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Effects of Ethanol and Phenobarbital on Hemoglobin Adducts Formation in Rats Exposed to Benzidine and Direct Black 38

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Abstract: The objective of this study was to evaluate the effects of pretreatment of ethanol (EtOH) and phenobarbital (PB), which are known to affect the metabolism of xenobiotics, in the formation of hemoglobin adducts in rats administered with benzidine (BZ) and Direct Black 38 (DB38). The experimental rats were divided into BZ and DB38 groups; each group was subdivided into control, EtOH, and PB groups. Blood samples were separated into hemoglobin and plasma immediately after obtaining and basic hydrolysis was done to convert the adducts into aromatic amines. Hydrolyzed BZ, monoacetylbenzidine (MABZ), and 4-aminobiphenyl (4ABP) were separated by reversed-phase liquid chromatography without derivatization. Then, quantitative analyses were performed using a high performance liquid chromatograph equipped with an electrochemical detector. The amount of metabolites was expressed in the hemoglobin binding index (HBI). As a result, the formations of hemoglobin in BZ-, MABZ-, and 4ABP-HBI of BZ-EtOH and BZ-PB groups were increased compared with those of BZ-control group. In DB38 group, all of HBIs except for BZ-HBI were increased more than those of DB38-control group regardless of the pretreatment. These results are attributable to the fact that EtOH and PB induced N-hydroxylation is related to the formation of hemoglobin adducts. They indicate that EtOH not only increases the adduct formation by inducing N-hydroxylation but also induces N-acetylation. PB induced N-hydroxylation and increased the adduct formation in BZ group, but decreased the adduct formation in DB38 group due to decreasing azo reduction. These results suggest that the effects of EtOH or PB should be considered in biochemical monitoring of BZ and DB38 for the assessment of intermittent exposure to BZ and DB38.

Key words: Ethanol, Phenobarbital hemoglobin adduct, Benzidine, Direct Black 38

Introduction

Benzidine (BZ) has been known for a long time as a carcinogen for bladder cancer. Recent studies also reported that the incidence of bladder cancer is higher in workers exposed to BZ compared to those who were not exposed[1–6]. The Ministry of Labor in Korea and American Conference of Governmental Industrial Hygienists (ACGIH) has confirmed BZ as an A1 human carcinogen with no occupational exposure limit[7, 8]. Azo dyes used widely as a raw material in the production of fabric, food and cosmetics and are produced in the world at 700,000 ton a year[9]. There are about 200 different benzidine based azo dyes, which are used in many products including color paper, fabric, leather, rubber, plastic, and fur[10]. BZ is a chemical substance absorbed through the skin[11] as Direct Blue 6 and Direct Black 38. A study confirmed that BZ and its metabolites were excreted through urine[12].

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Biochemical assessment on exposure to organic compounds generally includes analyses on urine metabolites, DNA adducts, and hemoglobin adducts[13]. The analysis on hemoglobin adducts is very effective since it not only shows the amount absorbed into the body but also can evaluate individual metabolism characteristics[14]. Furthermore, many studies reported that hemoglobin adducts are useful in the evaluation of exposure[15–17]. The Figure 1 shows that the formation process of adduct of aromatic amine through combination with hemoglobin in the body and degradation process of aromatic amine and hemoglobin by hydrolysis *in vitro*.

Assessment over exposure is not possible through only monitoring working environment for carcinogens that people get exposed to intermittently at low concentrations and could absorb through the skin such as BZ and DB38. Hence, it is critical to find the optimal method of assessment for long-term exposure[18]. The major metabolites of BZ include N-hydroxylation and N-acetylation. These metabolites are formed simultaneously and thus, counteract competitively each other. N-acetylation plays the role of detoxification since it lowers the concentration of N-hydroxyamine, which is a substance that induces genetic toxicity, through the metabolism of amines[19]. Thus, studies reported that the incidence of bladder cancer is high when N-acetylation is low[20–22]. Benzidine azo dye is absorbed through the body through a different BZ metabolic process. After this dye initially dissociates into BZ after azo reduction, it goes through N-hydroxylation and N-acetylation. Ethanol (EtOH) that workers get exposed to frequently due to their drinking habits would inhibit or induce oxidation[23]. and could increase N-acetylation when present together with aromatic amine[24]. On the other hand, phenobarbital (PB) was reported to induce oxidation but inhibit the activity of azo-reductase[25].

