

Dexamethasone Ameliorates Oxidative DNA Damage Induced by Benzene and LPS in Mouse Bone Marrow

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Mice were grouped to receive vehicle, dexamethasone (DEX), lipopolysaccharide (LPS), benzene (BZ, 200 mg/kg) and combinations: LPS + DEX, BZ + DEX, LPS + BZ, LPS + DEX + BZ. The DNA damage in bone marrow cells from BZ group was enhanced 2.8-fold measured by nuclear 8-hydroxy-2'-deoxyguanosine (8-oxodG) and 1.4-fold measured by Comet score (index of DNA breaks) ($p < 0.05$). In the BZ + DEX group, 8-oxodG level and the Comet score were lowered to 65% and 76% respectively of that in the BZ group ($p < 0.05$). The BZ + LPS caused a 3.9-fold increase in 8-oxodG and a 1.6-fold increase in the Comet score ($p < 0.05$). The LPS + DEX + BZ lowered 8-oxodG level and the Comet score to 50% and 78% of the values in the LPS + BZ group, respectively ($p < 0.05$). Nitrate/nitrite levels in serum were higher after BZ + LPS treatment than after all other treatments. Both 8-oxodG level and the Comet scores were correlated to the serum nitrate/nitrite level across all the treatments ($r = 0.55$, $p < 0.01$ and $r = 0.69$, $p < 0.01$, respectively). In bone marrow cells the 8-oxodG correlated with the Comet scores ($r = 0.80$, $p < 0.01$). We conclude that DEX administration can reduce the DNA damage from BZ treatment and from the combination of BZ and LPS. The correlation of DNA damage with nitrate/nitrite indicates the possible involvement of reactive nitrogen

species (RNS) in the interaction between BZ and the inflammatory reaction stimulated by LPS. The 8-oxodG determination is more sensitive than strand break analysis by the Comet assay in bone marrow *in vivo* in mice for measuring the BZ-induced DNA damage.

Keywords: Benzene, lipopolysaccharide (LPS), dexamethasone (DEX), 8-hydroxy-2'-deoxyguanosine (8-oxodG), single cell gel electrophoresis (Comet assay), reactive nitrogen species (RNS)

INTRODUCTION

Benzene (BZ) is a known leukaemia-inducing agent in humans and a multisite carcinogen in rodents.^[1] Benzene exposure elicits increased frequency of chromosome aberrations and micronuclei in peripheral blood and bone marrow cells both in epidemiological and animal studies.^[2,3] By means of alkaline single cell gel electrophoresis (Comet assay) BZ-induced DNA breaks in peripheral lymphocytes and bone marrow cells

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have been shown in animal studies.^[4,5] Similarly, BZ induces oxidative DNA damage assessed by nuclear 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) in target cells.^[6]

The mechanism underlying the genetic toxicity from BZ exposure is not fully elucidated. Inflammatory reactions may be triggered by BZ exposure,^[7] e.g. BZ administration to mice resulted in a 4-fold proliferation of granulocytic hyperplasia in bone marrow, the target tissue of BZ toxicity.^[8,9] Furthermore, it was demonstrated that BZ exposure enhanced nitric oxide production with enhanced expression of the inducible nitric oxide synthesis (iNOS) gene and an increase of other inflammatory mediators, resulting in haematosuppression.^[10–15] Recently, the DNA damaging effect of BZ was shown to be enhanced by lipopolysaccharide (LPS) treatment *in vivo* and by activation of human neutrophils with phorbol myristate acetate (PMA) *in vitro* (unpublished).

The data suggest that inflammatory reactions play an important role in BZ toxicity. Glucocorticoids act as anti-inflammatory agents by inhibiting the arachidonic acid cascade. Some of the effects are ascribed to selective inhibition of iNOS induction.^[16–18]

This study was designed to investigate the role of inflammatory reaction in the BZ-induced genotoxicity, the efficiency of intervention by dexamethasone (DEX) administration and possible involvement of reactive nitrogen species (RNS) in the BZ-induced genotoxicity and DEX intervention. LPS was used to mimic an inflammatory situation in mice. The 8-oxodG formation and DNA breaks assessed by the Comet assay in bone marrow nucleate cells were used as the indicators of genotoxicity.

