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Full Length Research Paper

The protective effect of plasma antioxidants during ozone autohemotherapy

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Ozone (O_3) therapy forms part of a group of complementary and alternative medical therapies and is gaining more and more interest worldwide. There is, however, some concern regarding O_3-toxicity and uncertainty about the effectiveness of O_3-therapy. In this study we investigated the possible protective effects of the plasma antioxidant defense system during O_3-AHT. Venous blood from six apparently healthy human donors was collected. In one part of the study a precise volume of blood was mixed with an equal volume of O_2/O_3 gas mixture containing 20 or 80 µg/ml O_3 for 20 min. In the other part, the plasma was washed out, the cells resuspended in a buffered phosphate solution and treated with same concentrations of O_3. Control samples was not treated or treated with O_2. Ozone-AHT caused increased plasma hydroperoxide levels and glutathione ratio. Antioxidant enzyme (catalase, glutathione reductase, glutathione peroxidase) activity of peripheral blood mononuclear cells (PBMC) decreased, whereas superoxide dismutase levels increased slightly. Plasma antioxidant capacity decreased. These effects were more evident in the absence of plasma antioxidants. Therefore the damaging effects of O_3 were quenched by the antioxidants present in plasma.

Key words: Ozone autohemotherapy, oxidant/antioxidant status, plasma antioxidants, glutathione, antioxidant enzymes.

INTRODUCTION

Alternative and complementary medicine are used when conventional medicine have failed and sometimes also used in conjunction with conventional medicine. Ozone (O_3) therapy is only one of many alternative approaches that have gained attention in the last couple of years. Claims are made that O_3 therapy can be used to treat various medical conditions, including diabetes mellitus, ischaemic disorders, malaria, open wounds and ulcerations (Al-Dalain et al., 2001; Ajamieh et al., 2002; Viebahn-Hansler et al., 2001; Jordan et al., 2002) and has even been proposed as a possible treatment for HIV/AIDS (Bocci, 1996; Shallenberger, 1998). With O_3-AHT, a specific volume of blood is drawn from the patient, mixed with a given volume of O_3/O_2 gas mixture having a predetermined O_3 concentration, and then returned to the patient. Once returned, ozonated blood is rapidly distributed to all tissues (Bocci, 2002).

Ozone can act as an oxidant either directly, when it dissolves in plasma and reacts with polyunsaturated fatty acids, antioxidants, cysteine-rich proteins and carbohydrates or indirectly, through the formation of reactive oxygen species (ROS), including hydrogen peroxide (H_2O_2) (Pryor et al., 1995). The purpose of O_3-AHT is to use O_3 *in vitro* as a drug for a brief period to achieve certain biological effects that can block an infection, improve O_2 delivery to anoxic tissue and upregulate antioxidant systems and so reduce chronic oxidative stress. This is done without directly exposing the patient to O_3 (Bocci, 2002). It has been shown that ROS, including H_2O_2 and lipid oxidation products (LOPs) generated by O_3-AHT, can enter the cells from the plasma and activate nuclear factor kappa B (NF-κB) to induce cytokine production in normal cells (Bocci, 1996) and so enhance the immune response (Larini and Bocci, 2005; Larini et al., 2001).

It is clear that the surfactant, the first compartment that comes in contact with O_3 when inhaled, is well equipped (under normal circumstances) with antioxidants to coun-

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ter harmful effects of inhaled oxidants. Unfortunately, O_3 remains toxic and inflammatory when it is inhaled (Mudway and Kelly, 2000), although it appears to be non-carcinogenic (Witschi et al., 1999). The antioxidant defence systems in blood are more than adequate to deal with ROS formed by O_3. It is not clear what the effects of O_3 are on blood that is directly exposed to O_3, such as in O_3-AHT. The aim of the study was therefore to determine if the *in vitro* exposure of blood to O_3 is toxic to blood or if it has certain beneficial effects in the blood. The strategy was to use a model where the effects of O_3 could be determined on PBMC in an intact antioxidant defence system (whole blood, including plasma antioxidants) and on PBMC where the antioxidant system was removed (without plasma antioxidants). In the latter case the plasma, and therefore the antioxidant defence systems, was washed out and replaced with phosphate buffered saline (buffered cells). These two groups were then exposed to O_2 and different O_3 concentrations to evaluate possible positive and/or negative effects. In contrast with previous studies which focused more on the release of cytokines from PBMC, this study focussed more on the levels of antioxidant enzymes in PBMC.

