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Effect of ozone on neutrophil function *in vitro*

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Summary The application of ozone is widely practised as a form of alternative medicine, particularly in Germany and Eastern Europe. Ozone major autohemotherapy (the return of a small amount of a patient's blood to the circulation after *ex vivo* exposure to ozone) has been reported to have a therapeutic effect in various pathological conditions, including ischemic, infectious, autoimmune and neoplastic disorders. Ozone has an effect on the expression of cytokines, adhesion molecules and acute phase reactants, which are responsible in part for the respiratory inflammatory response observed after exposure to this gas. The purpose of the present study was to investigate the effect of ozone administration *ex vivo*, at a concentration commonly used in major autohemotherapy, on peripheral blood neutrophil function *in vitro*. Blood drawn from healthy volunteers was studied for neutrophil adhesion, chemotaxis and O_2^- production before and after exposure to 30 μg/ml ozone. There was no significant difference in adhesion and chemotaxis of neutrophils exposed to ozone versus unexposed cells. O_2^- production was minimally decreased (20.3 ± 5.0 *vs.* 22.1 ± 5.5 nmol/10^6 cells/10 min, respectively; $P = 0.01$), a reduction of no clinical significance. This study confirms that major autohemotherapy with ozone is safe as far as neutrophil function is concerned. Combined with previous data, it seems that well-designed clinical trials to assess the efficacy of major autohemotherapy would pose no danger to blood cell populations, and should be encouraged.

Keywords Ozone, neutrophils, chemotaxis, adhesion, superoxide

Introduction

Ozone, in its gaseous state, is toxic to the airways and to the lungs (Bhalla, 1999). In solution, however, it may have therapeutic effects. Ozone's potential therapeutic effect is currently utilized in Germany and Eastern Europe within the scope of 'nonconventional' medicine for the treatment of a wide range of disorders, including amongst others, various neoplastic and inflammatory states, infections, burns, pressure sores and peripheral vascular disease in diabetic and nondiabetic patients (Paulesu *et al.,* 1991; Bocci, 1994). The treatment can be administered by different routes, including topically (in the form of ozonized water or oil), by major autohemotherapy (a procedure in which blood is drawn from a patient, exposed to ozone *ex vivo* and returned to the patient immediately after exposure), intravenously, intra-arterially, intra-articularly and intrathecally (Bocci, 1994). The therapeutic concentrations of ozone vary in relation to the mode of administration; the lowest concentrations (30–60 μg/ml) are used in major autohaemotherapy.

Although ozone is considered to be one of the most potent oxidants in nature, the mechanism of its therapeutic action is unclear. Several possible explanations have been proposed, including: generation of peroxides by ozonolysis with unsaturated fatty acids in cell membranes (Bocci, 1994; Verrazzo *et al.,* 1995), activation or generation of

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reactive oxygen species which function as physiological enhancers of various biological processes (including increased production of ATP) (Bocci, 1996a; Bocci, 1996b), and increased expression of intracellular enzymes with antioxidant activity (Bocci, 1996a; Bocci, 1996b). It has been reported that exposure to ozone results in a change in the level of a variety of biological factors, e.g. cytokines [IFNγ (Bocci & Paulesu, 1990), TNFα (Paulesu, *et al.,* 1991), TGFβ (Bocci *et al.,* 1994) and IL-8 (Bocci *et al.,* 1998)], acute phase reactants (Bocci *et al.,* 1993–94) and adhesion molecules from the integrin family such as CD11b (Hoffer *et al.,* 1999). Other reports suggested increased motility and adhesion of peripheral blood polymorphonuclear cells to epithelial cell lines derived from rat lung after exposure of rats to ozone (Bhalla *et al.,* 1993). Similarly, major autohemotherapy-induced leukocytosis and enhanced phagocytic activity of polymorphonuclear cells (Bocci, 1994) were reported.

Despite reports of its putative therapy and the relative safety of ozone therapy, and despite the low cost of this treatment, prospective, randomized, placebo-controlled studies, examining the possibility of integrating ozone major autohemotherapy into 'conventional' medicine, have not been undertaken. A requisite preliminary step is a demonstration of the safety of ozone treatment to the various blood cell populations, especially as the mechanism of ozone action is unclear. In the past, we have investigated the effect of ozone treatment *ex vivo* on red blood cell metabolism and haemolysis (Zimran *et al.,* 1999), and found no significant adverse effects. The purpose of the present study was to investigate the effect of exposure of blood to ozone *ex vivo* on certain neutrophil functions *in vitro*.

