

Full Length Research Paper

The effect of blood ozonation on mitochondrial function and apoptosis of peripheral blood mononuclear cells in the presence and absence of plasma antioxidants

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Ozone-autohemotherapy (O₃-AHT) has recently gained interest as a form of alternative and complementary medicine. There is, however, some concern regarding its toxicity and effectiveness. Ozone is a powerful oxidant and when introduced into biological fluids react with most cellular components including proteins, lipids and DNA. We assessed the effect of O₃-AHT on peripheral blood mononuclear cells (PBMC) viability, apoptosis and mitochondrial function in the presence and absence of plasma antioxidants. Exposure to ozone increased lactate dehydrogenase (LDH) release and caspase 3/7 activity in PBMC. A decrease in mitochondrial function was measured as a decrease in ATP levels and an increase in NADH/ NAD⁺ ratio. Complex IV (cytochrome c oxidase) of the respiratory chain was almost completely inhibited by ozone. These results indicated that the death of PBMC was probably through apoptosis. These effects were more evident in the absence of plasma antioxidants. Therefore, high concentrations of ozone were damaging to the cells, but this effect was diminished by antioxidants present in plasma. It is not certain if the *in vitro* damage will be propagated when ozonated blood is injected back into individuals. One must bear in mind that only a fraction of the total blood volume is ozonated.

Key words: O₃-AHT, alternative medicine, oxidative stress, mitochondrial function, apoptosis.

INTRODUCTION

Ozone is a powerful oxidant and its toxicity to the pulmonary system is well known (Pryor et al., 1995). Recently ozone has gained attention regarding its use as an alternative form of therapy. Ozone autohemotherapy (O₃-AHT) is a form of therapy where the blood of a patient is exposed to predetermined ozone concentration for a given

time and then reinfused back into the patient. The ozone dissolves in the plasma and reacts with organic molecules generating substances including lipid ozonation products (toxic aldehydes), free radicals (mainly H₂O₂) and ozonides. The current hypothesis is that these substances are responsible for the therapeutic response specifically, induction of cytokine release from PBMC (Bocci, 2002). These substances are also responsible for many toxic effects in PBMC including apoptosis (Larini et al., 2004) and DNA damage (Diaz-Llera et al., 2002).

Many studies have focused on the harmful effects of ozone in the lung and have claimed that the toxic effects observed after inhalation of ozone are due to the inefficient antioxidant capacity of the lungs. The surfactant of the lung, the first compartment that comes in contact with ozone when inhaled, contains slightly lower antioxidant concentrations than plasma (Mudway and Kelly, 2000),

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Abbreviations: O₃-AHT, ozone autohemotherapy; PBMC, peripheral blood mononuclear cells; LDH, lactate dehydrogenase; NADH, nicotinamide adenine dinucleotide (reduced form); NAD⁺, nicotinamide adenine dinucleotide (oxidised form); ROS, reactive oxygen species.

but is well equipped (under normal circumstances) with antioxidants to counter the harmful effects. But ozone remains toxic and harmful when it is inhaled. Therefore the question is whether ozone can be toxic to blood even if its antioxidant defence system is more than adequate to deal with the reactive oxygen species (ROS) that are generated by ozone.

Ozone reacts selectively with the haeme groups of haemoglobin, because it is recognized as oxygen (Cataldo and Gentilini, 2005). At the haemoglobin sites, it causes the oxidation of Fe (II) to Fe (III) in the heme groups and this is followed by a complete breakdown of the porphyrin rings of haemoglobin. This reaction is similar to the reaction of carbon monoxide (CO) with haemoglobin (Cataldo and Gentilini, 2005). CO also combines with the heme containing complex IV (cytochrome c oxidase) of the mitochondrial respiratory chain (Castoldi et al., 2005).

