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Genotoxic effect of ozone in human peripheral blood leukocytes

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Abstract

The genotoxic effect of ozone was studied in human leukocytes in vitro, using the single cell gel electrophoresis (SCGE) assay. Cell treatment for 1 h at 37 °C with 0.9–5.3 mM O_3 resulted in a dose-dependent increase of DNA damage, comparable to that induced by 4–40 mM of H_2O_2, used as a positive control. This effect of ozone was reversed by post-treatment incubation of the cells for 45–90 min at 37 °C, and prevented by pre-incubation of the cells with catalase (20 μg/ml). These results demonstrate that O_3 induces DNA-damage in primary human leukocytes. The damage is rapidly repaired, and probably mediated by the formation of H_2O_2. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Ozone; Human leukocytes; DNA damage; SCGE; Comet assay; Catalase

1. Introduction

The in vivo-genotoxicity of ozone is of interest because of the occurrence of ozone in polluted urban air, and because ozone therapy is used in several countries for the treatment of various conditions such as peripheral circulation disturbances, arthritis, retinitis pigmentosa, and bacterial and viral skin infections [1,2]. Studies of humans exposed to O_3 by inhalation have produced weak or inconclusive results with regard to genotoxic effects, and O_3 has been classified as a weak genotoxin to humans ([3] and references therein) and a weak clastogen in mammalian cells [4].

Most studies of the genotoxic effect of ozone in humans in vivo have been performed to evaluate the hazards of relatively low environmental exposures, and they have not provided clear evidence of such clastogenic activity [5–7]. However, other studies in animals, humans and human cells in vitro have reported increased frequencies of DNA strand breaks in O_3 exposed tissues [8–11]. With regard to the genotoxicity of ozone therapy, the single report that has been published is not conclusive [12]. This may partly be explained by the neutralising effect of antioxidants in plasma [13], and partly by the efficient antioxidant enzymatic defences of the leukocytes [14].

The genotoxic potential of ozone is caused by the reaction of O_3 and its reactive intermediaries (ORI) with cellular macromolecules. When O_3 is dissolved in biological fluids it decomposes and reacts instantly with unsaturated fatty acids in cell membranes generating ORI such as hydrogen peroxide (H_2O_2), aldehydes, ozonides and lipid peroxides [15,16]. Also proteins in plasma and cell membranes are oxidised, mainly in thiol groups. Nucleic acids are unlikely to be damaged

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by ozone itself, but the cascade of ORI that is generated may have genotoxic effects. It is well known, for instance, that H_2O_2 can cause DNA damage via the Fenton reaction [17].

Since ozone therapy may impose a potential genotoxic hazard for patients with insufficient antioxidant defence mechanisms and reduced DNA repair capacity, we considered it of interest to further explore the genotoxic effects of O_3 in human cells. The alkaline single cell gel electrophoresis (SCGE) assay [18] is a fast and sensitive method for the detection of DNA strand breaks. It was used in the present work to demonstrate that O_3 treatment gives rise to DNA damage in peripheral blood leukocytes in vitro. Furthermore, we studied the recovery of cells during post-treatment incubation, as well as the protective effect of catalase pre-treatment on O_3 induced DNA damage.

2. Materials and methods

2.1. Cells and treatments

Peripheral blood was obtained from six healthy, non-smoking volunteers, aged 22–48 years. The blood was diluted in PBS (60 μl blood in 1 ml PBS) and drawn into a 10 ml syringe. O_3 was produced in an O_3-generator (Ozomed, Cuba), and the O_3/O_2 gas mixture was introduced into the syringe, which was incubated while slowly rocking for 1 h at 37 °C. The O_3 concentrations ranged from 0.875 to 5.25 mM. In parallel experiments, H_2O_2 (Panreac, Spain) was added to the diluted blood at concentrations of 4 and 40 mM. Whole blood and blood diluted in PBS were used as non-treated controls (protocol 1). Cell viability determined with the Trypan blue exclusion method after treatment indicated that 80–98% of the cells were able to survive to O_3 and H_2O_2 concentrations used.

To assess post-treatment recovery, cells from four donors treated with the highest dose of O_3 (5.25 mM) were centrifuged, re-suspended in fresh PBS and incubated for 45 and 90 min after treatment (protocol 2). The effect of catalase was studied by pre-incubating the diluted blood from the other two donors with catalase (20 μg/ml, Sigma, USA) for 15 min before O_3 and H_2O_2-treatment as above (protocol 3).

