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Benzene-induced genotoxicity in mice in vivo detected by the alkaline comet assay: reduction by CYP2E1 inhibition

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Abstract

The myelotoxic and genotoxic effects of benzene have been related to oxidative DNA damage after metabolism by CYP2E1. Single cell gel electrophoresis (alkaline comet assay) detects DNA damage and may thus be a convenient method for the study of benzene genotoxicity. Benzene exposure to NMRI mice as a single oral gavage at 40, 200 or 450 mg/kg resulted in dose-related DNA damage indicated by an increased comet tail length of peripheral blood lymphocytes and bone marrow nucleated cells sampled 6 h after exposure. After a dose of 40 mg/kg, there was a 1.6-fold increase of 'tail length' in bone marrow nucleated cells in comparison with the control ($p < 0.01$). There was no significant increase in DNA damage in peripheral blood lymphocytes in the same animals. At 200 mg/kg, the tail length was 4.8-fold and 4.0-fold increased in the two cell types, respectively ($p < 0.01$). At 450 mg/kg, the tail length was further increased to 5.4-fold and 6.6-fold of the control values, respectively ($p < 0.01$). Pretreatment with propylene glycol (5 μl/g b.wt., twice with a 60-min interval), a selective CYP2E1 inhibitor, reduced the increase in the tail length by about half at all doses in both cell types ($p < 0.01$). By comparing our data with those from genotoxicity studies on benzene using other methods, we conclude that the 'alkaline comet assay' is a sensitive method to detect DNA damage induced by benzene. We also infer that CYP2E1 contributes, at least partly, to the formation of the 'comet'-inducing metabolites in the chosen cell types.

Keywords: Benzene; Single cell gel electrophoresis (SCGE, comet assay); Cytochrome P4502E1 (CYP2E1); Propylene glycol (PG)

1. Introduction

Benzene is widely used in industry. Although workplace exposure has been limited through strict safety regulation (Runion and Scott, 1985), benzene continues to be a component of gasoline, and low concentrations of benzene are present in cigarette smoke and cooking fumes (Lofroth et al., 1991).

Exposure to benzene has been shown to lead to aplastic anemia and acute myelogenous leukemia in humans and multiple forms of cancer in rodents (Aksoy, 1989; Huff et al., 1989). Benzene is metabolized in the liver to various phenolic metabolites which accumulate in the bone marrow (Rickert et al., 1979; Gad-El Karim et al., 1985). Myeloperoxidase and other heme–protein peroxidases present in the bone marrow may further convert the phenolic metabolites of benzene to semiquinone radicals (Subrahmanyam et al., 1991). This peroxidative metabolism and redox cycling of semiquinones can

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produce reactive oxygen species which may inflict oxidative damage on cellular DNA and induce genotoxic effects (Kolachana et al., 1993).

In vitro studies with microsomal systems indicate that CYP2E1 is the principal catalyst for the formation of the toxic metabolites (Johansson and Ingelman-Sundberg, 1988; Guengerich et al., 1991; Seaton et al., 1994). Indeed, in vivo treatment with a CYP2E1 inducer enhanced both the metabolism and toxicity of benzene (Nakajima et al., 1985). However, a reduction in benzene toxicity by blocking CYP2E1 has yet to be demonstrated for further support of this hypothesis. Recently, propylene glycol (PG) was shown to block CYP2E1 activity estimated by chlorzoxazone clearance in vivo as well as abolish the hepatotoxicity of paracetamol related to metabolic activation (Thomsen et al., 1995) by CYP2E1.

Considerable efforts have been focused on finding biomarkers which can earlier and more conveniently detect DNA damage induced by benzene. Recently, Plappert et al. (Plappert et al., 1994a,b) have demonstrated the ability of the SCGE assay to detect DNA damage induced in liver and blood cells of benzene exposed mice. The SCGE assay, also known as the comet assay, is a rapid, simple, visual and sensitive technique for measuring and analysing DNA modification (McKelvey-Martin et al., 1993) and appears to be a biomarker of benzene genotoxicity. In the alkaline comet assay, cells are embedded in a gel and subjected to an electric field after lysis of the membrane. Increased levels of DNA damage will allow the DNA to move more easily in the field, which can be detected after fluorescence staining of DNA and visual evaluation by fluorescence microscopy.