We investigated the effects of O₃-AHT with different concentrations of ozone on white blood cell apoptosis and mitochondrial function. We also investigated the effect of ozone on the respiratory chain complexes to determine if ozone reacts with complex IV. Study of the effects of ozone on cell cultures (e.g. lymphocytes) presents problems because due to the poor antioxidant capacity of media and serum results cannot be related to physiological antioxidant capacity (Leist et al., 1996). In this study the effects of ozone, in comparison to oxygen, on PBMC were investigated under conditions similar to that used during O₃-AHT.

EXPERIMENTAL PROCEDURES

Ozone generation and measurement

Ozone was generated from ultra pure oxygen (>99.9 % BOC special products, Afrox) using electrical corona discharge by an ozone generator developed and built by the School of Physics, Potchefstroom Campus of the North-West University (USA patent 09/914,199). The ozone concentration was determined by a UV/Vis spectrophotometer (Pharmacia Biotech Ultraspec 3000) by measuring the gas absorbance at 254 nm ($\epsilon = 3 \pm 0.03 \text{ mM}^{-1} \cdot \text{cm}^{-1}$). The ozone concentration was monitored in real time, using a specially designed quartz cell containing an inlet, where gas entered from the ozone generator and an outlet, where the gas left the cell and entered the syringe containing the blood.

Treatment protocol

The study was approved by the Ethics Committee of the North-West University (05M07). Blood (100 ml in EDTA) was collected from six apparently healthy human donors. The blood was divided into two samples. In the first sample, venous blood was used unchanged for the treatment. In the second sample the blood plasma was removed by differential centrifugation and the cells resuspended in phosphate buffered saline (PBS) before the treatment. These two samples of blood were then divided into four treatment groups that consisted of i) a control sample that received no treatment, ii) a sample treated with an equal volume of oxygen, and

samples treated with an ozone/oxygen gas mixture that contained either iii) 20 or iv) 80 $\mu\text{g/ml}$ ozone. The whole blood and the buffered cell samples were gently and continuously mixed with the gasses for 20 min. Thereafter the gas was carefully removed and the blood dispensed into test tubes for biochemical analyses or storage.

Isolation of PBMC and cell viability

PBMC were isolated from EDTA blood. The whole blood or buffered cells were layered onto to Histopaque (Sigma-Aldrich, St. Louis, MO, USA) and centrifuged at 50 x g for 30 min. The cells (buffy coat layer) were removed, resuspended in PBS and collected by centrifugation (50 x g for 30 min). This washing step was repeated twice. After removing the supernatant, the pellet was resuspended in RPMI1640 culture medium (Sigma-Aldrich, St. Louis, MO, USA). Cell viability was assessed by the trypan blue exclusion technique.

LDH release measurement

The lactate dehydrogenase (LDH) release was measured in plasma or buffered saline by an enzymatic reaction according to the method of Decker and Lohmann-Matthes (1988). In this reaction NAD⁺ is reduced to NADH through conversion of lactate to pyruvate by LDH. NADH then reduce a tetrazolium salt, INT (2-[4-iodophenyl]-3-[4-nitrophenyl]-5-phenyltetrazolium chloride) to formazan in the presence of electron coupling agents or diaphorase (Decker and Lohman-Matthes, 1988). The resultant formazan absorbs at 492 nm, which was spectrophotometrically measured in a BIO-TEK (FL600) microplate reader. The bicinchoninic acid (BCA) method for protein determination was done using bovine serum albumine (BSA) as a standard (Smith et al., 1985).

Caspase 3/7 assay

Cysteine aspartic acid-specific protease of caspases 3 and 7 were measured using the Apo-ONE[®] Homogenous Caspase-3/7 assay kit as instructed by the supplier (Promega, Madison, WI, USA). The gas-treated and isolated WBC were counted and seeded into 96-well plates with approximately 25 000 cells per well (final volume of 100 μl). As positive control, cells were incubated with 1 $\mu\text{g/ml}$ staurosporine for 2 h. Fluorescence was measured at 30 min intervals at excitation 485 and emission 530 nm for at least three hours.

