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The role of para-aminophenol in acetaminophen-induced methemoglobinemia in dogs and cats

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Acetaminophen (APAP) overdose in most species is associated with hepatotoxicity because of the metabolite *N*-acetyl-*p*-benzoquinoneimine (NAPQI). In dogs and cats, APAP overdose primarily causes methemoglobinemia and hemolysis. Although NAPQI has been proposed as the responsible intermediate in dogs and cats, it lacks chemical or pharmacokinetic characteristics that favor methemoglobin formation. We hypothesized that *para*-aminophenol (PAP) rather than NAPQI induces methemoglobinemia and that deficient arylamine *N*-acetyltransferase (NAT) activity in dogs and cats contributes to this species-dependent methemoglobinemia. Erythrocytes from dogs, cats, mice, and rats were exposed *in vitro* to APAP, NAPQI, and PAP. Only PAP induced methemoglobin and it induced more methemoglobin formation in dog and cat than rat and mouse erythrocytes. PAP also induced more methemoglobin in erythrocytes from Nat1/Nat2 knockout mice than wildtype (WT) mouse erythrocytes ($P < 0.05$), but less than in dog and cat erythrocytes ($P < 0.01$). APAP and PAP toxicity were compared *in vivo* in WT and Nat1/Nat2 knockout mice. APAP caused no hematotoxicity while PAP induced more methemoglobin in NAT1/NAT2 knockout mice than in WT mice ($P < 0.05$). These results support the hypothesis that PAP is the metabolite responsible for APAP-induced methemoglobinemia and that deficient NAT activity in dogs and cats contributes to this species-dependent toxicity.

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INTRODUCTION

Acetaminophen (APAP), a popular human analgesic and antipyretic is a common cause of canine and feline toxicoses reported to veterinary poison control centers (Jones *et al.*, 1992). In humans and most laboratory species, APAP is selectively hepatotoxic (Prescott *et al.*, 1971; Davis *et al.*, 1974), whereas dogs and cats primarily develop methemoglobinemia and hemolytic anemia after overdose (Nash *et al.*, 1984; Ilkiw & Ratcliffe, 1987; Schlesinger, 1995; Aronson & Drobatz, 1996; MacNaughton, 2003).

Acetaminophen is predominantly metabolized by sulfation and glucuronidation, followed by renal excretion of the conjugates in most mammalian species (Fig. 1; Prescott *et al.*, 1971; Savides *et al.*, 1984; Davis *et al.*, 1976; Gemborys & Mudge, 1981). A small percentage of APAP is oxidized by cytochrome P450 (CYP) enzymes to *N*-acetyl-*p*-benzoquinoneimine (NAPQI); (Davis *et al.*, 1976). At therapeutic doses of APAP, NAPQI binds to glutathione (GSH) and is then excreted in the urine as cysteine or mercapturic acid conjugates (Davis *et al.*, 1974). At toxic doses, sulfate and glucuronosyl transferases become saturated and increased NAPQI production occurs (Davis *et al.*, 1974). If GSH is depleted to <20% of its normal concentration, NAPQI binds covalently to cysteine groups on other hepatocellular proteins leading to cell death (Pumford *et al.*, 1990; Landin *et al.*, 1996).

In the veterinary literature, APAP-hematotoxicity in dogs and cats is often attributed to NAPQI (Allen, 2003; Taylor & Dhupa, 2003). It is postulated that cats may produce more NAPQI than other species as they have limited APAP glucuronidation (1.3% of excreted dose) (Savides *et al.*, 1984). The different target organ in cats has been attributed to the high numbers of exposed

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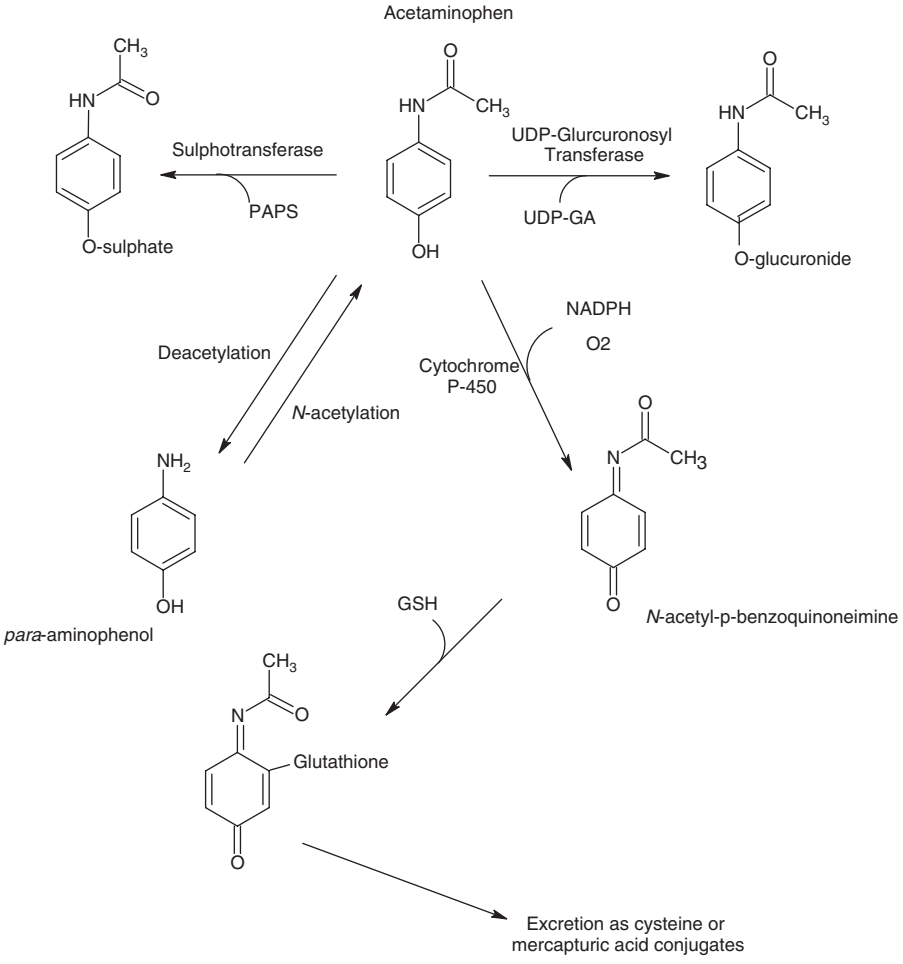