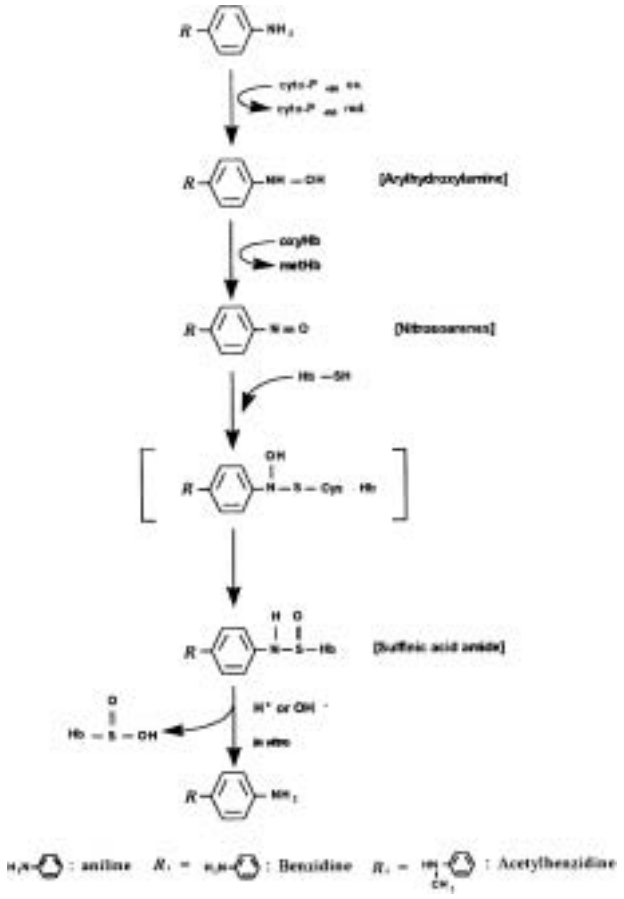

Fig. 1. Reaction which take place in erythrocytes when exposed to aromatic amine.

It is important to evaluate how adduct formation changes due to substances affecting on N-hydroxylation, N-acetylation and azo reduction in order to realize biological monitoring using hemoglobin adducts. In addition, the amount of adduct formed by DB38 could be different when it is exposed to BZ at the same molar amount since DB38, which is the most typical benzidine-based dye, goes through the BZ metabolism pathway after azo reduction[26]. Although many studies were done on hemoglobin adducts of BZ and azo dye[27–29], there has not been a study on comparing BZ and azo dye at the same time or evaluating the effects of exposure to these chemicals at the same time. Especially, the simultaneous experiment is necessary to assess the effect degree of ethanol and phenobarbital on azo reduction of DB38 if considering that airborne exposure of BZ and DB occurred at the same time in case of manufacturing or treating the dye.

The purpose of the present study was to evaluate the effects of hemoglobin adduct formation and N-acetylation with time and effects of DB38 on azo reduction in order to assess the effects of EtOH and PB as positive control on hemoglobin adducts formed with the administration of BZ and DB38 in rats.

Materials and Methods

Experimental groups and doses

The experimental rats were divided into the benzidine group (BZ group) and Direct Black 38 group (DB38 group). Each of these two groups was further subdivided into EtOH pretreatment group (EtOH group), PB pretreatment group (PB group) and control group with no pretreatment. Thus, total 6 groups were investigated and each group included 50 male *Sprague-Dawley* rats between 7 to 8 wk old weighing 230 ± 10 g. The pretreatment dose of EtOH was 1 g/kg body weight, which was 1/10 of the oral LD_{50} of 10.81 g/kg body weight in rats[30]. The pretreatment dose of PB was 80 mg/kg body weight. EtOH and PB diluted in distilled water at 10 ml/kg body weight were orally administered once 24 h

before the administration of BZ and DB38. Only distilled water was administered to the rats in the control group at this time. DB38 was administered orally by dissolving in 1,2-propanediol (Sigma, St. Louis, Missouri) by referring to the one-time oral dose of BZ to compare with the results of hemoglobin adduct formation due to BZ administration. Based on the previous report of Birner *et al*.[27], oral dose of BZ per one time was administered with 0.5 mmol BZ/kg body weight. The DB38 was also administered with same oral dose of BZ in order to objectively compare the difference in adduct formation between BZ and DB38.

Blood samples were obtained from the vena cava from 5 rats in each group before the oral administration of BZ and DB38; and at 30 min, 3 h, 6 h, 9 h, 12 h, 24 h, 48 h, 72 h, 96 h, and 144 h after the administration using heparin treated syringes. RBC were separated immediately after taking blood sample.

Experimental process

Preparation of blood and hydrolysis of hemoglobin adducts were done according to the methods suggested by Sabbioni and Neumann (1990)[16]. After recovering BZ, monoacetylbenzidine (MABZ), and 4-aminobiphenyl (4ABP), the analysis was done using a reversed-phase high performance liquid chromatograph (HPLC; Gilson 715 System, GILSON, Villier le Bel, France) equipped with an electrochemical detector (ICA-5212, TOA Electronics, Tokyo, Japan). The column equipped in the HPLC was HAISIL HL C_{18} (5 μm, 250 × 4.6 mm) and its operation temperature was 35˚C. The mobile phase of BZ and MABZ was Lithium chloride: Methanol (70 : 30) and the mobile phase of 4ABP was 0.2% Lithium chloride: Methanol (50 : 50). The potential values of electrochemical detector were set to 0.65V for BZ, 0.75V for MABZ and 0.85V for 4ABP, respectively. The detection limit was 1.82 ng/ml in BZ, 1.54 ng/ml in MABZ and 0.2 ng/ml in 4ABP, respectively.

