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Pyridoxine deficiency modulates benzene inhalation-induced hematotoxicity associated with hepatic CYP2E1 activity in $B_6C_3F_1$ mice

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ABSTRACT

Pyridoxine is a co-factor in many enzymatic reactions and impacts of deficiency have been observed in affected populations. A possible modifying effect of pyridoxine deficiency on benzene toxicity was assessed in male $B_6C_3F_1$ mice fed either a pyridoxine-deficient diet or a control diet. This treatment was combined with benzene inhalation exposure (100 ppm) or no benzene treatment. Pyridoxine-deficient mice exposed to 100 ppm benzene had significantly lower body, thymus and spleen weights. While total white blood cell counts, percentage of lymphocytes, hematocrit and hemoglobin levels were lower, the percentage of neutrophils was significantly higher in deficient and benzene-exposed mice compared to non-exposed controls. Hepatic CYP2E1 protein expression and activity in the deficient and exposed mice were also significantly higher compared to the non-exposed controls. A significant correlation between CYP2E1 activity and several hematological parameters was observed. These results demonstrated that pyridoxine deficiency significantly impacted benzene-induced hematotoxicity. Moreover, the observed agonistic effect of pyridoxinedeficiency and benzene inhalation exposure on CYP2E1 would seem to indicate an involvement of metabolism, but this needs to be further assessed.

1. Introduction

Pyridoxine deficiency is a nutritional problem in children, adolescents, pregnant women and vegetarians, particularly in developing countries [1–9]. Pyridoxine deficiency is usually caused by insufficient intake or malabsorption. The Recommended Dietary Allowance (RDA) for pyridoxine is 1.3 mg/day for adults (19–50 years), 1.9 mg/day during pregnancy, and 2.0 mg/day during lactation [10]. In the United States, most children, adolescents, and adults consume the recommended amount of pyridoxine, with the average intake being approximately 1.5–2.0 mg/day. However, when measured, 11 % of pyridoxine supplement users and 24 % of people who did not take supplements had low plasma pyridoxine concentrations [11]. A study in a Korean population reported that the mean dietary intake of pyridoxine is 1.9 ± 0.6 mg/day. Nevertheless, about 60 % of Korean adults in this study had plasma pyridoxine levels lower than 20 nmol/L, indicating a biochemical deficiency of pyridoxine; about 20 % of the same population had a marginal pyridoxine-deficient status [12].

Pyridoxine deficiency causes biochemical changes that are associated with microcytic anemia, dermatitis, depression and confusion, as well as a weakened immune system in humans, and seizures in infants [12–16]. Studies on the mechanisms by which pyridoxine deficiency causes toxic effects have shown that pyridoxine is required for the synthesis of δ-aminolevulinic acid, a precursor for heme synthesis. As a result, pyridoxine deficiency could give rise to hypochromic-microcytic anemia, which is characterized by smaller red blood cells with shorter life spans, and lower levels of hematocrit and hemoglobin [17–19].

In experimental animals, a deficiency in pyridoxine impacts growth and development, causes hematotoxicity, decreases white blood cell counts and impairs both humoral and cell-mediated immunity [20–23]. In addition, a deficiency of pyridoxine affects the activities of multiple pyridoxine-dependent enzymes, including S-adenosylmethionine hydrolase [24], RNA polymerases I and II [25], alanine aminotransferase [4], cystathionine β-synthase and cystathionine γ-lyase [26]. Pyridoxine deficiency also modulates the expression of several genes, including for phenylalanine hydroxylase, aspartate aminotransferase,

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glyceraldehyde-3-phosphate dehydrogenase, glycogen phosphorylase, and β-actin [27], as well as p21 mRNA levels [28].

Pyridoxine deficiency leads to modification of hepatic microsomal enzymes involved in the metabolic activation of toxic chemicals, including some carcinogens. Pyridoxine-deficient rats treated with N-nitrosodimethylamine had lower levels of the serum transaminase enzymes than rats receiving N-nitrosodimethylamine alone [29]; however, an increase in the activities of microsomal N-nitrosodimethylamine demethylase and NADPH cytochrome C reductase was observed [30]. Pyridoxine deficiency increased hepatic cytochrome P450 1A1 (CYP1A1) activity, as well as resulted in a slight increase in hepatic CYP2E1 activity, in male $B_6C_3F_1$ mice [23]. Both hepatic CYP1A1 and CYP2E1 are key enzymes in the metabolic activation of many pro-carcinogens found in the environment, including polycyclic aromatic hydrocarbons (PAHs) and benzene, respectively. Therefore, the modification of such enzymes potentially impacts the resultant toxicity of these chemicals.

Benzene is a known hematotoxic, myelotoxic and carcinogenic agent. It is oxidized in the liver by CYP2E1 to benzene oxide, the initial step in the bioactivation of benzene. Benzene oxide then undergoes non-enzymatic rearrangement to form phenol that is hydrolyzed to benzene dihydrodiol by microsomal epoxide hydrolase [31], with subsequent formation of hydroquinone and catechol, respectively. Hydroquinone and catechol are converted to 1,4-benzoquinone and 1,2-benzoquinone in the bone marrow. These reactive electrophiles and benzene oxide can form covalent bonds with cellular macromolecules, including proteins and DNA [32], and are involved in carcinogenesis by inducing bone marrow cell toxicity [33,34]. Benzene also causes oxidative DNA damage in humans, and levels of a urinary benzene metabolite (*t,t*-muconic acid) and 8-hydroxydeoxyguanosine have been used as biomarkers for the investigation of benzene exposure and early biological changes that could be indicative of increased risk for future disease, respectively [35]. Benzene exposure also induces anemia, neutrophilia, and lymphocytopenia in C57BL/6 mice [36] and increases the incidence of lymphoma and acute myeloid leukemia (AML) in the same animal model [37]. Acute inhalation exposure of CD-1 mice to 100 ppm benzene (6 h/d, 5 d) significantly depressed peripheral leukocyte levels [38] and repeated exposure of $B_6C_3F_1$ mice to the same benzene dose (100 ppm, 6 h/d, 5 d/wk, for 2 wk) decreased peripheral white blood cells numbers and bone marrow cells [39,40]. Lifetime inhalation exposure to 100 ppm benzene resulted in an increased incidence of anemia, neutrophilia, thymic lymphomas, and hematopoietic neoplasms in C57BL/6 mice [41,42].

