), and (E) activated Nuclear factor (erythroid-derived 2)-like 2 (Nrf2). All data are presented as mean arbitrary densitometric values relative to young control as 1 ± SEM of n = 5–9 animals per group with immunoblot representative of target protein and loading control results below graph. Statistically significant differences comparing against corresponding young group: *p < .05.
Figure 4. (Continued).
Currently, it is not known whether hepatic NQO1 activity is increased in old human livers. As our results on rats have consistently shown an increase in dicumarol-sensitive NQO1 activity, further research should investigate this finding in humans to determine whether an increase in NQO1 in old age may be a novel toxokinetic mechanism to protect against hepatotoxicity in older people.

Investigation into Nrf2 regulation of glutathione synthesis observed no change in nuclear activation or downstream protein expression of GCL and its catalytic or modulatory subunits between age and treatment groups. Activated Nrf2 has been previously reported to increase within hours following acetaminophen treatment in mice (45). We did not observe this in Fischer 344 rats, and this could be due to interspecies differences. We also did not observe the age-associated decline in Nrf2 or NQO1 as observed by Suh and coworkers (15) with the same species. This variation could be because our animals were fasted, which has been shown to alter glutathione levels (46) and affect many cellular processes that lead to Nrf2 activation (47). Surprisingly, we did observe increased protein expression and dicumarol-sensitive activity of NQO1, which is also regulated by Nrf2, and this may be mediated by other pathways such as the xenobiotic response element (48).

Old age was associated with higher serum levels of acetaminophen glucuronide, whereas serum acetaminophen sulfates was not significantly higher in old age rats compared with young rats (Figure 1A). These findings are consistent with both patient (38) and rodent studies (35) and suggest either increased conjugation activity or accumulation due to impaired renal clearance. Phase II metabolism is regulated by Nrf2, which did not vary with age or treatment in our study, and several studies have reported no change or a decline in conjugation activity with aging in humans (11) and rat livers (10). We observed elevated serum creatinine (a marker of renal function) following acetaminophen treatment in old rats. Many studies have reported

![Figure 4.](Continued)

![Figure 5.](Continued)
that acetaminophen can cause renal lesions and this risk is increased with age (35,36,49). Together with existing literature, our findings indicate that it is unlikely that Phase II metabolism is increased with aging, instead the elevation of serum conjugation metabolites is likely to be the result of impaired renal clearance from acetaminophen-induced nephrotoxicity.

In this study, a variety of methods were employed to investigate the major recognized metabolic pathways of acetaminophen. The animal model selected is a high-quality aging model free from age-associated obesity as discussed by Rikans in 1988 (14). We observed only a minor trend of increase (10%–15%) in body weights of old rats compared with young rats, whereas other models previously reported have found up to a 78% increase in body weight (22). Importantly, the observed lack of increase in susceptibility to acetaminophen hepatotoxicity in old age (39) and pharmacokinetic changes are consistent with human patient results (38), supporting the clinical relevance of our model. However, recently, rats have been proposed to be slightly more resistant to acetaminophen-induced hepatotoxicity than humans (6), and future research may be necessary to confirm these findings in lean mice, which have similar susceptibility to humans. Future research may also investigate other aging and antiaging models such as calorie-restricted and long-lived Ames dwarf mice and the effect of acetaminophen to better understand the mechanisms of acetaminophen-induced hepatotoxicity (50,51). The time of drug exposure is an important parameter for hepatotoxicity studies. We selected an incubation time of 4 hours as the half-life of acetaminophen is approximately 3 hours (52), which enabled us to observe changes in the concentrations of acetaminophen and its metabolites. We also were aware that glutathione levels deplete prior to this time point (6) and hence we expected hepatotoxicity and any protective responses to commence within this period. Our animals were also fasted to normalize their nutritional stores and potentiate hepatotoxicity (46).

**Conclusion**

In conclusion, old age is not associated with increased risk of acetaminophen-induced hepatotoxicity in male Fischer 344 rats despite higher serum levels of acetaminophen and its glucuronide metabolite. Detailed investigation of the potential mechanisms found that old age is associated with toxokinetic changes that favor reduced formation and enhanced metabolism of NAPQI, with no observed changes in Nrf2 mediated resistance with age or treatment. Although old age is associated with a reduced risk of acetaminophen liver toxicity, the changes in acetaminophen pharmacokinetics result in increased serum acetaminophen, which may increase the exposure of the kidneys and result in acetaminophen-induced nephrotoxicity.

**Funding**

The work was supported by the National Health and Medical Research Council (#570968, #570937) and the Geoff and Elaine Penney Ageing Research Unit, Royal North Shore Hospital. This research was supported in part by the Intramural research Program of the NIH, National institute on Aging.

**Acknowledgments**

The authors of the article would like to thank Dr Anneliese Rittau (AARF Laboratory, Concord Hospital and University of Sydney, Australia) for kindly measuring serum acetaminophen and acetaminophen conjugated metabolite levels and Dr Himesha Vandelbona (Liver and Digestive Diseases Laboratory, Kolling Institute of Medical Research, Australia) for kindly conducting primary hepatocyte experiments.

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