Materials and methods

Materials

Ozone was produced by a Dr Hänsler generator PM100 (Frankfurt, Germany). Dextran 70 (Macrodex 6% in 0.9% NaCl) was obtained from Pharmacia, Uppsala, Sweden. Horse heart cytochrome C (type IV), bovine erythrocyte superoxide dismutase (SOD), zymosan A, bovine serum albumin (BSA) and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma, St Louis, MO. Stock solution of PMA in dimethyl-sulfoxide (1 mg/ml) was stored at −70°C. To prepare activated serum, zymosan A was boiled in 1N NaOH solution for 10 min, then washed three times in Geys Balanced Salt Solution. After addition of freshly prepared human serum (15 mg zymosan/1 ml serum) the mixture was incubated for 20 min at 37°C, after which the zymosan was removed by centrifugation ($800 \times g$ for 10 min at 4°C). The serum was divided into aliquots and stored at −70°C. The buffer used was Dulbecco's phosphate-buffered saline (PBS). Where indicated, PBS with calcium and magnesium (PBS ++) and PBS ++ enriched with 6 mg/ml BSA and 1 mg/ml glucose (PBS +) were used. Blind-well Boyden chambers were purchased from Nucleopore Corporation, Bethesda, MD; corresponding filters (Membranafilter, 3 μm pore size) were purchased from Sartorius, Gottingen, Germany.

Methods

Subjects and ozone treatment

The study was performed on 15 healthy volunteers (age range 23–60) who were not receiving any form of medication. The study was approved by the Ethics Committee (IRB) of the Shaare Zedek Medical Center. Half of the blood drawn from each subject was exposed to ozone (the 'test' sample), and compared with the other half, exposed to room air (the 'control' sample). The colour of blood exposed to ozone is brighter than the colour of blood without such exposure. Thus, the adhesion assay, performed on whole blood, could not be blinded. The chemotaxis and O_2^- production assays were performed on purified neutrophil populations, and hence were blinded.

For the adhesion assay, 6 ml of blood was drawn into each of two heparin containing test tubes (a total of 12 ml blood per subject). Six millilitres of ozone at a concentration of 30 μg/ml was bubbled into one of the test tubes. For the chemotaxis and O_2^- production assays, 50 ml of blood was drawn from each subject into a siliconized vacuum bottle of the autohemotherapy set, containing 10 ml of 3.3% sodium citrate. Half of the blood was transferred to a 50 ml syringe (the 'control' sample); 25 ml of ozone, at a concentration of 30 μg/ml, was bubbled into the 25 ml of blood that remained in the bottle (the 'test' sample).

Separation of neutrophils and assessment of their function were performed by routine methods employed in research and in clinical practice (Matzner & Sallon, 1995).

Neutrophil function studies

Adhesion of neutrophils (exposed and unexposed to ozone) to 160 mg of condensed nylon fibres in a Pasteur pipette was performed using whole heparinized blood, as previously described, with minor modifications (MacGregor *et al.,* 1974). Neutrophil counts were made on whole blood incubated at 37°C for 15 min, before and after blood flowed

through a nylon fibre column. The results were expressed as the percent of neutrophils that adhered to nylon fibres as follows (ANC = absolute neutrophil count):

$$\text{Adhesion (\%)} = 100 - (\text{ANC in effluent/ANC in original blood}) \times 100$$

For the chemotaxis and O_2^- production assays, neutrophils were separated from whole blood by dextran sedimentation followed by hypotonic lyses and centrifugation (Boyum, 1968). This procedure left > 85% neutrophils in the purified sample.

Neutrophil migration was assayed by the leading front technique using blind-well Boyden chambers (Zigmund & Hirsch, 1973), with slight modifications (Matzner & Brzezinsky, 1984). The chemotactic agent used was 3% zymosan-activated serum (ZAS) as a source of C5a desarg. The upper chambers contained $1–2 \times 10^6$ cells per ml PBS +. Random motility was determined by measuring migration towards PBS +. Incubations were carried out at 37°C for 50 min, after which the filters underwent a staining cascade and reading at × 400 magnification for estimation of the migration distance from the leading front.