Sources of benzene exposure are present in both the indoor and outdoor environments, including releases from building materials and furniture, tobacco smoke, paint, incense burning, industrial processes, petroleum refining, waste incineration, automobile service stations, exhaust from motor vehicles, and industrial emissions [43]. The risk of adverse health effects from exposure to benzene in an occupational setting was evaluated and the results showed that workers who were exposed to benzene in a petroleum refinery had a higher risk for health effects compared to service station attendants [44]. There are certain sub-populations with pyridoxine deficiency where exposure to benzene has been reported, *e.g.*, plasma pyridoxal and pyridoxal-5'-phosphate concentrations in cigarette-smoking workers from a middle-income group were significantly lower compared to that in non-smokers [45]. The prevalence of pyridoxine deficiency and suboptimal pyridoxine status in Canadian women were 1.5 % and 10.9 %, respectively [46]. About 37 % of elderly subjects had low pyridoxal-5'-phosphate and more than 50 % of subjects failed to meet the recommended dietary intake [47]. It is therefore essential to study the combined effect of pyridoxine deficiency and benzene inhalation exposure, particularly the ability of pyridoxine deficiency to modify the hematotoxicity of benzene.

Though the effects of pyridoxine deficiency on the hematologic and immune systems have been reported, the concurrent effects of pyridoxine deficiency and benzene exposure on hepatic cytochrome P450 2E1 have not been studied. Our previous study showed that pyridoxine deficiency resulted in an increasing trend of hepatic CYP2E1 enzyme activity [23]. Another study demonstrated increases in rat hepatic CYP2E1 mRNA levels and protein expression following benzene exposure [48]. Understanding this potential interaction with regards to resultant toxicity will help identify those specific populations who are at increased susceptibility for developing adverse health impacts from benzene exposure, which can in turn help raise awareness about the importance of proper personal protection. This study was conducted to help improve our understanding of how pyridoxine deficiency could modify the hematotoxic effects of inhaled benzene.

2. Materials and methods

2.1. Animal model and diet-induced pyridoxine deficiency

Studies were conducted at the Chulabhorn Research Institute Laboratory Animal Center (Bangkok, Thailand), under the supervision of the Institutional Animal Care and Use Committee (IACUC-REF No. 012004). Male, weanling $B_6C_3F_1$ mice were individually housed in polycarbonate cages with wire mesh floor inserts. Animal rooms were maintained under strict hygienic and environmental conditions at a temperature of 23 ± 1 °C and a humidity of 55 ± 10 %, with a 12:12 h light-dark cycle. Weanling mice were acclimated for 2 weeks and then randomly assigned to one of two diet groups fed either a control diet (7 mg pyridoxine-HCl/kg diet, CD) or a pyridoxine-deficient diet (0 mg pyridoxine-HCl/kg diet, PD). The animal diet was prepared according to the American Institute of Nutrition formulation, AIN-93 G [49]. Food and water were provided *ad libitum*. Food intake and body weights were recorded daily, and pyridoxine status was determined at the end of the study using the erythrocyte aspartate aminotransferase activity coefficient (EAST-AC) based on data from weekly monitoring for 13 weeks [23]. An EAST-AC value greater than 1.6 was used as an indicator of pyridoxine deficiency.

2.2. Benzene inhalation exposure

At the end of the 13-week feeding period, mice were randomly assigned to one of four groups, designated CD-0, CD-100, PD-0, and PD-100 (n = 10/group) and were acclimatized for 5 days to nose-only inhalation exposure tubes. Mice in the CD-100 and PD-100 groups were exposed to 100 ppm benzene inhalation for 4 h/d (10:00 AM to 2:00 PM) for 10 consecutive days. The benzene concentration was selected following a range-finding pilot study (0, 10, or 100 ppm for 4 h/d for 10 days) to assess significant changes in the same endpoints selected for assessment of toxicity in this study (*i.e.*, hematology, immune system and bone marrow effects). Treated animals were observed daily, with no abnormal behavior being noted. Benzene-untreated mice (CD-0 and PD-0) were placed in nose-only tubes and exposed to unfiltered house air (T = 23 ± 1 °C, RH = 55 ± 10 %) for 4 h at the same period each day (*via* nose only-exposure tubes). Food and water were provided *ad libitum* except during the exposure periods. The nose-only inhalation exposure system (TSE, Germany) utilized real-time measurements for benzene concentration, relative humidity (50–60 %) and temperature (22−24 °C). Benzene concentration monitored in real-time was maintained at 100 ± 5 ppm throughout the exposures. After a 10-day benzene exposure period, mice were exsanguinated by cardiac puncture (under CO_2 euthanasia) within 24 h of the last exposure.

2.3. Hematological profiles and sample collection

For complete blood counts and white blood cell differential analysis, 300 μL of whole blood collected from each euthanized mouse was transferred into a tube containing K_3EDTA (BD Diagnostics, NJ) and evaluated using an automated blood cell analyzer (CELL-DYNE 3700, Abbott, Abbott Park, IL). The remaining blood (300 μL) was collected in

heparinized tubes. Each liver was quickly isolated from euthanized mice, weighed and transferred into ice-cold 0.9 % NaCl for further hepatic microsomal preparation. The spleen and thymus from each mouse were also aseptically harvested and weighed. The hematological parameters analyzed included total white blood cells, neutrophils, lymphocytes, monocytes, eosinophils, basophils, red blood cells, hemoglobin concentration, hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red cell distribution width (RDW) and platelet count.