Fig. 1. The metabolism of acetaminophen. Acetaminophen is metabolized in the liver. The predominant pathways in most species are sulfation or glucuronidation followed by renal excretion of the conjugates. A small percentage of acetaminophen is deacetylated to *para*-aminophenel or oxidized by cytochrome P450 enzymes to the reactive metabolite *N*-acetyl-*p*-benzoquinoneimine (NAPQI). NAPQI is short lived as it binds to glutathione (GSH) and is then excreted in the urine as cysteine or mercapturic conjugates. If the GSH supply is exhausted, NAPQI will bind to other hepatic proteins, leading to cell death.

sulfhydryl groups on feline hemoglobin predisposing it to oxidation and subsequent Heinz body formation (Desnoyers, 2000; Taylor & Dhupa, 2003). Dogs are the only other species known to develop APAP-induced hematotoxicity. In contrast to cats, glucuronidation is the predominant APAP biotransformation pathway (76%) in dogs (Savides *et al.*, 1984) and canine hemoglobin has fewer reactive sulfhydryl groups and is less sensitive to oxidation than feline hemoglobin (Snow, 1962). Therefore, a common explanation for APAP hematotoxicity in dogs and cats has not been found.

There is no direct evidence supporting NAPQI as the reactive metabolite responsible for APAP-induced hematotoxicity. Erythrocytes lack the CYP enzymes to produce NAPQI and circulating NAPQI is either an inactive cysteine conjugate or covalently bound to protein (Gillette *et al.*, 1981; Pumford *et al.*, 1990; Muldrew *et al.*, 2002). Therefore, erythrocytes are unlikely to be exposed to reactive NAPQI.

Methemoglobin-inducing chemicals such as dapsone hydroxylamine and phenylhydroxylamine, typically act through co-oxidation of oxyhemoglobin and subsequent redox cycling (Harrison & Jollow, 1987; Reilly *et al.*, 1999). Redox cycling occurs when the oxidized forms are reduced through unstable GSH conjugates or reduction by reductases found in erythrocytes. If NAPQI were able to reach erythrocytes, it would likely be removed by stable conjugation with GSH or covalent binding to cellular proteins (as in the liver), thus preventing the redox cycling typically associated with methemoglobin formation. Therefore, it seems unlikely that NAPQI is responsible for APAP-associated methemoglobinemia.

para-Aminophenol (PAP) is a minor APAP metabolite produced by deacetylation of APAP by hepatic microsomal carboxyesterases, that has been identified *in vivo* in cats, rats, and mice (Welsh *et al.*, 1966; Newton *et al.*, 1982; Klos *et al.*, 1992; Song & Chen, 2001) PAP has been shown to co-oxidize and redox cycle with oxyhemoglobin (Fig. 2) and is associated with aniline dye-induced methemoglobinemia (Bernheim *et al.*, 1937; Harrison & Jollow, 1986).

In rats and mice, >70% of PAP is removed as GSH and *N*-acetyl conjugates (Klos *et al.*, 1992; Song & Chen, 2001). *N*-acetylation of drugs is catalyzed by two closely related enzymes: *N*-acetyltransferase 1 and *N*-acetyltransferase 2 (NAT1 and NAT2; EC 2.3.1.5) (Grant *et al.*, 1989b). Cats only have NAT1 and dogs have no NAT enzymes (Trepanier *et al.*, 1997, 1998) making them deficient in or unable to *N*-acetylate arylamine-containing compounds respectively. Therefore, cats and dogs may be exposed to greater amounts of PAP following APAP exposure and, because NAT enzymes are present in erythrocytes, be less efficient at removal of PAP from within

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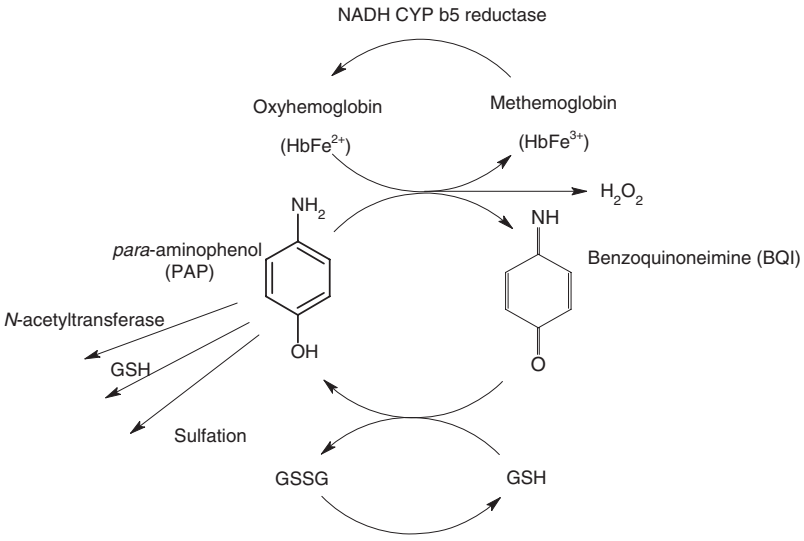

Fig. 2. Co-oxidation of *para*-aminophenol (PAP). PAP co-oxidates with hemoglobin. An electron is lost from both oxyhemoglobin and PAP, creating methemoglobin and oxidized PAP [*p*-benzoquinoneimine (BQI)]. The electrons in turn oxidize an oxygen species, creating superoxide or hydrogen peroxide. The methemoglobin is reduced to oxyhemoglobin by NADH cytochrome P450 b5 reductase and BQI is reduced to PAP by nonenzymatically binding to glutathione (GSH). PAP continues to redox cycle in the erythrocyte until it is removed by an alternative biotransformation pathway such as *N*-acetylation or conjugation with GSH or sulfate.

erythrocytes, making them more susceptible to PAP redox cycling and methemoglobin formation.

We hypothesized that PAP, but not APAP or NAPQI, would induce methemoglobin formation in erythrocytes *in vitro.* Moreover, we hypothesized that there would be differences in susceptibility to the methemoglobin forming potential of PAP related to *N*-acetylation capacity.

Therefore, we compared the *in vitro* deacetylation of APAP and *N*-acetylation of PAP in dogs, cats, rats, and mice. We also determined the *in vitro* methemoglobin forming potential of APAP, NAPQI, PAP, and species' sensitivity to *in vitro* PAP-induced methemoglobin formation. The *in vivo* APAP and PAP induction of methemoglobinemia in wildtype (WT) and *NAT1*/NAT2 double knockout C57BL/6 mice was also examined.

