Different Renal Chronotoxicity of Bromobenzene and Its Intermediate Metabolites in Mice

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Bromobenzene (BB) is known to pose a serious threat to human health. We previously demonstrated that BB showed chronotoxicity, that is, daily fluctuations in the severity of hepatotoxicity induced in mice. Although BB showed mild nephrotoxicity, a daily fluctuation was not observed in this toxicity. This might be attributed to the fact that BB-induced chronotoxicity is observed only in the liver and not in the kidneys and that the damage caused by BB is prominent in the liver, masking the daily fluctuation in nephrotoxicity. To confirm these two possibilities, we examined the daily fluctuations in nephrotoxicity due to BB intermediate metabolites that target the kidneys: 3-bromophenol, bromohydroquinone, and 4-bromocatechol. Mice were injected with 3-bromophenol, bromohydroquinone, or 4-bromocatechol intraperitoneally at six different time points in a day (zeitgeber time (ZT): ZT2, ZT6, ZT10, ZT14, ZT18, or ZT22). Mortality was monitored for 7 d post-injection. Mice were more sensitive to the acute toxicity of these metabolites around at ZT14 (dark-phase) exposure than around at ZT2 (light-phase) exposure. Furthermore, mice administered with a non-lethal dose of 4-bromocatechol showed significant increases in the levels of plasma blood urea nitrogen and renal malondialdehyde at ZT14 exposure. Moreover, glutathione peroxidase-4, a ferroptosis indicator, was attenuated at ZT14 exposure. These results indicate the toxicity of BB metabolites was higher during the dark-phase exposure, and demonstrate the reason why the diurnal variation of nephrotoxicity by BB was not observed in our previous report is that renal damage was masked due to severe hepatic damage.

Key words bromobenzene; diurnal variation; circadian rhythm; chronotoxicity; kidney

INTRODUCTION

Bromobenzene (BB) is a general industrial reagent that is usually used as a raw material for the synthesis of pharmaceuticals, dyes, and pesticides.1,2) BB is an organic pollutant that damages the natural environment and poses a serious threat to human health. One study state that BB induces severe hepatotoxicity and mild renal toxicity in mice.3) Although BB itself, a non-metabolite form, possesses low toxicity, its intermediate metabolites have toxic effects on the liver and kidneys. Hepatic CYP450s, such as CYP1A2, CYP1B1, and CYP2E1, metabolize BB to four metabolites: 3-bromophenol (3-BrP), bromohydroquinone (BrHQ), 4-bromocatechol (4-BrC), and BB-3,4-oxide. BB-3,4-oxide exhibits high hepatotoxicity and the other metabolites show nephrotoxicity,4–6) indicating that these metabolites have different target organs.

In this study, we targeted “chronotoxicology” as we have previously suggested the relationship between the administration timing and the toxicity severity of chemicals.7–9) Our previous study demonstrated notable daily fluctuations in the severity of toxic responses to BB in mice, i.e., mice were more tolerant to BB-induced hepatic injury during the dark-phase exposure (2:00) than during the light-phase exposure (14:00).8) Although BB showed mild nephrotoxicity, chronotoxicity was not observed in the kidneys. Thus, BB-induced chronotoxicity was possibly observed only in the liver and not in the kidneys.

In our previous study, streptomycin, an antibiotic known to induce defined nephrotoxicity, showed clear diurnal variation in the induced nephrotoxicity in mice.9) Based on the data available, we hypothesized that the chronotoxicity caused by BB is prominent in the liver rather than in the kidneys, thus masking the chronotoxicity in the latter organ. The metabolites of BB generated through various metabolic pathways occur in low concentrations and thus might contribute to negligible renal damage; owing to this, BB metabolite-induced nephrotoxicity cannot be estimated clinically. In this study, we examined the diurnal variation in the nephrotoxicity of BB intermediate metabolites: 3-BrP, BrHQ, and 4-BrC (Fig. 1).