Neutrophil O_2^- production was determined by measuring superoxide-dismutase (SOD) inhibitable cytochrome C reduction (Matzner *et al.*, 1982), using PMA as an inducer. Reaction mixtures contained 5×10^6 neutrophils, 500 ng PMA, 3.6 mg cytochrome C and (for measuring O_2^- independent cytochrome C reduction) 30 μg SOD in 2 ml PBS ++. Incubations were conducted at 37°C for 10 min. To examine the effect of ozone on unstimulated neutrophils, we repeated the assay in five subjects exactly as described above, replacing PMA with PBS ++.

Statistical analysis

The significance of differences between the test group and the control group was analysed using the paired *t*-test (two tailed). A *P*-value of < 0.05 was considered statistically significant.

Results

All the experiments gave results that fell within clinically normal ranges: 85–100% in the adhesion assay, 30–60 μm and 70–100 μm in the random and 3% ZAS induced migration assays, respectively, and 10–30 nmol/10^6 cells/10 min in the O_2^- production assay.

Results for the control and test samples in the adhesion assay were identical: 97.6 ± 2.2% and 97.5 ± 2.2%, respectively, with a range of 91.1–99.6% and 91.3–99.6%, respectively (Table 1).

In the chemotaxis assays a clinically significant difference of 20% between ozone-treated and untreated cells was defined as meaningful. Results of the control and test samples in the random chemotaxis assay were 46 ± 8 μm and 49 ± 6 μm, respectively, with a range of 30–60 μm and 39–57 μm, respectively. Results for the control and test samples in the 3% ZAS induced migration assay were 81 ± 9 μm and 83 ± 6 μm, respectively, with a range of 63–98 μm and 71–91 μm, respectively (Table 1). These differences were not statistically significant.

O_2^- Production

Results of the control and test samples in the PMA activated O_2^- production assay were 22.1 ± 5.5 and 20.3 ± 5.0 nmol/10^6 cells/10 min, respectively ($P = 0.01$), with a range of 10.4–26.8 and 10.4–26.8, respectively. Despite the statistical significance, the 8.1% decrease in the mean of the ozone exposed group lacks clinical importance, and all the levels remained in the normal range (Figure 1). Results of the control and test samples in the nonactivated (no PMA) O_2^- production assay were 0.3 ± 0.1 and 0.3 ± 0.3 nmol/10^6 cells/10 min, respectively, with a range 0.2–0.3 and 0.0–0.8, respectively.

Discussion

Major autohemotherapy with ozone, a common form of treatment in alternative medicine, has been reported to

Table 1 Effect of ozone on neutrophil functions. Neutrophils were isolated and studied after whole blood was exposed to room air [(−) ozone] or 30 μg/ml ozone [(+) ozone] as described in Materials and Methods. Results are expressed as mean ± SD. *n*, Number of experiments; each performed on a different healthy donor. *$P = 0.01$.

Function	*n*	(−) Ozone	(+) Ozone
Adhesion (%)	14	97.6 ± 2.2	97.5 ± 2.2
Migration (μm):			
Random	14	46 ± 8	49 ± 6
ZAS induced	14	81 ± 9	83 ± 6
O_2^- production: (nmol/10^6 cells/min)			
Uninduced PMN	5	0.3 ± 0.1	0.3 ± 0.3
PMA induced PMN	15	22.1 ± 5.5	20.3 ± 5.0*

