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Christopher J. Gordon [a], Reddy R. Gottipolu [b], Elaina M. Kenyon [a], Ronald Thomas [c], Mette C. Schladweiler [a], Cina M. Mack [a], Jonathan H. Shannahan [d], J. Grace Wallenborn [e], Abraham Nyska [f], Robert C. MacPhail [a], Judy E. Richards [a], Mike Devito [a] & Urmila P. Kodavanti [a]

[a] National Health and Environmental Effects Research Laboratory, ORD, U.S. Environmental Protection Agency , Research Triangle Park, North Carolina, USA

[b] National Research Council

[c] U.S. EPA NCBA/SEE Program

[d] Curriculum in Toxicology, University of North Carolina , Chapel Hill, North Carolina, USA

[e] Department of Environmental Sciences and Engineering, School of Public Health , University of North Carolina , Chapel Hill, North Carolina, USA

[f] Tel Aviv University , Tel Aviv, Israel

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AGING AND SUSCEPTIBILITY TO TOLUENE IN RATS: A PHARMACOKINETIC, BIOMARKER, AND PHYSIOLOGICAL APPROACH

Christopher J. Gordon[1], Reddy R. Gottipolu[2], Elaina M. Kenyon[1], Ronald Thomas[3], Mette C. Schladweiler[1], Cina M. Mack[1], Jonathan H. Shannahan[4], J. Grace Wallenborn[5], Abraham Nyska[6], Robert C. MacPhail[1], Judy E. Richards[1], Mike Devito[1], Urmila P. Kodavanti[1]

[1]National Health and Environmental Effects Research Laboratory, ORD, U.S. Environmental Protection Agency, Research Triangle Park, North Carolina, USA
[2]National Research Council
[3]U.S. EPA NCBA/SEE Program
[4]Curriculum in Toxicology, University of North Carolina, Chapel Hill, North Carolina, USA
[5]Department of Environmental Sciences and Engineering, School of Public Health, University of North Carolina, Chapel Hill, North Carolina, USA
[6]Tel Aviv University, Tel Aviv, Israel

Aging adults are a growing segment of the U.S. population and are likely to exhibit increased susceptibility to many environmental toxicants. However, there is little information on the susceptibility of the aged to toxicants. The toxicity of toluene has been well characterized in young adult rodents but there is little information in the aged. Three approaches were used: (1) pharmacokinetic (PK), (2) cardiac biomarkers, and (3) whole-animal physiology to assess whether aging increases susceptibility to toluene in the Brown Norway (BN) rat. Three life stages, young adult, middle aged, and aged (4, 12, and 24 mo, respectively), were administered toluene orally at doses of 0, 0.3, 0.65, or 1 g/kg and subjected to the following: terminated at 45 min or 4 h post dosing, and blood and brain toluene concentration were measured; terminated at 4 h post dosing, and biomarkers of cardiac function were measured; or monitor heart rate (HR), core temperature (Tc), and motor activity (MA) by radiotelemetry before and after dosing. Brain toluene concentration was significantly elevated in aged rats at 4 h after dosing with either 0.3 or 1 g/kg. Blood toluene concentrations were unaffected by age. There were various interactions between aging and toluene-induced effects on cardiac biomarkers. Most notably, toluene exposure led to reductions in mRNA markers for oxidative stress in aged but not younger animals. Toluene also produced a reduction in cardiac endothelin-1 in aged rats. Higher doses of toluene led to tachycardia, hypothermia, and a transient elevation in MA. Aged rats were less sensitive to the tachycardic effects of toluene but showed a prolonged hypothermic response. Elevated brain levels of toluene in aged rats may be attributed to their suppressed cardiovascular and respiratory responses. The expression of several cardiac biochemical markers of toluene exposure in the aged may also reflect differential susceptibility to this toxicant.

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Address correspondence to Dr. Christopher J. Gordon, B105-04, U.S. EPA, Research Triangle Park, NC27711, USA. E-mail: Gordon.christopher@epa.gov

Aging adults represent the fastest growing population segment in many countries. The percentage of adults in the United States that are age 65 yr and older is projected to increase from approximately 37% in 2004 to over 60% by 2024 (Centers for Disease Control: http://www.cdc.gov/nchs). Aging is associated with pathophysiological changes that would lead one to expect increased susceptibility to environmental toxicants. For example, increased formation of free radicals (Harman, 1992; Cantuti-Caselvetri et al., 2000), reduction in cardiovascular and respiratory capacity (Zhang & Sannajust, 2000), increased sensitivity to heat and cold stress (Kenney & Munce, 2003), and decreased ability to absorb, metabolize, and excrete drugs and toxicants (Cusak, 2004) are all exacerbated with aging.

There is remarkably little information regarding the risks of older adults exposed to environmental pollutants. The aged have been a focal point in the study of the susceptibility of the cardiovascular system to fine particulate matter (PM) and other forms of air pollution (Elder et al., 2007; Nadziejko et al., 2004). However, studies utilizing aged animals as a model system to evaluate the effects of exposure to environmental chemicals are limited. Investigations were undertaken to perform a multidisciplinary study of aging and susceptibility to an environmental toxicant with a substantial toxicological data base. To this end, the volatile organic solvent (VOCs) toluene was selected.

VOCs are prevalent in urban air and generated by several types of regulated sources. Many commercial products contain toluene, including paints, waxes, inks, cleaning solutions, and pesticides (Kenyon et al., 2008). The nervous and cardiovascular systems have been found to be susceptible to toluene and other VOC (Benignus, 1981; Greenberg, 1997; Kenyon et al., 2008). Toluene by oral or inhaled routes was found to elicit a marked tachycardia, hypertension, and hyperactivity in young adult Long-Evans (LE) rats monitored by radiotelemetry (Gordon et al., 2007). It was postulated that measuring cardiac biomarkers of oxidative stress, endothelial factors associated with cardiovascular function (i.e., endothelin), along with heart rate, core temperature, and motor activity, will provide a rounded assessment to determine whether age affects the susceptibility to toluene.

An oral gavage rather than inhalation route of exposure was used in the current studies to facilitate measurement of a variety of endpoints and comparison with previously generated data. Gavage exposure has often been used in acute toluene studies; a single oral dose of toluene elicits a prolonged elevation in blood toluene that can be used to approximate levels obtained during inhalation exposure (Gospe & Al-Bayati, 1994; Sullivan & Connolly, 1988; Kenyon et al., 2008). Hence, the overall aim of the current study was to assess the effects of aging on the pharmacokinetics, biochemical, and physiological effects of orally administered toluene in the Brown Norway rat.

MATERIALS AND METHODS

Animals and Toluene Exposure

Male rats of the Brown Norway (BN) strain were studied at ages 4, 12, and 24 mo, weighing approximately 300, 375, and 475 g, respectively. The animals were obtained from the National Institute of Aging colony maintained by Harlan Laboratories. The Brown Norway (BN) rat is a popular model for aging studies. The weight gain of the BN strain over its life time is limited compared to other strains, making it more amenable for neurobehavioral testing (Gordon, 2008).The rats were ordered in the three age groups and allowed to acclimate for several weeks in our animal facility (AAALAC Int. approved). All animals received standard Purina rat chow (Brentwood, MO) and water ad libitum. The rats were housed individually in polycarbonate cages lined with wood shaving bedding at an ambient temperature of 22°C, relative humidity of 50%, and 12:12-h L:D photoperiod (lights on at 6 a.m.). All experiments were approved by an Institutional Animal Care and Use Committee.

Gavage dosing of toluene (Burdock & Jackson, Muskegon, MI) with a dosing volume

of 4 ml/kg body weight was used in all studies. Rats used in the pharmacokinetic study were dosed with 0.3 or 1 g/kg in corn oil. Rats used in the cardiac biomarker and physiological studies were dosed with the corn-oil vehicle, and 0.3, 0.65, or 1 g /kg. Dosing for all three studies took place in the morning around 10 a.m. These doses are high relative to typical human environmental exposures. The doses were selected with the anticipation of observing effects that could be detected over the 4-h sampling period.

