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2-Nitropropane-induced DNA damage in rat bone marrow

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Abstract

DNA damage detected by the comet assay (single cell gel electrophoresis) and 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) formation in DNA in the bone marrow has been studied in groups of 6 male Wistar rats treated with a single i.p. injection of the carcinogen 2-nitropropane (2-NP, 100 mg/kg body weight) or vehicle. Twenty-four hours after 2-NP the average tail length in the comet assay in bone marrow cells was increased from 1.46 ± 0.27 to 9.61 ± 1.56 μm (mean ± SD, $p < 0.01$), and 8-oxodG levels in the DNA were increased from 1.04 ± 0.50 to 5.14 ± 2.42 per 10^5 dG ($p < 0.01$). There was a close correlation between the comet tail length and the 8-oxodG level ($r = 0.89$, $p < 0.05$). The results indicate that 2-NP inflicts DNA damage in the bone marrow cells and thus could be leukemogenic.

Keywords: 2-Nitropropane; Bone marrow; Comet assay; 8-OxodG

1. Introduction

2-Nitropropane (2-NP) has been widely used as an intermediate in chemical syntheses and in formulations of inks, paints, varnishes, adhesives and other coatings [1]. Although 2-NP has not been identified as a definite human carcinogen [2], an epidemiologic study found leukemia and non-Hodgkin's lymphoma among farmers exposed to solvents, including 2-NP [3]. 2-NP is hepatocarcinogenic in rats [4] and is genotoxic in vitro, inducing mutations in bacteria [5,6] and unscheduled DNA synthesis in hepatocytes [7]. It is thought that generation of reactive oxygen species via the metabolism of 2-NP-nitronate to acetone and nitrite plays an important role for the carcinogenic effect [8]. Indeed, in rat liver 2-NP causes mutagenic oxidative DNA damage in the form of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) [9,10]. Following i.p. injection, 2-NP and its carbon-containing metabolites are concentrated initially in fat and subsequently in bone marrow, adrenal glands and other internal organs [1,11]. In addition to liver damage, increased levels of 8-oxoguanosine and 8-oxodG were produced in the kidney of rats given 2-NP [12]. Moreover, DNA strands breaks measured by alkaline elution were observed in rat bone marrow cells after 2-NP administration [13]. Single cell gel electrophoresis (comet assay) of mouse bone marrow cells after in vivo exposure [14], and formation of 8-oxodG in isolated cells and mouse bone marrow indicate DNA damage after exposure to benzene [15]. This compound induces bone marrow depression in both rodents and humans and

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leukaemia in humans [16]. Accordingly, we studied the potential of 2-NP to induce DNA damage in the bone marrow in rats by means of the comet assay and formation of 8-oxodG in DNA.

2. Animals

Male Wistar rats, 6 weeks old and of 200–220 g body weight, were housed three to a cage with aspen wood bedding (Finntapwei, Finland) in an environmentally controlled animals facility operating on a 12:12 h dark/light cycle at 22–24°C and 55% humidity. The rats were given free access to tap water and standard laboratory diet (Altromin 1314, Lage, Germany). 2-NP (Aldrich Chemical Co., Milwaukee, WI, USA), 100 mg/kg body weight dissolved in corn oil (5 ml/kg), was injected i.p. to six rats. Another six rats received the vehicle alone. The 2-NP dose and sample time were chosen according to previous reports of oxidative DNA damage [9,12,13]. All rats were sacrificed 24 h after injection. Bone marrow from the two femurs of each rat was flushed out with 3×3 ml of cold phosphate-buffered saline (PBS, 0.1 M, pH 7.4) supplemented with 10 units/ml heparin. The harvested cells were separated and suspended in PBS, and washed twice with 1 ml of PBS. The cells were then resuspended and diluted to about 500 000 cells per ml. Twenty μl of this suspension was used for the comet assay. The remaining cells were stored at −20°C for analysis of 8-oxodG in the DNA.

