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The effect of ageing on isoniazid pharmacokinetics and hepatotoxicity in Fischer 344 rats

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Abstract

Isoniazid is the first-line treatment for tuberculosis; however, its use is limited by hepatotoxicity. Age-related differences in isoniazid pharmacokinetics and hepatotoxicity are uncertain. We aimed to investigate these in young (3 ± 0 months, $n = 26$) and old (23.0 ± 0.2 months, $n = 27$) male Fischer 344 rats following a low- or high-dose toxic regimen of isoniazid or vehicle (4 doses/day over 2 days; low: 100, 75, 75, 75 mg/kg; high: 150, 105, 105, 105 mg/kg i.p. every 3 h). Fifteen hours after the last dose, animals were euthanized and sera and livers were prepared for analysis. Isoniazid treatment increased serum hepatotoxicity markers (alanine and aspartate transaminase) in young animals but not in old animals, and only reached significance with the high dose in young animals. Isoniazid treatment caused a trend towards an increase in necrosis in young animals with both doses. In contrast, microvesicular steatosis was increased in old isoniazid-treated animals, reaching significance only with the low dose (steatosis prevalence in old: vehicle 1/9, isoniazid 4/5; $P < 0.05$). Among isoniazid-treated animals, concentrations of toxic intermediates acetylhydrazine and hydrazine were higher in old than young animals ($P < 0.05$). With both doses, hepatic cytochrome P450 2E1 activity was higher in young animals compared

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DISCLOSURE

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with old ($P < 0.05$). There were no other age effects seen on any of the other measured enzymes involved in isoniazid metabolism (N-acetyl transferase, amidase, glutathione-S-transferase). These results show age-related changes in isoniazid pharmacokinetics may contribute towards differential patterns of toxicity and confirm that standard hepatotoxicity markers do not detect isoniazid-induced microvesicular steatosis.

Keywords

ageing; isoniazid; liver; pharmacokinetics; toxicity

INTRODUCTION

Isoniazid is the first-line treatment for mycobacterium tuberculosis, one of the world's leading infectious causes of death, resulting in about 1.5 million deaths per year [1], of which an estimation of over 30% of tuberculosis-related deaths occur among old people, aged 65 and above [2,3]. Isoniazid is an effective therapy for tuberculosis but its use is limited by hepatotoxicity. Hepatotoxicity occurs in approximately 10% of patients treated with isoniazid with 1% developing clinical hepatitis [4]. One of the major risk factors for isoniazid-induced hepatotoxicity is old age [5–7]. However, the reason for this observation is unclear. A better understanding of isoniazid pharmacokinetics and toxicity in old age is required to establish safer management and therapeutic dosing guidelines for older adults.

Histologically, isoniazid-induced hepatotoxicity typically causes a necroinflammatory pattern of injury with steatotic, cholestatic and granulomatous patterns also described [8,9]. The underlying mechanism behind hepatotoxicity is not entirely clear; however, the toxic metabolites of isoniazid, acetylhydrazine [10], hydrazine [11] and the reactive metabolites produced from cytochrome P450 [12], are thought to be responsible for liver injury. These metabolites all contribute towards cell death via covalent binding [10], oxidative stress and mitochondrial dysfunction [13,14]. Hydrazine can also disrupt hepatic lipid regulation and cause steatosis [15]. In animal studies, Cytochrome P450 2E1 (CYP2E1) activity has been positively correlated with increased isoniazid-induced hepatotoxicity [16]. CYP2E1 is known to produce oxidative free radicals and highly reactive metabolites which cause liver damage [17–19]. There is some evidence to suggest that steatosis and necrosis develop independently [11,19].

Genotyping studies in humans have identified polymorphisms in several hepatic enzymes involved in the metabolism of isoniazid and production and clearance of its toxic metabolites, to be risk factors for isoniazid-induced hepatotoxicity. These enzymes include N-acetyltransferase 2 (NAT2, rat equivalent is NAT1) [20–23], Cytochrome P450 2E1 (CYP2E1) [12,20] and glutathione-S-transferase (GST) [21]. NAT2 metabolizes 50–90% of isoniazid to acetylisoniazid [8,24], while amidase metabolizes isoniazid to toxic hydrazine and acetylisoniazid to toxic acetylhydrazine [25,26]. These toxic metabolites can be cleared by NAT2 into a non-toxic intermediate, diacetylhydrazine, or alternatively oxidized by CYP2E1 to produce a reactive intermediate which is detoxified by GST [18–20,24].

