Full Length Research Paper

Ozone concentration dependent autohaemotherapy effects on baboon antioxidant capacity and DNA integrity and repair capacity of lymphocytes

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Although ozone is widely used as an alternative medicine, its safety and efficiency are met with scepticism. To shed some light on this, we assessed the effect of ozone-autohaemotherapy, using an O_2/O_3 gas mixture containing three different O_3 concentrations (20, 40 and 80 µg/ml), on the antioxidant status and lymphocyte DNA integrity in baboons. Ultra pure O_2 was used as a control because more than 95% of the O_2/O_3 gas mixture consisted of oxygen. Five percent of the blood volume of a baboon was withdrawn and treated with O_3. Baboons were anesthetized with intramuscular ketamine hydrochloride (±10 mg/kg). Blood was collected before ozone-autohaemotherapy and again 4, 24 and 48 h after treatment. In general, the changes in measurements were concentration dependant. Ozone-autohaemotherapy up regulated the antioxidant capacity as measured by the Oxygen Radical Absorbance Capacity assay. Total glutathione levels decreased after ozone-autohaemotherapy, most likely due to reaction with reactive oxygen species that was quenched by the GSH system. The activity of superoxide dismutase increased after ozone-autohaemotherapy with 20 µg/ml O_3. Ozone-autohaemotherapy caused a slight increase in DNA damage. However, very little DNA damage occurred following treatment with 80 µg/ml O_3, which also caused the highest increase in the antioxidant capacity. DNA repair capacity decreased and thus can have severe long term effects.

Key words: Ozone, ozone-autohaemotherapy, antioxidant capacity, DNA damage, comet assay.

INTRODUCTION

The use of ozone as an alternative form of medicine has been met with scepticism (Bocci, 2002; Halliwell and Auroma, 1991). Ozone, a potent oxidant, is highly toxic to the lung tissue when inhaled (Bocci, 1996). However, it is claimed that the careful use of ozone-treatment can have health benefits (Bocci, 1999). When ozone comes in contact with blood it immediately dissolves in the plasmatic water and reacts with biological molecules such as antioxidants, fatty acids and proteins (Bocci, 1999). The reaction implies two fundamental processes that include

the 'ozone initial reaction' and 'lipid peroxidation'. During the first reaction, reactive oxygen species (ROS) are generated to trigger several biochemical pathways in blood *ex vivo*. In the second reaction ozone then reacts with polyunsaturated fatty acids (PUFA's). One mole of ozone gives rise to two moles of aldehydes and one mole of hydrogen peroxide. These two reactions, completed within seconds following exposure of blood to O_3, use up the total dose of ozone. From this point on only ROS (mostly H_2O_2) and lipid oxidation products (LOP's) are responsible for the effects happening in the body (Foster et al., 2000).

It is important that the precise concentration of the O_3 must be known to be effective during O_3-autohaemotherapy (O_3-AHT). O_3-AHT has, similar to other drugs, a therapeutic window. Thus, if the concentrations are too low, it would have no effect while too high a concentration can be toxic (Bocci, 2006). All aerobic

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organisms produce ROS as part of their metabolism. ROS is potentially dangerous and can cause oxidative damage through its interaction with biological molecules (Bocci, 2005). In order to minimize the effects of ROS, aerobic organisms developed potent antioxidant systems to quench it (Bocci et al., 2000). Under normal conditions, these systems are sufficient to protect cells. Unfortunately, increased oxidative stress can overcome these mechanisms. Therefore when the ROS overwhelms the antioxidant defence systems DNA damage can follow. H_2O_2 can easily diffuse through cell membranes into the nucleus where it reacts with transient metal ions via the Fenton reaction to form highly reactive hydroxyl radicals ($OH^{\cdot}$) (Halliwell et al., 2000). Reactions of $OH^{\cdot}$ with DNA lead to a wide range of damage to all four bases and to the deoxyribose and can also cause DNA strand breaks (Aust and Eveleigh, 1999). Cells cannot tolerate damage to their DNA because it compromises the integrity and accessibility of essential information in the genome. To circumvent this, cells have a variety of repair strategies to restore DNA damage and these strategies depend on the type of DNA damage (Cooke et al., 2003; Giovannelli et al., 2006; Duthie and Collins, 1997; Labieniec and Gabryelak, 2005; Wood et al., 2001; Holz et al., 1995; Sharova, 2005).