Pharmacokinetics

Prior to use of the BN rats in pharmacokinetic (PK) studies, a barometric whole-body plethysmography system (Buxco Electronics, Inc., Sharon, CT) was used to obtain data on pulmonary ventilation parameters in the three age groups (n = 12/age group). These data were collected to facilitate interpretation of pharmacokinetic data, since pulmonary ventilation is a major determinant of overall toluene clearance. Parameters measured in the Buxco system included breathing frequency, tidal volume, and minute ventilation. The chamber was maintained at 22°C. Acclimation and training in the chamber followed the methods outlined by Kodavanti et al. (2005). Briefly, unrestrained, freely moving rats were housed in individual chambers and allowed 1 min acclimation time followed by a 5-min period of data collection.

The rats were allowed at least several days of recovery from the Buxco studies prior to the PK studies. Following dosing with 0.3 or 1 g/kg the rats were killed using CO_2 at 45 min or 4 h (n = 6 age group/dose level/time point). A 4-h time point was selected because it corresponds with the approximate time of peak elevation in blood toluene when administered orally in rats. Gospe and Al-Bayati (1994) found that oral toluene at a dose of 336 mg/kg peaked at 2–4 h; higher doses of 741 and 911 mg/kg peaked at 4–6 h. Another study by Sullivan and Conolly (1988) found similar relationships between dose and time to peak rise in blood toluene levels. The 4-h time point was selected for the cardiac biomarker study (see later description). Rats were decapitated while under CO_2 anesthesia and the brain was removed within 2 min after collection of blood via cardiac puncture in a heparinized syringe. Whole brains were excised, weighed, and homogenized in ice-cold phosphate-buffered saline (pH 7.4) and sealed in 20-ml vials with Teflon-lined caps within 4 min of decapitation. Brain homogenates and whole blood were shipped on ice to NMS Labs (Willow Grove, PA) for analysis. Toluene concentrations were assayed in blood and whole-brain homogenates by gas chromatography using a headspace sampling method as described previously (Bushnell et al., 2007). Analysis of variance (ANOVA) was used to evaluate age-group differences for each dose level and time point combination, followed by LSD tests for significance between group means as needed (SAS 9.1 Proc GLM, SAS Institute, Cary, NC).

Age-related differences in metabolic clearance of toluene were assessed *in vitro* using microsomes prepared from the livers of untreated BN rats aged 4, 12, or 24 mo (n = 6/group). Rat liver microsomal fractions were prepared as described previously (Devito et al., 1996; Lee et al., 2008) and stored at –80°C until use. Microsomal clearance of toluene was measured using an automated headspace technique as described by Tornero-Velez et al. (2004). Disappearance of toluene from the vial headspace was monitored utilizing a gas chromatograph (Hewlett-Packard model 5890 Series II GC) equipped with a 1/8-inch ID × 6 ft length stainless-steel column packed with 0.1% SP-1000 on 80/120 mesh Carbopack C (Supelco, Bellefonte, PA). The initial toluene concentration used was 30 ppm (selected to be below the K_m for toluene). Operating conditions were as follows: column/oven temperature 165°C, and flame ionization detector temperature 250°C. The flow of the helium carrier gas was 15 ml/min, 300 ml/min for air, and 30 ml/min for hydrogen. Toluene clearance was expressed as net metabolic clearance or clearance due solely to metabolism, i.e., corrected for loss of chemical due to nonspecific binding or minor chamber leakage. Net metabolic clearance is calculated from the

chamber loss curve once the cofactor (NADPH) is added to start microsomal metabolism

Cardiac Biomarkers

Rats were decapitated for necropsy without CO_2 anesthesia at 4 h after dosing for analysis of biomarkers. The heart was quickly excised, and half of the heart was longitudinally cut and fixed in neutral formalin for histological evaluation. Tissues were embedded in paraffin, sectioned, and hematoxylin and eosin (H&E) stained for blinded histological examination. From the other half of the heart, approximately 50 mg tissue was separated and frozen (–80°C) for later RNA isolation. The remaining left ventricle was used for isolation of mitochondria and cytosolic fractions.

Preparation of Cardiac Mitochondrion and Cytosolic Fractions Cytosolic and mitochondrion fractions were isolated by differential centrifugation from a small portion of freshly removed left ventricle. Briefly, tissues were homogenized in 10 ml of buffer A (210 m*M* mannitol, 5 m*M* MOPS, 70 m*M* sucrose, 1 m*M* ethylenediamine tetraacetic acid [EDTA], pH 7.4) on ice. After centrifugation at 300 × g for 20 min at 4°C, the supernatant was removed and re-centrifuged at 10,000 × g for 20 min at 4°C. The resulting supernatant (cytosolic fraction) was separated and the pellet was suspended in 1 ml of buffer B (210 m*M* mannitol, 5 m*M* MOPS, 70 m*M* sucrose, protease inhibitors [1:200 dilution], pH 7.4), sonicated for 10 s, and centrifuged at 800 × g for 10 min. The supernatant (mitochondrial fraction) was removed, frozen at –80°C and was used for protein and enzyme-activity assays.

Analysis of Cardiac Mitochondrial and Cytosolic Enzyme Activities The activities of cardiac mitochondrial enzymes were assayed using commercial kits (aconitase: OXIS International, Inc., Portland, OR; isocitrate dehydrogenase: Sigma Chemical Co., St Louis, MO; superoxide dismutase [SOD]: Randox Laboratories Ltd, Antrim, UK). Ferritin levels were determined using K-Assay from the Kamia Biomedical Company (Seattle, WA). NADH-Ubiquinone reductase (UBIQ-RD) activity was assayed following the method of Cormier et al. (2001). Glutathione peroxidase (GPx) and glutathione transferase (GTR) activities were assayed as described by Jaskot et al. (1983). These colorimetric assays were adapted for use on the KONLAB clinical chemistry analyzer (Thermo Clinical LabSystems, Espoo, Finland). Protein contents of mitochondrial and cytosolic fractions were determined using a Coomassie Plus Protein Assay Kit (Pierce, Rockford, IL) and bovine serum albumin (BSA) standards from Sigma Chemical Co. (St. Louis, MO).

RNA Isolation and Real-Time Polymerase Chain Reaction (PCR) Left ventricular RNA was isolated from control and high-dose (1 g/kg) toluene-exposed rats using TriReagent (Sigma, St Louis, MO), and snap-frozen in liquid nitrogen. RNA was further purified with Qiagen Rneasy mini columns (Qiagen, Valencia, CA) and resuspended in 50 µl diethylpyrocarbonate (DEPC)-treated water according to the manufacturer's protocol. RNA quantity was assessed with an Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA).

Real-time quantitative polymerase chain reaction (PCR) was performed using the Superscript III Platinum One-Step Quantitative RT-PCR System (Invitrogen Inc., Carlsbad, CA) to analyze mRNA expression levels for markers of oxidative stress, inflammation, thrombosis, vasoconstriction, and cardiac ion channels. PCR primers (20×, FAM-labeled) were obtained from Applied Biosystems (Foster City, CA) for selected markers and the housekeeping gene HPRT. The kit protocol was used for preparing samples and PCR was performed using the Applied Biosystems model 7900 Sequence Detection System (Applied Biosystems, Foster City, CA). The delta Ct values were converted to fold change according to the procedures described in the technical bulletin.