MATERIALS AND METHODS

Chemicals

Benzene (BZ) (Riedel-deHaen Co. Seelze, Germany. Purity: 99.7%, CAS No. 71-43-2), corn

oil (Sigma, St. Louis, MO), Dexamethasone (DEX) (Merck Sharp & Dohme B.V., Netherlands), Lymphoprep[™]-1.077 (Nycomed pharma AS Oslo, Norway), Eagle medium (GibcoBRL, Scotland), Mg⁺⁺ and Ca⁺⁺ free PBS (0.1 M, pH 7.4) (GibcoBRL, Scotland), fully frosted microscope slides (Labcraft, Houston), YOYO-1 (Molecular Probes, Netherlands) and alkaline phosphatase (Boehringer Mannheim, Germany) were purchased from the sources indicated. Histopaque-1.083, agarose type I-A and type VII, tritonX-100, lipopolysaccharide (LPS) from *Escherichia Coli Serotype* 026:B6, 2-deoxyguanosine (dG), nuclease P1 and 8-oxodG were purchased from Sigma, St. Louis, MO. The nitrate/nitrite colorimetric assay kit was purchased from Alexis Corporation (Århus, Denmark) and 30 kDa molecular weight cut-off filters were purchased from Whatman, Denmark.

Animal Treatment

The study was carried out with male NMRI mice of 20–22 g of body weight from the animal center, Panum Institute, Copenhagen. The animals were housed in an environmentally-controlled facility operating on a 12 h dark/light cycle at 22–24°C with free access to a standard diet and tap water. The vehicle + agents volumes given were kept constant at 10–15 µl/g BW in gavage, i.p. and s.c.

Mice were assigned to 8 groups of 7 animals. The groups were designed to receive vehicle control, DEX, LPS, LPS + DEX, BZ, BZ + DEX, BZ + LPS or LPS + BZ + DEX. DEX was injected i.p. at 40 mg/kg with phosphate-buffered saline (0.1 M, pH 7.4) as the vehicle. After 15 min, 5 mg/kg of LPS was given i.p. After another 15 min, 200 mg/kg of BZ was administered by gavage in corn oil as the vehicle. Six hours after BZ administration, blood samples were collected from the orbital vessels. The serum was used for nitrate/nitrite determination. The animals were killed and bone marrow was collected from the femurs for the Comet assay and 8-oxodG assay by methods described previously.^[5,19,20]

The epiphysial plates of the femurs were removed. The bone marrow was flushed out with 1 ml of cold Eagle medium supplemented with 10 units/ml heparin. An aliquot of 100 μ l was mixed with 1 ml Eagle medium. Two hundred μ l Histopaque-1083 was underlain. The samples were centrifuged at 200g, 3 min, 4°C. The nucleate cells were collected at the interface and washed twice with 1 ml of Eagle medium. The cell density was adjusted to about 10^5 per ml for the Comet assay. The residual part of the bone marrow was centrifuged at 800g, 10 min. The pellet was frozen at -80°C for subsequent 8-oxodG determination.

Analyses

The Comet assay under alkaline condition was conducted as described elsewhere.^[21,22] After neutralization, the slides were stained for 5 min with 85 μ l of 0.5 μM YOYO-1. An epifluorescence microscope equipped with an excitation filter of 490 nm from a 100 W mercury lamp and a barrier filter of 530 nm were used to obtain images for electronic storage. DNA breaks were evaluated with an Olympus Image Analysis System after coding the slides. One hundred randomly selected cells were classified visually as belonging to one of five classes of damage according to the ratio of the head and the tail, from 1 (no visible tail, i.e. zero detectable damage) to 5 (head of Comet very small, most of DNA in tail), and given a score of 1, 2, 3, 4, or 5 to each individual image respectively according to their classification.^[23] The evaluation was carried out with standard pictures of images with scores 1–5 on the screen. The results are presented as the average percentage of each score from 100 counted cells and total score from 100 counted cells in one slide. The total score is calculated as:

$$\begin{aligned} \text{Total score} &= (\text{number of cells with score } 1 \times 1 \\ &+ \text{number of cells with score } 2 \times 2 \\ &+ \dots + \text{number of cells with score } 5 \times 5). \end{aligned}$$

Thus, the score will range from 100 to 500 for each slide.

The nitrate/nitrite determination was based on the Griess reaction using a commercial kit.^[24] Plasma was filtered through a 30 kDa molecular weight cut-off filter. Fifty μ l ultrafiltrate of plasma was used in the assay.

Statistics

Groups were compared by means of one-way ANOVA test. Duncan's multiple range test was used for *post hoc* comparison of means. Differences were considered significant when $p < 0.05$. Linear regression was done by the least square method.

RESULTS

The Comet assay was successfully performed in all the samples ($n = 7$ in each group). Due to the limited amount of bone marrow cells left after selecting material for the Comet assay the remaining aliquots restricted 8-oxodG determination to 6 samples in the LPS and LPS + BZ + DEX groups; 5 samples in the control and BZ groups and 4 samples in the other groups. For similar reasons, the nitrate/nitrite analysis was limited: 5 in the LPS + DEX group; 6 in the control group, the LPS group and the BZ group and 7 in the remaining groups.