The objective of the present study was to use the alkaline comet assay to investigate benzene genotoxicity and the role of CYP2E1.

2. Materials and methods

2.1. Animals and treatment

Forty-eight male NMRI mice (21–25 g, age 6–7 weeks, Panum Institute, animal center, Copenhagen) with free access to a standard laboratory chow and tap water were randomly assigned to 8 groups of 6 animals. The animals were kept at controlled humidity (50–70%) and temperature (21–23°C), and light was controlled in a 12/12 h light dark schedule. Four groups were pretreated with propylene glycol (PG) (Nomeco Co. Copenhagen, Denmark, CAS No. 57-55-6), 50% (v/v) in water, 10 μl/g body weight twice by gavage with 60 min interval. Four groups received only water. Benzene (Riedel-deHaen Co. Seelze, Germany; purity, 99.7%; CAS no. 71-43-2) was administrated by gavage in doses of 0, 40, 200 and 450 mg/kg in corn oil (Sigma, St. Louis, MO). The vehicle plus benzene volume was kept constant at 15 μl/g b.wt. Six hours after benzene administration, decided from pilot experiments, blood samples were collected from the orbital vessels, the animals were killed, and the bone marrow from the femurs was collected. In an identical setting, 7 groups of 6 male Wistar rats with a body weight of about 200 g and age 6 weeks were dosed with vehicle benzene 50, 100 or 250 mg/kg body weight either as a single dose or 7 doses administered twice daily. Six hours after the single dose, the seventh dose or vehicle peripheral blood and femur bone marrow cells were used for the alkaline comet assay as described below.

2.2. Sample preparation

Twenty microliters of blood was diluted with 2% acetic acid for white blood cell counting, another 100 μl of blood was collected in an 1.5-ml Eppendorf tube and stabilized with 50 U of heparin. One ml of Eagle's medium (Gibco BRL, Scotland) was added and gently mixed. Two hundred microliters of Histopaque-1083 (Sigma, St. Louis, MO) was underlaid. The samples were centrifuged at 200 *g* for 3 min at 4°C. Lymphocytes were collected and washed twice with 1 ml of Eagle's medium. Three hundred microliters of Eagle's medium was added to obtain a cell suspension for the alkaline comet assay.

The bone marrow from the femurs was flushed out with 1 ml of cold Eagle's medium supplemented with 10 U/ml heparin in order to obtain the bone marrow cells. The cell density was adjusted to about 10^5 ml^{-1} for the alkaline comet assay.

2.3. Alkaline comet assay

Cells from pretreated, non-pretreated and control animals were run at the same time to allow compari-

son. The technique by Singh et al. (1988) was followed with minor modification. Eighty-five microliters of 1% normal melting agarose (type I-A, Sigma, St. Louis, MO) in Mg^{2+}- and Ca^{2+}-free PBS (0.1 M, pH 7.4) (Gibco BRL, Scotland) was dissolved and spread onto the frosted part of a glass slide (Socorex, Swiss) and covered with an 18 × 18 mm coverslip. Slides were kept at 4°C for 10 min. The coverslip was then removed and 10 μl of the cell suspension was mixed with 85 μl of 1% lower melting agarose (type VII, Sigma, St. Louis, MO) at 37°C and pipetted onto the first agarose layer. The gel was covered with a coverslip and stored at 4°C for 10 min. The slide was then immersed into a freshly prepared cold lysis solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris; pH 10 with 1% Triton X-100) at 4°C for 1 h. Electrophoresis buffer (1 mM Na_2EDTA and 300 mM NaOH, pH 13.0) was prepared just before use. The slides were placed in the tank side by side horizontally and buffer was added to a level of 0.25 cm above the slide, and the slides were left in the buffer for 30 min for the DNA to unwind. The electrophoresis was carried out at 25 V at room temperature for 25 min, and the electric current was adjusted to 300 mA by the buffer level. The slide was then neutralized 3 times in a solution of 0.4 M Tris buffer (pH 7.5) and stained for 5 min with 85 μl of 8.5 μg/ml acridine orange (Sigma, St. Louis, MO) in distilled water. An epi-fluorescence microscope equipped with an excitation filter of 490 nm from a 100-W mercury lamp and a barrier filter of 530 nm was used, and within 10 min of staining the DNA image was measured with a calibrated scale in the ocular at 400 × magnification. The measurements from 100 randomly selected cells per tissue per animal were averaged. Quantification of the DNA damage for each cell was calculated as:

comet tail length(μm)
= (maximum total length)
− (head diameter).