Data were analyzed by a two-way analysis of variance (ANOVA) with exposure and age as factors, using commercial software (SigmaStat Software, Inc., version 3.5; Point Richmond, CA). Pairwise group comparisons were made using the Holm–Sidak method (SigmaStat Software, Inc., Point Richmond, CA). Significance was ascribed to a test result at $p < .05$ or as specified in figure legends. PCR data were

analyzed using ABI sequence detection software (SDS), version 2.2. For each plate, cycle threshold (cT) was set to an order of magnitude above background. For each individual sample, target gene cT was normalized to control (HPRT) cT to account for variability in starting RNA amount. Expression of each exposure group was quantified relative to the change from control.

Physiology

Surgery Heart rate, core temperature, and motor activity were monitored in unrestrained rats using radiotelemetry (model TA11CTA-F40; Data Sciences International, St. Paul, MN). Details of the surgical procedure have been published (Gordon et al., 2007). Rats were anesthetized with a mixture of ketamine and xylazine (80/10 mg/kg; intraperitoneally). Once the desired plane of anesthesia was reached, a midline incision was made in the abdominal musculature and a new transmitter was implanted that had been refurbished by the manufacturer with a new battery and electrocardiogram (ECG) leads. The body of the transmitter was placed inside the abdominal cavity and sutured to the midline. The ECG leads were tunneled under the skin and positioned on the left and right sides of the thorax, allowing for the best detection of the ECG signal. The skin was closed with wound clips and the rat was administered an analgesic (buprenorphine; 0.03 mg/kg; SC) and an antibiotic (penicillin; approximately1000 U; im).

The battery of a new transmitter can operate continuously for approximately 6 mo. The transmitter has a magnetic switch allowing it to be turned on and off to conserve battery life. The rats in this study were monitored periodically for 8 mo after transmitter implantation. The temperature calibration of the transmitters was checked after extraction from the animals to assure proper operation of the units.

Protocol Heart rate, electrocardiogram (ECG) wave form, core temperature, and motor activity were recorded continuously by the telemetry system and analyzed with software provided by the manufacturer (Data Sciences Int., ART Gold, version 4.0). All animals were monitored remotely while housed in the vivarium. The ECG wave form was recorded for 10 s every 3 h. Heart rate, core temperature, and activity were recorded at 5-min intervals.

The rats were allowed to recover from surgery for 32 d prior to the first dosing with toluene. Prior to toluene exposure, rats were given a clean cage and left undisturbed for 5 d while baseline telemetry data were collected. Food and water were provided ad lib. Other than a daily visual inspection to ensure the animal's health and well being, the animals were left undisturbed during the monitoring period (see Gordon, 2008). Each rat was then administered the control vehicle, 0.3, 0.65, or 1 g/kg toluene at 10 a.m. with a 1-wk interval between dosings. Toluene treatments were randomized each week. The maximum dose of toluene was developed from previous study in this laboratory with Long-Evans rats (Gordon et al., 2007). It was found that a maximum oral toluene dose of 1200 mg/kg elicited marked elevation in heart rate and blood pressure that persisted for at least 6 h after dosing. Preliminary investigations with the BN rat, using motor activity as an endpoint, indicated a higher sensitivity to toluene in this strain compared to the LE strain. Hence, it was decided to limit the highest toluene dose to 1 g/kg. After dosing, the rats were observed for any signs of acute toxicity. Otherwise, rats were left undisturbed for at least 24 h after dosing. Mean ± SE body weights for the rats over the 4-wk period of toluene dosing were 347 ± 3 g, 383 ± 7 g, and 449 ± 9 g for the 4-, 12-, and 24-mo-old animals, respectively.

The response to toluene was analyzed using RMANOVA procedures (Sigma Stat). With little knowledge of the expected time course to toluene in the BN rat, we used a post hoc approach to study the time course changes in each of the telemetry parameters. The 5-min data were smoothed with a 5-point moving average technique prior to analysis. RMANOVA was used to analyze effects of toluene treatment and age on each of the telemetry parameters. Statistical analysis of the response of

each telemetry parameter to toluene was done by averaging the response over a given length of time after toluene exposure. The time of data averaging varied with each telemetry parameter because of differences in the onset and recovery described earlier. Heart rate was averaged over 120 min, motor activity over 60 min, and core temperature from 2 to 4 h after dosing.

RESULTS

Toluene Pharmacokinetic Studies

There were significant differences in the respiratory parameters across age groups. Absolute tidal volume increased with age but tidal volume normalized to body weight was significantly decreased. Respiratory frequency was affected by age, decreasing from 311 breaths/min in the 4-mo-old rats to 216 breaths/min in the 24-mo-old rats (Table 1). Both minute volume and minute volume normalized to body weight were significantly smaller in the oldest age group. For example, the normalized minute volume of the 24-mo-old rats was approximately 50% of the value measured in the 4-mo-old rats.

Toluene pharmacokinetics in the *in vivo* and *in vitro* studies were affected by age (Figures 1, 2, and 3). Toluene levels in the brain and blood were unaffected by age at the 45-min time point in rats dosed with 0.3 or 1 g/kg (Figures 1 A and 2 A). By 4 h post dosing, significant age differences in toluene concentration in the brain but not blood were observed in the 0.3- and 1-g/kg dose groups. Brain toluene at 4 h increased progressively with age in both dose groups. Brain toluene in the 24-mo-old animals was approximately 50%

TABLE 1. Effect of Age on Respiratory Parameters in Untreated BN Rats

Parameter	Units	4 mo	12 mo	24 mo
Body weight	g	296 ± 28^{a}	375 ± 29^{b}	479 ± 29^{c}
Frequency (F)	breaths/min	311 ± 46^{a}	254 ± 30^{b}	216 ± 35^{c}
Normalized F	breath/min/g	1.07 ± 0.24^{a}	0.68 ± 0.01^{b}	0.45 ± 0.08^{c}
Tidal volume (TV)	ml	1.79 ± 0.24^{a}	2.14 ± 0.28^{b}	2.35 ± 0.27^{b}
Normalized TV	ml/g	0.0061 ± 0.0007^{a}	0.0057 ± 0.0008^{a}	0.0049 ± 0.0004^{b}
Minute volume (MV)	ml/min	473 ± 44^{a}	$445 \pm 37^{a,b}$	419 ± 48^{b}
Normalized MV	ml/min/g	1.62 ± 0.24^{a}	1.19 ± 0.12^{b}	0.877 ± 0.10^{c}

Note. Data given as mean ± SD; $n = 12$ per age. Statistically significant differences between age groups are denoted by different letters. Groups with same letters are statistically insignificant. Parameters were normalized by dividing by body weight (g).

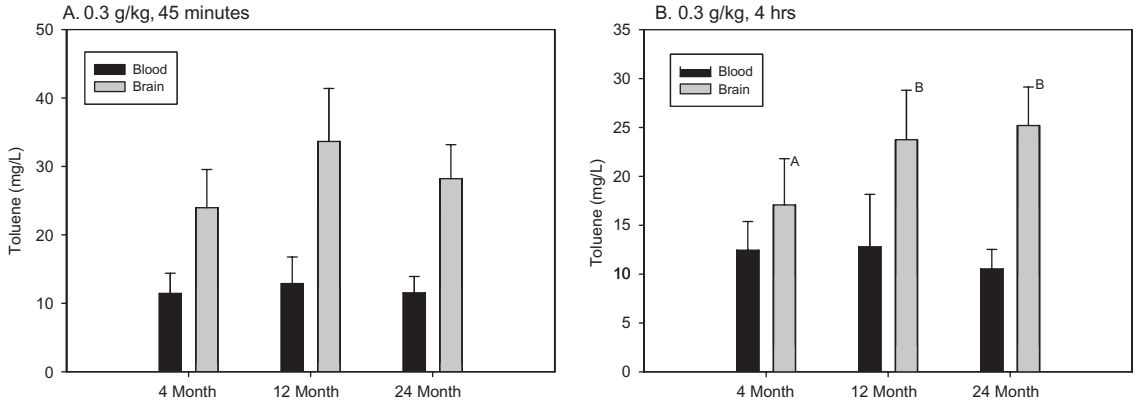

FIGURE 1. Effect of age on brain and blood toluene levels at 45 min (A) and 4 h (B) after oral dosing with 1 g/kg toluene. Values plotted as mean + SD ($n = 5$–6 per group).