2.2. The SCGE assay

The SCGE assay was performed as described by Singh et al. [18] with minor modifications [19,20]. After treatment, the cell suspensions were centrifuged. Re-suspended pellet (5 μl) was mixed with 75 μl of 0.5% low melting agarose (LMA, Fluka, USA) and added to conventional microscope slides pre-coated with 150 μl of 0.8% normal melting agarose. The slides were covered with coverslips and kept at 4 °C for 5 min to solidify the LMA. The coverslips were removed, a top layer of 75 μl LMA was added and the slides were kept again on ice for 2 min. After removal of the coverslips, the slides were immersed for 1 h at 4 °C in lysis solution; 2.5 M NaCl (Fluka), 100 mM EDTA (Sigma), 10 mM Tris (Fluka), 1% Triton X-100 (BDH, UK), 10% DMSO (Sigma), pH 10.0. The slides were then placed in an electrophoresis chamber containing 0.3 M NaOH (Sigma) and 1 mM EDTA at 4 °C for 20 min before electrophoresis (250 mA, 25 V, 30 min) in the same solution. The slides were washed 3× for 5 min in neutralising buffer (0.4 M Tris–HCl, pH 7.5) and let dry at 37 °C for 20 min before staining with ethidium bromide (Sigma, 20 μg/ml).

The slides were observed under a fluorescence microscope with a calibrated ocular micrometer. Images of 50 randomly selected cells (25 cells from each two replicate slides) were analysed from each treatment. From each cell, the length of the image (diameter of the nucleus plus migrated DNA) was measured in microns at a 400× magnification [19].

Differences between treatment means were tested for significance using Kruskall–Wallis and Dunn tests.

3. Results

Initial experiments showed that untreated cells in whole blood and PBS-diluted blood did not differ statistically with respect to DNA migration (image length) and percentage of damaged cells (data not shown). PBS-diluted blood was therefore used as control in subsequent experiments.

Tables 1 and 2 show the percentages of damaged cells and DNA migration measurements (image length in μm) after treatments of blood leukocytes from six healthy donors with O_3 and H_2O_2 in vitro. In spite of some individual variation with regard to the

Table 1
Percentages of damaged cells after in vitro treatment of peripheral leukocytes with H_2O_2 and O_3

Dose (mM)	Donor 1	Donor 2	Donor 3	Donor 4	Donor 5	Donor 6
Without catalase pre-treatment						
Control	8	10	10	6	36	20
H_2O_2 (4)	60	72	72	76	96	80
H_2O_2 (40)	92	76	100	94	96	98
O_3 (0.875)	50	78	82	78	84	76
O_3 (1.75)	64	84	92	90	88	80
O_3 (3.5)	66	86	94	88	90	96
O_3 (5.25)	90	94	100	98	92	96
Post-treatment incubation						
45 min	22	12	6	10	ND[a]	ND[a]
90 min	10	12	8	6	ND	ND
With catalase pre-treatment						
Control					16	2
H_2O_2 (4)					26	36
H_2O_2 (40)					26	34
O_3 (0.875)					28	20
O_3 (1.75)					34	30
O_3 (3.5)					36	36
O_3 (5.25)					28	36

Data shown are based on treatment means; 50 cells per treatment were measured.
[a] ND: not done.

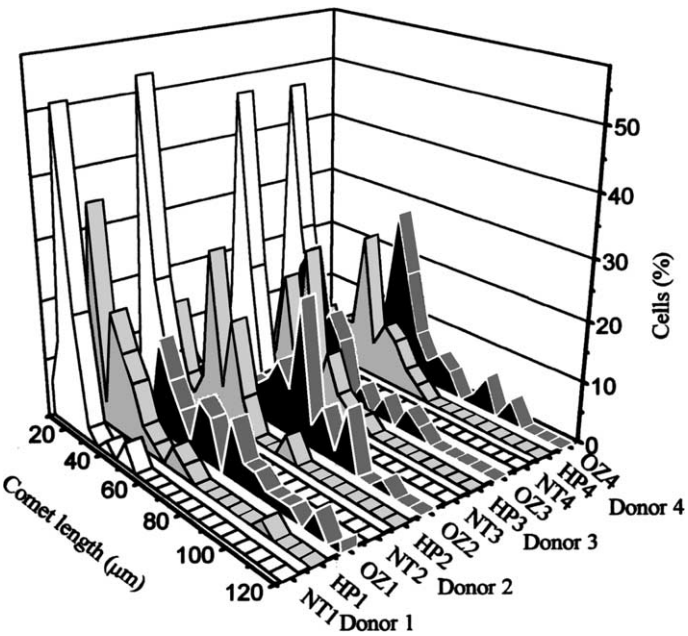

Fig. 1. Distribution of DNA image lengths in leukocytes of four donors after 1 h treatments in vitro with H_2O_2 (HP, 4 mM) and O_3 (Oz, 5.25 mM). Treatments were done at 37 °C for 1 h. Aliquots were taken to SCGE assay. Fifty cells per sample were registered. Data same as Table 2.