MATERIALS AND METHOD

Ozone generation and measurement

Briefly, O_3 was generated from ultrapure oxygen (> 99.9%) using electrical corona arc discharge by an ozone generator (School of Physics, North-West University, Potchefstroom, USA patent 09/914,199). This ozone generator allows the ozone concentration to be controlled in real time by photometric determination. Tygon polymer tubing (ozone-resistant) were used to ensure containment of ozone and consistency in concentrations.

Treatment protocol

The study was approved by the ethics committee of the North-West University (M0507). Venous blood in heparin (20 U/ml) was collected from six apparently healthy human donors. The blood was subdivided in siliconized glass syringes. Equal aliquots were exposed directly to oxygen or oxygen/ozone with different ozone concentrations (20 and 80 µg/ml) in a 1:1 ratio. The control sample received no treatment. The O_3 concentration was monitored during exposures, using a specially designed quarts cell containing an inlet, where gas entered from the O_3 generator and an outlet, where the gas left the cell and entered the syringe containing the blood. The blood was then briefly mixed with the gas.To determine the protective effect of plasma antioxidants, the plasma was removed by centrifugation. Briefly, the blood was centrifuged for 15 min at 180 x g. The plasma was then carefully removed without disturbing the plasma/cell interface. The plasma was replaced with the same volume of phosphate buffered saline (PBS). This wash procedure was repeated twice to give a total of three washes. The PBS suspended blood cells were then treated with oxygen or ozone as described, for whole blood.

Oxidative stress

Hydroperoxides in plasma or PBS buffer were measured with a commercially available kit (the d-ROMs test; DIACRON International, Grosseto, Italy). Changes in absorbance were measured kinetically at 485 nm every 3 min for 15 min using a Bio-Tek microplate reader (FL 600). The concentration of hydroperoxides in the sample was expressed as Carratelli units (CARR U) where 1 CARR U is equal to 0.08 mg/100 ml H_2O_2 (Iorio, 2002).

The ratio of reduced and oxidized glutathione was measured as described (Asensi et al., 1999), but with minor modifications. Briefly, a GSSG sample was prepared by adding a GSH-scavenger, M2VP (1-methyl-2-vinylpyridinium trifluoromethane sulfonate) to the samples. Metaphosphoric acid (5% v/v) was then added to the GSSG and total GSH samples to precipitate the proteins. DTNB was added to convert GSH into a spectrophotometrically detectable product followed by glutathione reductase and NADPH. GSH (3 µM) was used as standard. The absorbance was measured at 412 nm every min for 5 min using a Bio-Tek microplate reader (FL 600).

Antioxidant capacity

The total antioxidant capacity in plasma was measured by using the ferric reducing antioxidant potential (FRAP) assay (Benzie and Strain, 1999). A standard series of ascorbic acid (1mM) was used in the assay. Samples and acetate buffer (300 mM, pH 3.6) were added to the wells of a microtitre plate. FRAP reagent (acetate buffer, 10 mM TPTZ and 20 mM $FeCl_3.6H_2O$; v/v 10:1:1) was added to standards and samples. After the plate was incubated for 15 min at room temperature, the absorbance was measured at 593 nm with a Bio-Tek (FL 600) microplate reader.

Oxygen radical absorbance capacities (ORAC) were measured in the non-protein fractions of plasma essentially as described by Cao and Prior (1999). Trolox was used as standard series in the assay and plasma antioxidant capacity was expressed as Trolox equivalents (TE). Fluorescein (56 nM) was used as fluorescent decay marker. The reaction was started by adding 240 mM AAPH (2,2'-azobis(2-aminopropane)dihydrochloride). The fluorescence (excitation 485nm, emission 520nm) was recorded with a Bio-Tek (FL 600) microplate reader every 5 min for 2 h or until the last reading has decreased to less than 5% of the first reading. Polynomial regression analysis was used to determine antioxidant capacity.

Cellular antioxidant enzyme activity

The antioxidant enzyme analyses were done on isolated PBMC according to the methods of Ellerby and Bredesen (2000). The GR, GPx and catalase activity were expressed relative to the protein content. The linear increase in SOD activity was determined and the amount of protein that resulted in 50% inhibition of auto oxidation of 6-HD calculated. A decrease in 6-HD auto-oxidation therefore reflects an increase in the actual enzyme activity and vice versa.