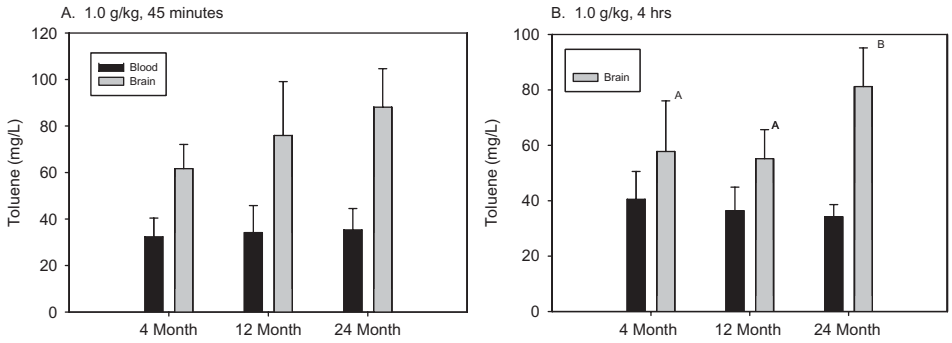

FIGURE 2. Effect of age on brain and blood toluene levels at 45 min (A) and 4 h (B) after oral dosing with 0.3 g/kg toluene. Values plotted as mean + SD (n = 5–6 per group).

higher than that of the 4-mo-old animals 4 h after dosing with 0.3 or 1 g/kg. *In vitro* metabolic clearance of toluene was also significantly affected by age. The net metabolic clearance of toluene increased by approximately 10% from 4 to 24 mo of age. The 24-mo *in vitro* metabolic clearance was significantly increased compared to the 4- and 12-mo measurements (Figure 3).

Cardiac Biomarkers

Some mitochondrial and cytsolic markers were affected by age and/or toluene exposure. Mitochondrial ferritin levels showed significant age-dependent increases in 12- and 24-mo relative to 4-mo rats; no changes were noted, however, due to toluene exposure (Figure 4). Mitochondrial aconitase activity remained unchanged with age and toluene exposure. Toluene increased UBIQ-RD activity in 12-mo-old rats but not in rats of other ages (Figure 4). The activities of cytosolic antioxidant enzymes SOD, GTR, and GPx were quantitatively lower in 24-mo animals relative to 4- and/or 12-mo rats (Table 2); these effects reached significance when all treatment groups were combined within an age group, due to the fact that no significant alterations were observed after toluene exposure. Cytosolic aconitase, unlike the mitochondrial aconitase, demonstrated a significant rise with age. Toluene exposure decreased the enzyme activity in 4- and 12-mo-old rats but not in 24-mo-old rats (Figure 5). Cytosolic ferritin levels were significantly higher in 24-mo-old rats relative to the other age groups, suggesting an iron overload in aged rats. Cytosolic ferritin increased with toluene exposure in 12-mo rats, however, these rats demonstrated high within-group variability. In the 24-mo-old rats, cytosolic ferritin decreased following toluene exposure (Figure 5).

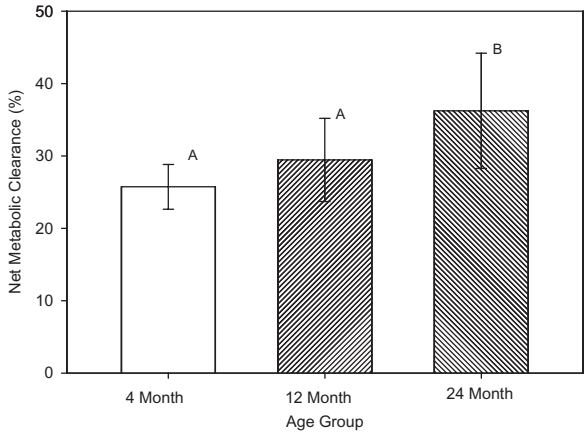

FIGURE 3. Effect of age on the *in vitro* metabolic clearance of toluene in hepatic microsomes. Values plotted as mean ± SD (n = 6 rats/group). Groups with the same letters are statistically not significantly different.

Gene Expression Changes Hearts from 24-mo-old rats (control and toluene-exposed) showed increased mRNA for heme oxygenase-1 (HO-1) relative to younger rats (Table 3). The expression of endothelin-1 (ET-1) and expression of its receptor B were lower in 12-mo relative to 4- or 24-mo rats (Table 3). Toluene exposure produced a decrease in ET-1 expression in 12- and 24-month old rats. The expression of ET receptor A (ET-A) did not show a

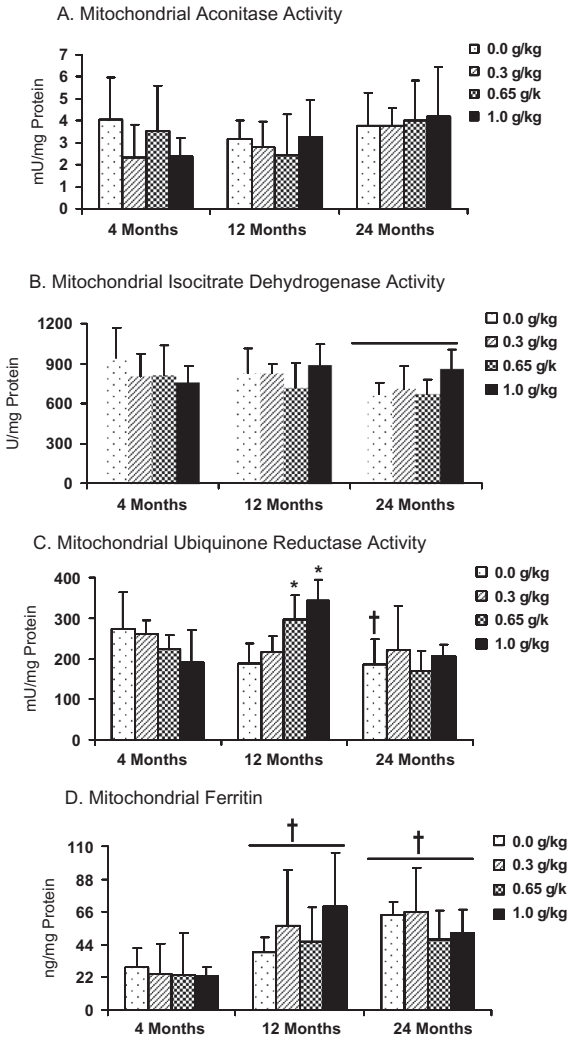

FIGURE 4. Effect of age and toluene on activity of cardiac oxidative stress sensitive mitochondrial enzymes and ferritin. Values represent mean + SD (n = 5–6 per group). Asterisk indicates significant ($p \leq .05$) toluene effect in relation to control within a given age group; † indicates significant age difference ($p \leq .05$) relative to 4-mo age. Line under significance level indicates the comparison is made with all animals within the age group regardless of toluene.

significant age or toluene effect. Expression of ET receptor B (ET-B) declined with toluene exposure in 12-mo in comparison to 4-mo and 24-mo rats. There were no significant effects of toluene exposure in mRNA expression of L-type calcium channel proteins (Table 3).

The cardiac expression of the blood coagulation and thrombosis markers such as tissue factor (TF), tissue plasminogen activator (t-PA), and plasminogen activator inhibitor-1 (PAI-1) was analyzed in each age group for control and high-dose toluene-exposed rats (Table 3). The expression of the coagulation marker PAI-1 was up-regulated in 24- relative to 4- or 12-mo-old rats. There was a trend for a decrease in PAI-1 expression in these rats; however, the difference was statistically not significant. There were no consistent age- or toluene-induced changes in TF and t-PA expression except for a decrease in 4-mo rats following toluene exposure.