MATERIALS AND METHODS

Animal Treatment Male ICR mice were purchased from...
Japan SLC Inc. (Shizuoka, Japan) and were maintained under standard conditions of controlled temperature (24 ± 1 °C), humidity (55 ± 5%), and light (12:12-h light/dark cycles, lights on at 08:00), with free access to water and food. Experimental treatments were conducted using 7-week-old animals. After completion of the experiments, the surviving mice were sacrificed using pentobarbital. All experiments were approved by the Institutional Animal Care and Experiment Committee of Kinjo Gakuin University.

Experimental Protocol For the mortality assay, 7-week-old male ICR mice were received a single intraperitoneal (i.p.) injection of 500 mg/kg of 3-bromophenol (3-BrP), 180 mg/kg of bromohydroquinone (BrHQ), or 220 mg/kg of 4-bromocatechol (4-BrC) at ZT2, ZT6, ZT10, ZT14, ZT18, or ZT22. Survival was noted at 7 d after injection in each group (A, C, E). Mean survival times (MSTs, expressed as days) were estimated until 7 d after the injection by Kaplan–Meier analysis (B, D, F). (A, D) Data for 3-BrP; (B, E) data for BrHQ; (C, F) data for 4-BrC.

Fig. 2. Diurnal Variation in BB Intermediate Metabolite-Induced Mortality

Male ICR mice (n = 5) were intraperitoneally injected with 500 mg/kg of 3-bromophenol (3-BrP), 180 mg/kg of bromohydroquinone (BrHQ), or 220 mg/kg of 4-bromocatechol (4-BrC) at ZT2, ZT6, ZT10, ZT14, ZT18, or ZT22. Survival was noted at 7 d after injection in each group (A, C, E). Mean survival times (MSTs, expressed as days) were estimated until 7 d after the injection by Kaplan–Meier analysis (B, D, F). (A, D) Data for 3-BrP; (B, E) data for BrHQ; (C, F) data for 4-BrC.
Western Blot Analysis  Protein samples (50 µg) extracted from the kidneys were separated via 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. 10) The primary antibodies used were as follows (all diluted 1:1500): mouse receptor-interacting protein (RIP) 1 monoclonal antibody, mouse RIP3 monoclonal antibody, and mouse glutathione peroxidase 4 (GPX4) monoclonal antibody (Santa Cruz Biotechnology, Dallas, TX, U.S.A.); rabbit cleaved caspase-3 polyclonal antibody (Cell Signaling Technology); and mouse β-actin monoclonal antibody (Medical & Biological Laboratories Co., Ltd., Aichi, Japan).

RESULTS AND DISCUSSION

In this study, we compared the chronotoxicity of three BB intermediate metabolites (3-BrP, BrHQ, and 4-BrC) that are known to induce renal toxicity. We first investigated the effect of injection time on BB intermediate metabolite-induced mortality. Figure 2 shows the number of surviving animals and the mean survival time (MST) 7d post-injection. When 3-BrP was administered at ZT10 and ZT14, all mice died (0%) within 4h (Fig. 2A). In contrast, when 3-BrP was administered at ZT6 and ZT22, three mice (60%) survived; when administered at ZT2 and ZT18, the number of surviving mice was one and two (20 and 40%), respectively. Following BrHQ administration, death of mice was observed only within 4h after the administration in all ZT groups. At 7d after the administration, no mice survived in the ZT14 and ZT18 groups and three (60%) survived in the ZT2 and ZT6 groups. In the ZT10 and ZT22 groups, the number of surviving mice was two and one (40 and 20%), respectively (Fig. 2B). Following 4-BrC administration, all the mice died within 3d when dosed at ZT14 or ZT18 (0%) and only one or two (20 or 40%) survived in other ZT groups (Fig. 2C). MST was almost consistent with the number of surviving mice in all groups (Figs. 2D–F). These results clearly indicate the lethal chronotoxicity of the three BB intermediate metabolites. Following administration at ZT14 (dark phase), all three metabolites showed high toxicity. Interestingly, the chronotoxicity patterns of these BB metabolites are different from those of BB itself, that is, mice exposed to BB in the dark phase had lower sensitivity to toxicity. 8) We previously reported that streptomycin, which shows nephrotoxicity, was highly toxic following dark-phase exposure and less toxic following light-phase exposure. 9) These findings of the previous studies do not contradict with those of the present study, suggesting that differences in the toxicity of BB and the three BB intermediate metabolites are attributed to their target organs. BB targets the liver, whereas its intermediate metabolites target the kidneys.