2.4. Pyridoxine status

Pyridoxine status for each mouse was determined from the erythrocyte aspartate aminotransferase activity coefficient (EAST-AC) as described previously [23]. Two hundred µl of blood hemolysate was mixed with either 1.98 or 2.0 mL of BAA (50 mM/2.5 mM/150 mM triethanolamine-EDTA-aspartic acid buffer, pH 7.5) to pyridoxine-stimulated and unstimulated tubes, respectively. Then, 50 µL of 11.2 mM nicotinamide adenine dinucleotide (NADH, Roche Diagnostics, Indianapolis, IN) and 50 µL of a 50 µg malate dehydrogenase/mL suspension (Roche Diagnostics) were added to all tubes. Twenty µl of pyridoxal-5'-phosphate (2 mg/mL in BAA; Sigma-Aldrich, St. Louis, MO) was then added to pyridoxine-stimulated tubes. All sample tubes were protected from light and incubated at room temperature for 30 min. Finally, 200 µL of 0.2 M α-oxoglutaric acid (pH 6.8) was added to initiate the enzymatic reaction. The EAST-AC activity was then calculated from the ratio of the reduction of NADH in stimulated to unstimulated samples for 10 min using a UV3100 spectrophotometer at 340 nm (Shimadzu, Kyoto, Japan).

2.5. Hepatic CYE2E1 activity (p-Nitrophenol hydroxylase [p-NPH])

Hepatic CYP2E1 activity was determined as described previously [23]. Briefly, the liver was perfused with ice-cold 0.9 % NaCl and then homogenized in buffer (1.15 % potassium chloride in 0.01 M potassium phosphate, pH 7.4). The liver homogenate was then centrifuged at 9,000 × *g* at 4 °C for 30 min. The resulting S9 fraction supernatant was transferred to a new tube for additional centrifugation at 105,000 x *g* at 4 °C for 1 h. Lastly, the microsomal pellet was resuspended in 0.01 M potassium phosphate and stored at −80 °C. The enzymatic reaction consisted of 1 mM *p*-nitrophenol (Sigma-Aldrich), 0.1 M potassium phosphate buffer (pH 7.4), 0.4 mg hepatic microsomes and water, was incubated at 37 °C for 3 min, and 10 µL of 100 mM NADPH (Sigma-Aldrich) was added before an additional incubation for 10 min. Two hundred µl of 1.5 N perchloric acid was added to terminate the enzymatic reaction (Merck, KGaA, Darmstadt, Germany). The precipitated proteins were discarded by centrifugation at 3,000 x *g* for 10 min and the supernatant was diluted at 10:1 with 10 N NaOH. Spectrophotometry was used to measure hydroxylation of *p*-nitrophenol (*p*-NPH) to *p*-nitrocatechol. The formation of *p*-nitrocatechol was measured using a UV3100 spectrophotometer at 546 nm (Shimadzu, Kyoto, Japan). Hepatic CYP2E1-*p*-NPH activity was calculated and expressed as nanomoles *p*-nitrocatechol formed/min/mg protein, using an extinction coefficient of 10.28 mM^{-1} cm^{-1}.

2.6. Immunoblotting of hepatic CYP2E1

Hepatic CYP2E1 protein expression was determined as described previously [23]. Briefly, hepatic microsomal proteins (5 µg) were denatured with sample buffer, loaded on a 10 % (w/v) SDS-polyacrylamide gel, and then electrophoresed (Mini-Protein III, Bio-Rad, CA). The gel contents were electrophoretically transferred to a PVDF-membrane (Hybond™-P, Amersham). The membrane was blocked in non-fat skimmed milk and incubated at 4 °C overnight with primary antibody at a 1:20,000 dilution (rabbit anti-rat CYP2E1, Chemicon International, Inc., Temecula, CA). After several washes in 0.1 % Tris Buffered Saline with Tween-20, the membranes were incubated with a 1:10,000 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (Chemicon) at room temperature for 1 h. The ECL Plus kit (Amersham) was used for the chemiluminescence reaction and exposure to Hyperfilm ECL™ (Amersham). GS-710 Calibrated-Imaging densitometer and Quantity One Quantitation software (Bio-Rad, Hercules, CA) were used for CYP2E1 protein analysis.

2.7. Statistical analysis

Data were expressed as mean ± SE. The Kolmogorov-Smirnov test was used to test for the normality of all data. To compare the 4 sets of parametric data, the one-way analysis of variance (ANOVA) was used to test the following parameters: EAST-AC, body and organ weights, erythrocyte-related parameters, and platelets using the Fisher's LSD test for post hoc analysis. The Kruskal-Wallis test was used to compare the 4 sets of non-parametric data, *i.e.*, white blood cell parameters, followed by the Mann-Whitney *U* test. The correlations for pyridoxine status, hepatic CYP2E1 activity and hematological parameters were performed using a Pearson Correlation Coefficient test. A *p-value* of *0.05* was considered statistically significant.

3. Results

3.1. Effects on pyridoxine status (EAST-AC values)

Mice fed the pyridoxine-deficient diet (PD-0 and PD-100 groups) had significantly higher EAST-AC values compared to the CD-0 controls ($p < 0.001$), and all values were higher than 1.6, indicating pyridoxine deficiency (Table 1). Inhalation exposure to benzene at 100 ppm did not affect pyridoxine status, as was observed by the lack of a significant difference between the EAST-AC values (PD-100 *vs.* PD-0 mice, $p = 0.48$). Additionally, in CD-100 mice fed the control diet and then exposed to benzene, the EAST-AC values were not significantly different from those of the untreated benzene CD-0 controls.

3.2. Effect on body and organ weights

Pyridoxine deficiency impacted growth and development of the animals during the study period, while there were no statistically significant differences in daily food intake and body weight gains between the CD and PD mice (7.78 ± 0.26 *vs.* 7.74 ± 0.21 g/d and 0.36 ± 0.13 *vs.* 0.01 ± 0.15 g/d, respectively, $p = 0.09$). This was assessed by measuring and comparing the body weights and organ weights of the treatment

Table 1
Erythrocyte-aspartate aminotransferase activity coefficients (EAST-AC), body weights and organ weights of mice fed either a control (CD) or pyridoxine-deficient (PD) diet (mean ± SE, n = 10/group).