Although clinically the risk of isoniazid-induced liver injury increases in old age and recent research has established a role of variation in metabolism in isoniazid-induced hepatotoxicity, the effect of ageing on isoniazid pharmacokinetics and hepatotoxicity mechanisms is poorly understood. Ageing is associated with many physiological changes that can affect the phase I and II metabolism of many drugs and risk of hepatotoxicity [27,28]. In older people compared with young treated with the same single dose of isoniazid daily for 2 months, serum isoniazid concentrations were unchanged with age. However, serum hydrazine levels were significantly increased in old age [29,30], which may imply older people could have increased susceptibility to hepatotoxicity caused by hydrazine. In humans, NAT2 activity is unaltered by age [30], but the effect of age on amidase activity is unknown. Rat studies have found decreased CYP2E1 activity in old age [31,32], which could result in reduced formation of the unidentified toxic intermediates and oxidative free radicals. The detoxification of these toxic intermediates may also be altered as GST activity has been seen to be either decreased [33] or unchanged with old age in rodents [34].

In patients, clinical data have elucidated the pharmacokinetics of isoniazid metabolism and toxic intermediates involved in the process of isoniazid liver toxicity. However, our understanding of the mechanism is limited due to ethical and experimental constraints in humans. Observational clinical studies are also confounded by age-associated factors that may impact susceptibility, which include increased polypharmacy and multimorbidities [28]. Therefore, controlled models are required to understand the mechanism of isoniazid hepatotoxicity and the effects of ageing. Currently no study has investigated the effect of ageing on isoniazid pharmacokinetics and toxicity in a controlled rodent model even though present evidence suggests that hepatic isoniazid metabolism changes occur with old age which may alter the risk of liver toxicity. Therefore, this rat study was undertaken to better understand the effect of ageing on isoniazid pharmacokinetics and hepatotoxicity. As there is no current consensus for animal models for isoniazid-induced hepatotoxicity, we choose Fischer 344 rats to be the animal model, as rats have been shown to metabolize isoniazid similar to humans [8], and this strain is commonly used for ageing studies [35].

MATERIALS AND METHODS

Animals

Young adult (3 ± 0 months, 380.9 ± 6 g, $n = 26$) and old (23.0 ± 0.2 months, 446.3 ± 5.5 g, $n = 27$) male Fischer 344 rats were obtained from Harlan Laboratories (Harlan, United States) or the National Institute on Aging (Bethesda MD). All young animals and the cohort of old animals for the lower dose vehicle or isoniazid regimen were housed at the Kearns Facility (Kolling Institute of Medical Research, Sydney, Australia), while the higher dose vehicle or isoniazid cohort of old animals were housed at the Gerontology Research Center (National Institute on Aging, Baltimore, United states). Animals were maintained on a 12-h light–dark cycle and had ad libitum access to food and water. The study was approved by the Royal North Shore Hospital Animal Care and Ethics Committee, the University of Sydney Animal Ethics Committee and the Animal Care and Use Committee of the National Institute on Aging.

Isoniazid treatment of animals and tissue collection

Animals were fasted overnight for 16 h before treatment with a low-dose (young control $n = 7$, young INH $n = 8$, old control $n = 7$, old INH $n = 5$) or high-dose (young control $n = 5$, young INH $n = 6$, old control $n = 8$, old INH $n = 7$) isoniazid regimen. Following method adapted from Sarich and colleagues [36], four doses (low-dose regimen: 100, 70, 70 and 70 mg/kg and high-dose regimen: 150, 105, 105 and 105 mg/kg) of isoniazid were injected intraperitoneally (i.p.) every 3 h for 2 days. This study was designed with lower and higher dose toxic regimens of isoniazid to better understand the pharmacokinetics and hepatotoxicity of isoniazid. Control groups of rats for each dose regimen were injected with vehicle sterile water over the same time frame. The initial dose was between 10 AM and 12 PM to avoid temporal variations in isoniazid metabolism. Fifteen hours following the last injection, animals were anaesthetized with an intraperitoneal injection of ketamine and xylazine (75 mg/kg and 10 mg/kg, respectively; Parnell Laboratories Pty Ltd, Sydney, Australia). A midline laparotomy was performed. Blood was taken from the inferior vena cava, and heparin (300U) was then injected into the same vessel. The portal vein was then cannulated with an 18G intravenous catheter (BD, Sydney, Australia), and 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) at a flow rate of 1–1.5 mL/min/g of liver 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. A section of the main lobe was also fixed in 10% neutral formalin (ProSciTech) for histology.