The analysis results were expressed with the hemoglobin binding index (HBI) to compare hemoglobin adducts of BZ, MABZ, and 4ABP at the same conditions. HBI was applied to objectively compare each hemoglobin adduct formed through administering BZ and DB38 with same dose under same condition and to assess the effect of EtOH and PB on azo reduction as a function of time. The HBI was the value obtained by dividing the number of mmol of BZ, MABZ, and 4ABP bind to 1 mol of hemoglobin by the amount of BZ or DB38 administered and its simplified formula is as follows.

HBI (hemoglobin adduct/amount administered)
= (mmol cleavage product/mol hemoglobin)
/ (mmol benzidine or Direct Black 38/kg body weight)

We assessed the ratio of N-acetylation with time in order to determine how N-acetylation of hemoglobin adducts changes due to EtOH and PB. The ratio of N-acetylation was obtained by dividing MABZ-HBI by BZ-HBI[29]. We assessed the HBI ratio of DB38 group by HBI of BZ group with time in order to determine the relative effects of EtOH and PB on azo reduction when DB38 and BZ were administered at the same oral dose (0.5 mmol/kg). Kruskal-Wallis test was performed to determine whether a difference was present in the amount of BZ, MABZ, and 4ABP bind to hemoglobin in EtOH group, PB group and control group with time. We used Tukey test, which is the multiple comparison method of ANOVA, after converting the resulting values in the order to determine the difference with each group.

Results

Effects of ethanol and phenobarbital on hemohglobin adducts

Effects of ethanol and phenobarbital in BZ group

No hemoglobin adduct was detected in the samples obtained before the administration of BZ. Table 1 shows BZ-HBI, MABZ-HBI, and 4ABP-HBI with time in BZ-control group, BZ-EtOH group, and BZ-PB group. Other than at 0.5 h, BZ-HBI was higher until 6 h in BZ-control group compared with in BZ-EtOH group and BZ-PB group. It was significantly higher in BZ-control group at 3 h, and in BZ-control group and BZ-EtOH group at 6 h (p<0.05). However, after 9 h, the average BZ-HBI was higher in BZ-EtOH group and BZ-PB group compared with BZ-control group. Significantly high BZ-HBI values were seen at 12 h and 144 h in BZ-EtOH group; and at 12 h, 48 h, 72 h, 96 h, and 144 h in BZ-PB (p<0.05). Other than at 0.5 h, MABZ-HBI was higher in BZ-control group until 6 h and was significantly high at 3 h (p<0.01). However, higher average MABZ-HBI values were seen after 9 h in BZ-EtOH group and BZ-PB group compared with BZ-control group. These values were significantly high in BZ-EtOH group at 48 h and in BZ-PB group at 72 h (p<0.05). 4ABP-HBI could not be obtained at 0.5 h in all groups. The average 4ABP-HBI value was higher after 9 h in BZ-EtOH group and BZ-PB group compared with BZ-control group. Compared with other groups, this value was significantly high in BZ-EtOH group at 12 h, 24 h, 72 h, 96 h, and 144 h; and in BZ-PB group at 3 h, 6 h, 9 h, 12 h, and 24 h (p<0.05).

Effects of ethanol and phenobarbital in the Direct Black 38 administered group

No hemoglobin adduct was detected in blood samples obtained before the administration of DB38. Table 2 shows BZ-HBI, MABZ-HBI, and 4ABP-HBI with time

Table 1. Hemoglobin binding index of cleavage product by ethanol or phenobarbital pretreatment 24 h before benzidine administration in rat