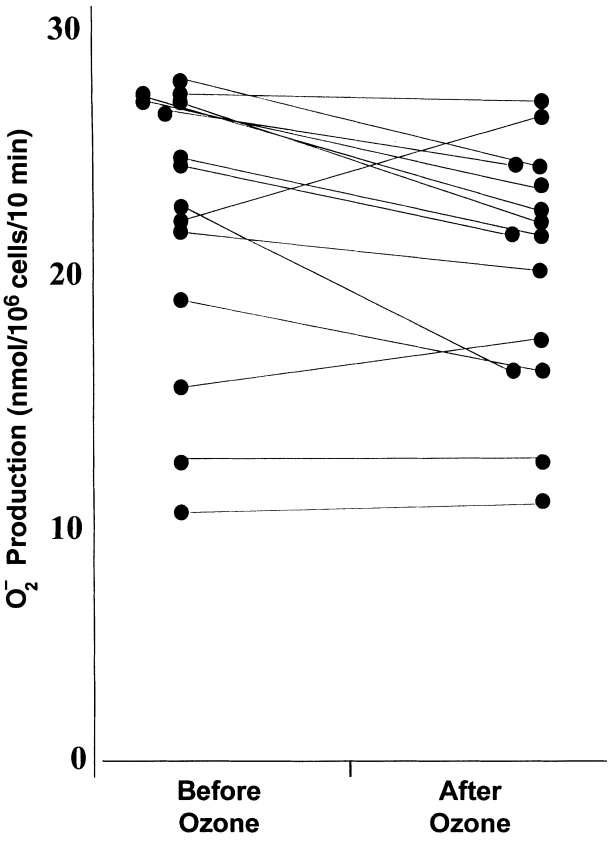

Figure 1. The effect of ozone on neutrophil O_2^- production. O_2^- production by PMA induced neutrophils after exposure of whole blood to room air (before ozone) or 30 μg/ml ozone (after ozone) was carried out as described in Materials and Methods. Each pair of dots represents an experiment performed on a different healthy donor.

have beneficial effects in a wide range of pathological conditions, including various inflammatory, ischaemic, infectious and neoplastic disorders, with minimal adverse side-effects (Paulesu *et al.*,1991; Bocci, 1994). Despite this data and the low cost of treatment, prospective, randomized, placebo-controlled clinical trials to examine the safety and efficacy of treatment have not been undertaken. Such studies would be in line with the recent American National Institutes of Health (NIH) initiative to support the investigation of alternative and complementary medicine (Jonas, 1998).

The present study investigated the effect of ozone administration, at a concentration commonly used in major autohemotherapy, on peripheral blood neutrophil adhesion, chemotaxis and O_2^- production in blood from healthy volunteers, before and after exposure to 30 μg/ml ozone. There was no significant difference between the neutrophils' various functions before and after exposure to ozone. The minimal decrease in PMA induced O_2^- production after exposure to ozone, though statistically significant ($P = 0.01$), has no clinical relevance. Therefore, autohemotherapy with ozone apparently has no damaging effects on adhesion, chemotaxis or O_2^- production of peripheral blood neutrophils, and may be considered safe in regard to these parameters. Accordingly, it seems reasonable to assume that there would be no damage to neutrophils in the systemic circulation after return of ozone treated blood to the patient, as these cells are exposed to comparably much lower concentrations of ozone due to > 0 : 100 dilution in the circulation.

Major autohemotherapy with ozone is claimed to have anti-inflammatory effects. One of the purposes of our study was to investigate whether exposure of blood to ozone induces the anti-inflammatory mechanism via depression of neutrophil functions. In view of the results of this study and assuming that an anti-inflammatory effect of ozone does in fact exist, it seems that an explanation other than depression of the neutrophil functions herein examined is to be sought.

Experiments in rats exposed to gaseous ozone (not in solution) revealed increased adhesion and chemotaxis of peripheral blood neutrophils to an epithelial cell line derived from rat lung (Bhalla *et al.,* 1993). In the present study, in which cells were exposed to ozone *in vitro*, the exposure did not result in alteration of adhesion, random migration or C5a directed migration. It has to be noted that the adhesion method used by us was designed to study only decreased adhesion; detection of increased adhesion requires different methods.

It is important to emphasize the limitations of this paper as an *in vitro* study of *ex vivo*-treated whole blood. The lack of change in chemotaxis represents a true lack of change in an *in vitro* setting, but may not faithfully represent the situation *in vivo*. The possibility that exposure to ozone *in vivo* may change neutrophil function due to alterations in factors tangential to the neutrophil, e.g. cytokine and chemoattractant levels or expression of adhesion proteins, was not excluded. Additionally, whilst this study evaluated the existing pool of mature circulating neutrophils, one cannot rule out that *in vivo* exposure to ozone may also have direct effect on myelopoiesis in the bone marrow, as suggested by the induction of leukocytosis after autohemotherapy with ozone (Bocci, 1994).

In view of the results of this study, combined with the results of the previous study, which showed no significant damaging effects on red cell enzyme levels and haemolysis, it may be inferred that therapeutic clinical trials to assess the efficacy of major autohemotherapy with ozone should pose no danger to blood cell populations. Such well-designed clinical studies may have a significant impact on

the future application of ozone therapy, and are to be encouraged.

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