Parameters	Control diet (CD)		Pyridoxine-deficient diet (PD)	
	CD-0	CD-100	PD-0	PD-100
EAST-AC	1.36 ± 0.56	1.35 ± 0.06	4.81 ± 0.73[b]	4.82 ± 0.64[b, c]
Absolute weights				
body (g)	31.4 ± 0.7	29.3 ± 0.4[a]	27.1 ± 0.6[b]	25.8 ± 0.5[b]
liver (g)	1.12 ± 0.04	1.06 ± 0.03	1.00 ± 0.03	1.08 ± 0.04
spleen (mg)	63.63 ± 1.33	47.28 ± 1.26[b]	53.60 ± 1.48[b]	45.76 ± 0.97[b, d]
thymus (mg)	28.68 ± 1.15	12.58 ± 0.79[b]	19.29 ± 1.25[b]	12.57 ± 0.56[b, d]

[a], [b] Significantly different from CD-0 controls at a $p < 0.05$ and b $p < 0.001$, respectively.
[c] Significantly different from the benzene-matched CD-100 group at $p < 0.001$.
[d] Significantly different from PD-0 controls at $p < 0.001$.

groups and the controls (Table 1). The pyridoxine-deficient mice (PD-0) had significantly lower body weights than the CD-0 controls (by 14 %, $p < 0.001$). Exposure to 100 ppm benzene inhalation for 10 days resulted in significantly lower body weights in the CD-100 mice compared to the CD-0 controls (by 7%, $p < 0.05$). The benzene-exposed pyridoxine-deficient mice (PD-100) also had significantly lower body weights compared to the CD-0 controls (by 18 %, $p < 0.001$); however, no significant difference was observed between the PD-100 mice and benzene-matched controls (CD-100).

The pyridoxine-deficient mice (PD-0) had significantly lower spleen weights and thymus weights than the CD-0 controls (by 16 %, and 33 %, respectively, $p < 0.001$). However, no statistically significant difference was observed in liver weights between the pyridoxine-deficient group (PD-0) and the CD-0 controls. Though absolute liver weights of the CD-100 and PD-100 mice appeared to be lower than those in the CD-0 controls, those differences were also not statistically significant. The spleen weights of benzene-exposed mice, the CD-100 and the PD-100 mice, were significantly lower than that of the benzene-unexposed CD-0 controls (by 26 % and 28 %, respectively; $p < 0.001$). The benzene-exposed pyridoxine-deficient (PD-100) mice also had significantly lower spleen weights (by 15 %) compared to the PD-0 controls ($p < 0.001$). The PD-100 mice had slightly lower spleen weights than those of their benzene-matched CD-100 counterparts ($p = 0.40$), and the difference in spleen weights in PD-100 and CD-100 *vs.* CD-0 mice was greater than that observed between PD-0 *vs.* CD-0 mice. However, a synergistic effect of pyridoxine deficiency and benzene inhalation exposure on body weight and/or spleen weight was not evident.

A similar trend was observed in thymus weights of the CD-100 and PD-100 mice that were significantly lower than the CD-0 controls (by 56 % in both cases; $p < 0.001$). The benzene-exposed pyridoxine-deficient (PD-100) mice also had significantly lower thymus weights compared to the PD-0 controls (by 35 %, $p < 0.001$), while the difference between the two benzene inhalation-exposed groups (CD-100 *vs.* PD-100) was not statistically significant.

3.3. Hematological effects

Both pyridoxine deficiency and benzene exposure individually resulted in significant hematological effects (Table 2). However, for certain parameters, the combination of pyridoxine deficiency and benzene inhalation exposure (PD-100) produced even greater effects than either treatment alone, suggesting a synergistic effect of the treatments. Pyridoxine deficiency alone (PD-0) significantly decreased total white blood cell (WBC) counts compared to the CD-0 controls (by 30 %, $p < 0.05$), while the PD-100 group exposed to benzene resulted in a significantly lower WBC number than in benzene-untreated PD-0 mice (by 45 %, $p < 0.05$). Benzene inhalation exposure in the CD-100 group resulted in a significant decrease in WBC numbers compared to the CD-0 controls (by 48 %, $p < 0.05$). The greatest reduction in WBC numbers (by 61 %, $p < 0.001$) was observed in the PD-100 group compared to CD-0 controls (Fig. 1A). However, there was not a statistically significant difference in WBC numbers between the two-benzene inhalation-exposed groups (numbers in PD-100 mice were 27 % lower than those in CD-100 mice; $p = 0.32$).

The percentages of neutrophils were not affected by pyridoxine deficiency (PD-0 *vs.* CD-0). Benzene inhalation exposure of mice fed a control diet resulted in significantly higher neutrophil counts compared to the benzene unexposed controls (CD-100 *vs.* CD-0, 2.7-fold higher, $p < 0.05$). However, an even greater effect of benzene exposure was observed in the pyridoxine-deficient mice compared to their respective diet-matched controls (PD-100 *vs.* PD-0, 3.0-fold higher, $p < 0.001$). Interestingly, the combination of pyridoxine deficiency and benzene exposure in the PD-100 mice yielded the greatest effect on neutrophil counts (3.8-fold higher, $p < 0.001$) compared to the CD-0 controls (Fig. 1B). The difference between the two benzene inhalation-exposed groups (CD-100 *vs.* PD-100) was statistically significant ($p < 0.05$).

Table 2
Hematological effects of benzene inhalation exposure in mice fed either a control (CD) or pyridoxine-deficient (PD) diet (mean ± SE, n = 10/group).