NADH/NAD⁺ assay

The NADH/NAD⁺ ratio was determined by the method of Zeres et al (1990). Fluoride treated blood (20 μl) was mixed with 1980 μl of a buffer solution containing 10 mM nicotinamide, 20 mM NaHCO₃ and 100 mM Na₂CO₃ at 4°C (pH = 7.5). The mixture was snap frozen in liquid nitrogen, thawed at room temperature in a water bath and promptly chilled to 0°C. To destroy the oxidized forms of NAD⁺, 1000 μl of this mixture was incubated at 60°C for 30 min in a dry heating block. The samples were stored at -80°C until assayed. The reaction mixture was added to the sample and the absorbance read at 570 nm in BIO-TEK microplate fluorescence (FL 600) plate reader after four min. A standard curve with NADH was generated and used to quantify the concentration of NADH and NAD⁺ in each sample (Zeres et al., 1987,1990).

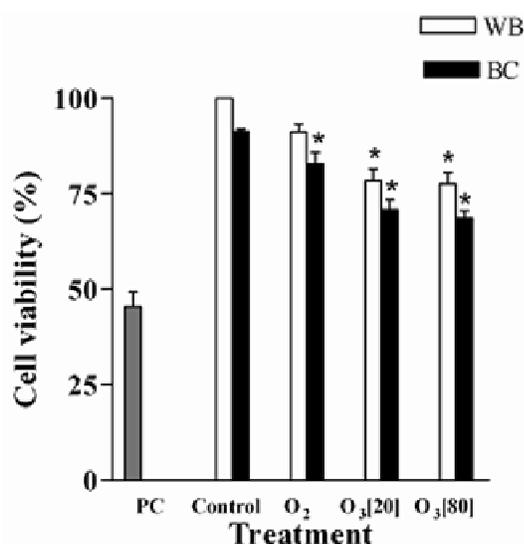


Figure 1. The viability of ozone treated PBMC. Results are given as mean \pm 1SEM (n=6) cell viability (%) for the positive control (PC), whole blood (WB) and the buffered cells (BC) at baseline and after exposure to oxygen and 20 and 80 μ g/ml ozone. $p < 0.05$ (Bonferroni test) relative to the control (*) of the whole blood. The PC cells were treated with 6% acetic acid.

ATP assay

The ATP bioluminescent somatic cell assay kit (Sigma-Aldrich, St. Louis, MO, USA) was used to determine ATP content of treated WBC. The assay was normalized to the number of viable cells because of rapid degradation of ATP after cell death. WBC were seeded in a microtitre plate containing approximately 20 000 viable cells/well. The assay was performed according to instructions of the manufacturer. An ATP standard curve was used to quantify the amount of ATP present in samples.

Isolation of rat liver mitochondria

Liver from healthy Sprague-Dawley rats were removed after decapitation, weighed and suspended in medium A (210 mM mannitol, 70 mM sucrose, 50 mM Tris-HCl, 10 mM EDTA, pH 7.4 at 4°C) to a end concentration of 10 % (w/v). The tissue was homogenized using a Potter-Elvehjem homogenizer set at 200 rpm for 15 strokes. The homogenate was centrifuged at 1000 \times g for five min at 4°C. The resulting supernatant was centrifuged at 7000 \times g for 10 min at 4°C. The supernatant was discarded at the mitochondrial-enriched pellet suspended in medium B (225 mM mannitol, 75 mM sucrose, 10 mM Tris-HCl, 0.1 mM EDTA, pH 7.4 at 4°C) and the centrifuge steps repeated. The final mitochondrial pellet was suspended at 10 mg/ml in medium B and kept on ice during assays respiration analysis which was performed immediately after isolation of mitochondria.

Mitochondrial respiration assay

Respiration was measured using a Mitocell™ (model MT 200) oxygen electrode and recordings were done using an Oxymeter™ (Strathkelvin Instruments LTD, UK) and Oxygen System software

(Strathkelvin 782, version 3.0). The reaction medium consisted of 150 mM mannitol, 250 mM sucrose, 200 mM KCl, 20 mM Tris-phosphate, 20 mM Tris-HCl (pH 7.5), 0.1mM EDTA and 0.25 mg/ml mitochondria in a reaction volume of 70 μ l. The substrates that were used were 10 mM glutamate + 10 mM malate and 25 mM succinate + 3 μ M rotenone. After recording state 2/4 respiration (ADP depleted) 2 mM ADP was added and state 3 respirations recorded.