We assessed the effects of O_3-AHT on the antioxidant capacity of baboons. DNA damage and repair was also measured to asses the changes, due to the challenge to the antioxidant capacity. These studies were done in an attempt to shed some light on the controversy of the beneficial effects of O_3-AHT on the one hand and the view that O_3-AHT is dangerous and non effective on the other hand.

MATERIALS AND METHODS

Preparation of ozone

Ultra pure oxygen (99.9%) was used to generate the ozone. The oxygen was passed through a flow rate regulator to ensure the correct flow rate to generate the required concentration of ozone. From the flow rate regulator, the oxygen was passed through a glass-isolated alternating current corona discharge ozonizer, developed and built by members of the School of Physical and Chemical Sciences of the North-West University (USA patent 09/914, 1996). The ozone concentration was determined by passing the O_3/O_2 gas mixture through a UV/Vis spectrophotometer (Pharmacia Biotech Ultraspec 3000). The O_3 concentration was measured at 254 nm. A special designed quartz cell was used to monitor the ozone concentration in real time. The quartz cell had an inlet and an outlet through which the gas passed. The gas was then pumped into a glass syringe containing the blood. Oxygen/ozone gas mixtures containing 20, 40 and 80 µg/ml O_3, were prepared.

Treatment of baboons

The study was approved by the Ethics Committee of the North-West University in accordance with the National Code for animal use in research, education, diagnosis and testing of drugs and related substances in South Africa (based on the 'Guide for the care and use of laboratory animals'; NIH85-23, Revised 1985). Twelve healthy baboons, weighing between 5 and 9 kg, were used. The baboons were housed at the Animal Research Centre of the Potchefstroom Campus of the North-West University. The baboons were individually caged in a room kept at constant room temperature and with automated day-night cycles. Water was available ad lib and baboons were fed standard laboratory chow. The baboons were anaesthetised with intramuscular ketamine hydrochloride (± 10 mg/kg) to enable handling, blood collection and the reinfusion of O_3 treated blood.

Baboons have 65 ml blood/kg (Kotzé et al., 1985). We collected five percent of the blood volume from the femoral vein in heparin containing polypropylene syringes. The blood was transferred to siliconised glass syringes and ozonated by gently mixing it for twenty minutes with the same volume of O_3/O_2 gas mixture containing 20, 40 or 80 µg/ml O_3 (1 - 5% O_3). The gas was then carefully removed. Directly before the ozonated blood was reinfused into the baboon, a control blood sample was collected. Blood samples were also collected at 4, 24 and 48 h after the ozonated blood was infused back into the baboon. In order to correct for possible effects of O_2 in the gas mixture, 5% of the blood volume of 6 baboons were treated also with O_2. Blood was also collected before and after 4, 24 and 48 h.

Antioxidant capacity

Reagents

1-Methyl-2-vinylpyridinium trifluoromethane sulfonate (M2VP) was obtained from Fluka (Buchs, Switzerland). Analytical grade NaH_2PO_4, Na_2HPO_4, K_2HPO_4, KH_2PO_4, 2,4,6-tripyridyl-s-triazine (TPTZ) and FeCl3.6H2O were obtained from Merck (Darmstadt, Germany). Reduced glutathione (GSH) and 5,5'Dithiobis-2-nitrobenzoic acid (DTNB) were obtained from Boehringer Mannheim, Germany. All other reagents were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA) and were of analytical grade.

Oxygen radical absorbance capacity (ORAC)

Plasma antioxidant capacity was determined by the ORAC assay (Ou et al., 2001). Trolox (a water soluble form of Vit E) was used to prepare a standard series and serum antioxidant capacity was expressed as trolox equivalents. Blood samples were collected in heparin vacutubes. AAPH (2,2'-azobis (2-aminopropane)dihydrochloride, 240 mM) was used as a peroxyl radical generator that oxidizes both lipophilic and hydrophilic antioxidants. The target for free radical attack was fluorescein (56 nM). Fluorescence decay was measured every 5 min for 2 h. Polynomial regression analyses were used to determine antioxidant capacity.