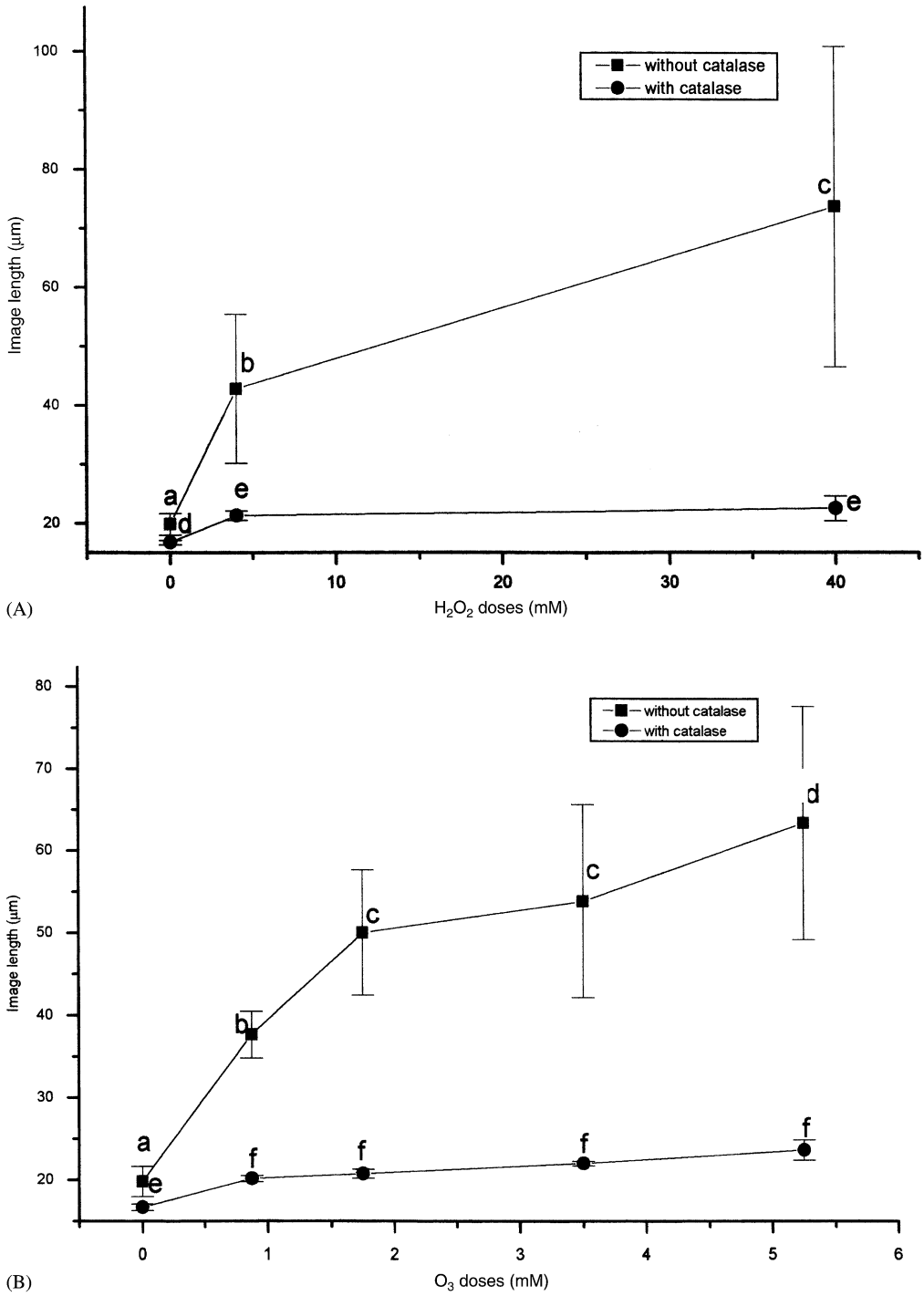

Fig. 2. Image length in human peripheral leukocytes after treatment with H_2O_2 (A) and O_3 (B). Mean ± S.D. of individual donors (pooled data from Table 2). Different letters indicate statistical differences between the treatments ($P < 0.05$); squares: no catalase pre-treatment ($N = 6$); circles: with catalase pre-treatment for 15 min before addition of H_2O_2 or O_3 ($N = 2$).