Body and Cardiac Weights and Histopathology Body weights of rats varied among age groups (controls: 4 mo, 323 ± 7 g; 12 mo, 369 ± 6.7 g; and 24 mo, 401 ± 14 g; mean ± standard error). Relative heart weight of the 24-mo-old rats was significantly greater than that of the 4- and 12-mo-old animals (Table 4). Toluene treatment exerted a significant effect on relative heart weight; the 1-g/kg toluene treatment elicited a significant reduction in the heart-to-body weight ratio in all three age groups (Table 4). Microscopic evaluation of cardiac tissues demonstrated scattered and limited foci of epicardial inflammation in both control and toluene-exposed rats regardless of age. However, myocardial degeneration (cardiomyopathy) and valvular myxomatous changes, although mild, were higher in old (24 mo) rats, regardless of toluene exposure (Table 5).

Physiology

Administration of the corn oil vehicle and toluene treatments elicited transient elevations in heart rate, core temperature, and motor activity. This response was attributed to the stress of handling and dosing. Examples of the time course to 1 g/kg toluene illustrate the tachycardia, hypothermia, and hyperactive responses (Figure 6). Heart rate of all 3 age groups increased with toluene, peaking at approximately 30 min after dosing and recovering within 90 min to control levels (Figure 6). Motor activity also rose abruptly after toluene but recovered to baseline levels in approximately 60 min after dosing (Figure 6). The time course for toluene-induced changes in body temperature was prolonged with the peak

TABLE 2. Activities of Cardiac Cytosolic Enzymes Involved in Antioxidant Compensation in Rats of Different Ages Exposed to Vehicle or Toluene

Marker/treatment	4 mo	12 mo	24 mo
Cytosolic superoxide dismutase, U/mg protein			
Control	3.79 ± 2.05	4.13 ±1.93	3.44 ± 1.98
0.3 g/kg	4.07 ± 1.56	4.44 ± 2.08	3.59 ± 1.57
0.65 g/kg	3.86 ± 1.38	3.78 ± 1.75	2.98 ± 1.61
1 g/kg	4.39 ± 1.19	5.46 ± 1.88	3.44 ± 2.13
Cytosolic glutathione transferase, IU/mg protein			
Control	0.055 ± 0.009	0.057 ± 0.004	0.049 ± 0.007†
0.3 g/kg	0.054 ± 0.004	0.057 ± 0.014	0.052 ± 0.003†
0.65 g/kg	0.057 ± 0.006	0.055 ± 0.005	0.045 ± 0.004†
1 g/kg	0.052 ± 0.007	0.063 ± 0.007	0.054 ± 0.009†
Cytosolic glutathione peroxidase, IU/mg protein			
Control	0.62 ± 0.08	0.56 ± 0.03†	0.52 ± 0.07†
0.3 g/kg	0.62 ± 0.05	0.58 ± 0.05†	0.55 ± 0.06†
0.65 g/kg	0.65 ± 0.10	0.56 ± 0.04†	0.48 ± 0.06†
1 g/kg	0.66 ± 0.06	0.64 ± 0.06†	0.53 ± 0.09†

Note. Values represent mean ± SD of 6 rats per group ($n = 5$ for 24 mo high dose toluene); †indicates significant age difference ($p \leq .05$) relative to 4 mo age.

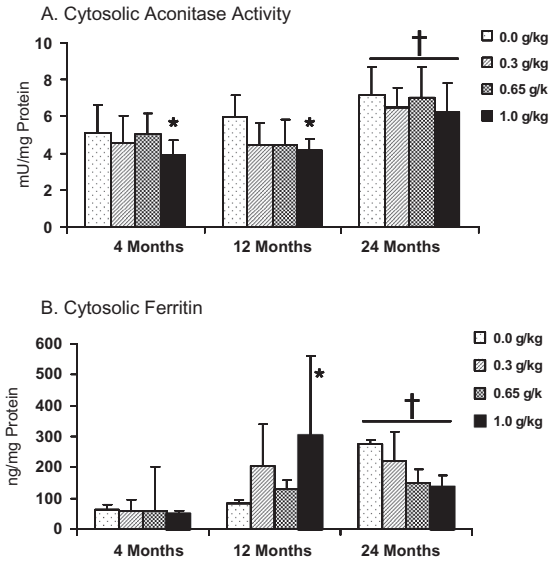

FIGURE 5. Effect of age and toluene on cardiac cytosolic aconitase activity and ferritin. Values represent mean + SD of 6 rats per group ($n = 5$ for 24-mo high-dose toluene). Asterisk indicates significant ($p \leq .05$) toluene effect in relation to control; † indicates significant age difference ($p \leq 0.05$) relative to 4- and 12-mo age. Line under significance level indicates the comparison is made with all animals within the age group regardless of toluene.

drop in core temperature occurring 1–2 h post dosing and recovery by approximately 6 h after dosing (Figure 6 C). The overall tachycardic effects of toluene were most profound in the two youngest age groups (Figure 7 A). Four-month-old rats exhibited significant elevations in heart rate at toluene doses of 0.65 and 1 g/kg. The 12-mo-old animals exhibited a significant response at a dose of 1 g/kg, whereas the response of the 24-mo-old animals failed to reach significance.

The effects of toluene on motor activity were also most pronounced in the youngest animals (Figure 7 B). Their motor activity was significantly elevated following doses of 0.65 or 1 g/kg, whereas motor activity of the 12-mo-old animals failed to reach significance at any dose. Motor activity of the 24-mo-old animals rose at only the highest dose of toluene. Because motor activity following administration of the corn oil vehicle was quantitatively reduced with increasing age, the percent elevation in motor activity exhibited an altered pattern compared to absolute activity (Figure 7 B). When normalized to control values, the rise in motor activity of the 4- and 24-mo-old animals was 100% more than that of the 12-mo-old

TABLE 3. Relative fold change in mRNA expression for Biomarkers of Microvascular Oxidative Stress, Inflammation, Vasoconstriction, and Thrombosis in Rats of Different Ages Treated With Vehicle or Toluene (1 g/kg)

	4 mo		12 mo		24 mo	
Biomarker	Control	Toluene	Control	Toluene	Control	Toluene
HO-1	1.21 ± 0.49	1.09 ± 0.14	1.13 ± 0.27	0.99 ± 0.26	2.35 ±0.47†	2.17 ± 0.68†
MIP-2	1.21 ± 0.30	0.73 ± 0.15	0.94 ± 0.19	1.24 ± 0.51*	2.16 ±1.22†	1.04 ± 0.25*
MMP-2	1.02 ± 0.17	0.78 ± 0.22	0.79 ± 0.13	0.49 ± 0.15	0.71 ±0.21†	0.61 ± 0.18†
ET-1	0.88 ± 0.53	0.82 ± 0.30	0.99 ± 0.14	0.76 ± 0.21*	1.41 ± 0.49	0.89 ± 0.19*
ET-A	0.94 ± 0.20	0.82 ± 0.13	0.72 ± 0.10	0.76 ± 0.12	1.00 ± 0.36	0.75 ± 0.13
ET-B	1.21 ± 0.42	0.97 ± 0.30	0.73 ±0.18†	0.49 ± 0.19*†	0.85 ± 0.15	0.71 ± 0.16
TF mRNA	0.97 ± 0.18	0.62 ± 0.06*	0.66 ± 0.15	0.65 ± 0.16	0.97 ± 0.41	0.77 ± 0.13
t-PA mRNA	1.03 ± 0.10	1.16 ± 0.07	1.26 ± 0.19	1.10 ± 0.18	1.16 ± 0.14	0.96 ± 0.08
PAI-1 mRNA	0.99 ± 0.12	1.21 ± 0.28	1.21 ± 0.39	0.80 ± 0.13	1.90 ± 0.98	1.07 ± 0.20

Note. Values represent mean ± SD of 6 rats per group (n = 5 for 24-mo high-dose toluene). Asterisk indicates significant ($p \leq .05$) toluene effect in relation to control within a given age group;

†indicates significant effect of age when compared to 4-mo-old group. TF, tissue factor.