Respiratory chain enzyme assays

All enzymatic determinations were done at 30°C on mitochondria isolated from liver homogenates as prepared for the respiration measurements. The complex I (NADH ubiquinone oxidoreductase), combined complex I and III (NADH:cytochrome c reductase), combined complex II and III (succinate-cytochrome c reductase) and complex IV (cytochrome c oxidase) activities were determined according to the methods described before (Rahman et al., 1996). Enzyme activities were normalized to citrate synthase activity (Robinson et al., 1987).

Statistical analysis

All statistical analyses was done using Graphpad Prism® (GraphPad Software Inc., San Diego, CA,USA, version 4) software package. Results were expressed as the mean \pm 1 standard error of 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 $p < 0.05$.

RESULTS

Cell viability, LDH release and caspase 3/7 activity

Following exposure to 20 and 80 μ g/ml O₃ cell viability as measured with trypan blue exclusion decreased significantly by 16 and 17% respectively (Figure 1). Without treatment viability of buffered cells decreased only slightly due to removal of plasma. This is important since removal of the plasma and resuspending the cells in PBS without further treatment did not markedly affect cell viability. Exposure to oxygen significantly decreased cell viability by approximately 11%. Ozone treatment caused further damage to the buffered cells since viability decreased by 24% and 26% after exposure of 20 and 80 μ g/ml ozone respectively. Although cell viability decreased, it remained significantly higher than in the positive control cells, which were treated with 6% acetic acid. LDH release is considered a sensitive assay to measure cell necrosis (Decker and Lohman-Matthes, 1988). In whole blood LDH released from cells increased by 49% after treatment with oxygen (Figure 2). Exposure to 20 μ g/ml and 80 μ g/ml ozone increased the LDH release significantly

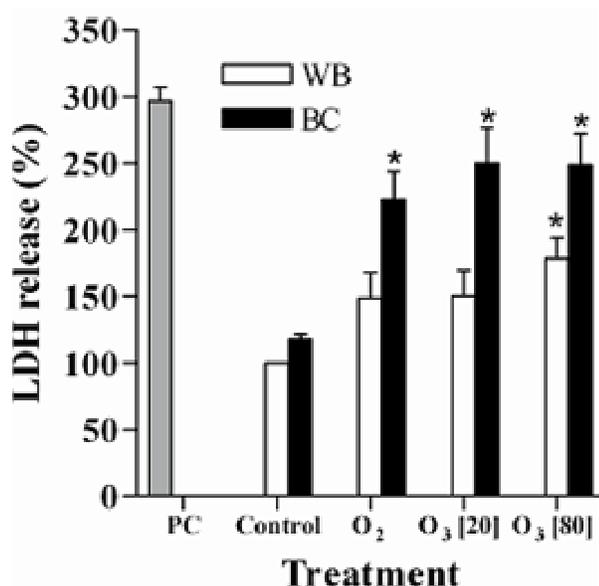


Figure 2. The LDH release from ozone treated PBMC. Results are given as mean \pm 1SEM (n=6) LDH release (%) for the positive control (PC), whole blood (WB) and the buffered cells (BC) at baseline and after exposure to oxygen and 20 and 80 μ g/ml ozone. $p < 0.05$, relative to the control (*) of the whole blood (Bonferroni test). The positive control (PC) was treated with 1% Triton X[®].