Time (h)	BZ-HBI			MABZ-HBI			4ABP-HBI		
	BZ-control	BZ-EtOH	BZ-PB	BZ-control	BZ-EtOH	BZ-PB	BZ-control	BZ-EtOH	BZ-PB
0.5	0.04 ± 0.02	0.070 ± 0.01	0.03 ± 0.01*	0.91 ± 0.18	1.21 ± 0.22	1.02 ± 0.22	ND	ND	ND
3	0.38 ± 0.01	0.14 ± 0.04	0.18 ± 0.02**	7.10 ± 1.72	3.10 ± 0.73	6.39 ± 1.53**	0.23 ± 0.08	0.13 ± 0.70	0.31 ± 0.08*
6	0.43 ± 0.03	0.40 ± 0.02	0.31 ± 0.02**	10.71 ± 4.97	9.09 ± 1.79	10.72 ± 1.70	0.55 ± 0.12	0.47 ± 0.07	0.77 ± 0.13*
9	0.57 ± 0.06	0.61 ± 0.09	0.61 ± 0.26	12.99 ± 5.07	15.33 ± 5.53	13.09 ± 1.29	0.54 ± 0.09	0.91 ± 0.16	1.37 ± 0.18**
12	0.63 ± 0.08	1.02 ± 0.19	1.07 ± 0.31*	15.81 ± 4.02	20.40 ± 4.05	18.75 ± 4.37	0.82 ± 0.17	1.18 ± 0.16	1.53 ± 0.29**
24	1.18 ± 0.49	1.29 ± 0.42	1.63 ± 0.45	20.02 ± 4.59	31.95 ± 9.74	24.70 ± 7.14	0.64 ± 0.11	1.90 ± 0.20	2.36 ± 0.41**
48	0.66 ± 0.09	0.84 ± 0.130	1.62 ± 0.26**	35.77 ± 5.50	50.01 ± 8.33	38.90 ± 5.01*	2.42 ± 0.20	2.78 ± 0.28	4.32 ± 1.24
72	0.58 ± 0.070	0.65 ± 0.02	1.26 ± 0.81**	29.14 ± 7.42	32.69 ± 8.02	46.37 ± 5.01*	1.71 ± 0.19	2.27 ± 0.30	2.39 ± 1.00*
96	0.49 ± 0.04	0.56 ± 0.07	0.91 ± 0.17**	22.22 ± 7.22	30.90 ± 9.89	36.77 ± 9.49	1.13 ± 0.16	1.68 ± 0.170	1.22 ± 0.17**
144	0.24 ± 0.04	0.47 ± 0.03	0.59 ± 0.21**	12.70 ± 3.92	13.88 ± 3.38	15.69 ± 2.29	0.59 ± 0.13	1.56 ± 0.19	1.19 ± 0.17**

BZ: benzidine; MABZ: monoacetylbenzidine; 4ABP: 4-aminobiphenyl;
HBI: hemoglobin binding index=(mmol cleavage product/mol hemoglobin)/(mmol benzidine/kg body weight);
BZ-control: no pretreatment, administration of 0.5 mmol benzidine/kg body weight only;
BZ-EtOH: pretreatment of 1 g EtOH/kg body weight;
BZ-PB, pretreatment of 80 mg phenobarbital/kg body weight;
*p<0.05 **p<0.01 by Kruskal-Wallis test; ND: not detected; Mean ± SD (N=5).

Table 2. Hemoglobin binding index of cleavage product by ethanol or phenobarbital pretreatment 24 h before Direct Black 38 administration in rat

Time (h)	DB38-HBI			MABZ-HBI			4ABP-HBI		
	DB38-control	DB38-EtOH	DB38-PB	DB38-control	DB38-EtOH	DB38-PB	DB38-control	DB38-EtOH	DB38-PB
0.5	ND	ND	ND	0.04 ± 0.04	0.04 ± 0.03	0.04 ± 0.04	ND	ND	ND
3	ND	ND	ND	0.04 ± 0.04	0.06 ± 0.07	0.06 ± 0.06	ND	0.13 ± 0.15	ND**
6	ND	ND	ND	0.04 ± 0.05	0.07 ± 0.09	0.05 ± 0.05	0.01 ± 0.01	0.14 ± 0.02	ND**
9	ND	0.01 ± 0.01	ND	0.09 ± 0.12	0.15 ± 0.15	0.10 ± 0.11	0.03 ± 0.02	0.13 ± 0.02	0.01 ± 0.01**
12	0.19 ± 0.13	0.16 ± 0.03	0.11 ± 0.04	0.87 ± 0.35	1.10 ± 0.51	0.90 ± 0.59	2.41 ± 0.47	8.89 ± 1.31	2.767 ± 0.43**
24	0.430 ± 0.34	0.58 ± 0.09	0.12 ± 0.05	1.68 ± 0.15	6.68 ± 4.31	3.31 ± 1.87	2.78 ± 0.21	13.10 ± 1.53	6.27 ± 0.90**
48	0.08 ± 0.05	0.21 ± 0.15	0.10 ± 0.05	0.52 ± 0.28	0.95 ± 0.22	1.00 ± 0.16*	1.98 ± 0.42	11.48 ± 1.46	2.38 ± 0.43**
72	0.05 ± 0.04	0.20 ± 0.15	0.07 ± 0.06	0.49 ± 0.15	0.52 ± 0.21	0.84 ± 0.06*	1.13 ± 0.20	1.55 ± 0.31	1.97 ± 0.39**
96	0.03 ± 0.05	0.10 ± 0.08	0.02 ± 0.01	0.26 ± 0.08	0.56 ± 0.12	0.64 ± 0.13**	0.14 ± 0.03	1.05 ± 0.18	0.84 ± 0.17**
144	0.03 ± 0.04	0.04 ± 0.06	0.01 ± 0.04	0.12 ± 0.11	0.16 ± 0.14	0.14 ± 0.16	0.10 ± 0.06	0.67 ± 0.13	0.01 ± 0.01**

