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SHORT COMMUNICATION

Generation of hydroxyl radicals during benzene toxicity

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Benzene has been recognized as an environmental carcinogen. The evidence of benzene being a human leukomogen is based on epidemiological studies of Aksoy [1], Vigliani [2], Infante *et al.* [3]. Toxic manifestations following benzene exposure are severe anemia, leucopenia, aplastic anemia and thrombocytopenia. Leucocytosis, eosinophilia and the presence of immature marrow cells in the blood have been also reported [4]. Bioactivation of benzene is believed to be important for its leukemogenic and toxic effects [5]. Khan *et al.* [6] reported accumulation of total iron in liver and bone marrow. Recently, we have reported accumulation of bleomycin detectable low molecular weight iron in bone marrow of benzene administered rats [7]. It has been suggested that free iron can generate hydroxyl radical by Haber Weiss and Fenton type reactions [8]. Dimethyl sulphoxide is a potent hydroxyl radical scavenging agent [9]. Klein *et al.* [10] observed that production of formaldehyde during metabolism of dimethyl sulphoxide is a good indicator of hydroxyl radical generating system. Also deoxyribose degradation by hydroxyl radical in various model systems has been successfully used to monitor the extent of hydroxyl radical formation [11]. In the present study, an attempt has been made to detect formation of formaldehyde and degradation of deoxyribose as indicators of hydroxyl radical generation during benzene toxicity.

Materials and methods

Female albino rats (Swiss Wistar Strain, bred in ITRC colony) weight approximately 100 ± 10 g maintained on standard Lipton pellet diet were used throughout the experiment. The animals were divided into three groups of 20 each, namely control (normal saline administered), 5 days and 10 days benzene administered (0.5 mL/kg body wt, i.p., daily) groups. After 24 hr of last treatment, the rats were killed by cervical dislocation, femurs were taken out immediately and flushed with 50 mM phosphate buffer, pH 7.4 (0.5 mL per femur). The bone marrow flushings of three animals were combined, homogenized and the 9000 *g* supernatant (S-9 fraction) was prepared by standard centrifugation procedure. Protein content was determined by the method of Lowry *et al.* [12].

Production of formaldehyde from dimethyl sulphoxide during NADPH-dependent electron transfer by bone marrow S-9 fraction was measured by the procedure of Klein *et al.* [10]. Briefly, the standard reaction mixture consisted of 83 mM potassium phosphate buffer, pH 7.4, 0.1 mM EDTA, 10 mM $MgCl_2$, 0.3 mM NADPH, 1–2 mg of S-9 fraction protein, and 33 mM dimethyl sulphoxide in a final volume of 3.0 mL. The fraction was initiated by the addition of NADPH and was terminated by the addition of 1.0 mL of ice-cold 17.5% (w/v) trichloroacetic acid after incubation for 10 min at 37° with shaking. A 2.0 mL aliquot was then assayed for formaldehyde according to the spectrophotometric method of Nash [13].

Deoxyribose degradation by S-9 fraction of bone marrow was assayed essentially by the method of Puppo and Halliwell [11]. Briefly, the assay mixture consisted of 25 mM KH_2PO_4/KOH buffer, pH 7.4, 5.6 mM deoxyribose, various scavengers (where applicable) and S-9 fraction of bone marrow (1–2 mg protein). After incubating the reaction mixture for 1 hr at 37° with shaking, 1.0 mL of ice-cold 2.8% (w/v) trichloroacetic acid and 1.0 mL of 0.67% thiobarbituric acid were added. Tubes were then kept in boiling water for 10 min. Samples were cooled at room temperature and 5.0 mL of butanol was added for colour extraction which was measured spectrophotometrically at 532 nm.

Results and discussion

NADPH-dependent electron transfer by rat bone marrow S-9 fraction was used on $OH^{\cdot}$ generating system. Data from Table 1 show that formaldehyde production from dimethyl sulphoxide by bone marrow S-9 fraction was increased at least 10 times in the presence of NADPH after 5 days of benzene exposure which persisted for up to 10 days of treatment (data not shown). Using degradation of deoxyribose as another model for an $OH^{\cdot}$ generating system, we were able to notice that degradation of deoxyribose was also enhanced (4-fold) by bone marrow S-9 fraction of 5 days benzene treated rats (Table 1).