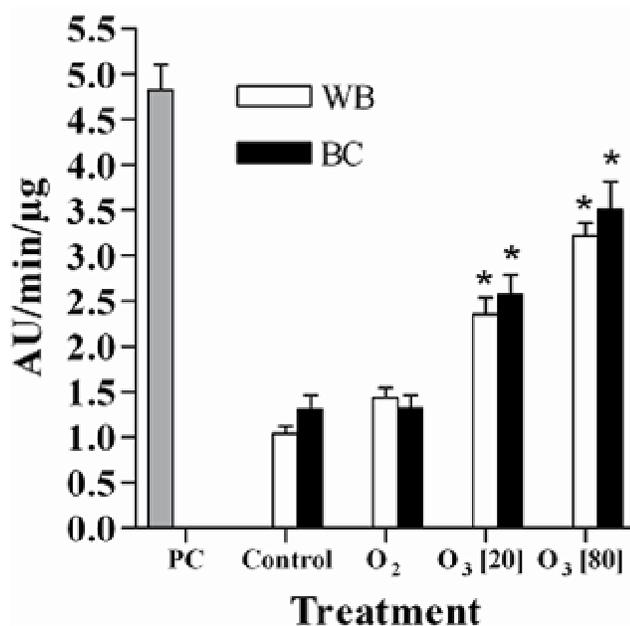


Figure 3. The caspase 3/7 activity of ozone treated PBMC. Results are given as the mean \pm 1SEM (n=6) caspase 3/7 activity (AU/min/ μ g protein) for whole blood (WB) and buffered cells (BC) at baseline and after exposure to oxygen and 20 and 80 μ g/ml ozone. $p < 0.05$ with respect to control (*) of the whole blood group (Bonferroni test). The positive control (PC) was treated with 1 μ g/ml staurosporine.

by 50 and 79%, respectively. LDH release from untreated buffered cells did not differ significantly from that of whole blood. Treatment of buffered cells with oxygen and the different concentrations of ozone increased LDH release significantly by 23, 50 and 49% after treatment with oxygen and 20 and 80 μ g/ml ozone, respectively.

The mean control levels of caspase 3/7 activity in the whole blood group was 1.04 AU/min/ μ g and after oxygen treatment this increased by approximately 16% to 1.24 AU/min/ μ g (Figure 3). In whole blood the caspase activity increased significantly after exposure to 20 and 80 μ g/ml ozone by approximately 56 and 68% respectively, when compared to the control. The ozone treated groups also differed significantly from the oxygenated samples. The mean control caspase activity of the buffered cells was approximately 20% (1.30 AU/min/ μ g) higher than that of the whole blood. This was not significantly different. The oxygenated samples were also not significantly different. The caspase activity of the ozone treated buffered cells increased by 60 and 56% with 20 and 80 μ g/ml ozone respectively.

Mitochondrial function

The NADH/NAD⁺ ratio: In this study the baseline NADH/NAD⁺ ratios ranged between 0.6 and 1.9. After treatment with oxygen and 20 μ g/ml ozone the ratios in whole blood increased, but not significantly (Figure 4). With 80 μ g/ml ozone treatment the ratio increased by 40%. In the buffered cells the ratios increased significantly following treatment with oxygen and 80 μ g/ml ozone. The increase in the NADH/NAD⁺ ratio was brought about by both an increase in NADH and a decrease in NAD⁺.

ATP levels: The ATP levels in the PBMC in whole blood and in buffered cells decreased slightly, but significantly after treatment with oxygen, 20 and 80 μ g/ml ozone (Figure 5). The treated samples in the buffered cells also contained significantly less ATP than the oxygenated sample in whole blood.

Mitochondrial respiration: To further determine the effects of ozone on mitochondrial function, respiration in ozone treated liver mitochondria was measured. The amount of mitochondria required for this and the respiratory chain complex enzyme analyses could not be obtained from blood. State 3 respiration using substrates entering reducing equivalents either through complex I (glutamate and malate) or II (succinate plus the complex I inhibitor, rotenone) were determined and the data is summarized in Figure 6. As can be seen respiration through complex II had in general higher respiration than through complex I, which is the time-dependent step of the respiratory chain. Oxygen treatment increased respiration which may be due to a relatively better supply of