Hepatotoxicity measurements

The collected blood was allowed to clot at room temperature before separating the serum by centrifugation at 10 000 rpm (7378 g) for 10 min (Sigma centrifuge 1–14; John Morris Scientific Pty Ltd, Australia). Sera were then stored in aliquots at –80 °C. Serum liver function tests (alanine transaminase (ALT), aspartate transaminase (AST), protein, albumin and alkaline phosphatase (ALP)) and creatinine concentrations were measured to assess liver and kidney injury. 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) [37].

Fixed liver tissue was embedded in paraffin, and 5- μ m sections were mounted on slides. Slides were processed for haematoxylin and eosin staining in the National Association of Testing Authorities accredited hospital laboratory for the Pathology department of Royal Price Alfred Hospital, Sydney, Australia. Histopathology was described by an anatomical pathologist (C.M.), who was blinded to the sample groups. Microvesicular steatosis was graded with a standard pathology scale from 0 to 3, where each number represented <5%, 5–33%, 34–66% and 67–100%, respectively. Images were taken on an Olympus BX51 microscope connected to a Olympus DP26 camera (Olympus, Sydney, Australia). Animals with histopathologic evidence of age-related hepatic malignancy, lymphoproliferative disease or other liver disease were excluded ($n = 4$ old vehicle-treated and $n = 2$ old low-dose regimen isoniazid treatment).

Serum isoniazid, acetylhydrazine and hydrazine levels

Concentrations of isoniazid, acetylhydrazine and hydrazine were measured in the serum of treated animals only using a method adapted from Sarich et al. [11]. Samples were prepared following the methods of Sarich, with minor adjustments such as centrifuging for 10 min at 15300 rpm (21 460 *g*) (Sigma centrifuge 3–16pk, John Morris Scientific Pty Ltd, Australia) and filtering through a RC Membrane, 0.2- μ m, 4-mm syringe filter (#AFO-3203-12, Phenomenex, Sydney, Australia) prior to mixing with the derivatizing agent (8.22 mM methoxybenzaldehyde, 1 mM fluorenone and 7.5% (v/v) formic acid) at a ratio of 4:1. After incubation of the mixture for 2 h in the dark at room temperature, the sample was injected onto the column.

Isoniazid, acetylhydrazine and hydrazine were chromatographically measured using an Xbridge BEH C18 2.5 μ m 4.6 $\times$ 100 mm column XP (Waters Australia, Sydney, Australia, part number 186006039), with safety guard holder (#KJ0-4282, Phenomenex, Sydney, Australia) and cartridges (#AJ0-4321, Phenomenex, Sydney, Australia) and an Agilent HPLC (Agilent 1100 series, Agilent Technologies, Victoria, Australia) fitted with a UV detector set to 300 nm. The program followed is in Table I, with a flow rate was 600 μ L/min. Mobile phase A consisted of 20% acetonitrile, 10% methanol in 5 mM sodium acetate pH 5 and mobile phase B consisted of 40% acetonitrile, 40% methanol in 5 mM sodium acetate pH 5. All eluents were filtered and degassed before use.

The calibration curves of isoniazid, acetylhydrazine and hydrazine concentration (mol/L) versus the ratio of isoniazid, acetylhydrazine, hydrazine to 9-fluorenone resulted in a correlation coefficient of at least $r^2 = 0.811$, $r^2 = 0.872$ and $r^2 = 1.000$ ($n = 3$), respectively, for three different concentrations (25, 50 and 100 μ M). The acetylhydrazine, isoniazid, hydrazine and fluorenone had the following retention times 7.88, 8.869, 15.318 and 13.212 min, respectively. The peak of isoniazid coincided with the tail section of the acetylhydrazine peak. The peak of acetylhydrazine was symmetrical so the area was determined by taking twice that of the first half of the peak. The area of the isoniazid peak was calculated by subtracting the total area of both acetylhydrazine and isoniazid peaks from the calculated acetylhydrazine peak only.