MATERIALS AND METHODS

The following compounds were purchased from Sigma-Aldrich Inc (St Louis, MO, USA): NAPQI, $K_3Fe(CN)_6$), KCN, K_2HPO_4, 4-2-hydroxyethyl-1-piperazineethanesulfonic acid (HEPES; free acid), $NaH_2PO_4.H_2O$, Me_2SO (DMSO; anhydrous dimethyl sulfoxide), *para*-aminobenzoic acid (PABA), cimetidine, triethanolamine–HCl, bovine serum albumin, 4-acetamidobenzoic acid (Nac-PABA), PAP, leupeptin, phenylmethylsulfonyl fluoride (PMSF), 4-aminophenol hydrochloride, 2,6-di-tert-butyl-4-methylphenol (BHT), HCl, acetyl DL-carnitine hydrochloride, carnitine acetyltransferase, acetyl coenzyme A (AcCoA), triethylamine (TEA), DL-dithiothreitol (DTT), potassium chloride (KCl), APAP, butyric anhydride, and ethylenediaminetetraacetic acid (EDTA).

Anhydrous dextrose, NaCl, Na_2HPO_4, $MgSO_4.7H_2O$, $CaCl_2.2H_2O$, PCA, acetonitrile, and glacial acetic acid were purchased from Fisher Scientific (Fairhaven, NJ, USA). KH_2PO_4 was purchased from BDH Inc., Toronto, ON, Canada. The Bio-Rad DC Protein Assay was purchased from Bio-Rad Laboratories (Hercules, CA, USA).

Animals

Written informed consent was obtained from owners of healthy pet cats and dogs for blood sampling. Canine and feline livers were obtained immediately post euthanasia from mature healthy animals deemed inappropriate for adoption by the Prince Edward Island Humane Society. Male Sprague-Dawley rats aged 10–12 weeks, and male and female WT (C57BL/6) mice aged 6–7 weeks were purchased from Charles River, St Hyacinthe, QC, Canada and acclimatized for two or more weeks. C57BL/6 double knockout mice deficient in *Nat1* and *Nat2* (Sugamori *et al.*, 2003, 2007) were maintained in a breeding colony at the University of Prince Edward Island. All procedures were performed in accordance with the guidelines of the Canadian Council on Animal Care and with the approval of the University of Prince Edward Island Animal Care Committee.

Rats were sedated with 5 mg/kg xylazine (20 mg/mL Rompun7; Bayer, Toronto, ON, Canada) and 70 mg/kg ketamine (100 mg/mL Vetalar 7; Bioniche Animal Health Canada, Belleville, ON, Canada) intraperitoneally (IP) for venipuncture of the ventral tail artery. Euthanasia of rats was by inhalation of CO_2 and exsanguination by cardiac puncture. Euthanasia of mice was by IP injection of 80 mg/kg pentobarbital (diluted Euthansol® 340 mg/mL; Schering-Plough Animal Health, Pointe Claire, QC, Canada) followed by cardiac puncture and exsanguination for blood collection followed by removal of livers.

Liver fractionation

Livers were homogenized on ice (Tissue Tearor, Model 398; Biospec Products Inc., Dremels, WI, USA) in four volumes of TEDK buffer (10 mM triethanolamine–HCl, 1 mM EDTA, 1 mM DTT, 50 mM KCl, pH 7.0) with 10 μM leupeptin, 100 μM

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PMSF, and 18 μM BHT (Grant *et al.*, 1990). Cytosol and microsomes were separated by differential centrifugation (Allegra X-15R Centrifuge, Beckman Coulter L8-60M Ultracentrifuge; Beckman Coulter, Fullerton, CA, USA) (Grant *et al.*, 1989a, 1990). Protein concentrations were determined by BioRad DC Protein Assay (BioRad, Hercules, CA, USA), a modified Bradford technique (Bradford, 1976).

N-*acetyltransferase assay*

The assay for *N*-acetyltransferase was performed as previously described (Andres *et al.*, 1985; Grant *et al.*, 1989a; Cribb *et al.*, 1991), using 9–10 acceptor substrate concentrations ranging from 2.5 to 500 μM and an acetyl-CoA concentration of 100 μM. All assay conditions were adjusted to ensure linear formation of metabolites. *N*-acetylated PABA and APAP were measured by high performance liquid chromatography (HPLC) using UV detection at 270 nm by a Beckman Ultrasphere ODS C-18 column (150 × 4.6 mm, 5 μm particle size) and a Shimadzu SCL-10A solvent delivery system (Shimadzu Scientific, Columbia, MD, USA). In all cases, 50 μL of the supernatant was injected into the HPLC without further preparation. The mobile phase for NacPABA consisted of water:acetonitrile:glacial acetic acid:TEA at a ratio of 89:11:1:.05 (v/v) (Grant *et al.*, 1991). The mobile phase for analysis of APAP was water:acetonitrile:glacial acetic acid:TEA at a ratio of 96:4:1:0.05 (v/v). Both mobile phases were used isocratically at 1.25 mL/min. The retention times of APAP and NacPABA were 6.3 and 6.7 min, respectively.

Deacetylation of APAP

Deacetylation of APAP to PAP by hepatic microsomes was assessed by HPLC measurement of the product. Final protein concentrations ensuring linear formation of PAP, with <10% consumption of 5,000 μM APAP were used. Following incubation of the microsomes and APAP, the reactions were stopped by the addition of *n*-butyric anhydride so as to form the stable derivative 4-hydroxybutyranilide (BPAP) (Gemborys & Mudge, 1981). The isocratic mobile phase for BPAP consisted of water:acetonitrile:glacial acetic acid:TEA at a ratio of 91:9.0:1.0:0.05 (v/v) at 1.25 mL/min. Detection was at 245 nm. The retention time of BPAP was 10.2 min.

In vitro *exposure of erythrocytes*

Erythrocytes from all species were washed in PBS 1–2 times and resuspended in HEPES-buffered medium [15 mM 4-(2-hydroxyethyl)-1-piperazine ethane sulphonic acid, pH 7.4, 125 mM sodium chloride, 6 mM KCl, 1.2 mM magnesium sulphate, 1 mM sodium phosphate, 1 mM calcium chloride, and 10 mM glucose] to yield packed cell volumes (PCV) of 35–50% (Fairbanks & Klee, 1987). Lysed erythrocytes were prepared by suspension in distilled water at a 1:1 ratio v/v and vortexing.