Table 2
Tail image length (μm) in human leukocytes after H_2O_2 and O_3 treatment. (DNA migration mean of 50 cells ± S.E. per data treatment point)

Dose (mM)	Donor 1	Donor 2	Donor 3	Donor 4	Donor 5	Donor 6
Without catalase pre-treatment						
Control	19.95 ± 6.75	19.71 ± 5.47	20.83 ± 6.25	19.69 ± 4.86	22.13 ± 10.63	16.63 ± 7.25
H_2O_2 (4)	29.59 ± 15.31*	34.53 ± 13.94*	40.18 ± 15.66*	42.96 ± 16.46*	66.29 ± 16.87*	42.80 ± 17.70*
H_2O_2 (40)	56.44 ± 21.43*	41.98 ± 19.20*	99.52 ± 31.71*	50.01 ± 14.78*	103.71±43.12*	90.36 ± 32.80*
O_3 (0.875)	33.51 ± 17.95*	38.30 ± 14.48*	39.55 ± 13.84*	40.25 ± 14.39*	39.56 ± 16.81*	34.61 ± 14.40*
O_3 (1.75)	46.37 ± 29.13*	50.29 ± 16.70*	39.86 ± 13.02*	46.17 ± 15.21*	60.20 ± 19.13*	57.54 ± 9.75*
O_3 (3.5)	39.47 ± 22.43*	55.42 ± 18.38*	45.04 ± 12.49*	49.43 ± 17.42*	62.88 ± 23.39*	71.16 ± 13.65*
O_3 (5.25)	55.82 ± 25.48*	71.73 ± 19.67*	43.78 ± 14.60*	55.78 ± 16.42*	83.00 ± 26.90*	70.49 ± 22.12*
O_3 (5.25 mM) post-treatment incubation						
45 min	23.28 ± 14.02	23.40 ± 9.04*	19.87 ± 6.21	19.56 ± 5.07	ND[a]	ND[a]
90 min	21.01 ± 9.0	19.75 ± 5.84	19.83 ± 6.44	20.10 ± 5.58	ND[a]	ND[a]
With catalase pre-treatment						
Control					16.98 ± 8.71	16.43 ± 6.85
H_2O_2 (4)					20.75 ± 9.54*	21.81 ± 11.88*
H_2O_2 (40)					20.95 ± 10.41*	23.92 ± 15.41*
O_3 (0.875)					20.39 ± 8.53*	19.88 ± 9.41*
O_3 (1.75)					21.10 ± 7.12*	20.34 ± 9.95*
O_3 (3.5)					21.82 ± 10.42*	22.18 ± 9.57*
O_3 (5.25)					22.77 ± 12.68*	24.51 ± 18.48*

[a] ND: not done.
* $P < 0.05$ (significantly different with respect to non-treated control).

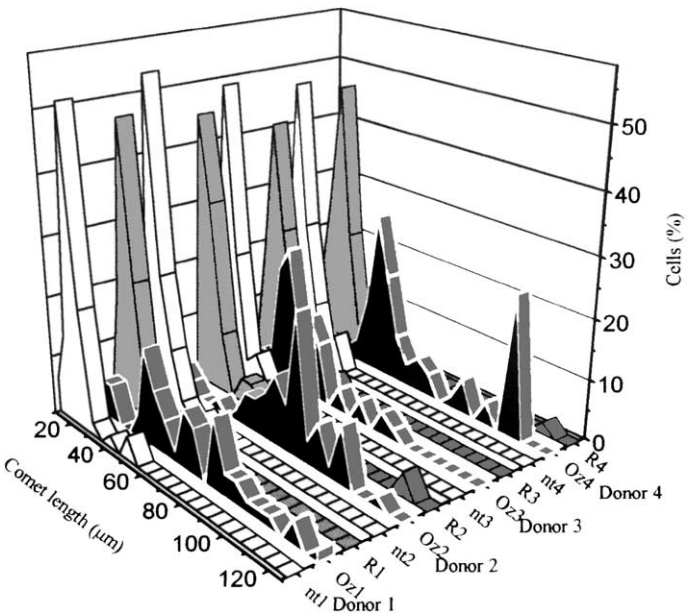

Fig. 3. Distribution of cells based on image length in four donors studied to determine damage reversal after O_3 treatment. Oz: treatment with O_3 (5.25 mM) for 1 h at 37 °C; R: damage reversal in cells incubated for further 45 min after treatment; nt: non-treated control cells. Data same as Table 2.

distribution of comet lengths (Fig. 1), there was a clear effect of both treatments with respect to the fraction of damaged cells as well as the comet length. The compiled data from the six donors showed a dose dependent effect for both H_2O_2 (Fig. 2A) and O_3 (Fig. 2B).