Total GSH (GSHt)

GSHt is the sum of reduced GSH and oxidised GSSG. Blood samples were collected in EDTA vacutubes. Proteins were precipitated with metaphosphoric acid (MPA, 5%). The MPA extract was diluted with 500 mM $NaPO_4$ buffer (500 mM $NaPO_4$, 1 mM EDTA, pH 7.5). 5,5-Dithiobis(2-nitro-benzoic acid), 0.3 mM) was added to the final dilution to convert GSH into a spectrophoto-metrically detectable product which was followed by adding glutathione reductase and 1 mM NADPH. 3 µl of GSH was used as a standard (Tietze, 1969). The absorbance was measured at 412 nm at 1 min intervals for 5 min using a Bio-Tek (FL 600) microplate reader. GSHt was determined by calculating the linear slope of the increase in absorbance.

Superoxide dismutase (SOD)

Superoxide dismutase activity was measured by monitoring the auto-oxidation of 6-hydroxydopamine (6-HD) in a spectrophotometer (Sharova, 2005). Erythrocytes were isolated and diluted with cold water and added to an extraction medium (ethanol/chloroform). The supernatant was used to determine the protein content (Smith et al., 1985). Supernatant containing 1 µg/ml protein was used to determine the SOD activity. A diethylenetriaminepentaacetic acid (DETAPAC, 0.1 mM) buffer was added to the sample in a microplate, followed by 6-HD (10 mM). The auto-oxidation of the 6-HD was recorded at 490 nm for 4 min at 1 min intervals using a Bio-Tek (FL600) microplate reader. The amount of protein required to inhibit 50% of the auto-oxidation of 6-HD was determined.

Comet assay

Materials

Low melting point agarose (LMPA) was obtained from Roche and H_2O_2, Na_2HPO_4 and KH_2PO_4 from Merck (South Africa). The rest of the chemicals were all purchased from Sigma Aldrich Co., St Louise, USA and were of analytical grade.

Isolation and preparation of lymphocytes

Blood samples were collected in vacutest tubes containing heparin. In order to isolate lymphocytes, whole blood (2 ml) was added on top of 2 ml Histopaqe® in a polypropylene tube and then centrifuged at 5000 x g for 30 min at room temperature. The buffy coat containing the lymphocytes was removed, transferred to a 1.5 ml centrifuge tube and washed twice with PBS at 600 x g for 5 min. The final lymphocyte pellet was suspended in 500 µl PBS.

Single cell gel electrophoresis (Comet assay)

The comet assay was done under alkaline conditions as previously described (Singh et al., 1988) with minor modifications (Van Dyk et al., 2005). Briefly, 40 µl of the cell suspension was mixed with 150 µl one percent low melting point agarose at 37^oC. Of this, 100 µl was evenly spread onto microscope slides pre-coated with 300 µl 1% high melting point agarose. Slides were kept on ice to allow the agarose to solidify. The remaining cells were exposed to 60 µM H_2O_2 for 20 min at 37^oC. The H_2O_2 was removed and the cells incubated in 450 µl HAMS F10 for 10, 20 and 30 min at 37^oC. At each time point an aliquot of cells was collected and spread on the slides. The slides were immersed in chilled lysis buffer containing 5 M NaCl, 0.4 M EDTA, 1% Triton X-100, 10% DMSO in ddH_2O at 4^oC and kept overnight. The slides were then placed in a horizontal electrophoresis tank containing freshly prepared and chilled electrophoresis buffer (0.3 M NaOH, 0.05 M EDTA and ddH_2O). The slides were left for 30 min to allow DNA unwinding. Electrophoresis was then done at 30 V and 300 mA at 4^oC for 45 min. The slides were rinsed with water and placed in a neutralizing buffer (0.5 M TrisHCl pH 7.5) for 15 min at 4^oC. The slides were stained with ethidium bromide. Pictures of the comets were taken with an Olympus IX70 fluorescence microscope (200x magnification) and for each sample a minimum of 50 comets were randomly chosen to determine the percentage tail DNA with CASP® (Comet Assay Software Project v.1.2.2). The data were processed in Excel®.