DB38: direct black 38; MABZ: monoacetylbenzidine; 4ABP: 4-aminobiphenyl;
HBI: hemoglobin binding index=(mmol cleavage product/mol hemoglobin)/(mmol Direct Black 38/kg body weight);
DB38-control: no pretreatment, administration of 0.5 mmol Direct Black 38/kg body weight only;
DB38-EtOH: pretreatment of 1 g EtOH/kg body weight;
DB38-PB, pretreatment of 80 mg phenobarbital/kg body weight;
*p<0.05 **p<0.01 by Kruskal-Wallis test; ND: not detected; Mean ± SD (N=5).

in DB38-control group, DB38-EtOH group and DB38-PB group (Table 2). BZ-HBI could not be obtained in DB38-control group and DB38-PB group at 9 h; and in DB38-EtOH group at 6 h. This value was high in DB38-control group only at 12 h. At all other times, the average BZ-HBI value was higher in DB38-EtOH group. Other than 0.5 h, the MABZ-HBI value was higher at all other times in DB38-EtOH group and DB38-PB group compared with DB38-control group. This value was significantly high in DB38-EtOH group at 48 h and 96 h; and in DB38-PB group at 48 h, 72 h, and 96 h (p<0.05). 4ABP-HBI could not be obtained in DB38-control group until 3 h, in DB38-EtOH group until 0.5 h, and in DB38-PB group until 6 h. Other than at 72 h, the average 4ABP-HBI value was higher in DB38-EtOH group and DB38-PB group at all times compared with DB38-control group. This value was significantly high at 3 h, 6 h, 9 h, 12 h, 24 h, 48 h, 96 h, and 144 h in DB38-EtOH group; and at 24 h, 72 h, and 96 h in DB38-PB group (p<0.01).

Effects of ethanol and phenobarital on N-acetylation

Changes of N-acetylation in BZ group

Although the ratio of N-acetylation was between 16.92 to 25.02 between 0.5 h to 24 h in BZ-control group, it

increased significantly after 24 h with the highest value of 54.15 at 48 h. The ratio of N-acetylation showed no significant changes in BZ-EtOH group with the value being between 17.37 to 25.08 between 0.5 to 24 h. However, it increased significantly after 24 h with the highest value being 59.41 by 48 h. This N-acetylation ratio was higher in BZ-PB group at 0.5 h, 3 h, and 6 h compared with BZ-control group and BZ-EtOH group. Contrarily, this ratio was lower in BZ-PB group after 24 h compared with BZ-control group and BZ-EtOH group with the highest ratio being 40.28 at 96 h (Fig. 2).

Changes of N-acetylation in DB group

The highest ratio of N-acetylation was 10.30 at 72 h in DB38-control group; was 11.56 at 24 h in DB38-EtOH group; and was 28.03 at 24 h in DB38-PB group (Fig. 3).

Effects of ethanol and phenobarbital on azo reduction of DB 38

The cases that any HBI was not detected in DB38 group and BZ group were not presented in Table 3. Since the BZ-HBI ratio and MABZ-HBI ratio in control group, EtOH group and PB group (HBI in DB38 group/HBI in BZ group) was lower than 1 at all times, hemoglobin

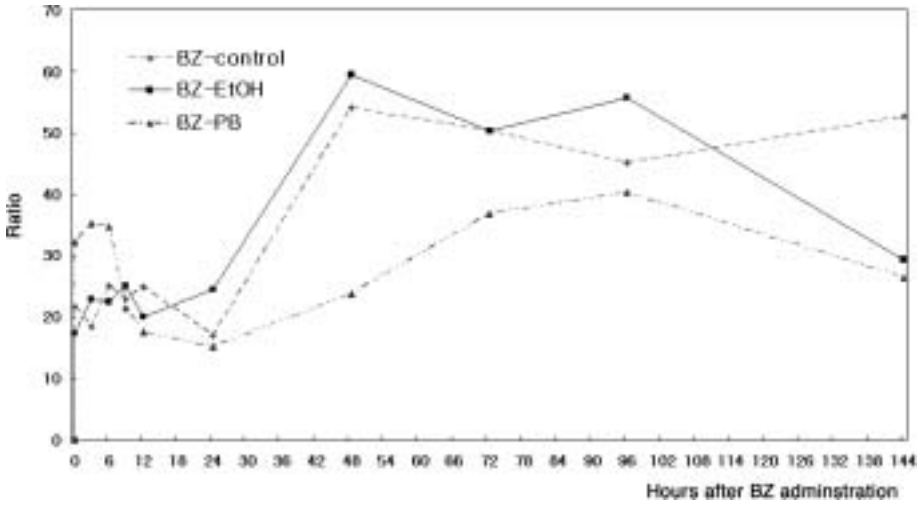

Fig. 2. The ratio of N-acetylation (monoacetylbenzidine-hemoglobin binding index/benzidine-hemoglobin binding index) by ethanol or Phenobarbital pretreatment 24 h before benzidine administration in rat.