BZ treatment induced a 2.8-fold increase in the nuclear 8-oxodG in the bone marrow cells in comparison with the control group ($p < 0.05$). In the BZ + DEX group, the 8-oxodG level was lowered to 1.8 times the value in the control group ($p > 0.05$) and was 65% of the level of BZ group ($p < 0.5$). LPS treatment by itself tended to increase the 8-oxodG level but not significantly ($p > 0.05$). In contrast, LPS + BZ caused a 3.9-fold increase as compared with the control ($p < 0.05$). The LPS + BZ + DEX reduced the increase in 8-oxodG level to 2.0 times the control value ($p > 0.05$) and 0.5 times the LPS + BZ group ($p < 0.05$) (Figure 1).

The results of the Comet assay showed similar effects of the treatments on both the distribution of the classification and the total score of the Comets in each animal. Treatment of mice with BZ shifted the distribution of 100 counted cells to a higher Comet classification (more DNA breaks) and increased the total score of the Comet assay by 1.4 times the control value ($p < 0.01$). Treatment with BZ + DEX resulted in a decrease in the number of cells with higher Comet classification, and the total score was only 1.1 times the control value ($p > 0.05$) and 0.8 times the total score from the BZ group ($p < 0.05$). Treatment with LPS + BZ led to a considerable increase in the fraction of cells with higher Comet classification and

increased the total score to 1.6 times the control value ($p < 0.05$) and 1.1 times the value from the BZ group ($p < 0.05$). In the LPS + BZ + DEX group the distribution of Comets was shifted to lower classifications; and the total score was only 1.3 times the control value ($p < 0.05$) and was 0.78 times the total score from the LPS + BZ group ($p < 0.05$) (Table I).

Treatment with LPS or BZ led to a slight and non-significant increase in the nitrate/nitrite levels in the serum ($p > 0.05$). Nitrate/nitrite levels were higher after LPS + BZ treatment (2.5-fold higher, $p < 0.05$). The LPS + BZ + DEX lowered the nitrate/nitrite levels by 37% from the LPS + BZ ($p < 0.05$) (Figure 2).

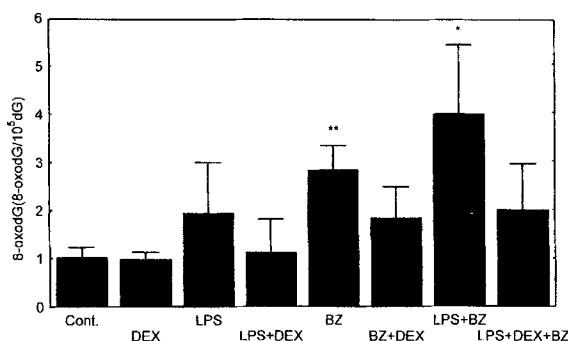


FIGURE 1 The nuclear 8-oxodG from mouse bone marrow cells after *in vivo* treatment of BZ, LPS and DEX and their combinations. * $p < 0.05$ in comparison with all other group. ** $p < 0.05$ in comparison with control, DEX or LPS + DEX group. The columns and bars indicate the means and SD from 6 samples in LPS group and LPS + BZ + DEX group, 5 samples in control and BZ groups and 4 samples in the other 4 groups.

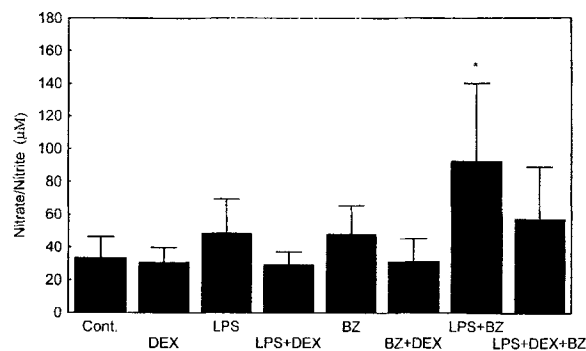


FIGURE 2 Nitrate/nitrite levels in serum of the mice after various treatment. * $p < 0.05$ in comparison with the levels of other 7 treatments. The columns and bars indicate means and SD of 5 samples from LPS + DEX group, 6 samples from control, LPS group and BZ group and 7 samples from the other groups.