Tissue extraction for enzyme activity assays

For enzymatic activity assays, liver tissue was homogenized with a dounce homogenizer (Sigma-Aldrich) in sucrose-Tris buffer (0.25 M sucrose, 10 mM Tris-HCl, pH 7.4), and the cells were sequentially fractionated. In brief, liver homogenate was centrifuged at 12 000 *g* for 20 min. The pellet was discarded, while half of the remaining supernatant (cytoplasmic fraction) was collected and stored at -20°C and was used to analyse amidase and NAT1 activity. The other half was further centrifuged at 100 000 *g* for 1 h. The supernatant containing the cytosolic fraction was collected for measurement of GST activity. The pellet was washed with the sucrose-Tris buffer once more and spun at 100 000 *g* for another 30 min. The pellet containing the microsomes was dissolved in 20% (v/v) glycerol and 0.1M Tris-HCl, pH 7.4 and used to analyse CYP2E1 activity.

CYP2E1 activity was measured on the extracted liver microsomes as described by Roberts [38], via aniline hydroxylase activity, as conversion of aniline to p-aminophenol occurs. P-aminophenol absorption was measured at the wavelength of 620 nm.

Rat NAT1 corresponds to human NAT2 and is the major enzyme involved in clearance of isoniazid. Following the method used by Kawamura et al. [39], NAT1 activity was measured using 100ug of cytosolic protein by the conversion of Acetyl-CoA and isoniazid into Coenzyme A, which is quenched by guanidinium-HCl and reacts with 5,50-dithio-bis (2-nitrobenzoic acid (DTNB) producing a product detectable at 405 nm.

Using a method adapted from Ko and colleagues [40], amidase activity was measured using the substrate p-acetaminophenol to produce p-aminophenol which was reacted with o-cresol solution to develop a detectable indophenol measured at 615 nm. Briefly, amidase activity was determined in a reaction mixture containing 750ug liver cytoplasm, 0.1M Tris buffer pH 8.4 and 100 mM p-acetaminophenol. After 1 h of reaction time at 37 °C, 2 volumes of o-cresol colour developing mixture (34.25 mM o-cresol, 1.39 mM CuSO₄, 29.8 mM NH₄OH) was added to stop the reaction and this was left for 10mins at room temperature before measuring absorption at 615 nm.

Following the method used by Habig and colleagues [41], GST activity was measured over 5 min using the substrate 1, 2-Dichloro-4-nitrobenzene and glutathione (GSH) to produce a detectable conjugate measured at 340 nm absorbance at room temperature.

Statistical analysis

Statistical analysis was conducted using IBM SPSS statistics version 20. Data were analysed using analysis of variance (ANOVA) with post hoc Tukey test. *P* values less than 0.05 were considered significant. Data are presented as mean ± standard error of the mean.

Correlations were determined using Pearson's test. Analysis of serum metabolites were compared among young and old isoniazid-treated animals only, while all other analysis included all groups.

RESULTS

Animal details

Animal ages, body weights (pre- and postfasting), liver weights, hepatic histopathology and serum biochemistry are shown in Table II. Two of the nine old animals treated with high-dose isoniazid died after four and five doses respectively and were unable to be necropsied. The role of isoniazid in their death was unknown and they were excluded from the analysis.

Hepatotoxicity markers

Figure 1 shows representative images of histology of young and old animals treated with the low-dose isoniazid regimen or vehicle controls. Similar observations were observed at the higher dose. Histopathological examination revealed (Table I) a trend of higher prevalence of necrosis in young isoniazid-treated animals compared with young vehicle controls (*P* > 0.05). No significant difference in the prevalence of necrosis was observed in old animals treated with either regimen compared with vehicle.

Compared with the corresponding vehicle-treated control animals, microvesicular steatosis was more prevalent in old low-dose isoniazid-treated animals (old: vehicle 1/9, low-dose isoniazid 4/5; $P < 0.05$). There was a trend towards increased microvesicular steatosis in the old higher dose isoniazid group, compared with corresponding vehicle-treated animals (old: vehicle 3/8, high-dose isoniazid 5/7; $P > 0.05$). However, there was no microvesicular steatosis seen in the young vehicle-treated groups, with only minor signs of microvesicular steatosis in the isoniazid-treated groups (low-dose 1/8, high dose 2/6). On necropsy, the rats did not display any other abnormal histology in other organs, including lung tumours.