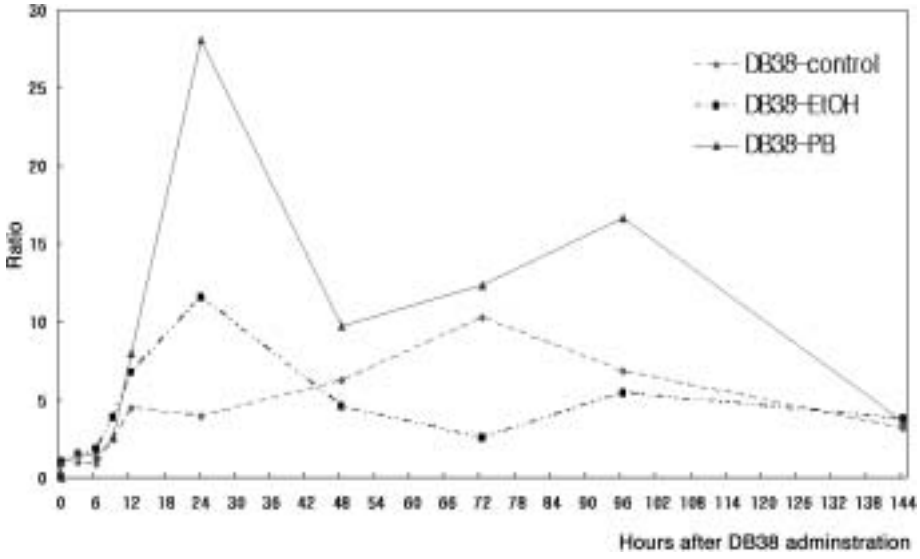

Fig. 3. The ratio of N-acetylation (monoacetylbenzidine-hemoglobin binding index/benzidine-hemoglobin binding index) by ethanol or Phenobarbital pretreatment 24 h before Direct Black 38 administration in rat.

Table 3. The ratio of Direct Black 38 group's HBI to benzidine group's HBI by ethanol or phenobarbital pretreatment 24 h before benzidine or Direct Black 38 administration in rat

Time (h)	Control			EtOH			PB		
	BZ	MABZ	4ABP	BZ	MABZ	4ABP	BZ	MABZ	4ABP
0.5	-	0.0471	-	-	0.0295	-	-	0.0375	-
3	-	0.0050	-	-	0.0184	1.0026	-	0.0095	-
6	-	0.0033	0.0236	-	0.0078	0.2979	-	0.0049	-
9	-	0.0072	0.0597	0.0058	0.0097	0.1457	-	0.0076	0.0005
12	0.3035	0.0548	2.9604	0.1567	0.0540	7.5592	0.1058	0.0480	1.8118
24	0.3605	0.0841	4.3448	0.4474	0.2091	6.9051	0.0724	0.1340	2.6533
48	0.1271	0.0146	0.8158	0.2453	0.0189	4.1363	0.0632	0.0259	0.5499
72	0.0813	0.0167	0.6619	0.3111	0.0158	0.6830	0.0541	0.0181	0.8210
96	0.0643	0.0118	0.1224	0.1869	0.0183	0.6290	0.0263	0.0173	0.6825
144	0.1218	0.0094	0.1637	0.0865	0.0112	0.4262	0.0088	0.0088	0.0110

HBI: hemoglobin binding index=(mmol cleavage product/mol hemoglobin)/(0.5 mmol benzidine or Direct Black 38/kg body weight); Control: no pretreatment; EtOH: pretreatment of 1 g EtOH/kg body weight; PB: pretrearment of 80 mg phenobarbital/kg body weight; BZ: the ratio of Direct Black 38 group's benzidine-HBI to benzidine group's benzidine-HBI; MABZ: the ratio of Direct Black 38 group's monoacetylbenzidine-HBI to benzidine group's monoacetylbenzidine-HBI; 4ABP: the ratio of Direct Black 38 group's 4-aminobiphenyl-HBI to benzidine group's 4-aminobiphenyl-HBI.

adducts were formed less when DB38 was administered compared to when BZ was administered regardless of pretreatment. Especially, the MABZ-HBI ratio was relatively lower compared with the BZ-HBI ratio. However, the 4ABP-HBI ratio was higher than 1 in DB38-control group at 12 h and 24 h; in DB38-EtOH group at 3 h, 12 h, 24 h, and 48 h; and in DB38-PB group at 12 h and 24 h, indicating that formation of hemoglobin adducts in DB38 group was more than in BZ-control, BZ-EtOH and BZ-PB groups (Table 3).