Statistical analyses

All statistical analyses were done with Graphpad Prism® (version 4). Results were expressed as the mean ± 1SEM. In order to measure the level of statistical difference, the distribution of the data was determined with normal probability plots. To determine the variation between the means values of each of the groups, an analysis of variance (ANOVA) was done. The repeated measure ANOVA was used because different treatments were given to the same individual and each individual served as his/her own control. In cases where significant differences were measured, the Bonferroni post-hoc test was used to determine the extent of significance between the different groups. Differences in mean values were considered significant at $p < 0.05$.

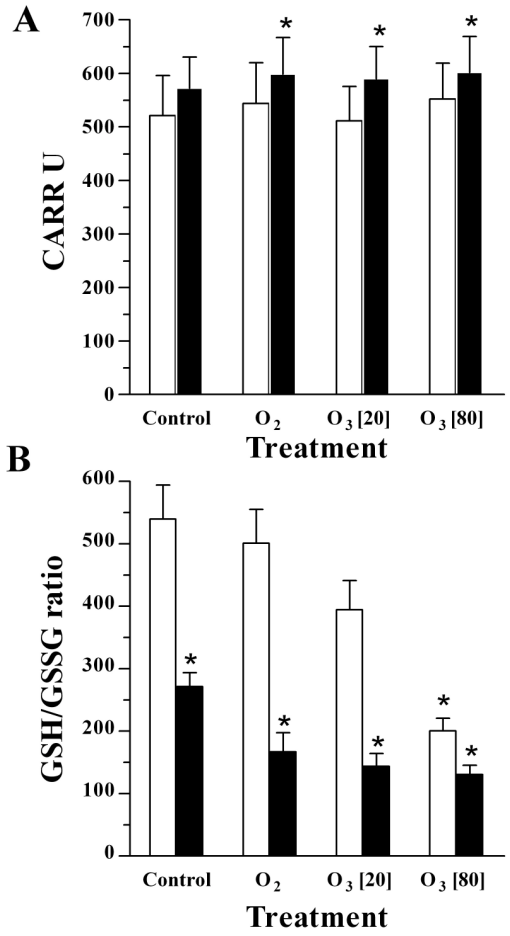

Figure 1. Characterization of the oxidative stress status after treatment. A) Levels of hydroperoxides measured in the two intervention groups Results are given as mean ± 1SEM (n=5)CARR U for whole blood (open bars) and the buffered cells (black bars) at baseline and after exposure to oxygen and 20 and 80 µg/ml ozone. B) The GSH/GSSG ratio measured in the two intervention groups. Results are given as the mean ± 1SEM (n=6) GSH/GSSG ratio for whole blood and buffered cells at baseline and after exposure to oxygen and 20 and 80 µg/ml ozone. $p<0.05$ with respect to control (*) of the whole blood group (Bonferroni test).

RESULTS

Oxidative stress

Figure 1A summarize the effect of O_3 exposure on hydroperoxide levels. Following exposure to O_2, hydroperoxide formation in whole blood increased by approximately 4%. Following exposure to 80 µg/ml O_3 mean values increased by approximately 6%. In the buffered cells, exposure to O_2 and both O_3 concentrations increased hydroperoxides significantly by approximately 13%. Significant increases were seen in the buffered cells that is, in the absence of antioxidants. The increase varied from 12 - 14%.

Treatment with O_2 caused a slight decrease (7%) in the mean GSH/GSSG ratio (Figure 1B). After treatment with the different O_3 concentrations, the ratio decreased significantly by 27% and 63% respectively from the control. The decreases in the ratio between O_2 and O_3 treated whole blood were also significant. The ratio in the control sample of the buffered cells was significantly lower than the control sample of the whole blood (Figure 1B). In the buffered cells, the ratio decreased by 69% after treatment with O_2 and by 73% and 76% after treatment with 20 and 80 µg/ml O_3 respectively. All these ratios were significantly less than that measured in the whole blood control and the O_2 treated sample.

Antioxidant capacity

Figure 2 summarizes the effect of O_3 on the antioxidant capacity. Following treatment with O_2 the ORAC value decreased slightly by approximately 7% (Figure 2A). Treatment with 20 and 80 µg/ml O_3 decreased the ORAC by 17% and 18% respectively. In the buffered cells exposure to O_2 and O_3 (20 and 80 µg/ml) caused a slight increase, but not significant. The significant differences between the buffered cells and the whole blood were expected. It can also be ascribed to the removal of the plasma antioxidant capacity by removing the plasma

The ORAC assay measures the ability of the plasma to scavenge free radicals, whilst the FRAP measures the reducing ability of plasma. The mean FRAP values of the O_2 treated samples did not differ much from the control values (Figure 2B). The average FRAP values decreased significantly by 35 and 43% to with treatment with 20 and 80 µg/ml O_3 respectively. Similar to the ORAC assay, the control values of the buffered cells were much lower than in whole blood. The treated samples decreased even further and differed significantly from the whole blood control and O_2 treated samples (Figure 2B).