Parameters	Control diet (CD)		Pyridoxine-deficient diet (PD)	
	CD-0	CD-100	PD-0	PD-100
White blood cell (K/μl)	3.6 ± 0.4	1.9 ± 0.2[a]	2.5 ± 0.5[a, c]	1.4 ± 0.2[b, d]
Neutrophil (%)	11.1 ± 1.0	30.2 ± 5.0[a]	14.1 ± 1.0	41.6 ± 6.7[b, c, e]
Lymphocyte (%)	81.3 ± 1.4	63.0 ± 5.5[a]	76.8 ± 2.6[a]	48.3 ± 7.2[b, c, e]
Monocyte (%)	3.7 ± 0.5	5.1 ± 0.7	5.2 ± 1.6	6.8 ± 1.1[a]
Eosinophil (%)	1.1 ± 0.3	0.3 ± 0.1[a]	1.7 ± 0.5	0.4 ± 0.1[a]
Basophil (%)	2.7 ± 0.3	1.4 ± 0.2[a]	2.1 ± 0.4	1.9 ± 0.2[a]
Red blood cell (M/μl)	10.2 ± 0.2	9.4 ± 0.1[b]	10.6 ± 0.1	9.3 ± 0.1[b, e]
Hemoglobin (g/dl)	16.9 ± 0.4	15.6 ± 0.2[b]	14.2 ± 0.1[b]	12.5 ± 0.2[b, c, e]
Hematocrit (%)	50.0 ± 1.0	46.4 ± 0.7[a]	41.3 ± 0.4[b]	36.8 ± 0.5[b, c, e]
MCV (fl)	49.0 ± 0.2	49.5 ± 0.2	38.9 ± 0.2[b]	39.6 ± 0.3[b, c]
MCH (pg)	16.6 ± 0.0	16.7 ± 0.1	13.4 ± 0.1[b]	13.4 ± 0.1[b, c]
MCHC (g/dl)	33.9 ± 0.1	33.6 ± 0.1	34.4 ± 0.1[a]	33.8 ± 0.1[d]
RDW (%)	20.1 ± 0.5	19.4 ± 0.5	26.3 ± 0.6[b]	25.3 ± 0.6[b, c]
Platelet (K/μl)	819.1 ± 49.3	925.5 ± 28.9[a]	944.3 ± 33.8[a]	1023.7 ± 32.0[b]

Abbreviations: MCV: mean corpuscular volume, MCH: mean corpuscular hemoglobin, MCHC: mean corpuscular hemoglobin concentration, RDW: red cell distribution width.
[a, b] Significantly different from CD-0 controls at [a] $p < 0.05$ and [b] $p < 0.001$, respectively. [c] Significantly different from benzene-matched CD-100 group at $p < 0.001$.
[d, e] Significantly different from PD-0 controls at [d] $p < 0.05$ and [e] $p < 0.001$, respectively.

Either pyridoxine deficiency (PD-0) or benzene inhalation exposure (CD-100) alone significantly reduced lymphocyte values compared to the CD-0 controls (by 6 % and 22 %, respectively, $p < 0.05$, Fig. 1C). However, benzene inhalation exposure of pyridoxine deficient mice (PD-100) yielded the greatest effect (41 % lower than in the CD-0 controls, $p < 0.001$).

The percentages of circulating eosinophils and basophils were unaffected by pyridoxine deficiency alone, while benzene exposure alone significantly decreased the numbers of eosinophils (by 73 %) and basophils (by 51 %) compared to the CD-0 control ($p < 0.05$) and CD-100 treatment groups. The PD-100 mice had significantly lower numbers of eosinophils (by 63 %) and basophils (by 32 %) compared to the CD-0 controls ($p < 0.05$). The differences in the number of eosinophils between the PD-100 and PD-0 groups ($p = 0.094$), and between the two benzene-exposed groups (CD-100 *vs.* PD-100, $p = 0.568$), were not statistically significant (Fig. 1D). A significant difference in monocyte values was observed only in PD-100 mice compared to benzene-unexposed CD-0 controls ($p < 0.05$).

Red blood cell (RBC) counts were unaffected by pyridoxine deficiency alone, but benzene inhalation exposure in either the CD-100 or PD-100 groups resulted in significantly lower circulating RBC numbers than the values observed in the CD-0 controls (by 8 % and 9 %, respectively, $p < 0.001$). A combination of benzene exposure and pyridoxine deficiency in PD-100 mice also resulted in lower RBC numbers compared to benzene unexposed PD-0 mice ($p < 0.001$). However, the difference between the two benzene-exposed groups (CD-100 *vs.* PD-100) was not statistically significant.

Both pyridoxine deficiency and benzene exposure alone significantly reduced hemoglobin values (by 16 % and 8 %, respectively) and reduced hematocrit values (by 17 % and 7 %, respectively; $p < 0.001$) compared to CD-0 controls. However, the combination of pyridoxine deficiency and benzene inhalation exposure in the PD-100 group had the greatest effect (26 % reduction in both hematocrit and hemoglobin values)