Multiple factor analysis of variance (MANOVA) was used for statistical analysis, considering blood and bone marrow cells as repeated measures of the dependent variable, and dose and propylene glycol as independent variables. Differences were considered significant when $p < 0.05$, 95% confidence intervals were calculated for the relative change. The analysis was based on individual animal responses.

3. Results

The analysis of variance showed significant differences between cell types ($p < 0.0001$), between doses ($p < 0.0001$), and between treatment with propylene glycol and vehicle ($p < 0.0001$) Treatment of mice with a single dose of benzene by oral gavage increased the tail length of both peripheral lymphocytes and bone marrow nucleated cells in a dose-dependent manner (Fig. 1 and 2). At 40 mg/kg, the comet tail length was 1.6-fold increased in nucleated bone marrow cells (Fig. 2); whereas, no significant change was observed in lymphocytes (Fig. 3). At 200 mg/kg, the tail length was 4.8-fold and 4.0-fold increased in the two cell types, respectively ($p < 0.01$). At 450 mg/kg, the tail length was further increased to 5.4-fold and 6.6-fold of the control values, respectively ($p < 0.01$).

Pretreatment with propylene glycol reduced the increase in the tail length by 45.1% (95% CI, 30.8–59.4) at 200 mg/kg and 41.0% (95% CI, 30.8–51.2) at 450 mg/kg of the induced tail length of peripheral lymphocytes ($p < 0.01$). In bone marrow nucleated cells, PG pretreatment reduced the increase in the tail

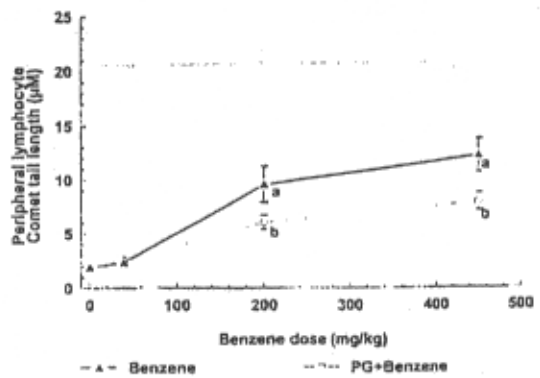

Fig. 1. Dose–effect relationship of a single gavage dose of benzene with and without propylene glycol pretreatment with respect to the comet tail length of peripheral blood lymphocytes in mice, symbols and bars represent means and standard errors of the mean. a, significantly different from the control at $p < 0.01$; b, significantly different from the same dose group without PG pretreatment at $p < 0.01$.

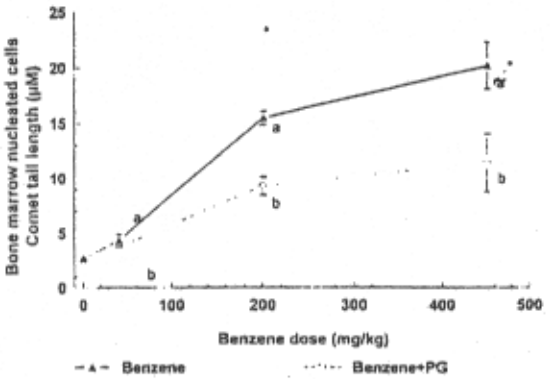

Fig. 2. Dose–effect relationship of a single gavage dose of benzene with and without propylene glycol pretreatment on the comet tail length of bone marrow nucleated cells in mice. Symbols and bars represent means and standard errors of the mean. a, significantly different from the control at $p < 0.01$; b, significantly different from the same dose group without PG pretreatment at $p < 0.01$.