In young animals treated with the high-dose regimen of isoniazid, serum ALT and AST were significantly increased compared with the corresponding young vehicle control group, and young low-dose isoniazid regimen-treated group (Figure 2a and b, $P < 0.05$). No difference in serum ALT and AST was observed in the low- or high-dose old isoniazid-treated animals compared to vehicle-treated old animals.

Isoniazid, hydrazine and acetylhydrazine metabolite levels

Old animals treated with low-dose isoniazid had significantly higher serum acetylhydrazine levels compared with the corresponding young group Figure 3 ($P < 0.05$), while this observation was not significant for the old high-dose group. In contrast, serum hydrazine increased by 8.4 fold in old rats treated with the higher dose compared with their corresponding young group ($P < 0.05$), with a nonsignificant 1.8 fold increase for old animals treated with the low dose. Serum isoniazid was only detected in old animals treated with high-dose isoniazid ($P > 0.05$).

Enzyme activity

Isoniazid treatment significantly increased CYP2E1 activity relative to vehicle (Figure 4a, 240%, 309%, low- and high-dose regimen, respectively) in a dose-dependent manner (Figure 4a, $P < 0.05$). CYP2E1 activity was increased in old animals treated with the low-dose regimen (2.8 fold, $P < 0.05$) compared with the corresponding vehicle group, while this observation was not significant for the high-dose and corresponding vehicle groups. With both isoniazid dosing regimens, young animals had higher CYP2E1 activity compared with old animals ($P < 0.05$), while CYP2E1 activity was not significantly higher in young than old vehicle-treated animals ($P > 0.05$). CYP2E1 activity was moderately positively correlated with serum ALT ($r^2 = 0.375$, $P < 0.001$), AST ($r^2 = 0.4148$, $P < 0.001$, $n = 24$) and weakly negatively correlated with serum acetylhydrazine levels ($r^2 = 0.1692$, $P < 0.05$, $n = 26$).

N-acetyltransferase 1, glutathione-S-transferase activity did not change with age or treatment group (Figure 4b, d, $P > 0.05$). Amidase activity did not change with age, but was reduced in young animals treated with the lower dose of isoniazid (Figure 4c, $P < 0.05$).

DISCUSSION

The present study demonstrates for the first time that age-related changes in isoniazid pharmacokinetics in Fischer 344 rats may alter the pattern of hepatic injury caused by isoniazid. In young animals, the isoniazid insult resulted in elevation of serum hepatotoxicity

markers and increased prevalence of hepatic necrosis. Interestingly, in old animals there was no increase in serum hepatotoxicity markers or in the incidence of necrosis; however, they had a higher prevalence of microvesicular steatosis. Following isoniazid treatment, young animals displayed increased CYP2E1 activity, while old animals had lowered CYP2E1 activity and an accumulation of hydrazine and acetylhydrazine in the serum. Taken together, these data show that following exposure to a toxic regimen of isoniazid, age-related changes in pharmacokinetics may contribute to altered pathologies of isoniazid-induced hepatotoxicity in old age. Our results also show that current standard hepatotoxicity markers are unable to detect all patterns of isoniazid-induced liver injury, as the serum toxicity markers ALT and AST did not detect the microvesicular steatotic damage induced by isoniazid in old rats.

In humans, clinical research has shown that isoniazid-induced liver injury causes pathology consisting of necrosis, steatosis and an inflammatory reaction, and the risk of isoniazid-induced liver injury increases with old age [5,6,8,9]. Consistent with this, we observed necrosis and steatosis in our rat model of isoniazid-induced hepatotoxicity. However, our study shows that following a toxic regimen of isoniazid, differential patterns of toxicity exist in young and old animals. Young animals are more susceptible to necrotic patterns of injury, which are detectable by increased serum hepatotoxicity markers, while old animals are more susceptible to microvesicular steatosis. Our current findings strongly suggest that there are changes with ageing that may contribute to altered risk of isoniazid toxicity in old age. Interestingly, these results are contrary to clinical observations. This may suggest that rather than physiological changes of ageing, other confounding issues, such as the age-related increase in polypharmacy and multimorbidities, may be responsible for the increased risk of isoniazid hepatotoxicity in old age observed clinically. Future studies should comprehensively investigate these other age-associated factors to determine their role and confirm these findings in other species/strain models.