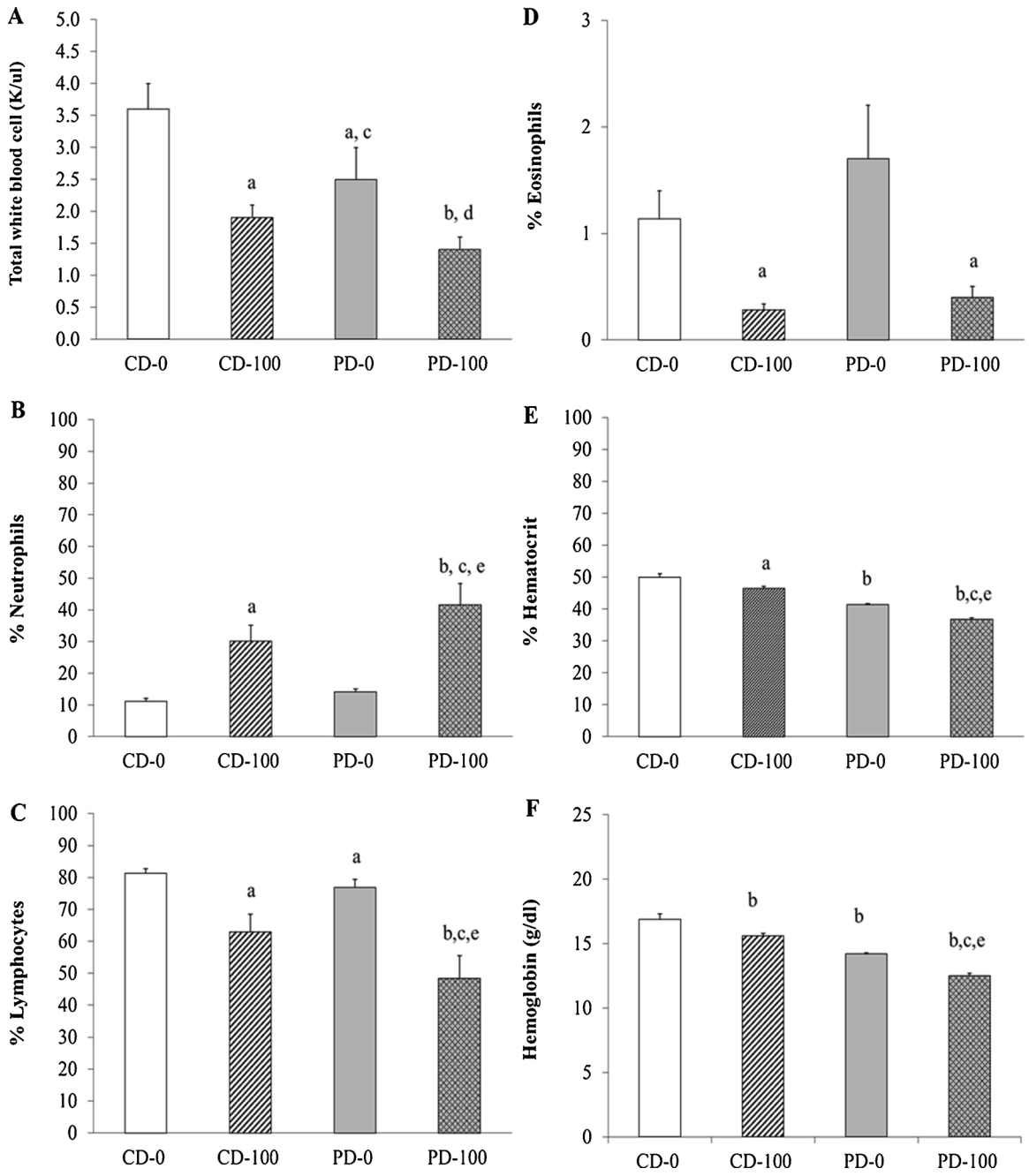

Fig. 1. The effects of pyridoxine deficiency and benzene inhalation exposure (0 or 100 ppm) on white blood cell counts, percentages of neutrophils, lymphocytes and eosinophils, hematocrit and hemoglobin. Values are expressed as mean ± SE (n = 10/group).
[a, b] Significantly different from CD-0 controls at [a] $p < 0.05$ and [b] $p < 0.001$, respectively. [c] Significantly different from benzene-matched CD-100 group at $p < 0.001$. [d, e] Significantly different from PD-0 controls at [d] $p < 0.05$ and [e] $p < 0.001$, respectively.

compared to the benzene unexposed CD-0 mice ($p < 0.001$, Fig. 1E, F).

Pyridoxine deficiency resulted in significantly lower mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCH) values in both the PD-0 and PD-100 mice compared to the CD-0 mice ($p < 0.001$). However, benzene exposure alone (CD-100) did not affect MCV compared to the CD-0 controls and no significant differences in MCV values between the PD-100 and PD-0 mice were observed ($p = 0.050$). Significantly higher mean corpuscular hemoglobin concentration (MCHC) and red cell distribution width (RDW) were observed in pyridoxine-deficient mice (PD-0 *vs.* CD-0, $p < 0.05$ and PD-0 *vs.* PD-100, $p < 0.001$) compared to controls. However, no significant differences in mean corpuscular hemoglobin (MCH) values between the PD-0 and PD-100 mice were observed. Finally, platelet numbers in the PD-0 and CD-100 mice were higher than the CD-0 controls (by 15 % and 13 %, respectively, $p < 0.05$). Overall, the greatest difference in the number of platelets was seen in the PD-100 group compared to those in the CD-0 controls (by 25 %, $p < 0.001$).

3.4. Effect on hepatic cytochrome P450 2E1

3.4.1. CYP2E1 protein expression

CYP2E1 protein expression measured in liver microsomes from each animal is shown in Fig. 2A. Protein band intensities were analyzed in terms of their relative intensity compared to the control values (CD-0) that are adjusted to 100 %. Neither pyridoxine deficiency (PD-0) nor benzene exposure (CD-100) alone had a significant effect on CYP2E1

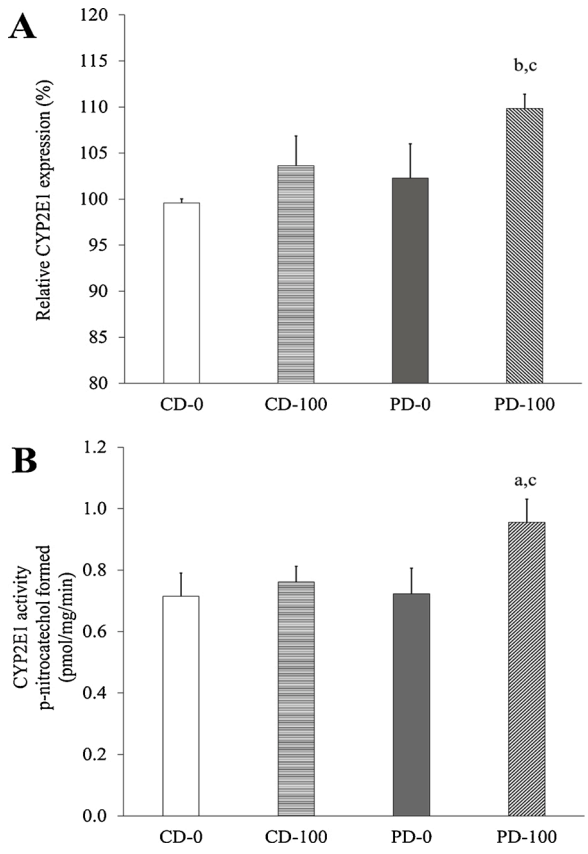

Fig. 2. The effects of pyridoxine deficiency and benzene inhalation exposure (0 or 100 ppm) on hepatic CYP2E1 protein expression and CYP2E1 activity (mean ± SE, n = 10/group). **Panel A**: The level of CYP2E1 expression was measured as the intensity of protein bands, while that of the controls (CD-0) was arbitrarily assigned a value of 100 % and the intensities of the protein bands in other lanes were expressed relative to that of the CD-0 controls samples using densitometry. **Panel B**: The effects of pyridoxine deficiency and benzene inhalation exposure (0 or 100 ppm) on hepatic CYP2E1 activity.
[a, b] Significantly different from CD-0 controls at [a] $p < 0.05$ and [b] $p < 0.001$, respectively.
[c] Significantly different from PD-0 controls at $p < 0.05$.