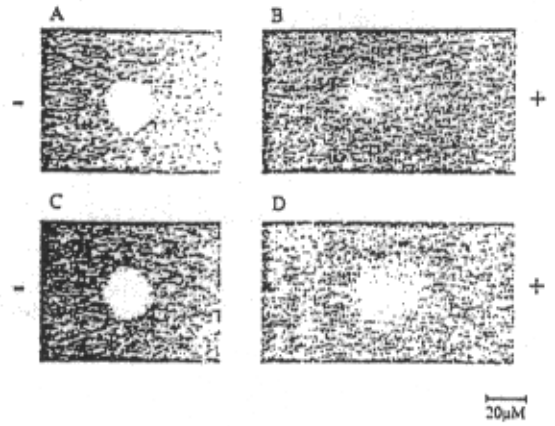

Fig. 3. Fluorescence photomicrographs showing individual mice lymphocyte and bone nucleated cells from the comet assay. A: comet image of lymphocyte at basal level. B: comet image of lymphocyte with a long tail. C: comet image of nucleated cell at basal level. D: comet image of nucleated cell with a long tail.

length by 34.5% (95% CI, 20.9–48.1) at 40 mg/kg, 48.1% (95% CI, 44.6–51.6) at 200 mg/kg and 50.0% (95% CI, 28.2–71.8) at 450 mg/kg ($p < 0.01$).

Liver, spleen weights and white blood cell (WBC) counts were not significantly different between any group (Table 1).

There were no comets formed in rat peripheral blood cells or in bone marrow cells. The average comet length was 2.65 ± 0.63 micrometer in peripheral lymphocytes and 2.85 ± 0.74 in bone marrow nucleated cells. In the benzene-treated groups the average ranged from 2.62 to 2.78 and from 2.85 to 2.93, in peripheral lymphocytes and bone marrow nucleated cells, respectively, and with SD between 0.72 and 0.90 ($p > 0.05$ by analysis of variance).

4. Discussion

The present study demonstrated that genotoxic effects of benzene given in vivo in mouse blood and bone marrow cells can be detected sensitively with the alkaline comet assay. Furthermore there was a relationship between the benzene in vivo *dose* and the tail length without significant changes in WBC, liver weight, and spleen weight (Table 1). The benzene-induced genotoxicity could be reduced by pre-

Table 1
The effect of oral benzene doses with and without PG pretreatment on WBC, liver and spleen weight in mice

	n	Control	40 mg/kg	200 mg/kg	450 mg/kg	*F*-value [a]	*p*-value [a]
WBC/mm^3							
No pretreatment	6	11133 ± 864	11166 ± 1713	11233 ± 638	9967 ± 650		
PG pretreatment	6	10233 ± 585	10366 ± 344	9366 ± 516	11417 ± 553	2.04	0.07
LW/BW(×100)							
No pretreatment	6	4.65 ± 0.26	4.57 ± 0.30	4.59 ± 0.29	4.33 ± 0.27		
PG pretreatment	6	4.42 ± 0.20	4.46 ± 0.57	4.32 ± 0.33	4.49 ± 0.42	0.69	0.68
SW/BW(×100)							
No pretreatment	6	0.49 ± 0.04	0.50 ± 0.07	0.53 ± 0.06	0.55 ± 0.07		
PG pretreatment	6	0.46 ± 0.05	0.48 ± 0.04	0.48 ± 0.03	0.47 ± 0.04	2.06	0.07

WBC, white blood cell count; LW, liver weight; SW, spleen weight; BW, body weight.
[a] From MANOVA testing difference between no and PG treatment.

treatment with propylene glycol (PG) supporting the role of CYP2E1 in bioactivation of benzene. The genotoxicity occurred early after benzene exposure, i.e. after 6 h.

The present data are in accordance with a report that toluene reduced benzene toxicity in blood and bone marrow cells (Plappert et al., 1994a); the mechanism is not clear from that study, but toluene could compete for CYP2E1 metabolism and reduce benzene metabolism as does propylene glycol (Thomsen et al., 1995). Also, Plappert et al. reported that inhalation of 100 ppm benzene for 5 days (6 h/day) and 300 ppm for 3 days induced a significant increase of the comet moment in liver cells and in peripheral blood cells in mice, respectively (Plappert et al., 1994b). These doses would correspond to more than 150 mg/kg/day for 3 days by oral gavage according to the benzene dosimetry study by Henderson (Henderson et al., 1989). Accordingly, lymphocytes and bone marrow nucleated cells may be more susceptible to benzene challenge than other cell types, assuming that the dosimetry study is correct.