TABLE I The effect of lipopolysaccharide (LPS) and dexamethasone (DEX) on the formation of benzene (BZ)-induced DNA breaks in mouse bone marrow nucleate cells was evaluated by the formation of DNA breaks with the Comet assay. The mice were given *in vivo* treatment of BZ, LPS, DEX and their combinations. The results are presented by averaging the percentage of each Comet class from 100 counted cells in one slide and total score for each slide. The data showed the mean \pm SD from 7 samples for each treatment

	Control	DEX	LPS	LPS + DEX	BZ	BZ + DEX	BZ + LPS	BZ + LPS + DEX
Class 1	92.7 \pm 2.0	92.9 \pm 3.1	84.5 \pm 2.5	88.4 \pm 4.7	68.6 \pm 5.5	88.8 \pm 2.0	57.3 \pm 5.4	78.5 \pm 4.8
Class 2	4.6 \pm 0.8	5.1 \pm 2.4	11.1 \pm 3.7	9.0 \pm 4.1	15.4 \pm 3.9	8.2 \pm 2.9	19.6 \pm 5.9	10.7 \pm 4.3
Class 3	2.3 \pm 1.1	1.6 \pm 1.1	3.7 \pm 1.0	2.7 \pm 1.7	7.3 \pm 3.0	2.5 \pm 1.0	9.7 \pm 2.5	4.7 \pm 1.1
Class 4	0.4 \pm 0.8	0.4 \pm 0.2	1.4 \pm 1.1	0.5 \pm 0.7	6.1 \pm 4.4	0.7 \pm 0.8	6.1 \pm 3.3	3.7 \pm 2.1
Class 5	0	0	0	0	2.4 \pm 1.2	0	5.7 \pm 3.9	2.0 \pm 1.9
Total scores	110.4 \pm 4.2	109.6 \pm 4.6	123.6 \pm 4.4	116.9 \pm 8.6	158.4 \pm 14.7	120.3 \pm 13.7	178.7 \pm 24.5*	139.0 \pm 11.2*

* $p < 0.01$ in comparison with all the other treatment.

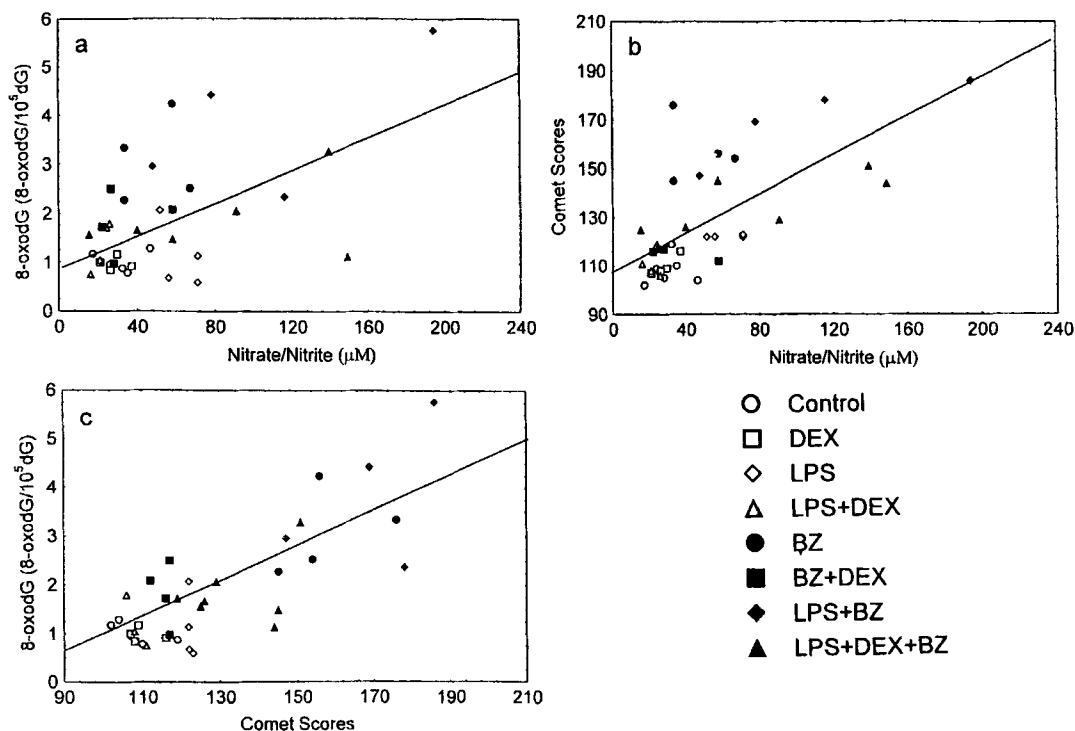


FIGURE 3 The correlation among 8-oxodG, Comet scores of bone marrow nucleate cells and serum nitrate/nitrite concentration in mice after *in vivo* treatment of BZ, LPS, DEX and their combinations: (a) nitrate/nitrite with 8-oxodG: $r=0.55$, $p < 0.01$; (b) nitrate/nitrite with the Comet score: $r=0.69$, $p < 0.01$; (c) 8-oxodG with the Comet scores: $r=0.80$, $p < 0.01$.