Stock solutions of APAP, NAPQI, and PAP were prepared in 100% DMSO immediately prior to use and diluted with HEPES. Vehicle control consisted of 0.5% DMSO in HEPES. Duplicate samples were incubated in a shaking hot water bath at 37 °C for 5 or 60 min.

Methemoglobin concentrations for *in vitro* studies were determined as previously described, modified to perform in a 96 well plate spectrophotometer (Spectra Max 340; Molecular Devices, Sunnyvale, CA, USA) (Fairbanks & Klee, 1987).

In vivo *studies in knockout mice*

For the APAP trials, male and female WT and knockout mice were injected IP with either 10 mL/kg of saline or 250 or 400 mg/kg APAP in saline at 40 °C. Mice were killed and sampled at 0, 3, 24 and 48 h post-treatment ($n = 4$ for each group at each time-point).

For the PAP studies, a killing/sampling time of 5 min was chosen based on preliminary data. Control and PAP WT and knockout mice were injected with either 10 mL/kg of saline or 400 mg/kg PAP IP. For fasting studies, food was withheld for 16 h prior to exposure.

Whole blood was collected via cardiac puncture into 400 μL Vacutainers® containing lithium heparin (Becton Dickinson, Franklin Lakes, NJ, USA). Whole blood was centrifuged for 5 min to measure the PCV (Haemofuge, Heraeus Christ, West Germany). Blood smears were stained with Wright Giemsa (Shandan Instant Hemotoxylin; Fisher Scientific, Fairhaven, NJ, USA) or New Methylene Blue stains (J.T. Baker, Phillipsburg, NJ, USA). Plasma aspartate aminotransferase (AST) activity and alanine aminotransferase (ALT) activity were measured by a Hitachi 917 (Roche Diagnostics Canada, Laval, QC, Canada).

Histological sections of livers were prepared and stained with hematoxylin and eosin stains (Hematek® Stain Pack; Bayer HealthCare, Toronto, ON, Canada) using standard techniques. The methemoglobin concentration was measured by co-oximetry (Radiometer Copanhagan ABL-700; Radiometer Canada, London, ON, Canada) at the Queen Elizabeth Hospital, Charlottetown, PE, Canada.

Statistical analysis

Comparison of species deacetylation and *N*-acetylation was by one-way ANOVA and Bonferroni *t*-tests. Comparison of *in vitro* PAP-induced methemoglobin was by repeated measures one-way ANOVA's and Dunnett *t*-tests. Species comparison of methemoglobin production by 100, 250, and 500 μM PAP in dogs, cats, and rats was by one-way ANOVA and Bonferoni *t*-tests at each concentration. The comparison of methemoglobin generation in lysed and intact erythrocytes was also by one-way ANOVA followed by Bonferoni multiple comparison tests. Methemoglobin generation in WT and knockout mice following incubation with 500 μM PAP was compared using a two-tailed *t*-test.

All statistical calculations were performed using GraphPad Prism version 3.00 for Windows, GraphPad Software (San Diego, CA, USA). Unless otherwise stated, means are expressed as ±SEM (standard error of the mean) and differences were considered statistically significant when $P < 0.05$.

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RESULTS

In vitro *N-acetylation and deacetylation*

N-acetylation of PABA by hepatic cytosol was used as a positive control to confirm the methodology and previously reported species differences in *N*-acetylation activity. *N*-acetylation of 100 μM PABA and 100 μM PAP were linear with protein concentration and time in rats, WT mice, and cats. The mean *N*-acetylation activity at 100 μM PABA and 100 μM PAP are summarized in Table 1. No activity was detected in dogs or knockout mice. WT mouse PABA and PAP *N*-acetylation was significantly greater than feline, canine and knockout mouse PABA and PAP *N*-acetylation ($P < 0.001$). Rat PABA and PAP *N*-acetylation was significantly greater than cats, dogs, and knockout mice ($P < 0.01$), The *N*-acetylation of PAP across a range of concentrations was determined in rats, WT mice and cats. WT mice and rats displayed curvilinear Eadie-Hofstee transformations, consistent with the known presence of multiple *N*-acetyltransferase enzymes in these species. In contrast, cats displayed linear Eadie-Hofstee transformation, consistent with a single enzyme (Fig. 3).

Hepatic microsomes isolated from all species exhibited deacetylation of 5000 μM APAP. There was no significant difference in the deacetylation velocity between species ($P > 0.05$) (Table 1).

In vitro *methemoglobin generation*

There was no significant methemoglobin induction following exposure of erythrocytes of any species to 0.25% DMSO in HEPES *in vitro*. Preliminary studies of PAP-induced methemoglobin formation showed a rapid initial formation, followed by a decreasing rate such that maximal methemoglobin formation (plateau) was achieved by approximately 60 min. As a 5-min incubation period was the shortest practical exposure, this was chosen to represent the initial rate of methemoglobin formation. Sixty minutes was chosen to represent the maximum methemoglobin formation.

Table 1. Velocity of the *N*-acetylation of 100 μM PABA and 100 μM PAP and deacetylation of 5000 μM APAP

Animals	Final protein concentration (mg protein/mL)	Velocity of PABA N-acetylation (nmol/mg protein/min)	Velocity of PAP *N*-acetylation (nmol/mg protein/min)	Velocity of APAP deacetylation (nmol/mg protein/min)
Rats ($n = 4$)	1.30	0.436 ± 0.027	0.5 ± 0.022	0.80 ± 0.005
Cats ($n = 4$)	1.0	0.058 ± 0.006	0.058 ± 0.015	0.050 ± 0.018
Wildtype mice ($n = 6$)	1.15	1.234 ± 0.205	1.276 ± 0.478	0.078 ± 0.007
Dogs ($n = 4$)	1.0	Below detectable limits	Below detectable limits	0.050 ± 0.018
NAT1/NAT2 knockout mice ($n = 6$)	1.30	Below detectable limits	Below detectable limits	–

Hepatocyte cytosol was incubated with 100 μM PABA or PAP in the presence of 100 μM AcCoA and an AcCoA regenerating system at 37 °C for 10 min to determine the *N*-acetylation velocity. Hepatic microsomes were incubated with 5000 μM acetaminophen (APAP) at 37 °C for 10 min to determine deacetylation velocity. There was no significant difference in species deacetylation ($P > 0.05$).
PAP, *para*-aminophenol; PABA, *para*-aminobenzoic acid; APAP, acetaminophen.