TABLE 4. Effect of Toluene on Relative Heart Weight (mg/g Body Weight)

Treatment	4 mo	12 mo	24 mo
Control	0.289 ± 0.035	0.291 ± 0.04	0.323 ± 0.031†
0.3 g/kg	0.270 ± 0.009	0.271 ± 0.01	0.303 ± 0.01†
0.65 g/kg	0.283 ± 0.023	0.267 ± 0.017	0.312 ± 0.024†
1 g/kg	0.271 ± 0.01*	0.267 ± 0.01*	0.282 ± 0.016*†

Note. Values represent mean ± SD of 6 rats per group (n = 5 for 24-mo high-dose toluene). Asterisk indicates significant ($p \leq .05$) toluene effect in relation to control within a given age group;

†indicates significant age difference ($p \leq .05$) relative to 4-mo and/or 12-mo rats.

TABLE 5. Histopathology Incidence Table Demonstrating Number of Animals With Pathology in Rats of Different Age Groups

Cardiac pathology	4-mo	12-mo	24-mo
Epicardium: mononuclear cell infiltration	10/24	4/24	5/23
Myocardium: degeneration and inflammation (cardiomyopathy), focal	3/24	2/24	10/23
Left atrioventricular valves: myxomatous degeneration, focal	0/24	2/24	6/23

Note. Values indicate number of animals demonstrating the abnormality per total number of rats examined. Note that the severity of lesions in each case was rated 1 based on the scale of zero being no abnormality to 4 being severe lesions. Since there were no toluene-related effects within a given age group, all rats of the same age were combined.

animals. The hypothermic effects of toluene were most pronounced in the oldest rats (Figure 7 C). A significant reduction in core temperature in the 24-mo-old rats was observed at toluene doses of 0.65 or 1 g/kg, whereas significant hypothermia in the younger two groups was only observed following 1 g/kg. It is interesting to note that core temperature of the oldest animals was consistently higher than that of the younger animals across all doses of toluene. The relative drop in core temperature following toluene exposure is higher in the oldest animals because of their elevated baseline core temperature (Gordon, 2008).

DISCUSSION

Data suggested an increase in susceptibility of the aged BN rat to toluene. The pharmacokinetic facet of the study showed that older rats sustain greater toluene loads in the brain within 4 h after dosing. This coincided with the time when the biochemical cardiac markers were assessed, and several of these biomarkers were significantly affected by toluene in aged but not younger animals. The maximal elevations in heart rate and motor activity occurred at approximately 30 min post dosing. Interestingly, age-related elevations in brain and blood toluene were indistinguishable at 45 min post dosing, which coincided closely with the peak changes in heart rate and motor activity.

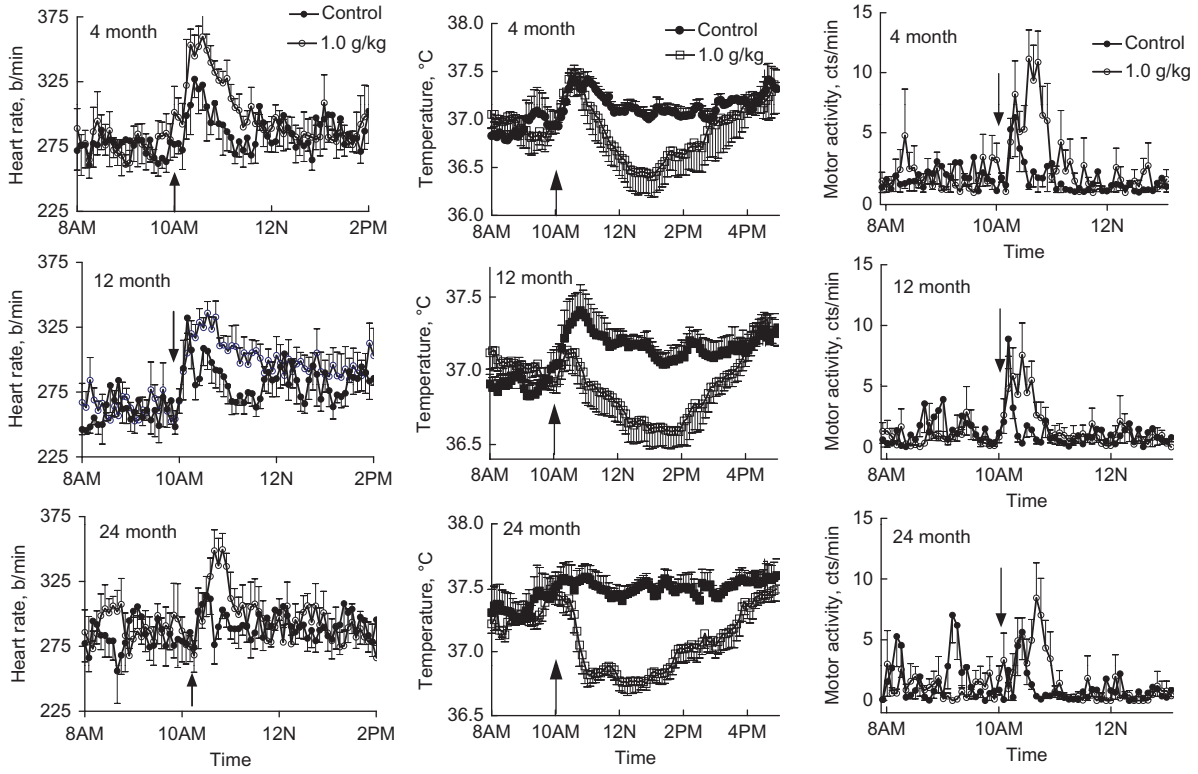

FIGURE 6. Time course of heart rate, core temperature, and motor activity in BN rats dosed with corn oil and 1 g/kg toluene. Arrow indicates administration of vehicle or toluene. Data plotted as mean ± SE; $n = 5$ for 4-mo-old, 6 for 12-mo-old, and 5 for 24-mo-old animals.

Indeed, the toluene-induced changes in heart rate and motor activity recovered prior to the peak rise in brain and blood toluene levels (i.e., at 4 h). On the other hand, the hypothermic effects of toluene were accentuated in the aged animal and recovery from hypothermia took several hours. Overall, it appears that the aged rats were unable to mount as robust a tachycardic and respiratory response to toluene, with the result being slower clearance of toluene and a prolonged hypothermic effect compared to younger animals.