Both the 8-oxodG and total Comet scores showed positive correlation with the nitrate/nitrite level across all the treatments ($r=0.55$, $p < 0.01$ and $r=0.69$, $p < 0.01$, respectively) (Figure 3(a) and (b)). The 8-oxodG level and total Comet scores in bone marrow cells were positively correlated ($r=0.800$, $p < 0.01$) (Figure 3(c)).

DISCUSSION

In the present study BZ-induced oxidative DNA damage in bone marrow cells was enhanced by LPS and ameliorated by DEX. The two measures of oxidative DNA damage, the 8-oxodG level and DNA breaks assessed by the Comet assay, correlated with serum nitrate/nitrite concentrations. These data suggest an important role of inflammatory reactions in the bone marrow toxicity of BZ.

Previous studies have demonstrated that BZ administration leads to increased formation of DNA breaks in lymphocytes and bone marrow cells in mice.^[4,22] Benzene exposure *in vitro* and *in vivo* can also result in oxidative DNA damage shown as the formation of 8-oxodG.^[6] LPS *per se* has been reported to induce DNA breaks and 8-oxodG.^[25-27] No significant effect of LPS was seen in this respect in the present study although the data showed the same trends. However, the enhanced effect of BZ after LPS pretreatment indicates an *in vivo* interaction of LPS and BZ in oxidative DNA damage.

Recent data from our laboratories have shown that BZ could not induce a significant increase in 8-oxodG level in DNA from liver and spleen cells after *in vivo* treatment (data not shown), whereas the Comet assay showed significantly increased DNA breaks in bone marrow cells.^[22] The general and consistent pattern in the present

experiment indicates a more substantial increase in the 8-oxodG level than in DNA breaks detected by Comet assay in bone marrow cell after BZ only or the combined treatment of BZ and LPS. Although oxidative attack can lead to the formation of DNA breaks, some oxidised bases may not result in strand breaks.^[28] The use of the formamidopyrimidine DNA glycosylase (FPG) to induce nicks at 8-oxodG sites could have potential for increased sensitivity of the Comet assay^[28] but was not available for the present study.

LPS administration to mice can produce a typical acute phase response involving the enhanced expression of iNOS,^[29,30] which can be estimated by measuring nitrate/nitrite in body fluids as the end products.^[31] Since DEX is an inhibitor of iNOS,^[16–18] the abrogation of enhanced BZ-induced damage by LPS with DEX further supports the hypothesis of the involvement of RNS in the mechanism.

The DNA damage in the group treated with BZ + LPS was significantly decreased by DEX in comparison with the group treated with BZ + LPS. Furthermore, the indices of DNA damage correlated with nitrate/nitrite formation across all treatments. Besides the inhibition of iNOS by DEX, it has been reported that DEX can also inhibit the generation of superoxide anion both *in vitro* and *in vivo* through a mechanism that remains unclear.^[32–35] We have proposed that hydroxylation and nitration of BZ via peroxynitrite are involved in BZ genotoxicity.^[36] Thus, recent data indicate that peroxynitrite can modify BZ non-enzymatically to hydroxylated as well as nitrated aromatic compounds, including phenol, nitrophenols and nitrobenzene in a non-biological incubation system. Phenol and *p*-nitrophenol were also generated by incubation of BZ with human neutrophils pretreated with PMA accompanied with the increase of 8-oxodG in nuclear DNA in neutrophils.^[36] Reduced generation of both nitric oxide and superoxide anions by DEX will limit the formation of peroxynitrite. Thus a simultaneous

suppression of both iNOS and the generation of superoxide anion by DEX supports the possible involvement of peroxynitrite or similar RNS/ROS in the mechanism of BZ genotoxicity. A similar mechanism may explain that indomethacin inhibited BZ toxicity.^[37–40]

It is well known that CPY 2E1 catalysed metabolism is involved in BZ genotoxicity.^[41,42] Although DEX is also an inducer of CYP2E1,^[43–46] that effect is relatively tissue specific, occurs later after administration and is probably of less importance in the present study.^[43,47]

In conclusion, DEX administration ameliorated LPS-enhanced and BZ-induced oxidative DNA damage which correlated with nitrate/nitrite production. These data indicate an important role of inflammatory reactions in BZ bone marrow toxicity.

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