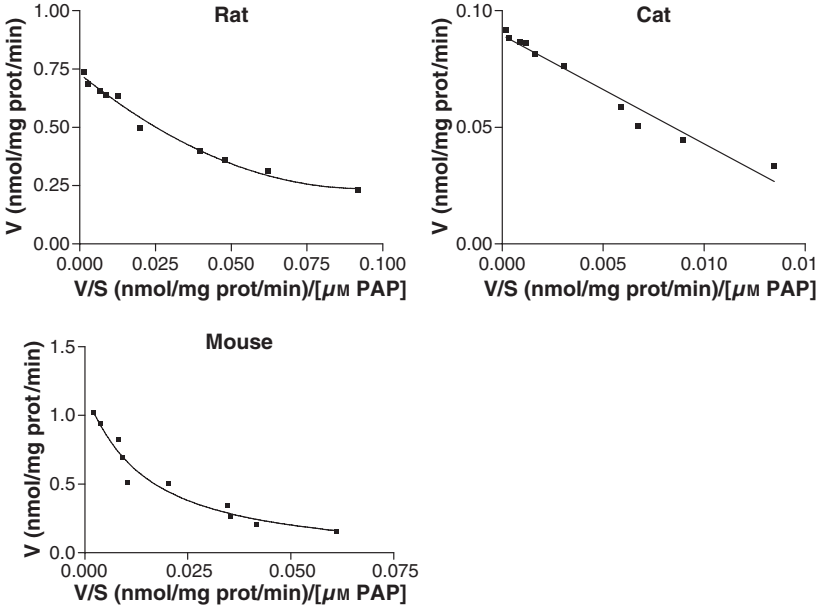

Fig. 3. Eadie-Hofstee plots of *in vitro* *N*-acetylation of 100 μM *para*-aminophenol by hepatocyte cytosol of individual animals. The curvilinear pattern of the Eadie-Hofstee graphs of the rat and mouse indicate there are two or more enzymes catalyzing the reaction. The linear pattern of the feline *N*-acetylation indicates there is a single enzyme catalyzing the reaction.

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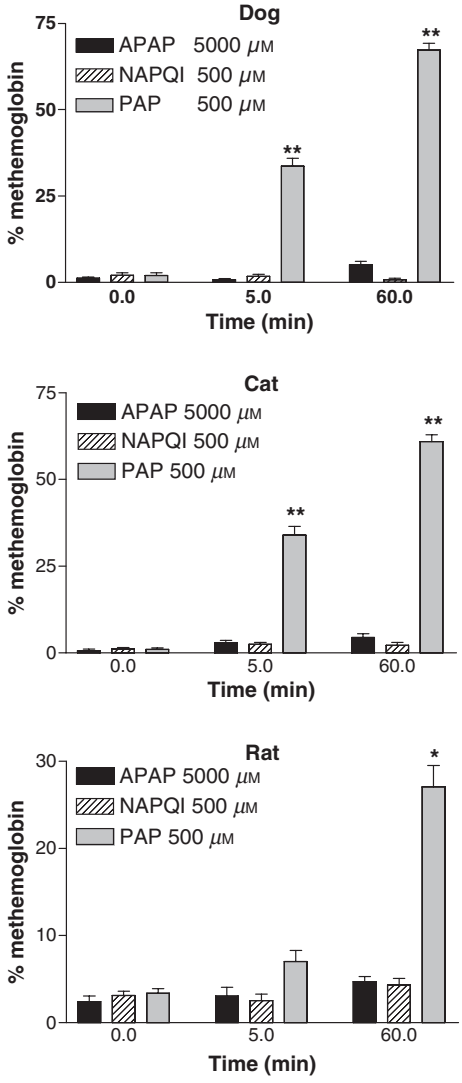

Fig. 4. Acetaminophen (APAP), *N*-acetyl-*p*-benzoquinoneimine (NAPQI) and *para*-aminophenol methemoglobin induction at 5 and 60 min. Methemoglobin formation following 0, 5, and 60 min exposure to APAP, NAPQI and PAP in dog (top), cat (middle) and rat (bottom) erythrocytes *in vitro* ($n = 6$) ($^*P < 0.05$; $^{**}P < 0.01$).

There was no significant methemoglobin generation following 5 or 60 min incubation with up to 5000 μM APAP or 500 μM NAPQI in feline, rat or canine erythrocytes, but 500 μM PAP induced significant methemoglobin formation in all species at 60 min (Fig. 4). Dose-dependent *in vitro* methemoglobin formation in rat, cat, and dog erythrocytes following 5 and 60 min of incubation with PAP is shown in Fig. 5. Methemoglobin induction by 500 μM PAP at 60 min was significantly greater in canine and feline erythrocytes than rats, WT and knockout mice (Fig. 6). While methemoglobin formation in knockout mice erythrocytes was significantly greater than in WT mice erythrocytes, this was still much lower than in canine and feline erythrocytes (Fig. 6).

In vitro methemoglobin formation was compared in lysed vs. intact erythrocytes exposed to 250 μM PAP (Fig. 7). There was significantly greater methemoglobin induction in rat and WT mouse lysed compared with intact erythrocytes, whereas