Cellular antioxidant enzyme activity

Levels of antioxidant enzymes decreases when there is a state of acute oxidative stress. We therefore also investigated the effect of O_3 on the antioxidant enzyme activities in PBMC (Figure 3). In whole blood, after treatment with 20 and 80 µg/ml O_3, catalase activity decreased significantly by approximately 11 and 14% respectively. In the buffered cells, treatment with O_2 and the different concentrations of O_3 decreased catalase levels significantly by 9, 14 and 18% (Figure 3A). The mean levels of SOD activity (Figure 3B) increased significantly after treatment with 20 and 80 µg/ml by approximately 17% in both cases. Treatment of the buffered cells with O_2 and the different concentrations of O_3 caused a significant increase of approximately 15, 20 and 17% respectively. Treatment of whole blood with O_2 and O_3 had no significant effect on GPx levels (Figure 3C). In the buffered cells, exposure to 80 µg/ml O_3 signi-

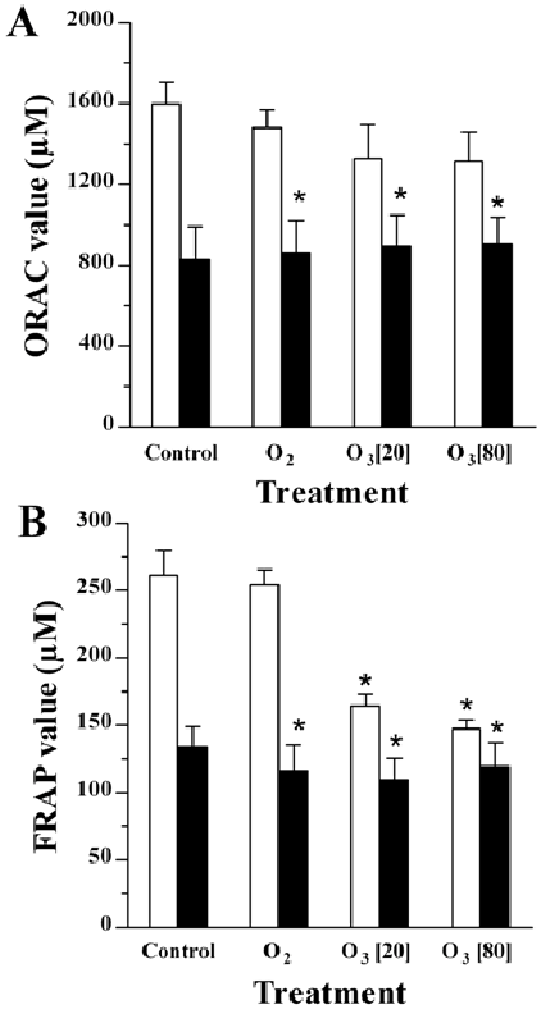

Figure 2. Characterization of the antioxidant status after treatment. A) The ORAC values measured in the two intervention groups. Results are given as the mean ± 1SEM (n=6) ORAC value (µM) for whole blood (open bars) and buffered cells (black bars) at baseline and after exposure to oxygen and 20 and 80 µg/ml ozone. B) The FRAP values measured in the two intervention groups. Results are given as the mean ± 1SEM (n=6) FRAP value (µM) for whole blood and buffered cells at baseline and after exposure to oxygen and 20 and 80 µg/ml ozone. $p<0.05$ with respect to control (*) of the whole blood group (Bonferroni test). The ORAC and FRAP was measured in plasma for the whole blood and cell homogenate for the buffered cells

ficantly decreased GPx levels by approximately 17%. GR levels did not change significantly after treatment with O_2 and 20 µg/ml O_3 in both treatment groups (Figure 3D). After treatment with 80µg/ml O_3, the level of GR decreased significantly by 15% in whole blood and 20% in buffered cells (Figure 3D).