Statistical analysis

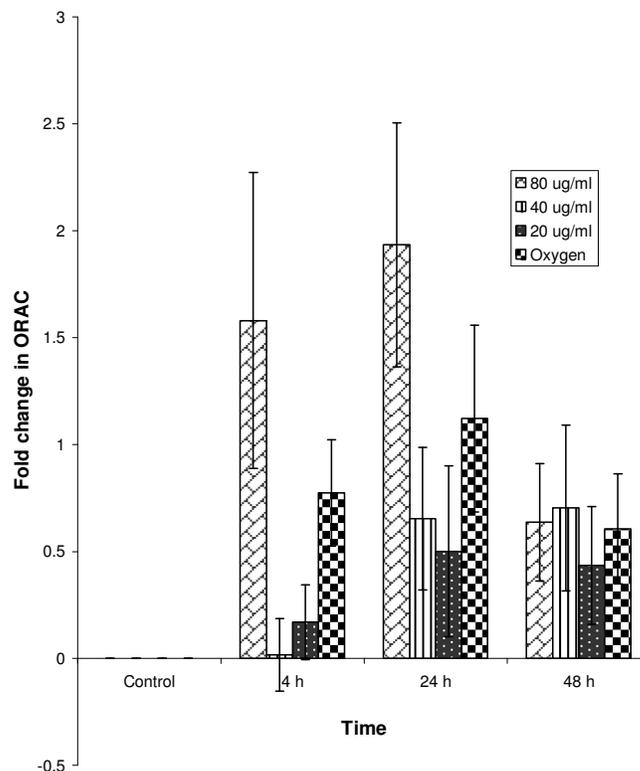Data in the text are presented as the mean ± SEM. The results

Figure 1. Changes in serum ORAC after autohaemotherapy. The serum ORAC was measured before autohaemotherapy (control) and again 4, 24 and 48 h after autohaemotherapy with oxygen and a O_2/O_3 gas mixture containing 20, 40 and 80 µg/ml O_3. Values are given as a mean ± SEM (*p < 0.05).

were statistically analyzed using the Student's t-test for paired and unpaired samples. Results were considered significant if p < 0.05.

RESULTS AND DISCUSSION

Antioxidant capacity

The serum ORAC assesses the antioxidant capacity in the blood since it measures the total radical scavenging ability of the serum (Cao et al., 1998). There are hydrosoluble antioxidants such as uric acid, ascorbic acid and albumin present. Proteins and lipids can also have radical scavenging characteristics. The ORAC was presented as the fold change from baseline because the control values from the four different treatment groups differed somewhat. The changes after O_3-AHT with 80 µg/ml O_3 was more pronounced than the changes in the other groups (Figure 1). After treatment with 40 and 20 µg/ml O_3, the ORAC did not differ significantly both within groups and over time. The treatment with O_2 was done to balance for the effects of the oxygen in the O_2/O_3 gas mixture because more than 95% of the gas mixture consisted of oxygen. Surprisingly AHT with oxygen caused a more pronounced increase in serum ORAC than the 20 and 40 µg/ml O_3 after 4 and 24 h. The fact
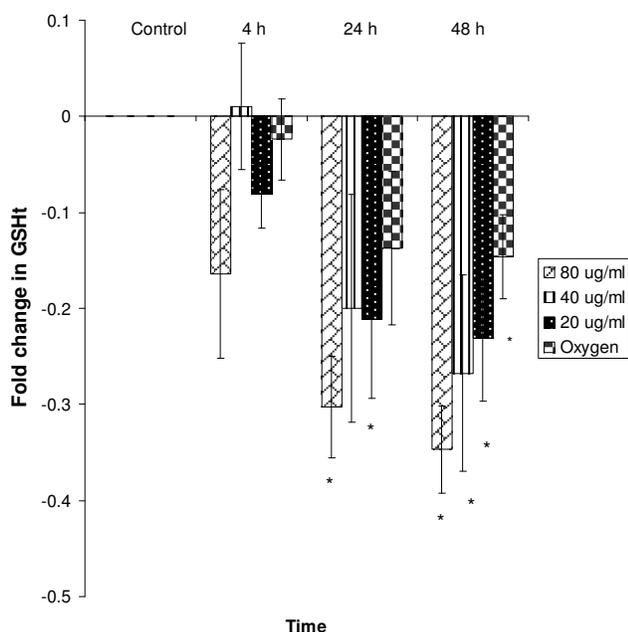
Figure 2. Changes in total GSH after autohaemotherapy. The total GSH was measured before autohaemotherapy (control) and again 4, 24 and 48 h after autohaemotherapy with oxygen and a O_2/O_3 gas mixture containing 20, 40 and 80 µg/ml O_3. Values are given as a mean ± SEM (*$p < 0.05$).