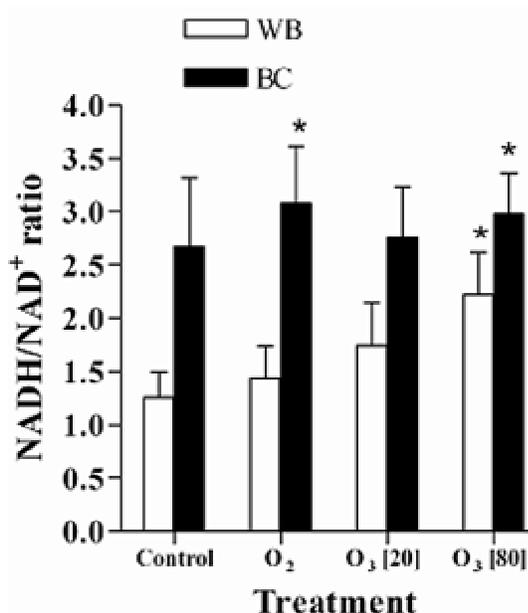


Figure 4. The NADH/NAD⁺ ratio measured in the two intervention groups. Results are given as the mean ± 1SEM (n=6) NADH/NAD⁺ ratios for whole blood (WB) and buffered cells (BC) at baseline and after exposure to oxygen and 20 and 80 µg/ml ozone. p<0.05 with respect to control (*) of the whole blood group (Bonferroni test).

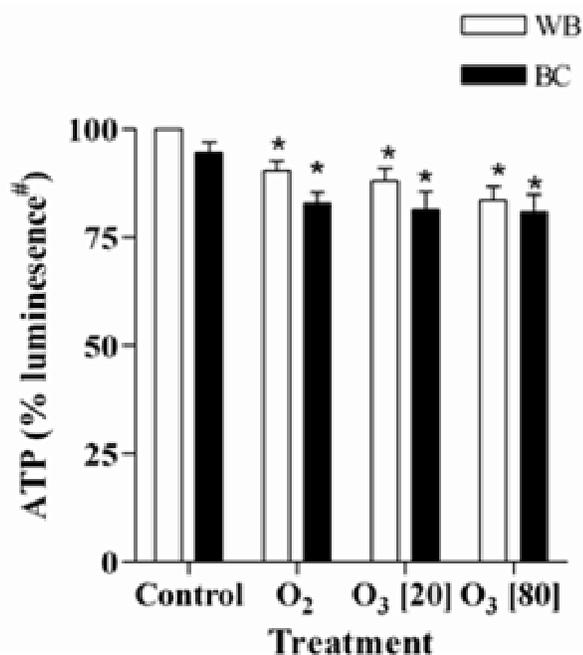


Figure 5. The ATP levels measured in the two intervention groups. Results are given as the mean ± 1SEM (n=6) ATP levels for whole blood (WB) and buffered cells (BC) at baseline and after exposure to oxygen and 20 and 80 µg/ml ozone. p<0.05 with respect to control (*) of the whole blood group (Bonferroni test).[#] % luminescence/µg cells.

oxygen in the reaction. However, using both substrates, ozone treatment at 20 µg/ml significantly reduced respiration by approximately 68% (succinate) and 40% (glutamate and malate). With 80 µg/ml ozone treatment the decrease was even more significant with 89% and 80% for the two substrates respectively.

Respiratory chain enzyme activities: The direct effect of ozone treatment on individual enzyme complexes of the respiratory chain was investigated and summarized in Table 1. The same treatment protocol as for the respiration measurements was used. Treatment with oxygen caused significant decreases in complex I activity, combined complex II+III activity and complex IV activity, by 32, 27 and 50% respectively. This may be due to oxidation of reducing elements in these complexes. Treatment with the two ozone concentrations had no significant effect on complex I or combined complex I+III activity, but decreased complex II+III activity significantly. However, complex IV activity was even more significantly (~95%) inhibited by even the lowest ozone concentration.