3. Comet assay

The comet assay was performed as described previously [14,17] with minor modifications of the technique described by Singh et al. [18]. Eighty-five μl of 1% normal melting agarose (type I-A, Sigma, St. Louis, MO, USA) in Mg^{2+}- and Ca^{2+}-free PBS (0.1 M, pH 7.4) (Gibco BRL, Scotland, UK) was dissolved and spread onto fully frosted microscope slides (Labcraft, Houston) and covered with a 18×18 mm coverslip. After 10 min at 4°C the coverslip was removed and 10 μl of cell suspension mixed with 85 μl of 1% lower melting agarose (type VII, Sigma) at 37°C was pipetted onto the first agarose layer. After 10 min at 4°C with a coverslip the slide was 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. The slides were placed horizontally in a tank with electrophoresis buffer (1 mM Na_2EDTA and 300 mM NaOH, pH 12.7) for 30 min to unwind DNA. 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 three times in a solution of 0.4 M Tris buffer (pH 7.5) and stained for 5 min with 85 μl of 0.5 μM YOYO-1 (Molecular Probes, the Netherlands) in PBS (0.1 M, pH 7.4). Ten minutes after staining, DNA comets were measured with an Olympus image analysis system (Olympus, Denmark) using an excitation filter of 490 nm and a barrier filter of 530 nm. Cells were identified at $400 \times$ magnification. Brightness and contrast were adjusted to clearly define the comet head and tail borders. One coded slide was evaluated per rat and the observer was unaware of the treatment. Fifty randomly selected cells were measured from each slide. Quantification of the DNA damage was calculated as: Comet tail length (μm) = (maximum head–tail length) − (head diameter).

4. Determination of 8-oxodG

Cells were suspended in 1.8 ml ice-cold TE buffer (150 mM NaCl, 10 mM Tris, 10 mM Na_2EDTA, pH 8.0), and 200 μl 1% dodecyl sulfate sodium salt (SDS) was added. After vortexing for 30 s and incubation in a water bath at 37°C for 10 min, 200 μl 3 M sodium acetate (pH 5.2) and 550 μl 5 M sodium perchlorate were added. Chloroform/isoamyl alcohol (24:1) 2 ml was used to purify DNA. After rotation in an extraction bench for 10 min the samples were centrifuged at 3500 rpm for 10 min and 2 volumes of ice-cold 96% ethanol were slowly added to the separated supernatant (non-organic phase). The DNA was allowed to precipitate at −20°C overnight followed by centrifugation at 3000 rpm for 5 min. The DNA precipitate was washed with 70% ethanol, dried with a stream of nitrogen gas, dissolved in 200 μl 20 mM sodium acetate (pH 4.8), and digested to nucleoside level at 37°C with

Table 1
Levels of 8-oxodG in DNA and DNA migration in the comet assay in rat bone marrow nucleated cells 24 h after administration of 2-nitropropane (2-NP, 100 mg/kg b.wt.) or vehicle

	2-NP	Vehicle	*t*-Test
8-oxodG/10^5 dG	5.14 ± 2.42	1.04 ± 0.50	$p = 0.000094$
Comet tail length (μm)	9.61 ± 1.56	1.46 ± 0.27	$p = 0.000187$

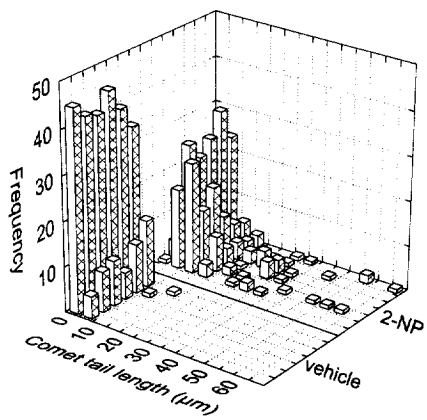

Fig. 1. Distribution of the comet tail length (single cell gel electrophoresis) in 50 individual bone marrow cells collected from each of 12 rats 24 h after treatment with vehicle ($n = 6$, left–front) or 2-nitropropane (2-NP 100 mg/kg; $n = 6$, right–back).