Hydroxyl radicals ($OH^{\cdot}$) are widely implicated as major damaging species in free radical pathology [14, 15]. Unequivocal evidence of $OH^{\cdot}$ formation in a particular process *in vitro* or *in vivo* is difficult to obtain [16]. There are many reports in which inhibition of a particular reaction by one or more compounds known to scavenge $OH^{\cdot}$ is taken as evidence of $OH^{\cdot}$ involvement. In the present study, both NADPH dependent production of formaldehyde from dimethyl sulphoxide and degradation of

Table 1. Effect of benzene administration (0.5 mL/kg body wt daily) on generation of formaldehyde from dimethyl sulfoxide during NADPH-dependent electron transfer and deoxyribose degradation by S-9 fraction of bone marrow of rats

Treatment	Formaldehyde production (nmol/mg protein/10 min)	Deoxyribose degradation
None (Control)	4.81 ± 0.29	0.071 ± 0.007
Benzene (5 days)	51.33 ± 3.98*	0.293 ± 0.11*

Values are expressed as mean ± SE of at least four separate experiments.
* Significant from control $P < 0.001$ as evaluated by Student's *t*-test.

Table 2. Effect of hydroxyl radical scavenging agents on the production of formaldehyde from dimethyl sulfoxide and on deoxyribose degradation by rat bone marrow S-9 fraction

Addition	Concentration	Formaldehyde production (nmol/mg protein/10 min)		% Inhibition of deoxyribose degradation	
		Control	Benzene treated (5 days)	Control	Benzene treated (5 days)
None	—	4.73 ± 0.28	50.36 ± 4.26	0	0
Butanol	100 mM	2.26 ± 0.11	15.79 ± 0.62	53.10 ± 3.79	47.35 ± 4.01
Ethanol	100 mM	2.45 ± 0.21	22.07 ± 0.63	36.30 ± 2.82	69.97 ± 5.62
Mannitol	100 mM	4.35 ± 0.12	16.59 ± 1.64	79.10 ± 5.39	62.20 ± 4.92
Urea	100 mM	6.96 ± 0.56	95.42 ± 8.20	No inhibition	No inhibition
SOD	58 μg/mg	9.87 ± 0.54	227.20 ± 19.15	0.89 ± 0.03	3.18 ± 0.26
Catalase	67 μg/mL	0.76 ± 0.06	1.24 ± 0.12	96.46 ± 8.29	84.45 ± 7.32
Desferal*	50 μM	2.41 ± 0.16	2.21 ± 0.34	95.58 ± 7.59	98.23 ± 6.59
DETAPAC	0.067 mM	4.38 ± 0.27	30.80 ± 2.98	87.62 ± 6.29	82.33 ± 5.92
Sodium benzoate	25 mM	4.21 ± 0.41	19.31 ± 1.93	39.80 ± 3.43	53.70 ± 5.29
Sodium azide	1 mM	4.38 ± 0.34	137.16 ± 13.67	No inhibition	No inhibition

Values are expressed as mean ± SE of at least four separate experiments.
* Desferrioxamine mesylate.

deoxyribose as indicators of $OH^{\cdot}$ radical generation was enhanced by S-9 fraction of rat bone marrow after benzene administration.

The effects of $OH^{\cdot}$ scavenging agents, both on the production of formaldehyde and degradation of deoxyribose have been evaluated. Production of formaldehyde was suppressed by butanol, ethanol, mannitol, catalase, desferal, DETAPAC and sodium benzoate. However, urea and azide showed no inhibitory effect (Table 2). Similarly, inhibition in deoxyribose degradation was maximum with desferal (98.23%). On the basis of effects of catalase which almost completely inhibited formaldehyde production as well as deoxyribose degradation and no inhibitory effect of azide (an inhibitor of catalase) indicate that H_2O_2 serves as a precursor of $OH^{\cdot}$ generation. The effect of SOD through the dismutation unit ion is to increase the level of H_2O_2 and consequently an increase in $OH^{\cdot}$ production is obtained. Similarly azide may be inhibiting catalase besides other peroxidases such as myeloperoxidases from diminishing the H_2O_2 and consequently the level of H_2O_2 again increases. The production of $OH^{\cdot}$ in both the system appears to be mediated by iron due to the inhibition by DETAPAC or by desferal (Table 2).

The presence *in vivo* of such a "Low Molecular Weight Iron Pool" has been suggested for many years [17]. The iron source first to be considered is iron chelated by nucleotides, citrate, or other small molecules. Our earlier report [7], indicated that free bleomycin detectable iron was accumulated in bone marrow which may only be able to carry out a modified Haber Weiss reaction [17] or a Fenton reaction [14] in the marrow environment. Present results of generation of hydroxy radicals *in situ* during benzene toxicity gives additional validation. Recently Pryor [18] suggested that the hydroxyl radical is the only free radical that has the capacity to add to nucleotides in DNA because of its high vicinity of DNA *in situ* with stereo- and regio-selectivity to cause site specific damage to DNA. On the basis of these data, it is suggested that hydroxyl radicals are generated *in situ* during benzene toxicity.

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