(4-BrC) Plasma BUN and AST levels were determined using the BUN Wako Test and transaminase CII-Test Wako (FUJIFILM Wako Chemicals, Osaka, Japan), according to the manufacturer’s instructions. 8) Total renal MDA levels were measured via a colorimetric microplate assay (Oxford Biomedical Research, MI, U.S.A.), according to the manufacturer’s instructions.

We next examined the effect of injection time on the severity of organ toxicity using a non-lethal dose of 4-BrC (165 mg/kg). As early death within 4h was observed with 3-BrP and BrHQ injections, described as above, we only selected 4-BrC for the subsequent experiments. For experimental convenience, we chose ZT2 and ZT14 as injection times. In the ZT2 group, the plasma level of BUN, an indicator of nephrototoxicity, was not increased following 4-BrC exposure; however, BUN level was markedly increased in the ZT14 group (Fig. 3A). In parallel with BUN, we measured the plasma AST level, an indicator of hepatotoxicity. We found that 4-BrC administration did not affect the plasma AST levels both in ZT2 and ZT14 (Fig. 3B), suggesting that 4-BrC was toxic only to the kidneys. Additionally, we estimated the levels of MDA as an oxidative stress indicator in the kidneys. The administration of 4-BrC significantly increased the renal MDA level in the ZT14 and ZT2 groups. However, MDA level in the ZT2 injection group was significantly lower than that in
mainly mediated necrosis. To investigate the type of cell death induced by 4-BrC, we analyzed the levels of proteins related to cell death: cleaved caspase-3 for apoptosis, RIP1/3 for necrosis, and GPX4 for ferroptosis. No obvious changes were observed in cleaved caspase-3, RIP1, and RIP3 levels in any group, suggesting that apoptosis and necrosis are not induced by 4-BrC (Fig. 4). In contrast, a decrease in GPX4 level was observed after administering 4-BrC at ZT14 (Fig. 4). We did not observe the 4-BrC-induced renal necrosis by histopathological analysis (data not shown). This suggested that 4-BrC concentration used in this study caused mild renal damage and did not lead to necrosis. From these results, it can be suggested that 4-BrC causes ferroptosis, which shows diurnal variation.

In this study, we showed BB intermediate metabolite-induced chronotoxicity in the kidney. However, our previous result showed the BB-induced nephrotoxicity. However, the renal chronotoxicity was not changed between the light phase and the dark phase. Although critical reason in difference of previous and current research is not cleared yet, we think it is occurred to mask the renal chronotoxicity by inducing severe hepatic injury. In the future experiment, we need to measure accumulated amounts of each BB-intermediate metabolites since these amounts might alter by CYP and/or injury levels.

Many biological factors show circadian rhythm in their expression levels. For example, Xu et al. have reported diurnal variation in hepatic antioxidant gene expression in mice. Glutathione (GSH) is an antioxidant that is critical for maintaining health and protecting against toxic compounds. Both 4-BrC and 3-BrP have been reported to deplete the renal GSH level. Ferroptosis was proposed in 2012 as the form of cell death induced by erastin, which inhibits the import of cystine, leading to GSH depletion and GPX4 inactivation. Our results showed that GPX4 was only repressed at ZT14 by 4-BrC. Therefore, GSH-related proteins or genes may be associated with chronotoxicity.

To conclude, our present study demonstrates that BB induces renal damage as well as hepatic damage through its metabolites, such as 3-BrP, BrHQ, and 4-BrC. The reason why the diurnal variation of nephrotoxicity by BB was not observed in our previous report is that renal damage was masked due to strong hepatic damage.

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Conflict of Interest The authors declare no conflict of interest.

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