Discussion

Biological monitoring using hemoglobin adducts is being used in ethylene[31, 32], propylene oxide[33], acrylamide[34], vinyl chloride[35], and benzo(a)pyrene[36, 37]. Active studies are being conducted on hemoglobin adducts of nitrosoamine and 4-aminobiphenyl which are related with smoking[38, 39]. Aromatic amines oxidize into arylhydroxyamine through N-oxidation when absorbed into the body. Arylhydroxyamine is converted to nitrosoaren due to oxidation reaction. Nitrosoaren then produces sulfinic acid amide by binding with -SH group of glutathione or -SH group of hemoglobin cysteine[29].

DB38 shows color due to azo bonding(-N=N-) and is synthesized through coupling with azo compounds. The resulting dye is non-volatile and very stable at room[40]. However, when the diazo linkage of dye is broken by an enzyme[41] or heat[42], it converts to an aromatic amine. Dye goes through BZ metabolic process by azo reduction within the body with studies detecting BZ, MABZ and DABZ in urine of DB38 exposed workers[43–45]. We also confirmed BZ and its metabolites binding with hemoglobin after oral administration of DB38 in rats in the present study. There has not been a study on comparing BZ and azo dye at the same time or evaluating the effects of exposure to these chemicals at the same time.

Aromatic amines go through N-hydroxylation in order to bind with hemoglobin. N-hydroxylation is the process in which OH-group is added to amine group[46] and CYP-450 plays a major role during this process[47–51]. CYP 1A1 and 1A2 are the enzymes that are involved in N-hydroxylation of BZ. These enzymes are inhibited by α-naphthoflavone, ellipticine[50], furafylline[52, 53], and 6-methyl-1,3,8-trichlorodibenzofuran[54] and induced by β-naphthoflavone[55, 56], 2,3,7,8-tetrachlorodibenzo-p-dioxine[54], and triethylphosphorothioate[57].

Other than at 3 h and 6 h, the average BZ-HBI was higher in BZ-EtOH group and BZ-PB group compared with BZ-control group. Furthermore, MABZ-HBI was high at all times except at 3 h and 4ABP-HBI was high at all times. We believe that these results were due to EtOH[23, 58]. and PB[25] inducing CYP-450, which participates in N-hydroxylation, increasing hemoglobin adducts formed at the time of BZ administration. The average BZ-HBI formed due to the administration of DB38 was relatively high at all times after 9 h except at 12 h in DB38-control group and at 12 h in DB38-EtOH group. However, unlike BZ-PB group, the average BZ-HBI was lower at 12 h, 24 h, and 144 h in DB38-PB group compared with DB38-control group due maybe to PB not only activating N-hydroxylation but also inhibiting azo reduction[59], which is the initial metabolism process of DB38. Other than at 0.5 h, the average MABZ-HBI in DB38-

EtOH group and DB38-PB group was higher compared with in DB38-control group. The average 4ABP-HBI was also high at all times compared with DB38-control group. As in the case of BZ group, these results were probably due to EtOH and PB increasing hemoglobin adducts with the administration of DB38.

N-acetylation plays the role of increasing solubility by adding acetate group into nitrogen and oxygen in aromatic amine or hydroxyamine[60]. We relatively evaluated the ratio of N-acetylation by dividing MABZ-HBI by BZ-HBI in order to assess N-acetylation occurring in hemoglobin adducts. The N-acetylation ratio was the highest in BZ-control group with 54.15 at 48 h, in BZ-EtOH group with 59.41 at 48 h, and in BZ-PB group with 40.28 at 96 h. A study reported that N-acetylation increases when exposed to EtOH together with aromatic amines[23, 57]. We could not find any significant difference in the ratio of N-acetylation although MABZ-HBI in BZ-EtOH group was actually higher than MABZ-HBI in BZ-control group due to BZ-HBI in BZ-EtOH group relatively larger than BZ-HBI in BZ-control group since EtOH increased N-hydroxylation and N-acetylation about the same rates[23, 24]. BZ-PB group showed a relatively smaller ratio of N-acetylation after 24 h compared with that in BZ-control group. However, we could not simply conclude that N-acetylation occurred less in BZ-PB group since BZ-HBI in BZ-PB group was relatively larger than BZ-HBI in BZ-control group after 24 h.

The N-acetylation ratio in DB38-control group was the highest at 72 h with 10.30; in DB38-EtOH group at 24 h with 11.56; and in DB38-PB group at 24 h with 28.03. When the N-acetylation ratio was compared between BZ group and DB38 group, it was relatively small in DB38-control, -EtOH, and -PB groups compared with BZ-control, -EtOH, and -PB groups. The ratio of N-acetylation in DB38-PB group was higher than in DB38-control and DB38-EtOH groups since azo reduction of DB38 was inhibited due to PB[58] according to relatively smaller BZ-HBI in DB38-PB group compared with BZ-HBI in DB38-control and DB3-EtOH group. The ratio of N-acetylation was more than 1 in BZ-control, -EtOH, and -PB groups and in DB38-control, -EtOH and -PB groups at all times, showing that MABZ-HBI was higher than BZ-HBI. This result confirmed that 4-nitroso-4'-N-acetylbiphenyl is formed the most within RBC when exposed to BZ or DB38.