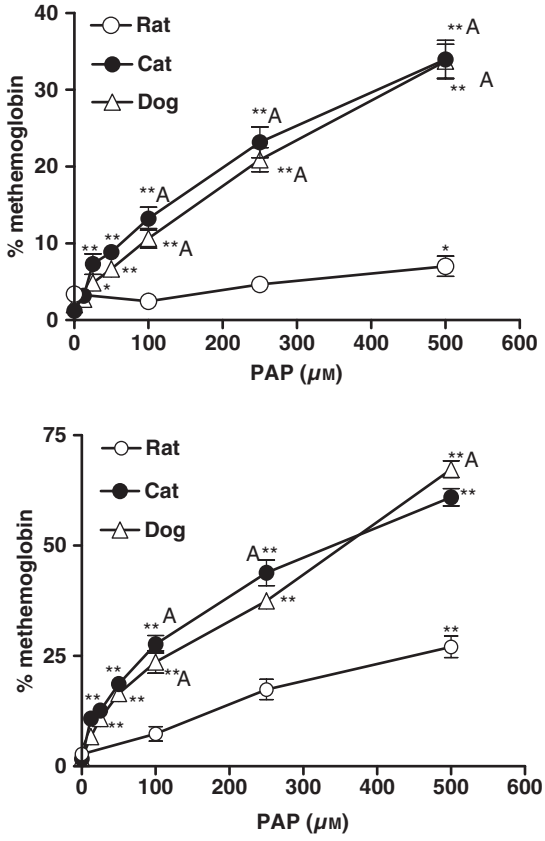

Fig. 5. Dose-dependent methemoglobin induction by *para*-aminophenol (PAP) at 5 and 60 min. Dose-dependent methemoglobin induction by PAP at 5 (top) and 60 (bottom) min *in vitro* in canine, feline and rat erythrocytes ($n = 6$). $^*P < 0.05$, $^{**}P < 0.01$ describe significant methemoglobin induction compared with control of 0.25% DMSO in 4-2-hydroxyethyl-1-piperazineethanesulfonic acid (HEPES) for that species. 'A' indicates methemoglobin induction is significantly greater than rat methemoglobin induction.

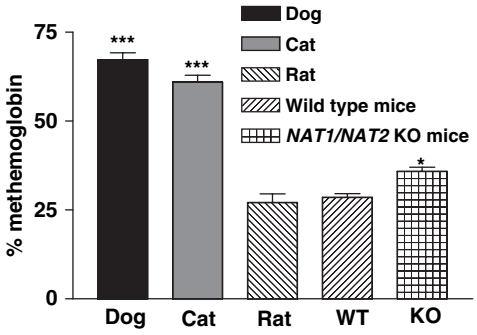

Fig. 6. Comparison of *in vitro* induction of methemoglobin formation in dogs, cats, rats, wildtype (WT) C57BL/6 and *NAT1*/NAT2 knockout mice by 500 μM *para*-aminophenol at 60 min. The methemoglobin formation in dog and cat erythrocytes was significantly greater than rat, WT and NAT1/NAT2 knockout mouse (KO) erythrocytes ($^{***}P < 0.001$). Methemoglobin generation in KO erythrocytes was significantly greater than in WT erythrocytes ($^*P < 0.05$) ($n = 6$ in dogs and cats, $n = 4$ in WT and knockout mice).

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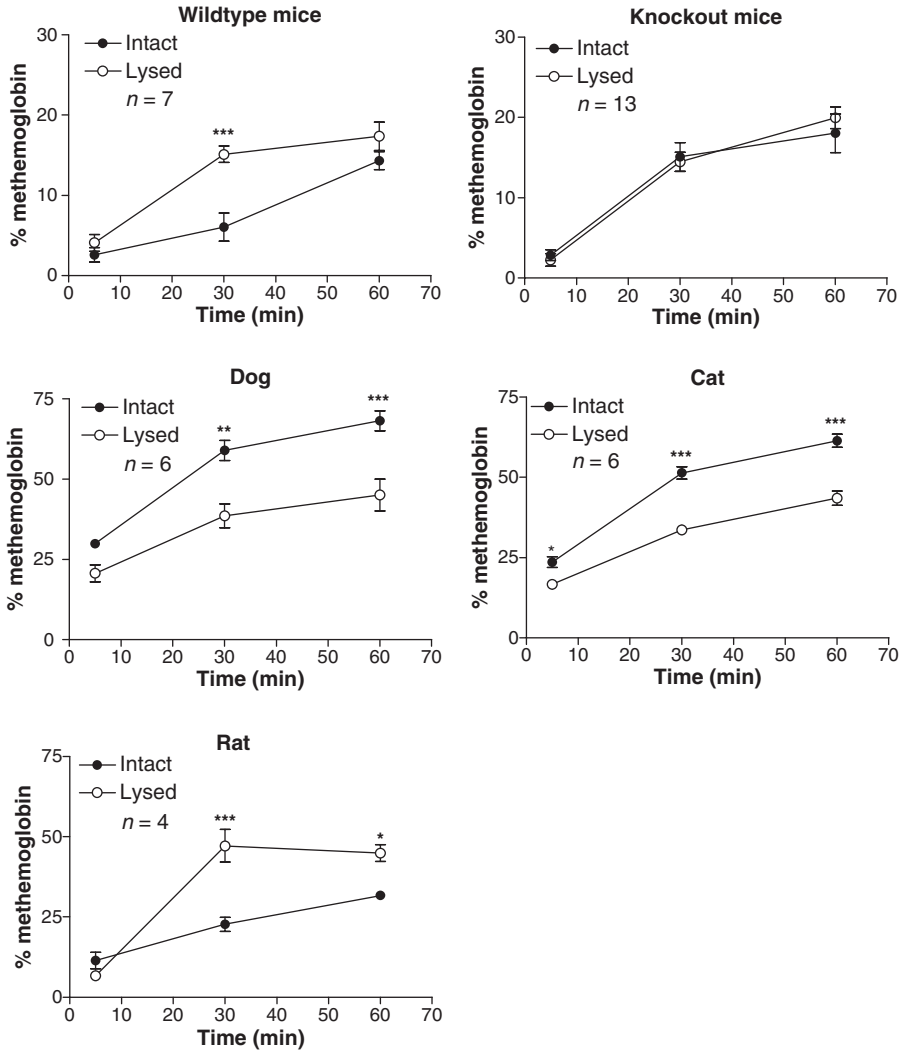

Fig. 7. Methemoglobin generation in intact and lysed erythrocytes following incubation with 250 μM *para*-aminophenol (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). Lysed erythrocytes in rats and wildtype mice formed more methemoglobin than intact red blood cells whereas intact erythrocytes developed more methemoglobin than lysed erythrocytes in dogs and cats. There was no significant difference in methemoglobin formation in lysed and intact cells in knockout mice.

there was significantly greater methemoglobin induction in canine and feline intact compared with lysed erythrocytes. There was no significant difference in methemoglobin production in lysed vs. intact erythrocytes of knockout mice ($P > 0.05$).

In vivo *results*

Wildtype and knockout mice ($n = 4$ per group with equal numbers of males and females) were injected with saline or APAP (250 or 400 mg/kg). At 3 h postinjection, AST and ALT activities were 161 ± 23.5 and 40.5 ± 6.4 IU/L respectively in saline injected mice compared with mean activities of 456.3 ± 138.3 and 1004 ± 312 IU/L in 250 mg/kg treated mice. Elevated ALT and AST were also present in APAP-injected mice at later time-points.