Many studies have shown that the toxic intermediates of isoniazid contribute to isoniazid-induced hepatotoxicity [10,11,42,43]. In this study, we found an increase in serum acetylhydrazine and hydrazine in old isoniazid-treated animals, but not in young treated animals, indicating age-related changes in isoniazid pharmacokinetics. Importantly, such an increase in serum hydrazine has been previously documented in older, but not in younger patients after taking a therapeutic dose of isoniazid and demonstrates that our rat model shows consistency with observations in humans [29]. However, contrary to other in vitro studies [42,43], the increase in serum hydrazine did not translate into increased toxicity. Instead, this may play a role in the manifestation of steatosis in vivo. Administration of toxic doses of hydrazine to rats causes lipid dysregulation affecting lipid uptake [44], triglyceride synthesis [45], lipoprotein synthesis [15,46] and mobilization of peripheral fat [15] storages resulting in steatosis [15]. This may explain the increased incidence of microvesicular steatosis observed in our old rats following two days of isoniazid treatment. Together with toxicity results, our results support the idea that necrosis and steatosis may be occurring via independent mechanisms as previously proposed [11,19]. Interestingly, we observed elevated serum acetylhydrazine and hydrazine in old rats but no significant finding of cellular death as reported in in vitro and in vivo animal studies following direct exposure to these toxic metabolites [10,47,48]. This suggests that complex metabolic regulatory

pathways are involved in the mechanism of toxicity, which may be affected by ageing physiology.

Several pharmacogenomic studies have identified CYP2E1 activity to be a major determinant of isoniazid- induced hepatotoxicity [20,21,49]. Consistent with the study by Yue et al. [16], we identified a positive correlation between CYP2E1 activity and the serum hepatotoxicity markers ALT and AST. In both age groups, we also observed the induction of CYP2E1 activity with isoniazid treatment, as has been noted previously [50,51]. This increase was significantly greater in young rats compared with old. CYP2E1 is known to produce oxidative free radicals and highly reactive metabolites which cause liver damage [17–19]. The finding of increased activity of this enzyme in young animals may explain the observed increase in necrotic hepatotoxicity. This is in line with human genotype studies where overactive CYP2E1 activity increases susceptibility to isoniazid-induced hepatotoxicity [12,20]. On the other hand, the decrease in CYP2E1 activity in old rats is consistent with slower clearance and the accumulation of hydrazine and acetylhydrazine, which would hinder acute necrotic damage but promote the induction of steatosis. There were no other major age or treatment effects seen on any of the other enzymes involved in isoniazid metabolism. These results highlight age-related changes in CYP2E1 activity as a major determinant of the pattern of isoniazid liver toxicity.

In addition, this study confirms that although the currently clinically used serum hepatotoxicity markers, such as ALT and AST, are able to detect necrotic liver damage they do not detect steatotic damage. This is consistent with the study by Vivekanandan [15] in which neither marker was capable of detecting microvesicular steatotic damage. This finding raises issues with the use of standard hepatotoxicity markers to identify all forms of isoniazid liver injuries and indicates the imperative need to develop more specific novel hepatotoxicity markers to detect both necrotic and steatotic injury to assist in better management of isoniazid-induced hepatotoxicity.

One of the limitations of this study is the comparability of the dosing regimen and route used in this study to that used clinically [7]. We acknowledge the dose given in this study is greater than a clinical therapeutic dose; however, this was necessary to cause hepatotoxicity in Fischer 344 rats. Previous studies have shown that animals treated with long-term (5 weeks) low isoniazid doses via oral drug administration fail to show any sign of hepatotoxicity [13]. Therefore, an acute toxicity method utilizing intraperitoneal injections that was previously reported to successfully cause isoniazid-induced hepatotoxicity was chosen [36]. It is possible that the necrosis observed in young animals in this study was exacerbated by saturation of NAT1 clearance of isoniazid and its metabolites at the high dose used, which was previously observed by Mitchell et al. [8]. In addition, isoniazid, the first-line treatment for tuberculosis, is commonly taken in combination with other antituberculosis agents, and these drug combinations were not investigated in this study [52]. Our animals were healthy and not tuberculosis infected or unwell, unlike patients, and this may affect pharmacokinetics and toxicity outcomes. Furthermore, the cohort of old animals treated with the higher dose isoniazid regimen and vehicle were not tested concurrently in the same housing as the lower dose for logistical reasons; however, we observed similar patterns of toxicity between different dosing regimens even under different housing. Finally,

future studies could investigate the role of the immune response, which is involved in isoniazid-induced hepatotoxicity [53–56] and is also affected by ageing [57].