DISCUSSION

When O_3 is comes into contact with biological fluids, it apparently acts in two ways. The first is an O_3-olefin reaction where O_3 reacts with the double bonds in organic substances such as fatty acids to produce H_2O_2, aldehydes and other peroxides. The second mechanism is an O_3-electron donor reaction where a hydroxyl radical (OH^-) is formed (Pryor et al 1995). The hypothesis behind O_3-AHT is that the generated reactive oxygen species (ROS) are involved in upregulation of the antioxidant defence mechanisms, which can combat chronic oxidative stress (Bocci, 2002). In this study we found small (5%), but significant increases in hydroperoxide formation in whole blood when compared to published results (Bocci, 2002). When the plasma was removed, hydroperoxides formation increased approximately threefold. This clearly demonstrates that the antioxidants in plasma were sufficient to inhibit the formation of hydroperoxides. The result also indicated that lipid peroxidation occurred.

The GSH/GSSG ratio decreased markedly because GSH decreased and GSSG increased. This suggested that the small increases in hydroperoxides, could be attributed to GSH acting as a sacrificial antioxidant. Oxidative stress was markedly increased when the plasma was removed. The depletion of the GSH when O_3 is introduced can at least in part be responsible for cell death, because of the decreased antioxidant capacity (Higuchi, 2004; Pastore et al., 2003). This is consisted with previous results which indicated that O_3 depletes GSH in red blood cells (Bocci, 2002) and in plasma of baboons (Van Helden, 2005).

O_3-AHT decreased both the radical scavenging capacity and the ferric reducing ability of plasma. It is likely that the ROS depleted the antioxidants present in plasma. This is supported by the finding that hydroperoxide formation was not markedly increased in whole blood, but increased by approximately threefold when the plasma antioxidants were removed. The antioxidant capacity decreases when human plasma is exposed to chemically produced peroxyl radicals or to other ROS. This is usually accompanied by a decrease in the onset of lipid peroxidation (Berman and Brodaty, 2004; Cross et al., 1992). Similar decreases in antioxidant capacity have been found when plasma is exposed to O_3 (Bocci et al., 1998; Cross et al., 1992; Bocci et al., 2001). In this case the water soluble antioxidants probably act as sacrificial antioxidants and decreased as the O_3 increased. The most important scavenger of O_3 in human plasma seems to be uric acid, which is also most abundant in plasma (Cross et al., 1992). The decreases in antioxidant capacity can contribute to a state of oxidative stress, which can cause oxidative damage to proteins, lipids and DNA. The results with the antioxidant capacity analyses support our results of the levels of hydroperoxides, which ultimately shows that O_3 caused a state of increased oxidative stress.

Cellular antioxidant activity is of critical importance in the defence of cells against ROS. When cells are

exposed to H_2O_2 over a long period there is a dose dependent increase in catalase, SOD and GPx mRNA