DISCUSSION

The main purpose of this study was to evaluate the effect of ozone on white blood cell viability and mitochondrial function under conditions similar to O₃-AHT. It has been reported that ozone affects cell viability of cultured monocytes (Klestadt et al., 2005). Exposure of PBMC to ozone also caused a dose dependent increase of DNA damage (Diaz-Llera et al., 2002). Under conditions of oxidative stress, ROS can damage chromosomal DNA and other cellular components. This can result in DNA degradation, protein denaturation and lipid peroxidation (Higuchi, 2004). Thus, increased oxidative stress caused by ozone is likely to be responsible for the increased death of PBMC that we observed, since mechanical stress was not present. Ozone exposure increases the formation of H₂O₂ which can damage cells via the Fenton reaction. The ozone also reacts with polyunsaturated fatty acids (PUFAs) in the membranes of the cells, generating lipid ozonation products (LOP). This can further lead to the increased cell necrosis and can explain why LDH release increased. On the other hand, membrane damage due to ozone binding to PUFAs can damage the cell membrane and so increase LDH release, without causing necrosis to the cells. The LOP, particularly 4-hydroxynonenal (HNE), is able to activate biochemical and immunological pathways. Studies proved that HNE cause apoptosis and the release of caspase 3 (Larini et al., 2004). Many cell death events displays apoptotic and necrotic features, which can make it difficult to distinguish between the two forms of cell death (LaCasse et al., 2005). Since caspase 3/7 levels increased three fold due to ozone treatment increased death of PBMC was proba-

Table 1. The effect of ozone treatment on the activity of different respiratory chain complexes in rat liver mitochondria.

Complex	Control	O ₂	O ₃ [20]	O ₃ [80]
complex I	308±22	210±38*	376±23	409±20
complex I+III	430±20	383±4	413±28	482±35
complex II+III	478±6	348±41*	249±14*	291±35*
complex IV	2797±167	1396±101*	131±12*	129±7*

The mean ± 1SEM enzyme activity in nmole/min/UCS (n=3) are shown. p<0.05 (*) versus the control (Bonferroni test).

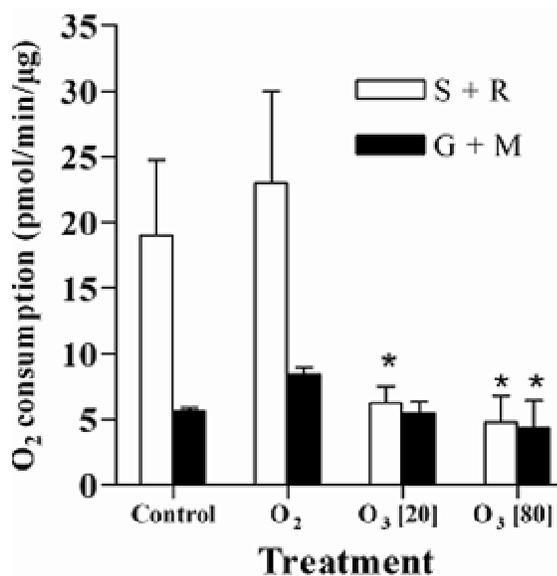


Figure 6. Effects of ozone treatment of rat liver mitochondrial state 3 respiration. The bars represent the mean ± 1SEM state 3 respiration (μmol/min) measured after the addition of 2 mM ADP (n=3). p<0.05 versus the control (*) of the different substrates added. S = succinate; R = rotenone; G = glutamate; M = malate.

by caused by apoptosis. In this case it would prove useful to measure the amount of LOP formed, in order to determine the relationship between the toxic aldehydes and caspase activity.

ROS, cell death and apoptosis can not be mentioned in the same sentence without including the role of the mitochondria. Treatment with ozone decreased ATP levels (Figure 4) increased the NADH/NAD⁺ ratio (Figure 3) and caspase activity (Figure 2). This indicated that mitochondrial function was affected. Mitochondrial oxidative phosphorylation is the major ATP synthetic pathway in eukaryotes. Electrons liberated from reducing substrates (NADH) are delivered to oxygen via a chain of respiratory complexes (complex I-IV). The energy from the electrochemical gradient ($\Delta\psi$) across the inner mitochondrial membranes is used to drive ATP production by complex

V (Wallace, 1999; 2001). The measurement of ATP levels is therefore a good indicator of mitochondrial function.