The route of exposure has profound effects on the magnitude of micronuclei in the bone marrow, presumably due to the differences in obtained benzene levels (Tice et al., 1989). The pretreatment with PG may possibly have changed the pharmacokinetics of benzene. However, if a considerable inhibition did occur, it would result in higher benzene levels for a longer time. This would imply a higher toxicity by a to CYP2E1-related mechanism which cannot invalidate the finding of reduced toxicity. It is possible that the reduction in toxicity observed is underestimated if higher benzene concentrations increase other activation pathways.

In comparison with in vivo genotoxicity studies of benzene with other techniques such as micronuclei, sister chromatid exchanges, chromosomal aberrations and 8-hydroydedeoxyguanosine in DNA, (Tice et al., 1980; Harper et al., 1984; Sabourin et al., 1990; Kolachana et al., 1993; Chen et al., 1994) the comet assay appears equally sensitive. Again, if we extrapolate our oral dosing to the equivalent inhalation exposure studies according to the benzene dosimetry study by Henderson et al. (1989), the alkaline comet assay also appeared equally sensitive to other genotoxicity markers after benzene given by inhalation (Styles and Richardson, 1984; Luke et al., 1988; Tice, 1988).

The alkaline comet assay is a sensitive technique for detecting DNA damage in a small number of cells. The assay detects single and double strand breaks as well as alkali-labile sites of the DNA. It has been reported that as few as 0.1 DNA changes per 10^9 Da can be detected (Gedik et al., 1992). However, DNA breaks can originate from the process of DNA repair (excision), replication, recombination or during the process of cell apoptosis, or by the direct modification by chemical agents and their metabolites (Eastman and Barry, 1992). Thus, further study of the genotoxicological meaning of the alkaline comet assay in comparison with conventional genotoxicological techniques is warranted, especially with regard to whether the assay detects in vivo strand breaks after benzene exposure.

The bioactivation of benzene to the toxic metabolites phenol and hydroquinone is thought to be primarily catalysed by CYP2E1. In metabolism studies, induction of CYP2E1, confirmed by Western blotting, induced the formation of benzene metabolites, including a 9-fold increase of hydroquinone formation which is considered as a major contributor to the myelotoxicity (Kalf, 1987; Schrenk et al., 1992). We have recently demonstrated that propylene glycol inhibits CYP2E1 activity to 10% or less (Thomsen et al., 1995). The present study used the same protocol for CYP2E1 inhibition by propylene glycol and this can partly prevent the induced DNA damage in peripheral blood lymphocytes and bone marrow nucleated cells from mice. We also challenged rats, however, even after 7 repeated doses, up to 250 mg/kg. we could not detect any significant change of the comet tail length. These results of species difference are in accordance with a benzene carcinogenicity study in mice and rats (Huff et al., 1989). The reason for this species difference in susceptibility may be that mice metabolize more of the benzene by pathways leading to the toxic metabolites than does rat (Henderson et al., 1989; Medinsky et al., 1989). This is also consistent with the fact that mice have about twice the CYP2E1 enzyme activity of rats, but only about 25% of the activity of detoxifying enzymes, e.g. epoxide hydrolase and glutathione-*S*-transferase. Thus, mice will activate benzene more readily and detoxify the resulting reactive products

more slowly than rats (Cox, 1991). A metabolism study showed that 10 times higher internal doses of phenol and catechol in the lung, hydroquinone in blood, and muconic acid in liver were achieved from the dose of benzene administered to mice as compared with rats (Henderson et al., 1989; Medinsky et al., 1989). We infer that the difference of metabolism between two species results in the difference of formation of 'comet'-inducing metabolites. At the dose of propylene glycol given we achieved an inhibition of CYP2E1 activity to about 16%, as measured by the clearance of chlorzoxazone in vivo (Thomsen et al., 1995). Although the inhibition of CYP2E1 by PG appears quite specific we cannot rule out that other enzymes important for benzene genotoxicity could be influenced. However, the well documented role of CYP2E1 in the role of benzene genotoxicity and the reduced genotoxicity after CYP2E1 inhibition make us conclude that metabolic activation by CYP2E1 contributes, at least partly, to the formation of the 'comet'-inducing metabolites in the studied cell types.

In addition, we conclude that the alkaline comet assay is a sensitive and convenient method for further studies of benzene genotoxicity in peripheral lymphocytes and nucleated bone marrow cells in mice.

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