protein levels compared to the control values (PD-0 *vs.* CD-0, $p = 0.83$ and CD-100 *vs.* CD-0, $p = 0.81$ respectively). In contrast, an effect was observed in the pyridoxine-deficient animals exposed to 100 ppm benzene inhalation (PD-100), resulting in significantly greater CYP2E1 protein levels compared to the unexposed CD-0 and PD-0 animals ($p < 0.001$ and $p < 0.05$, respectively). Although the mean value of CYP2E1 protein expression for the PD-100 group appears to be higher than that of CD-100, the difference was not statistically significant.

3.4.2. CYP2E1 activity

Hepatic CYP2E1 activity in the pyridoxine-deficient mice (PD-0) was not significantly different from that in the CD-0 mice ($p = 0.94$, Fig. 2B). Benzene exposure alone caused slightly higher CYP2E1 activity (by 6 %) in exposed mice; however, this difference was not statistically significant when compared to either the benzene unexposed CD-0 or PD-0 mice ($p = 0.66$ and 0.73, respectively). Hepatic CYP2E1 activity in the benzene exposed PD-100 mice was significantly greater when compared to the unexposed pyridoxine-deficient (PD-100 *vs.* PD-0) mice (by 32 %, $p < 0.05$). In addition, 100 ppm benzene exposure of the pyridoxine-deficient mice (PD-100) resulted in significantly higher hepatic CYP2E1 activity as compared to that in the benzene unexposed CD-0 mice (by 34 %, $p < 0.05$).

3.5. Correlation between CYP2E1 and pyridoxine status for hematological parameters

The possible correlations between hepatic CYP2E1 activity and pyridoxine status (EAST-AC) for hematological parameters were determined by regression analysis (Table 3). For CYP2E1, results showed that the total white blood cell counts, percentage of lymphocytes, hemoglobin, and percentage of hematocrit were negatively correlated with CYP2E1 activity ($p < 0.05$), while the percentage of neutrophils and monocytes were positively correlated with hepatic CYP2E1 activity ($p < 0.05$).

In terms of pyridoxine status, only the red blood cell-related hematological parameters were significantly correlated, *i.e.*, hemoglobin, percentage of hematocrit, mean corpuscular volume (MCV), and mean corpuscular hemoglobin (MCH) were negatively correlated with EAST-AC values ($p < 0.001$), while red cell distribution width (RDW) was positively correlated. However, no statistically significant correlation was observed between pyridoxine deficiency and white blood cell parameters.

4. Discussion and conclusion

EAST-AC levels have been widely used as an indicator of pyridoxine status in animal and human studies [3,8,23,50–52]. Administration of a pyridoxine-deficient diet for 13 weeks significantly increased EAST-AC values and decreased hemoglobin and hematocrit by 18 and 22 %, respectively [23]. In this study, pyridoxine deficiency resulted in increased EAST-AC levels (> 1.6) in the pyridoxine-deficient mice unexposed to benzene (PD-0; 4.81), as well as in the pyridoxine-deficient mice exposed to 100 ppm benzene through inhalation (PD-100; 4.82). Interestingly, exposure to 100 ppm benzene (*i.e.*, PD-100 *vs.* PD-0) did not affect pyridoxine status. This could indicate that any potential interaction among the two factors in downstream toxicity does not involve benzene modifying pyridoxine status. Importantly, while it was observed that diet-induced pyridoxine deficiency in these experimental animals resulted in lower body weight gains and organ weights compared to the controls, this was not statistically significant. Nevertheless, this is similar to observations in other studies [20–22,24,26,53–55].

Both pyridoxine deficiency alone (PD-0 mice) and benzene exposure alone (CD-100 mice) resulted in decreased body weights and lower spleen and thymus weights when compared to the CD-0 controls. Benzene exposure in the pyridoxine-deficient mice (PD-100) also resulted in

Table 3
Correlations between hepatic CYP2E1 activity or pyridoxine status (EAST-AC) and hematological parameters.

Hematological Parameters	CYP2E1 activity		EAST-AC	
	r	*P value*	*r*	*P value*
White blood cell (K/μl)	**-0.496***	**0.002**	−0.218	0.189
Neutrophil (%)	**0.334***	**0.040**	0.096	0.567
Lymphocyte (%)	**-0.383***	**0.018**	−0.154	0.357
Monocyte (%)	**0.356***	**0.028**	0.205	0.216
Eosinophil (%)	−0.159	0.341	0.055	0.744
Basophil (%)	0.225	0.174	−0.033	0.842
Red blood cell (M/μl)	−0.281	0.079	0.054	0.742
Hemoglobin (g/dl)	**-0.366***	**0.020**	**-0.602****	**0.000**
Hematocrit (%)	**-0.347***	**0.028**	**-0.620****	**0.000**
MCV (fl)	−0.200	0.215	**-0.705****	**0.000**
MCH (pg)	−0.227	0.159	**-0.714****	**0.000**
MCHC (g/dl)	−0.170	0.293	0.261	0.104
RDW (%)	0.168	0.300	**0.599****	**0.000**

Abbreviations: MCV: mean corpuscular volume, MCH: mean corpuscular hemoglobin, MCHC: mean corpuscular hemoglobin concentration, RDW: red cell distribution width.
*Statistically significant at $p < 0.05$.
**Statistically significant at $p < 0.001$.

lower body weights and spleen and thymus weights compared to the CD-0 controls. However, there was no significant difference between the two benzene-exposed groups (CD-100 and PD-100). This finding suggests that there are no synergistic effects of diet-induced pyridoxine deficiency and benzene inhalation exposure on animal body weight or organ weights.