Pre-incubation of cells from two donors with catalase for 15 min significantly decreased the percentages of damaged cells and comet length, even in the controls, compared with the corresponding treatments without catalase ($P < 0.001$), suggesting that H_2O_2 is the main mediator of these effects. However, the damage levels in the catalase-pre-treated cells exposed to O_3 and H_2O_2 were still significantly higher ($P < 0.05$) than in the untreated catalase control, suggesting that other intermediaries in addition to H_2O_2 were causing DNA damage as well (Tables 1 and 2).

Cells from four donors treated with the highest dose of O_3 (5.25 mM) were allowed to recover in the incubator for 45 or 90 min. In all cases, this post-treatment incubation reduced the percentages of damaged cells (Table 1) and the image length (Table 2, Fig. 3) to control levels, indicating that the cells recover rapidly from the genotoxic effect of O_3 treatment.

4. Discussion

Our present results show that exposure of blood leukocytes to O_3 gives rise to an immediate and dose-dependent increase of DNA damage that is rapidly reversed during prolonged incubation, and it is likely to be mediated mainly by H_2O_2, because most of the effect is prevented by pre-incubation with catalase.

It is doubtful that O_3 itself is the agent causing DNA damage. However, in reactions with unsaturated fatty acids and thiol groups in proteins, a cascade of intermediaries with H_2O_2 as one of the final products are generated [15,21].

On the other hand, the ability of O_3 treatment to cause DNA damage in vivo via H_2O_2 formation and the Fenton reaction has been questioned, because of the enormous quantities of oxidant sequestering agents present in whole blood [22]. It is well known that H_2O_2 generates DNA single strand breaks at concentrations as low as 25 μM, and produces a clear dose effect relationship at higher concentrations in different cells types [23–28]. Our results in this study demonstrate that the DNA strand breaks induced by O_3 treatment are likely to be mediated by H_2O_2 because of the efficient protective effect of catalase (Fig. 2B). The use of catalase to protect against the genotoxic effects of H_2O_2 has also been applied in other in vitro systems with similar results [26,29,30].

It should be noted that catalase pre-treatment decrease DNA damage levels also in untreated controls. This result could be due to the endogenous damage inactivation or removal as a consequence of the cellular metabolism that generates H_2O_2 between other reactive oxygen species or exogenous oxygen intermediaries provided by cell manipulation [31,32].

Although H_2O_2 is a rather stable molecule in aqueous solution, it can cause strand breaks in purified DNA [33]. In biological systems, the genotoxic effects of H_2O_2 (DNA strand breaks and DNA adducts) are mediated by the very reactive $OH^{\bullet}$ radical that is produced by the redox cycle properties of transition metals [23,29,34]. It has been reported that quaternary structure of chromatin depends on copper and iron DNA–protein associations [35]. However, Akman et al. [36], reported that the oxidative base damage depends mainly on primary DNA sequence and that the role of transition metals associated with chromatin is not relevant. Nevertheless, when oxidative stress is extreme (50 mM H_2O_2) transition metal ions may be liberated from extranuclear proteins and transported into the nucleus, thereby allowing transition metal catalysed reactions in the close vicinity of DNA.

The kinetics of DNA strand break repair caused by oxidant agents in different cell systems has been studied extensively. There is no general agreement regarding the time needed to complete DNA chain ligation by the DNA repair mechanisms involved and recovery times range from a few minutes till 2 h [27,37–42]. The almost complete recovery of the DNA oxidative damage in so short time is reported for a number of oxidative modifications such as 8-OH-guanosine, 5,6-diOH-uracil, 2-OH-adenine, 5-OH-cytosine, 5-OH-methyluracil. All these oxidative modifications can produce breaks in the sugar phosphate bond and then generate single or double strand breaks [41]. Nevertheless, it seems that DNA strand break rejoining and base excision repair, the principal mechanisms involved in the repair of oxidative DNA damage, are completed in a few hours after

introduction of the damage [27,37,38,42]. In our case this short time of recovery could indicate that the induced lesions are mainly base or pentose oxidations (Tables 1 and 2).

In summary, we have demonstrated that O_3 induces DNA damage in human peripheral leukocytes in vitro. The DNA damage is likely to be dominated by DNA strand breaks, it is reversible, and mainly mediated by the formation of H_2O_2. Further studies of the effect of ozone in blood cells in vivo are warranted in order to assess the possible risk of genotoxic effects associated with human ozone therapy.

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