There were no light microscopic changes in histological sections of livers of mice treated with saline. There was variable hepatic necrosis in livers of mice treated with 250 mg/kg APAP at 48 h, whereas all livers from mice treated with 400 mg/kg APAP developed severe, bridging, centrolobular hepatic necrosis by 48 h.

There were no significant changes in the PCV or methemoglobin concentration, nor any Heinz bodies or eccentrocytes in blood from any WT or knockout mice injected with saline or 250 or 400 mg/kg APAP, at any of the time-points sampled (3, 24, and 48 h).

Mice were treated with saline or 400 mg/kg PAP IP and sampled at 5 min postexposure. There was no significant difference in the PCV of control or PAP-treated mice in fasted and nonfasted experiments ($P = 0.34$). Direct blood smears of whole blood at 5 min postinjection were normal.

There was a significantly higher ($P = 0.01$) methemoglobin concentration in nonfasted knockout mice compared with nonfasted WT mice following PAP injection (Fig. 8a) because of significantly greater methemoglobin in female knockout vs. female WT mice ($P = 0.005$).

There was no significant difference in the methemoglobin concentration of fasted knockout and WT mice following PAP injection ($P = 0.79$). There was significantly greater methemoglobin in fasted than nonfasted WT mice postinjection of PAP ($P = 0.01$) but no significant difference between fasted and nonfasted knockout mice (Fig. 8b).

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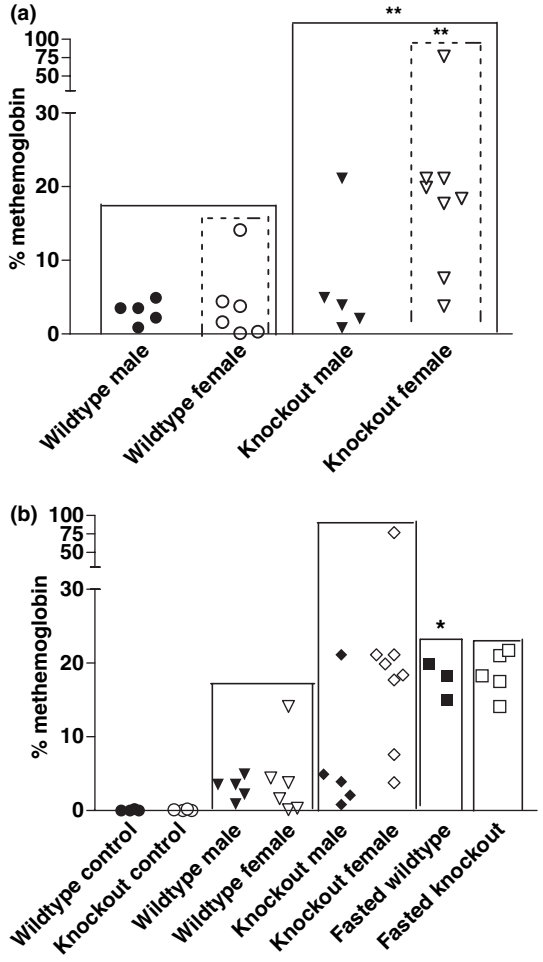

Fig. 8. (a) The percentage methemoglobin in nonfasted wildtype (WT) and knockout mice 5 min postinjection of 400 mg/kg *para*-aminophenol (PAP). Knockout mice had significantly greater methemoglobin than WT mice following injection of PAP because of significantly greater methemoglobin in female knockout mice vs. WT female mice. $^{**}P < 0.01$. (b) The percent methemoglobin in nonfasted and fasted WT and knockout mice 5 min postinjection of 400 mg/kg PAP. Control mice were injected with saline. The methemoglobin generation in fasted mice was not significantly different than in nonfasted mice however, fasted WT mice did generate significantly greater methemoglobin than did nonfasted WT mice; $^{*}P < 0.05$.

DISCUSSION

Dogs and cats are the only reported species in which APAP toxicity is associated with hematotoxicity, primarily methemoglobinemia and hemolytic anemia. The reaction has often been attributed to NAPQI, the toxic metabolite responsible for APAP-induced hepatotoxicity in humans (Desnoyers, 2000; Taylor & Dhupa, 2003). Concentrations of APAP two- to eightfold above the feline and canine hematotoxic threshold (Savides *et al.*, 1984) did not induce significant methemoglobin *in vitro* indicating that the parent compound APAP likely undergoes biotransformation to induce methemoglobinemia. Further, NAPQI did not induce methemoglobin *in vitro* and therefore is unlikely to be the reactive metabolite responsible for APAP-induced methemoglobinemia in dogs and cats.

The minor APAP metabolite PAP has been overlooked as a possible reactive metabolite responsible for APAP-induced hematotoxicity despite being known to contribute to aniline dye-induced methemoglobinemia in humans (Harrison & Jollow, 1987). However, PAP was capable of inducing methemoglobin *in vitro* in erythrocytes from all species tested (Figs 4–6) and *in vivo* in mice under certain conditions (Fig. 8). These results support the hypothesis that the most likely APAP metabolite responsible for hematotoxicity in dogs and cats is PAP.

para-Aminophenol has been observed as an *in vivo* APAP metabolite in cats, rats, and mice (Welsh *et al.*, 1966; Newton *et al.*, 1982; Klos *et al.*, 1992; Song & Chen, 2001), but has not been observed in dogs. To confirm the potential for *in vivo* formation of PAP, we determined that hepatic microsomes from all species tested were capable of deacetylating APAP to PAP (Table 1).

The half-life of PAP is <5 min in the rat (Harrison & Jollow, 1987) largely because of efficient *N*-acetylation. Rats have three functional NAT enzymes: NAT1, NAT2, and NAT3 (Walraven *et al.*, 2006). Mice also have three NAT enzymes, however mouse NAT3 is poorly functional (Fretland *et al.*, 1997; Estrada-Rodgers *et al.*, 1998). Dogs completely lack NAT enzymes and cats only have NAT1 (Cribb *et al.*, 1996; Trepanier *et al.*, 1997, 1998). We confirmed the species differences in *N* -acetylating activity previously reported using PABA as a probe substrate (Table 1) and demonstrated that cats have a markedly reduced ability to re-acetylate PAP to APAP, while dogs had no ability to reacetylate APAP. While extrapolation of *in vitro* metabolism to *in vivo* metabolism can be misleading because of variations in co-factors and alternative metabolic pathways, these *in vitro* results are consistent with previous *in vitro* and *in vivo* results of acetylation of arylamine substrates in these species. These results are also consistent with the hypothesis that relative exposure to PAP following APAP in dogs and cats is likely to be much higher than in other species.