Figure 3. Changes in SOD activity after autohaemotherapy. The SOD activity was measured before autohaemotherapy (control) and again 4, 24 and 48 h after autohaemotherapy with oxygen and a O_2/O_3 gas mixture containing 20, 40 and 80 µg/ml O_3. Values are given as a mean ± SEM (*$p < 0.05$).

that the high concentration of O_3 upregulated the antioxidant capacity may suggest that O_3-AHT produced a significant amount of ROS and LOP's. This probably prompted the body to produce more of these radical scavenging molecules, especially since the increase was maintained for 24 h.

Glutathione is a vital redox buffer in the cell. Total glutathione (GSH + GSSG) was measured and is presented in Figure 2 as the fold change from baseline.

Total GSH decreased in all treatment groups following AHT. The decrease was more pronounced in the group treated with 80 µg/ml O_3 than in the other groups. It is possible that the GSH molecules served as sacrificial molecules during interaction with the ROS and LOP's formed by AHT. This finding supports the suggestion that the higher O_3 concentration generated more ROS and LOP's and therefore caused the upregulation of the antioxidant capacity. The decrease was significant in all four treatment groups 48 h following AHT with the smallest decrease in the oxygen group. Our results suggest that the negative effect of the AHT on the total glutathione levels were dose-dependant.

Superoxide dismutase (SOD) catalyses the dismutation of superoxide into oxygen and hydrogen peroxide. It therefore forms an important antioxidant defence in nearly all cells that are exposed to ozone and oxygen. SOD activity was determined as the concentration of protein (µg) that inhibits autooxidation of 6-OH-dopamine by 50%. The results are summarized in Figure 3.
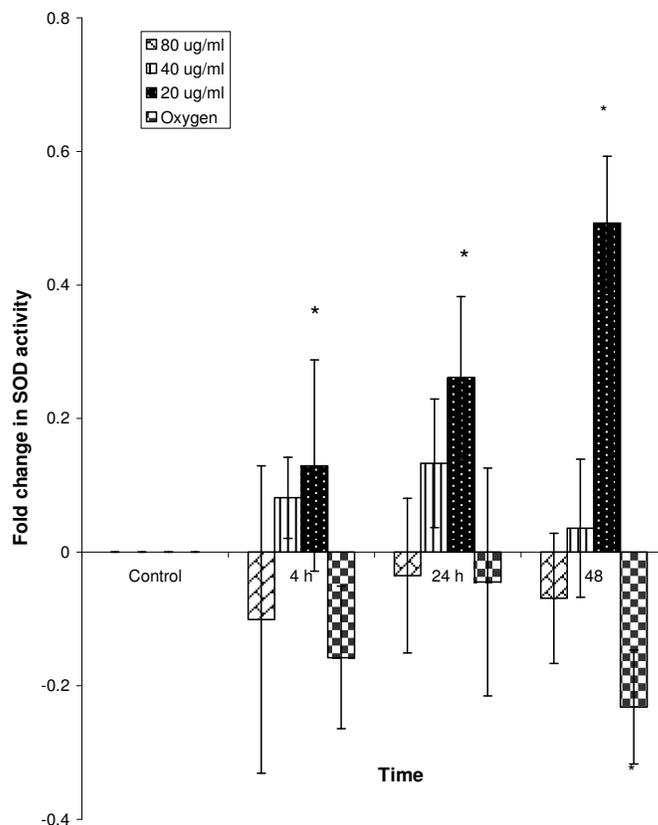
O_3-AHT with 40 and 80 µg/ml O_3 had no effect on SOD activity. The activity in the 20 µg/ml O_3 group increased significant over the 48 h period. This finding was surprising because it was expected that the higher concentrations of O_3 would upregulate activity of SOD. It is possible that the upregulation of the radical scavenging molecules after O_3-AHT with the higher O_3 concentrations (Figure 1) reduced most ROS in the blood before it could have had any effect on the SOD activity. Treatment with 20 µg/ml O_3 resulted in almost no change in the antioxidant capacity (Figure 1). This may suggest that the effect of the generated ROS during O_3-AHT with 20 µg/ml O_3 was not enough to trigger the upregulation of the first line of defence antioxidants but it had an effect on the SOD activity.