CONCLUSION

In conclusion, age-related changes in isoniazid pharmacokinetics result in altered patterns of toxicity, with young animals developing a necroinflammatory pattern of injury and old animals developing microvesicular steatosis following a toxic regimen of isoniazid. Detailed investigation found higher CYP2E1 activity in young treated animals, which correlated with increased serum hepatotoxicity markers. In contrast, old animals had an accumulation of hydrazine in their blood which caused steatosis, likely due to decreased CYP2E1 clearance. These results indicate that CYP2E1 activity may be an important determinant of the histopathology of isoniazid-induced liver toxicity. In addition, we showed that measurement of serum ALT and AST cannot detect isoniazid-induced steatosis. Future research should focus on better understanding these toxicity mechanisms to identify therapeutic targets for prevention and treatment of hepatotoxicity, optimize dosing and develop more accurate hepatotoxicity markers for all patterns of isoniazid-induced hepatotoxicity for patients of all ages.

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ABBREVIATIONS

INH	Isoniazid
ALT	Alanine transaminase
AST	Aspartate transaminase
CYP2E1	Cytochrome P450 2E1
NAT	N-acetyltransferase
GST	Glutathione-S-transferase

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Figure 1. Representative histopathological observations in liver sections from young (3 months) and old (24 months) rats after treatment with low vehicle or isoniazid (INH) regimen via intraperitoneal injection. Young vehicle (a), young isoniazid treated (b), old vehicle (c) and old isoniazid treated (d). High-dose regimen resulted in the similar observations. Images are in 200× magnification. Black arrow indicates patches of necroinflammation. Regions of microvesicular steatosis are represented by *.

Figure 2.

Serum hepatotoxicity markers in young (3 months) and old (24 months) rats 15 h after treatment with vehicle control (low dose: solid black bar, high dose: solid grey bar) or isoniazid (low dose: open bar, high dose: open striped bar) regimen via intraperitoneal injection. Serum alanine transaminase (ALT) (a) and aspartate transaminase (AST) (b). All data are presented as mean $\pm$ SEM of $n = 5-8$ animals per group. Statistically significant differences comparing against corresponding age vehicle control group: * $P < 0.05$. Statistically significant difference comparing against corresponding age lower dose group: ** $P < 0.05$

Figure 3.

Serum acetylhydrazine and hydrazine concentrations in young (3 months) and old (24 months) rats 15 h after treatment with isoniazid (low-dose regimen: open bars, high-dose regimen: solid black bar) via intraperitoneal injection. Serum acetylhydrazine (a) and hydrazine (b). All data are presented as mean $\pm$ SEM of $n = 5-8$ animals per group. Statistically significant differences comparing against corresponding young and same dose group: * $P < 0.05$.

Figure 4.

Isoniazid metabolizing enzyme activities in young (3 months) and old (24 months) rats 15 h after treatment with a low- or high-dose regimen of vehicle (low dose: solid black bar, high dose: solid grey bar) or isoniazid (low dose: open bar, high dose: open striped bar) regimen via intraperitoneal injection. N-acetyltransferase 1 (NAT1) activity (a), amidase activity (b), Cytochrome P450 2E1 activity (CYP2E1) (c) and Glutathione-S-transferase (GST) activity (d). All data are presented as mean $\pm$ SEM of $n = 5-8$ animals per group. Statistically significant differences comparing against corresponding age vehicle control group: * $P < 0.05$. Statistically significant difference comparing against corresponding age lower dose group: ** $P < 0.05$. Statistically significant difference comparing against corresponding young isoniazid group with same dose *** $P < 0.05$.

Table I

The gradient program used to measure serum isoniazid, acetylhydrazine and hydrazine.