Amines with one amine group such as aniline and 4ABP are more useful when acetylation can be explained in the evaluation of exposure to hemoglobin adducts[17] since the risk for bladder cancer increases when N-hydroxylation increases competitively due to slowly progressing N-acetylation[61, 62]. However, it is difficult to explain for toxicity and solubility only with N-acetylation because deacetylation and N-acetylation occur at the same time in amines with more than 2 amine groups such as BZ[29]. The rate of N-acetylation progression differs significantly according to races. N-acetylation progresses the fastest in Asians, followed by Blacks and Caucasians showing the slowest N-acetylation[17].

We determined how much of DB38 was metabolized by going through BZ by azo reduction within the body by comparing BZ and HBIs of its metabolites in DB38 and BZ groups. For this purpose, we assessed the ratio of HBI in DB38 group divided by HBI in BZ group. BZ-HBI ratio and MABZ-HBI ratio in DB38 group were less than 1 at all times of observation regardless of pretreatment. Especially, MABZ-HBI ratio was relatively lower than BZ-HBI ratio, suggesting that DB38 was absorbed into the body and goes through only part of BZ metabolism process. When Direct Red 46, which was an azo dye similar to DB38, was administered in rats, the results showed that BZ formed hemoglobin adducts at low concentrations[28], similar to the result shown in the present study. However, 4ABP-HBI was higher than 1 in DB38-control group at 12 h and 24 h; in DB38-EtOH group at 3 h, 12 h, 24 h, and 48 h; and in DB38-PB group at 12 h and 24 h, with the formation at higher concentrations compared with BZ group. These results were similar to the study done by Birner *et al*. (1990)[27] who used the BZ azo dye, Direct Black 29. This cause could not be explained clearly since the mechanism of 4ABP production with BZ metabolites has not been elucidated[64], needing further studies in the future.

When same doses of BZ were administered according to BZ azo dyes in dogs that have a similar carcinogenesis mechanism as humans, the results showed that the amount of urinary BZ was less BZ administered with the administration of Direct Black 4 and Direct Red 28 but was similar with the administration of Direct Green 1 and Direct Orange 8[64]. Direct Brown showed more urinary BZ compared with BZ group[65]. Therefore, it is difficult to apply the study result that hemoglobin adducts produced through the exposure to DB38 was less than hemoglobin addcuts produced through the exposure to BZ in all BZ dyes.

The concentration of hemoglobin adducts was very low 24 h after the azo dye Direct Red 46 and the azo pigment Yellow 17 were administered in *Wistar* rats, with especially low concentration in the less carcinogenic Yellow 17 pigment[28]. When hemoglobin adducts were measured 24 h after the oral administration of BZ and 3,3´-dichlorobenzidine at 0.2 mmol each in *Wistar* rats, the results showed that hemoglobin adducts formed very little with the administration of less carcinogenic 3,3´-dichlorobenzidine compared with BZ[29], indirectly explaining the toxicologic significance of hemoglobin

adducts.

Because BZ and DB38 were orally administered, a limit of this study was that a difference could be present in the absorption and adduct formation in workers at actual work sites who absorb these chemicals through the respiratory system and skin. Furthermore, N-acetylation occurs faster in the experimental animal *Sprague-Dawley* rats compared with other rats[66]. and adduct formation is different according to the type of experimental animals[67]. Therefore, MABZ-HBI could have been higher in our study compared with other studies. Although BZ causes bladder cancer in humans[6], it causes liver cancer in mice and rats[68]. However, the results more applicable to humans could be obtained when studies are to be conducted in dogs since BZ causes bladder cancer in dogs who have similar levels of N-acetylation as in people[69].

Conclusion

The formations of hemoglobin in BZ-, MABZ-, and 4ABP-HBI of BZ-EtOH and BZ-PB groups were increased compared with those of BZ-control group. In DB38 group, all of HBIs except for BZ-HBI were increased more than those of DB38-control group regardless of the pretreatment. These results are attributable to the fact that EtOH and PB induced N-hydroxylation is related to the formation of hemoglobin adducts. They indicate that EtOH not only increases the adduct formation by inducing N-hydroxylation but also induces N-acetylation. PB induced N-hydroxylation and increased the adduct formation in BZ group, but decreased the adduct formation in DB38 group due to decreasing azo reduction. These results suggest that the effects of EtOH or PB should be considered in biochemical monitoring of BZ and DB38 for the assessment of intermittent exposure to BZ and DB38.

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