DNA damage and repair capacity

The results are summarized in Figure 4. The mean tail DNA % decreased 4 h following O_3-AHT with 80 µg/ml O_3. No changes were measured in the 40 µg/ml O_3 group (Figure 4). Treatment with 20 µg/ml O_3 and O_2 increased
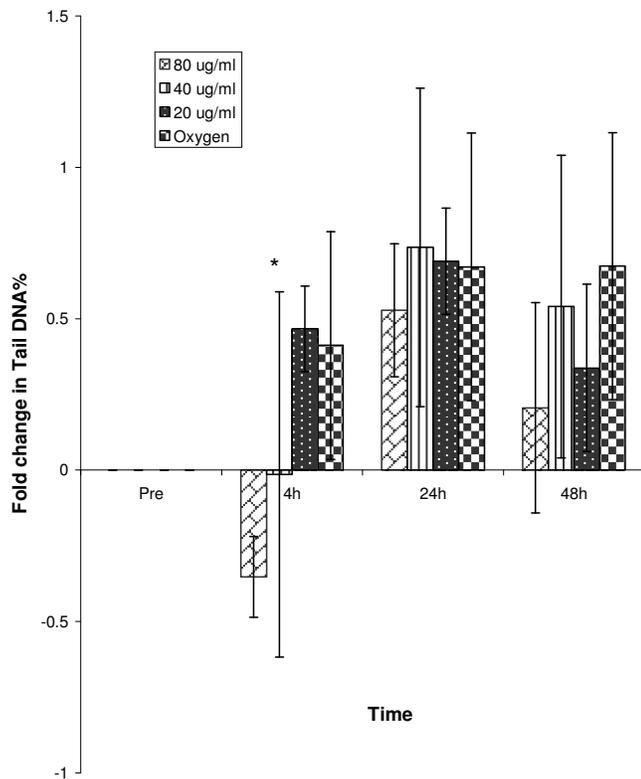
Figure 4. Changes in tail DNA after autohaemotherapy. The tail DNA% was measured before autohaemotherapy (control) and again 4, 24 and 48 h after autohaemotherapy with oxygen and a O_2/O_3 gas mixture containing 20, 40 and 80 µg/ml O_3. Values are given as a mean ± SEM (*$p < 0.05$).
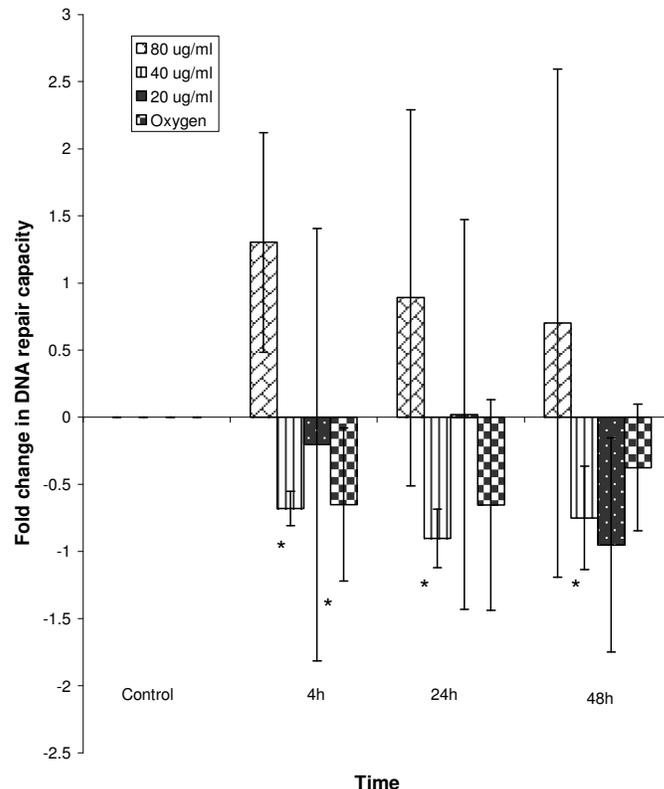