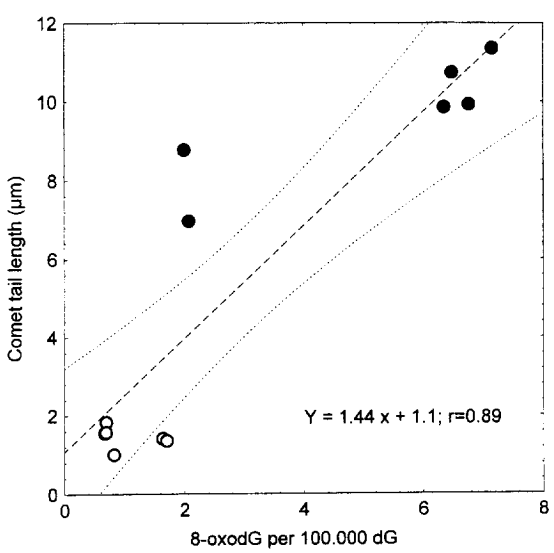

Fig. 2. Relationship between formation of 8-oxodG in DNA and comet tail length (single cell gel electrophoresis) in bone marrow nucleated cells from rats 24 h after i.p. injection of 2-nitropropane 100 mg/kg b.wt. (●) or vehicle (○). The dashed line indicates the regression line with 95% confidence limits (dotted lines).

20 μl (5 U) per sample of Nuclease P1 (Sigma) for 30 min and 20 μl (1 U) per sample of alkaline phosphatase (Boehringer Mannheim, Germany) for another 60 min. The ratio of 8-oxodG to deoxyguanosine (dG) was measured using a HPLC system with electrochemical and UV detections previously described [19].

5. Statistics

2-NP and vehicle treated rats were compared on a group basis by means of *t*-tests. Probability values less than 0.05 were considered statistically significant.

6. Results

The results of this study demonstrated that 2-NP inflicts damage to DNA including oxidative modification of guanine in rat bone marrow cells. Twenty-four hours after a single i.p. dose of 2-NP, rat bone marrow nucleated cells showed an 8-fold increase in the average tail length in the comet assay, indicating DNA damage, although with variation in response between individual cells (Table 1; Fig. 1). The 8-oxodG level in the DNA was increased 5-fold by 2-NP (Table 1). Interestingly, the tail length in the comet assay and the 8-oxodG level in nucleated bone marrow cells were closely correlated ($r = 0.89$, $p < 0.05$) and the corresponding regression line had an intercept not different from zero (Fig. 2).

7. Discussion

The comet assay provides a method for determining conformational and other changes in DNA in individual cells [18]. 8-OxodG is a mutation-prone product of deoxyguanosine oxidation, and a molecular hallmark of 2-NP exposure in the liver [9,10,12,20]. The close correlation between these two assays demonstrates the suitability of the comet assay to detect oxidative DNA damage. As the cause of 8-oxodG formation by 2-NP, involvement of hydroxy radicals produced via metabolism of 2-NP-nitronate has been proposed [9,21]. This metabolism

could be catalysed by myeloperoxidase in the bone marrow, similarly to the toxic effects of benzene metabolites [14–16]. Recently it was suggested that 2-NP metabolism may also generate NO radicals [22]. Although 2-NP has mainly been shown to be hepatocarcinogenic in rats following exposure by inhalation and by gavage, we found substantial DNA damage in bone marrow by the comet assay and 8-oxodG formation. This is in contrast to the reported lack of effect of 2-NP in the mouse bone marrow micronucleus test [1]. The reason for this discrepancy is not clear but is most likely due to species difference [23]. Thus, 2-NP is hepatocarcinogenic only in rats and not in mice [2]. Moreover, 2-NP induced much less 8-oxodG in the liver and is less carcinogenic in rabbits than in rats [24]. In addition, the effects of 2-NP on the bone marrow cells are likely to be time and concentration dependent but the present study with only a single dose and time point do not allow assessment of the maximum effects. The various cell population in the bone marrow may also respond differently to 2-NP as suggested by a variable tail length increase in the comet assay. Alternatively, the micronucleus test could be insensitive or unspecific to detect DNA damage in the form of 8-oxodG and comet formation. However, that is unlikely since there is excellent agreement between these assays with respect to the effect of benzene on mouse bone marrow [14–16]. In conclusion, our study indicates that the comet assay is sensitive for detection of oxidative DNA damage and 2-NP inflicts DNA damage in the bone marrow in rats and could thus be leukemogenic.

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