Pharmaco-kinetics

Toluene is eliminated by two principal pathways: metabolic biotransformation in the liver and pulmonary exhalation (Ogata et al., 1970). The relative amount of toluene excreted through these two pathways depends on dose, route, and species. Ogata et al. (1970) concluded that in humans and animals 60–75% of the absorbed toluene is accounted for as urinary hippuric acid (a by-product of hepatic deactivation) within 12 h. Because metabolic biotransformation of toluene, as assessed by measurement of hepatic microsomal clearance, was elevated in the aged animals, it might be postulated that the increased toluene concentration in brain at 4 h post exposure in aged animals was partially attributable to decreased breathing rate and minute volume in aged rats. However, if this was the predominant explanation, it would be expected that blood toluene concentration would also be elevated in aged animals, and this was not observed. Another possible explanation for the difference observed in brain concentration but not blood concentration is age-related changes in

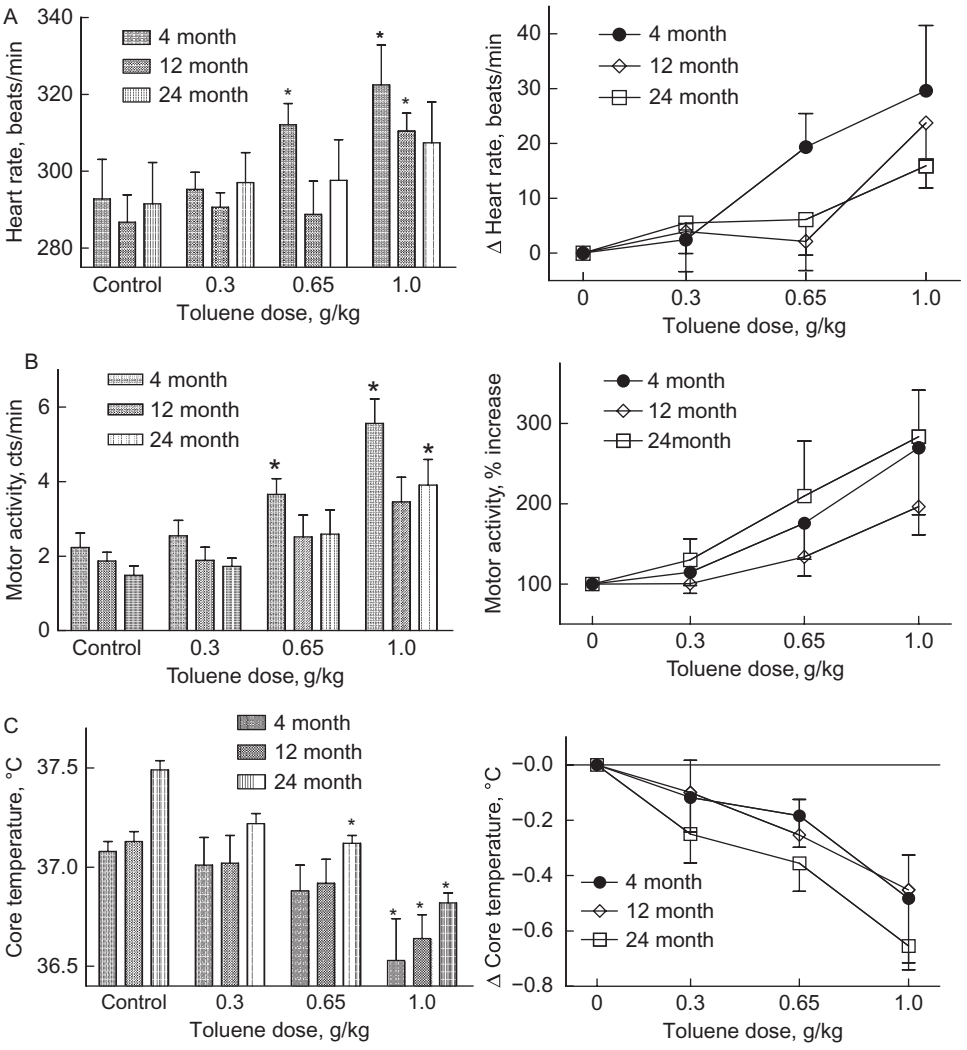

FIGURE 7. Overall effect of toluene on heart rate (A), motor activity (B), and core temperature (C) in the three age groups. Upper plots are absolute data and lower plots are relative changes from baseline. Asterisks indicate significant difference from control group. RMANOVA results: heart rate, treatment ($F = 10.2$, $p < .05$); age (NS), treatment-age (NS); motor activity, treatment ($F = 17.7$, $p < .05$), age (NS), treatment-age (NS). Temperature, treatment ($F = 15.3$, $p < .05$); age (NS), treatment-age (NS). Data plotted as mean ± SE.

partitioning of toluene into brain. For example, Mahale et al. (2007) reported that the blood:air partition coefficient for several volatile solvents such as benzene, chloroform, perchloroethylene, and trichloroethylene is significantly increased in aged compared to adult or 10-d-old rats. Thus, the most plausible explanation is that a combination of pharmacokinetic factors, including age-based changes in respiratory parameters and brain partition coefficient, contributed to the significantly elevated toluene brain concentration in aged rats in this study.

A central question in this study is: Are the effects of aging on the physiological response to toluene attributed to pharmacokinetic and/or pharmacodynamic differences? For example, aged Fischer 344 rats show altered clearance rates to ethanol compared to young adults (Ott et al., 1985). The hypothermic and behavioral effects of ethanol in young and aged rats were attributed in part to differences in age-dependent metabolism and clearance of ethanol in aged animals. As in other rodents, the proportion of body fat of the BN strain increases while the

proportion of lean body mass decreases with aging. The percentage of body fat of the 26-mo-old male is approximately double that of the 5-mo-old (Wolden-Hanson et al., 1999). In view of the differences in lean mass and body fat in young and aged rats, one would expect the pharmacokinetics of a lipophilic molecule such as toluene to vary more in the aged animal.

Cardiac Biomarkers

ET-1 is a potent vasoconstrictor and has been associated with an increase in systolic blood pressure (Honore et al., 1992). Since oral toluene in rats of the Long-Evans strain was shown to elicit an elevation blood pressure lasting several hours after dosing (Gordon et al., 2007), a reduction in ET-1 might be expected as a compensatory response. The inhibition of ET-1 mRNA expression in all age groups at 4 h post toluene exposure in the present study is associated with increase in blood pressure during that time (Gordon et al., 2007). It is also likely that the effects on ET-1 and its receptors might be manifested directly by toluene acting on myocardial cells (Magnusson et al., 1998), resulting in deregulation of its production.

A decline in cardiac mitochondrial and cytosolic antioxidant enzymes was observed together with increases in ferritin and cytosolic aconitase activity in control 24-mo relative to 4-mo rats, which is consistent with oxidative stress and iron overload in aged humans (Galaris et al., 2008). Aging hearts also demonstrated increases in gene expression for biomarkers of oxidative stress, inflammation, and thrombosis, and decreases in ion-channel proteins. However, as observed previously in rats (Robert et al., 1997), matrix metalloprotease (MMP)-2 mRNA levels fell with age. Overall, toluene did not exacerbate biomarker changes seen in the 24-mo rats. Some effects of toluene were seen, however, including significant decreases in wet cardiac weights in all age groups, increases in mitochondrial UBIQ-RD activity in 12-mo rats, a fall in cytosolic aconitase activity in 4- and 12-mo rats, and inhibition of macrophage inflammatory protein-2 (MIP-2) and plasminogen activator inhibitor-1 (PAI-1) in 24-mo rats. Thus, toluene may not accelerate age-related cardiac oxidative stress and inflammation but rather may inhibit vasoconstriction and might even offer protection by reducing baseline thrombosis, inflammation, and vasoconstriction in BN rats.

Mitochondrial enzymes such as aconitase, isocitrate dehydrogenase (ICDH), SOD, and UBIQ-RD are highly sensitive to inhibition by reactive oxygen species (ROS). ICDH activity is highest in heart (Popova et al., 2007) and is crucial for cardiomyocyte energy. The loss of activities of these enzymes is associated with increased superoxide generation and oxidative damage in a variety of pathological conditions (Bulteau et al., 2004) including aging (Judge & Leeuwenburgh, 2007) and cardiovascular disease (Sastre et al., 2003; Judge & Leeuwenburgh, 2007). The current study demonstrated that the mitochondrial enzymes sensitive to oxidative stress were inhibited by age but were not further reduced by toluene exposure. The activity of UBIQ-RD was increased in 12-mo rats, suggesting that acute toluene does not exacerbate age-related mitochondrial oxidative stress.