Figure 5. Changes in DNA repair capacity after autohaemotherapy. The DNA repair capacity was measured before autohaemotherapy (control) and again 4, 24 and 48 h after autohaemotherapy with oxygen and a O_2/O_3 gas mixture containing 20, 40 and 80 µg/ml O_3. Values are given as a mean ± SEM (*$p < 0.05$).

the tail DNA % at 4 h. After 24 and 48 h an increase in tail DNA % was measured in all four treated groups. The tail DNA % in the 80 µg/ml group increased the least, indicating that there was less DNA damage. This supports the suggestion that the higher concentration of O_3 upregulated the antioxidant capacity and therefore most of the harmful ROS was neutralized before it could cause any damage to the DNA. Although the tail DNA % in the other treatment groups was more pronounced, the increase from baseline values was still not significant.

To determine the DNA repair capacity of the lymphocytes, they were treated with H_2O_2 to deliberately damage DNA. The lymphocytes were then incubated in HAMS F10 to allow DNA repair to take place. A linear regression using the H_2O_2 treatment and 10, 20 and 30 min data points were fitted to the results to determine the rate at which tail DNA% decreased. The rate was regarded as the DNA repair capacity over 30 min. The results are summarized in Figure 5.

O_3-AHT affected the repair capacity in all treatment groups. The DNA repair capacity was significantly de-creased 4 hours following O_2-AHT and O_3-AHT with 40 µg/ml O_3. At the same time point the DNA repair capacity was unchanged in the 20 µg/ml group. O_3-AHT with 80 µg/ml O_3 increased DNA repair. This increase was main-

tained over the 48 hour period. After 24 and 48 h following O_3-AHT with 40 µg/ml O_3, a significant decrease was measured. The repair capacity results are difficult to explain and deserve further investigation.

Conclusion

The ORAC strongly suggested that ozone-autohaemotherapy upregulated the antioxidant capacity in the blood of the baboons. The decreasing levels of the total GSH shows that they reacted with the ROS and LOP's that formed during O_3-AHT. It strongly supports the notion that the GSH antioxidant system forms part of the group of molecules acting as the first line of defense against ROS (Weinstein et al., 2000; Raza et al., 2002). SOD activity was only upregulated after treatment with the lowest O_3 concentration. The results also suggested that the first line of defense antioxidants was able to protect DNA from ROS and LOP's and no severe damage was caused after reinfusion of the ozone treated blood. However, the decrease in DNA repair capacity suggested that O_3-AHT is still not very safe to use and that it may have severe long term effects on the integrity of the DNA when repeated treatments are done (Biard,

2007). A reason for the decrease in DNA repair capacity may be due to inactivation of proteins involved in DNA repair by ROS and LOP's formed during O_3-AHT. It is well established that lipid radicals and ROS can oxidize proteins and alter their function in the body (Stadtman, 1992).

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