Pyridoxine deficiency causes hypochromic-microcytic anemia in experimental animals, a specific type of anemia that is characterized by low MCV, small RBC and hypochromia in blood smear samples [56–58]. Our study showed similar results, where pyridoxine deficiency significantly reduced hematocrit, hemoglobin and MCV values in both the PD-0 and PD-100 mice compared to the CD-0 controls, while benzene exposure alone (CD-100) significantly reduced hemoglobin and hematocrit values in exposed mice. The combination of pyridoxine deficiency and benzene exposure in the PD-100 group had the greatest effect on reducing hematocrit and hemoglobin levels compared to the CD-0 controls, suggesting a synergistic effect of pyridoxine deficiency on benzene hematotoxicity. The hematotoxic effects of pyridoxine deficiency observed in this study were similar to those noted in a previous study [23]. However, hematotoxicity resulting from the combination of pyridoxine deficiency and benzene exposure, particularly on expression and activity of CYP2E1 in response to benzene exposure, is of great interest.

Benzene is well-known to induce hematotoxicity, bone marrow toxicity and hematopoietic neoplasia in humans and mice [59–61]. It causes cancer in humans, namely acute myelogenous leukemia (AML), and is associated with an increased risk of lung cancer [59,62]. The hematotoxicity of benzene has been observed as a decreased production of peripheral erythrocytes, leukocytes and platelets that causes anemia, leukopenia, and thrombocytopenia, which can be called pancytopenia and, in more severe cases, aplastic anemia [59].

In this study, benzene inhalation exposure for 10 days resulted in lower total white blood cell counts, percentages of lymphocytes, eosinophils, and basophils, as well as decreased red blood cell counts, hemoglobin and hematocrit levels, and higher percentages of neutrophils and platelets. The hematotoxic effects observed in this study are similar to those reported elsewhere that showed that benzene is a hematotoxin and induces anemia, lymphocytopenia, and neutrophilia in mice by disruption of granulocytic and erythroid progenitor cells in the bone marrow [41,63]. In other animal studies, exposure to 100 ppm benzene through inhalation for 2 weeks decreased hemoglobin concentration, platelet count, and percentage of lymphocytes and eosinophils in mice [64], while chronic intermittent inhalation exposure to benzene at 100 and 300 ppm (6 h/d, 5 d/wk, 26 wk) increased incidence of myeloid leukemia compared to the sham controls [37]. Inhalation exposure to 100, 300, and 400 ppm benzene in CBA/Ca mice produced dose-dependent decreases in blood lymphocytes and cellularity of bone marrow, the target organ for benzene-induced hematotoxicity [65].

Possible mechanisms for benzene-induced toxicity have been reported [31]. Hepatic CYP2E1 enzyme levels appear to be required for the oxidation of benzene to express hematotoxicity, myelotoxicity and genotoxicity. Although the production of benzene metabolites initially occurs in the liver, benzene toxicity is primarily expressed in the bone marrow [31]. Benzene is reported as a weak CYP2E1 enzyme inducer, with relatively high exposure levels of benzene (300 ppm; 6 h/d for 2-wk) required to increase bone marrow CYP2E1 gene expression by 2.3-fold compared to control mice [61]. These results support our findings. In our study, neither exposure to 100 ppm benzene nor diet-induced pyridoxine deficiency alone strongly induced hepatic CYP2E1. However, a combination of pyridoxine deficiency and exposure to 100 ppm benzene resulted in significantly higher levels of hepatic CYP2E1 protein expression, as well as higher levels of CYP2E1 activity that were concomitant with a significant difference in hematological parameters. Additionally, statistically significant correlations between hepatic CYP2E1 activities and hematological parameters were observed. These results would seem to support the hypothesis that pyridoxine deficiency can affect the CYP2E1-dependent metabolism of benzene and thus possibly contribute to increased hematotoxicity.

While the exact mechanisms underlying benzene-induced hematotoxicity are not yet fully understood, benzene metabolism is a proposed mechanism. Although bone marrow is the primary target of benzene toxicity, the effects of pyridoxine on RBC-related parameters have been shown in other studies [62,65]. We propose that a possible mechanism for the interaction of diet-induced pyridoxine-deficiency and benzene exposure involves induction of hepatic CYP2E1 protein expression and activity. This is based on our observation that while neither factor alone resulted in significant induction of CYP2E1 protein expression and/or activity, a combination of both factors resulted in a significant induction. As such, the results herein seem to indicate an increased susceptibility to the hematotoxic effects of benzene for people with pyridoxine deficiency. Meanwhile, for countries and sub-populations where pyridoxine deficiency is an issue, an increased awareness is needed for the supplementation of pyridoxine in the diet and/or the minimization of exposure to high concentrations of benzene to minimize risks of serious health effects. Monitoring of specific health endpoints related to benzene toxicity in these populations might also be recommended. Clearly, additional studies are needed to confirm the mechanistic basis for the observed synergistic effect, *i.e.*, the levels of urinary benzene metabolites as biomarkers of benzene exposure, phase II hepatic enzyme activity, and analysis of cDNA microarrays for gene expression.

In conclusion, the combination of diet-induced pyridoxine deficiency and exposure to 100 ppm benzene by inhalation resulted in significantly greater effects on certain hematological parameters than either treatment alone. Diet-induced pyridoxine deficiency enhanced the hematotoxic effects of benzene exposure on peripheral blood cells, as well as increased the severity of anemia. The mechanism(s) for this apparent synergy is currently unknown. However, there is evidence to suggest that the two factors, *i.e.*, pyridoxine deficiency and benzene, share potential target tissues, and the agonistic effect of pyridoxine deficiency and benzene inhalation exposure on CYP2E1 observed in this study would seem to indicate an involvement of metabolism, but this needs to be further assessed.

Author statement

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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