Time (min)	% A	% B
0	0	100
1	0	100
6	40	60
11	100	0
18	100	0
19	0	100
35	0	100

Table II

The body and liver weights and serum biochemistry of young and old animals treated with lower (100, 70, 70, 70 mg/kg for 2 days) or higher (150, 105, 105, 105 mg/kg) dosing regimens of isoniazid or vehicle. Data are presented as mean $\pm$ SEM.

	Young vehicle		Young isoniazid		Old vehicle		Old isoniazid	
	Low	High	Low	High	Low	High	Low	High
Dose regimen								
<i>N</i>	7		7	5	5	6	8	7
Age (months)	3.0 $\pm$ 0	3.0 $\pm$ 0	24.0 $\pm$ 0.0	3.0 $\pm$ 0.0	24.0 $\pm$ 0.0	3.0 $\pm$ 0.0	22.2 $\pm$ 0	22.2 $\pm$ 0
Prefast weight (g)	376.2 $\pm$ 5.6	372.0 $\pm$ 6.5	449.3 $\pm$ 5.7 [*]	383.2 $\pm$ 9.3	442.1 $\pm$ 11.3 [*]	396.7 $\pm$ 5.0	NR	NR
Experiment day weight (g)	359.0 $\pm$ 6.9	350.2 $\pm$ 5.1	432.0 $\pm$ 6.3 [*]	372.6 $\pm$ 10.4	410.9 $\pm$ 9.6 [*]	365.9 $\pm$ 4.9	426.4 $\pm$ 11.6	404.4 $\pm$ 15.9
Liver weight (g)	11.7 $\pm$ 0.4	10.8 $\pm$ 0.1	13.5 $\pm$ 1.0	11.6 $\pm$ 0.2	13.7 $\pm$ 0.64	13.0 $\pm$ 0.4	12.9 $\pm$ 0.9	14.2 $\pm$ 0.9
Histology								
Necrosis (2-5%)	1 (2%)	5 (1-5%)	3 (5%)	3 (1%)	1 (5%)	6 (2-5%)	4 (5%)	1 (1%)
Microvesicular steatosis (grade)	0 (0)	1 (1)	1 (2)	0	4 (2-3) ^{*,**}	2 (2)	3 (1-2)	5 (1-3)
Serum biochemistry								
Protein	65.9 $\pm$ 0.9	62.5 $\pm$ 2.1	63.9 $\pm$ 2.1	65.8 $\pm$ 0.6	62.4 $\pm$ 0.5	62.5 $\pm$ 0.9	58.3 $\pm$ 1.1 [*]	51.3 $\pm$ 1.6 ^{*,**,*}
Albumin	36.6 $\pm$ 1.0	34.75 $\pm$ 1.3	30.3 $\pm$ 1.6 [*]	35.2 $\pm$ 0.6	29 $\pm$ 0.8 [*]	35.5 $\pm$ 0.8	25.4 $\pm$ 0.8 ^{*,***}	23.6 $\pm$ 1.0 ^{*,***}
Bilirubin	1 $\pm$ 0	1 $\pm$ 0	2.3 $\pm$ 0.5 [*]	1 $\pm$ 0	1.6 $\pm$ 0.4	1 $\pm$ 0	2.7 $\pm$ 0.2 [*]	3.2 $\pm$ 0.4 ^{*,***}
ALP	135.4 $\pm$ 4.8	126.5 $\pm$ 4.0	94.4 $\pm$ 17.8	133.6 $\pm$ 7.2	94.4 $\pm$ 12.4	111.5 $\pm$ 3.5	162.6 $\pm$ 20.1 ^{***}	125.1 $\pm$ 15.5
Creatinine	41.4 $\pm$ 3.8	38.14 $\pm$ 2.3	39.3 $\pm$ 1.7	38.2 $\pm$ 1.9	47.8 $\pm$ 3.5	40.2 $\pm$ 1.6	47.0 $\pm$ 1.1	53.0 $\pm$ 6.7

* $P < 0.05$ compared to corresponding young group.

** $P < 0.05$ compared to corresponding age vehicle group.

*** $P < 0.05$ compared to corresponding group in lower dose.

Microvesicular steatosis was graded using a standard pathology scale (0: $<5\%$, 1: 5–33%, 2: 34–66%, 3: 67–100%).

NR: Data was not recorded.