In addition to deficient *N*-acetyltransferase activity possibly leading to increased systemic exposure to PAP, it could also further enhance susceptibility to hematotoxicity of PAP by decreasing removal of PAP from within erythrocytes. Consistent with this hypothesis, canine and feline erythrocytes showed significantly greater species sensitivity to the methemoglobin-induction effects of PAP. However, the difference in methemoglobin formation in knockout vs. WT mouse erythrocytes following PAP exposure, while significant, was much less than the species differences in susceptibility observed *in vitro.* These results suggest that deficient *N*-acetylation, while an important factor, is unlikely to be the sole determining factor for susceptibility to APAP-induced methemoglobinemia and that other susceptibility factors likely exist within the erythrocytes of cats and dogs.

Comparing methemoglobin induction in intact vs. lysed erythrocytes *in vitro* provides further evidence for a possible role of *N*-acetyltransferase at the level of the erythrocyte. Lysing the

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erythrocytes removes penetration of erythrocytes by PAP as a variable and removes the contribution of cellular enzymes such as CYP b5 reductase and NAT. In rat and WT mice erythrocytes, lysing erythrocytes increased the relative formation of methemoglobin. This was likely because of the loss of detoxification by NAT and of methemoglobin reduction by CYP b5 reductase (Bernheim *et al.*, 1937; Harrison & Jollow, 1986). In contrast, knockout mice erythrocytes showed similar methemoglobin formation in lysed and intact erythrocytes, confirming a role for NAT in influencing PAP-induced methemoglobin formation.

Cat and dog erythrocytes displayed significantly greater methemoglobin formation in canine and feline intact erythrocytes. The defect in *N*-acetylating activity in these species likely leads to prolonged redox cycling of PAP in intact cells, and hence increased methemoglobin formation. In lysed cells, this redox cycling would be lost and hence the formation of methemoglobin would be less. There is evidence for relatively poor methemoglobin reductase activity in feline and canine erythrocytes (Stolk & Smith, 1966) and the results seen here are consistent with this observation. If there is relatively poor reductase activity in dog and cat erythrocytes, lysing the erythrocytes would have a smaller effect on protection against methemoglobin formation, as observed here. Together, these results are consistent with a role for deficient NAT and a second susceptibility factor possibly related to other protective pathways such as methemoglobin reductase. The predictions from the *in vitro* experiments were borne out by the *in vivo* experiments. Hepatotoxic doses of APAP did not cause methemoglobinemia or hemolysis in C57BL/6 WT and *Nat1*/Nat2 double knockout mice, confirming that a NAT deficiency alone is insufficient to cause APAP-hematotoxicity. However, when WT and knockout mice were treated directly with PAP *in vivo*, significantly higher methemoglobin concentrations were observed in knockout compared with WT mice. This is consistent with the hypothesis that a deficiency of NAT enzymes may contribute to species sensitivity to PAP-induced methemoglobinemia, but confirms that additional factors are important in determining susceptibility.

Another major detoxification pathway of PAP is conjugation with GSH. All mice in our experiments were fed the same diet for two or more weeks and all sampling was performed in the morning to avoid dietary or diurnal variation of GSH (Schnell *et al.*, 1984). Female mice unexpectedly showed significantly greater methemoglobin generation than males. This may have been as a result of lower [GSH] in females as female C57BL/6 mice have been reported to have lower [GSH] than males (Dai *et al.*, 2006).

Fasting decreases GSH and other biotransformation factors such as sulfhydryl groups (Walker *et al.*, 1982). Fasted WT mice developed significantly more PAP-induced methemoglobin than non fasted WT mice again suggesting that [GSH] may contribute to PAP effects. While these observations indicate that GSH concentrations may be important, differences in GSH concentrations are unlikely to explain the species differences in hematotoxicity.

Over-all, while not directly assessed, the results observed here are consistent with another susceptibility factor at the concentration of the erythrocyte. Differences in reductase activities within the erythrocyte are a possible explanation. It is also likely that the decreased glucuronidation activity in cats contributes to over-all susceptibility to APAP toxicoses in cats, although it is not likely a contributing factor in determining target-organ susceptibility. There were limitations to the studies reported here. We did not compare the *in vivo* PAP formation between species nor did we establish a direct *in vivo* link of APAP deacetylation to the development of APAP-hematotoxicity in cats and dogs. PAP is quite unstable and accurate *in vivo* quantification is difficult. To directly demonstrate the importance of deacetylation and lack of acetylation in dogs and cats, it will be necessary to either inhibit the enzymes responsible for the deacetylation of APAP or block deacetylation by some other means. An alternative but more difficult approach would be to restore acetylating activity in those species. While the mice studies provided some additional insights, the clearly demonstrated differences from cats and dogs in responses at the concentration of the isolated erythrocytes confound their use as a model. Mouse NADH CYP b5 reductase activity is reportedly greater than many other species and this may explain the difference (Stolk & Smith, 1966; Srivastava *et al.*, 2002).

Differences in individual or species' reactions to drug toxicity are typically multifactorial. We have shown that PAP is most likely the reactive metabolite responsible for the methemoglobinemia induced by APAP *in vivo*. Dogs and cats likely experience hematotoxicity instead of hepatotoxicity because of the greater accumulation of systemic PAP in the absence of effective *N*-acetylation. Efficient clearance of APAP to PAP may also decrease the risk of hepatotoxicity, although other factors such as decreased CYP-dependent bioactivation to NAPQI may also be involved. PAP is not directly hepatotoxic and is known to induce methemoglobinemia. Further, deficient *N*-acetyltransferase at the concentration of the erythrocyte may make them more sensitive to the effects of PAP, likely through a decreased clearance of the metabolite and subsequent prolonged co-oxidation. Limited feline glucuronidation may contribute to this species' low threshold for APAP toxicity by resulting in more deacetylation rather than more oxidation as was previously thought, but likely does not contribute to the target-organ specificity. Other factors such as inherently greater reactivity of canine and feline oxyhemoglobin with PAP, availability of GSH or decreased CYP b5 reductase activity are also likely involved.

Future studies such as species comparison of *in vivo* PAP production, evaluation of CYP b5 reductase activity and the effect of GSH are needed to better define this species-specific toxicity.

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