The mitochondrial function of the liver homogenates indicated that ozone treatment decreased the rate of state 3 respirations. It also inhibited complex II+III and especially complex IV activity, probably by interacting with the Fe-S clusters in the respiratory complexes. Acute ROS exposure can inactivate the iron sulphur (Fe-S) centres of the electron transport chain complexes, resulting in the shutdown of mitochondrial energy production (Wallace, 1999). The decreased complex IV activity could have been caused by ozone directly binding to complex IV, similar to its reaction with haemoglobin (Cataldo et al., 2005). This corresponds to previous results where low concentrations of ozone caused 20% inhibition of complex IV activity in fibroblasts (Van der Zee et al., 1987). It is important to note that control of mitochondrial oxygen consumption and ATP synthesis is shared by several steps of oxidative phosphorylation and this can vary according to the type of tissue. This implies that there is no limiting step in the regulation of respiration (Rossingnol et al., 2000). Control of oxidative phosphorylation in the liver is mainly done at the phosphorylation level by ATP synthase and the phosphate carrier. Therefore, decreases in complex IV activity have to exceed a critical value before a decrease in mitochondrial respiration can be observed. This is because there is an excess of active respiratory chain complexes that can serve as a reserve to compensate for a deficit (Rossingnol et al., 2003). This implies that, although ozone inhibits complex IV by 95%, respiration still takes place through the other complexes that was not inhibited, resulting in a less significant inhibition of respiration by ozone (Figure 6).

Another important consideration is that ATP can act as a switch between apoptosis and necrosis. Cells that are more dependent on ATP from oxidative phosphorylation are more likely to undergo necrosis, whereas cells that are more dependent on ATP from glycolysis are more likely to undergo apoptosis when the ATP levels decrease (Li et al., 2003). PBMC obtain most of their ATP by metabolism and oxidation of glucose (Arslan et al., 1984), but it is not clear whether they depend more on oxidative

phosphorylation or glycolysis for ATP. Apoptosis is common in PBMC, but is dependent on the specific subtype of cells, with T-lymphocytes more likely to undergo apoptosis than other cells (LaCasse et al., 2005). The inhibited mitochondrial function and decreased ATP levels could however have contributed to the initiation of apoptosis. This is achieved by the opening of the mitochondrial permeability transition pore (MPT) located in the inner mitochondrial membrane. A number of cell death-promoting factors are located in the mitochondrial inner membrane space, including cytochrome c, apoptosis-inducing factor and caspases. The opening of the MPT causes collapse of the membrane potential, swelling of the mitochondrial inner membrane and release of the death promoting-factors. Opening of the MPT can also be mediated by excessive calcium uptake (Wallace, 1999). This, together with the elevated caspase activity argues strongly in favour of apoptosis as the mechanism of cell death in PBMC after exposure to ozone. Unfortunately the results as to the mechanism of cell death are inconclusive. As a follow up, we therefore propose to design a study that will specifically investigate if ozone causes necrosis or apoptosis. This will have to include long term exposure with a follow up of several hours after blood is exposed to ozone. Such a study will include an in depth investigation in morphological and biochemical changes brought about by ozone.

Conclusions

In conclusion, these results clearly indicates a significant inhibition of mitochondrial respiration, specifically through inhibition of cytochrome c oxidase (complex IV), that may then lead to reduced ATP production, increase of ROS, increased NADH/NAD⁺ ratio and apoptosis and/or necrosis. Antioxidants in plasma dampened these effects of ozone. This highlights the importance of the plasma antioxidant defence system in protection against ROS. It is not certain that these *in vitro* effects will propagate when ozonated blood is reinjected back into the individual undergoing O₃-AHT.

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