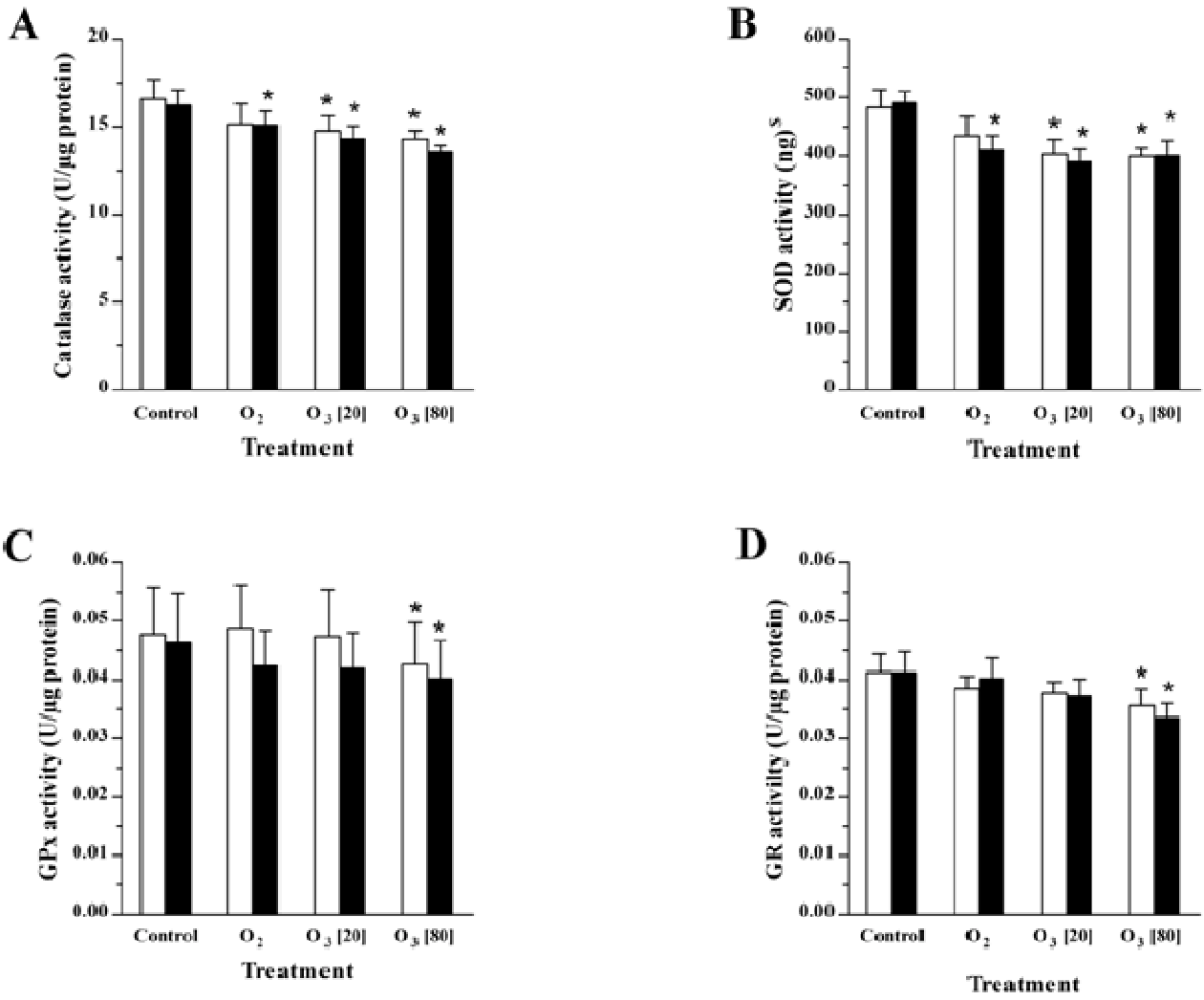

Figure 3. The levels of antioxidant enzymes in PBMC after treatment. A) Catalase activity (U/µg protein) B) SOD activity (ng) C) GPx activity (U/µg protein) and D) GR activity (U/µg protein) for whole blood (open bars) and buffered cells (black bars) at baseline and after exposure to oxygen and 20 and 80 µg/ml ozone. Results are given as the mean ± SEM (n=6). $p<0.05$ with respect to control (*) of the whole blood group (Bonferroni test). $^{\$}$ng = the amount of protein that results in 50% inhibition of 6-HD auto-oxidation.

and more enzymes are produced. Tissues are protected from ROS by expression of genes encoding antioxidant enzymes, but this usually occurs only after chronic oxidative stress (Mates et al., 1999). Jurkat T cells treated with O_3 concentrations ranging from 12 to 72 µg/ml increased their levels of SOD, GPx and GR (Larini et al., 2003). This upregulation of the enzyme synthesis was only observed after 24 h, with even more pronounced effects after 72 h. Results in baboons treated with a single dose of O_3 (80 µg/ml) indicated decreased activity of catalase with increases in SOD activity of red blood cells (Van Helden, 2005). Our results indicated a decrease in catalase, GPx and GR activity, but increases in SOD activity of PBMC. Reduction of catalase, SOD and GPx can cause accumulation of H_2O_2 or $O_2^{\cdot-}$, which in turn can react with metal catalyst to form even more reactive species. This can ultimately lead to a state of increased oxidative stress, which can alter the redox state. The increase observed in the SOD activity could indicate an initial stimulation of activity to protect the cells against oxidative stress. These results in PBMC indicate that after repeated treatments with O_3, these cells could follow the same trend of upregulated antioxidant defence, as Jurkat T cells in culture (Larini and Bocci, 2005).

Conclusion

In conclusion, the results indicate that the plasma antioxidant defences plays a major role in protecting

against the possible harmful effects of O_3 during O_3-AHT. Further research will necessitate to repeat the *in vitro*

study but to use blood of patients with diseases associated with chronic oxidative stress. Comparing the results from patients with increased oxidative stress with those where people have increased antioxidant capacity may give new insights into the mechanisms of oxidative damage to cells. This will also give further insight into the effectiveness of O_3-AHT.

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