Mitochondria are the site for heme synthesis, and iron (Fe) homeostasis is tightly regulated (Fontenay et al., 2006). Mitochondrial ferritin plays an important role in the regulation of Fe homeostasis and oxidant production (Campanella et al., 2004; Harrison & Arosio, 1996). It was postulated that as a result of Fe overload with aging, toluene would impact Fe homeostasis differently in the heart. The increase in mitochondrial ferritin in 24-mo rats relative to 4-mo rats suggested that there is an increased need for sequestering free Fe within mitochondria.

Cytosolic aconitase functions as an Fe chaperone protein (Martelli et al., 2007), and serves to maintain free Fe homeostasis within the cytoplasm (Harrison & Arosio, 1996; Levi & Arosio, 2004). The increase in cytosolic aconitase in 24-mo relative to 4-mo rats suggests that there is greater need for sequestration of Fe. The increase in cytosolic aconitase activity

together with ferritin protein in 24-mo rats suggests an Fe overload in aged rats, as has been observed in aging humans (Saito et al., 2003). Surprisingly, toluene produced inhibition of cytosolic aconitase activity in 4- and 1- mo rats. The mechanism for this inhibition may be related to acute toluene-induced effects on cell membranes and receptors (Magnusson et al., 1998).

Cytosolic antioxidant enzymes such as SOD, GPx, and GTR are known to scavenge superoxide free radicals and offer protection (Mak & Newton, 2001). The age-related decrease in the activity of these enzymes may indicate a decrease in compensatory capacity. Inhibition of SOD was noted with aging (Reiss & Gershon, 1976). HO-1 is transcriptionally activated in response to increased free radicals in cells (Alam & Cook, 2003). Higher baseline expression of this marker among 24-mo-old relative to 4- and 12-mo rats further supports the role of oxidative stress in the heart. Despite its known acute cardiophysiological effects (Gordon et al., 2007), toluene did not impact these parameters, suggesting that old-age-related abnormalities are not exacerbated by acute toluene exposure.

MMP play an important role in regulation of extracellular matrix components and age related degenerative changes in the heart. Elevated levels of circulating MMP (MMP-2 and MMP-7) in humans are associated with aging (Bonnema et al., 2007); however, the mechanisms by which MMP may modulate cardiac aging are unknown. It was noted that MMP-2 mRNA expression declined with age in BN hearts, with toluene producing no effect. Consistent with our finding, MMP-2 mRNA and activity in cardiac tissues was shown to decline with age in rats (Robert et al., 1997) and also in cardiomyopathic hamsters (Masutomo et al., 1999). The role of each MMP in aging needs to be further investigated.

It was presumed that some mitochondrial carriers are impaired upon aging, leading to a decline in membrane potential, possibly because of reduced energy production by cardiomyocytes (Sastre et. al., 2003). Toluene exposure was shown to alter ion channel function in neuronal cells (Magnusson et al., 1998; Shafer et al., 2005). Therefore, gene expression of the potassium channel protein kv1 and the L-type calcium channel protein, which might reflect the level of activity of these proteins, was analyzed. Decreased mRNA expression of these channel proteins in 24- relative to 4-mo rats suggests a transcriptional effect of toluene on channel genes; mRNA expression was, however, not affected by toluene exposure.

The aging heart undergoes structural and functional alterations leading to a compensatory hypertrophy and ultimately atrophy (Suh et al., 2003). The increase in cardiac inflammation, lesion frequency, and upregulation of inflammatory genes in aged rats is in agreement with the higher risk for age-related cardiovascular diseases in humans (Edelberg et al., 2004). This level of cardiac impairment was not expected to be enhanced by toluene exposure; however, wet cardiac weights were quantitatively reduced by toluene exposure. The mechanism of this reduction is uncertain, but one can postulate that reduction in cardiac tissue fluid retention may relate to the effect of toluene on cell membranes (Magnusson et al., 1998).

Physiology

It was assumed at the onset that the autonomic response of the BN strain would be similar to that of other rat strains dosed with toluene. However, compared to the LE rat, the cardiovascular response of the BN rat to toluene was relatively weak but the thermoregulatory response was exacerbated. LE rats monitored by radiotelemetry underwent a marked elevation in heart rate and blood pressure that persisted for up to 6 h after oral toluene (Gordon et al., 2007). For example, heart rate of LE rats dosed with 0.8 g/kg toluene increased by over 100 beats/min within 30 min and remained elevated by 25–50 beats/min for approximately 6 h. In comparison, heart rate of 4-mo-old BN rats dosed with 1.0 g/kg toluene increased by approximately 50 beat/min and was fully recovered within 2 h. In addition, the core temperature response to toluene in the LE rat differs considerably from the BN strain; a

toluene dose of 1.2 g/kg led to <0.5°C decrease in core temperature within 1 h post dosing in the LE strain. This was followed by a prolonged rise in temperature that persisted for several hours after dosing. The acute hypothermic response of the 4-mo-old BN rat was more than double that of the LE strain. Interestingly, both strains exhibited equivalent elevations in motor activity following acute toluene exposure. Overall, the marked difference in cardiovascular and thermoregulatory response to toluene between the two rat strains was unexpected. It is also of interest to note that basal heart rate of the BN rat over a 24-h period is much lower than for other commonly used strains, including the LE, Sprague-Dawley, Fischer 344, and Wistar strains (Gordon & Watkinson, 1995). On the other hand, the body temperatures and circadian rhythms were similar between strains.

The rightward shift in the toluene heart-rate dose response in the aged rats suggests the possibility of a breakdown in the cardiovascular response to an environmental insult. Studies in aged rats have showed impairment in baroreflex control of heart rate and blood pressure. For example, 24-mo-old Wistar rats displayed a 49% reduction in the tachycardic response to methylscopolamine compared to 10-wk-old animals but no difference in response to propranolol, a drug that blocks sympathetic tone (Irigoyen et al., 2000). The incidence of cardiac arrhythmias is increased in aged rats exposed to particulate, gaseous, and concentrated air particles (Elder et al., 2007; Nadziejko et al., 2004).

The longer hypothermic response to toluene in the aged rats may have resulted from impaired thermoregulatory response to toluene. A moderate hypothermic response was shown to improve recovery and survival to a variety of toxicants (Gordon, 2005). Moderate hypothermia reduces toxicity through a variety of mechanisms. Hence, one might argue that the hypothermic response in the aged animals may be a means of reducing susceptibility to the toxicant. On the other hand, the higher brain levels of toluene in the aged animals may have led to a more prolonged hypothermic response in the aged rats. As rodents age, their ability to control body temperature under resting conditions remains preserved but their ability to withstand cold and heat stress is compromised (Gordon, 1993). The ability to develop a fever in response to an injected pyrogen is also attenuated with aging (Buchanan et al., 2004). The cardiovascular response of aged rats (24 mo) to acute hypothermia was inadequate compared to younger animals (Helwig et al., 2006). If the hypothermic response to toluene is a result of impairment in thermal effectors for controlling heat production and heat loss, then one might expect that the aged rat would be more affected by toluene than younger animals, resulting in a more prolonged hypothermic response. In addition, the attenuated tachycardic response to toluene may well represent a weakened cardiovascular response to toluene-induced hypothermia in the aged rat (Figure 7 C).

In conclusion, the pharmacokinetic, biochemical, and physiological studies provide insight into the susceptibility of the aged rodent to a toxicant. There were significant differences in the autonomic response to toluene in aged rats that were manifested at a time with no significant differences in brain and blood toluene levels. These impaired autonomic responses (i.e., hypothermia and lack of tachycardia) may have affected the clearance of toluene, which, by 4 h post dosing, was significantly different in young versus aged animals. The significant differences in expression of cardiac biomarkers at 4 h may reflect increased brain/blood levels of toluene. The use of pharmacokinetics along with determination of biochemical markers and physiological responses strengthens the understanding of how aging increases susceptibility.

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