African Journal of Biotechnology Vol. 8 (5), pp. 844-848, 6 March, 2009
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2009 Academic Journals

Full Length Research Paper

Ozone autohaemotherapy protects against ketamine hydrochloride® induced liver and muscle damage in baboons

Liezl Gibhard[1]*, Marietjie Meyer[2] and Herculaas F. Kotzé[1]

[1]School for Chemistry and Physical Science, North-West University, Private Bag X6001, Potchefstroom, 2520, South-Africa.
[2]AMPATH, Mooimed Private Hospital, Albert Luthuli Street, Potchefstroom, 2520, South Africa.

Accepted 5 September, 2008

Ozone is currently under scrutiny because of various claims of beneficial effect in disease. In order to shed some light on this we assessed the acute and chronic effect of O_3 autohaemotherapy (AHT) on liver and muscle damage in baboons. Five percent of the total blood volume of a baboon was treated with O_2 and O_3. Eleven baboons were acutely treated with an O_2/O_3 gas mixture containing 20, 40 and 80 µg/ml ozone. Five were treated with pure O_2 and three received no treatment to assess the effect of the ketamine hydrochloride anaesthesia. Blood samples were collected before treatment and after 4, 24 and 48 h. Anaesthesia increased aspartate aminotransferase (AST), alanine aminotransferase (ALT) and creatine kinase (CK) levels markedly. O_3-AHT had a protective effect, since enzyme levels were lower. O_2-AHT had no protective effect on liver and muscle damage. An O_2/O_3 gas mixture containing 40 µg/ml O_3 was used for chronic O_3-AHT (n=6) treatment. The animals were treated at 0, 24 and 48 h. Blood was collected before treatment and again after 4, 24, 28, 48, 52, 72 and 96 h. ALT levels increased and remained elevated. AST levels increased during the four hours following each treatment and remained elevated. CK levels increased markedly during the four hours following treatment, but decreased after treatment was stopped. The magnitude of changes was small and does not support the view that infusion of ozonated of blood is toxic.

Key words: O_3-AHT, O_2-AHT, liver damage, muscle damage.

INTRODUCTION

Ozone is a strong oxidant and much more reactive than oxygen (Bocci, 1999). When ozone comes into contact with biological fluids, it dissolves and undergoes rapid degradation at pH > 5. Olefin reacts with ozone to produce aldehydes, the lipid oxidation products (LOPs) and reactive oxygen species (ROS). The ROS is the same as those that are produced via normal biochemical processes in the body (Bocci, 2002). It is these LOPs and ROS, and not the ozone that are responsible for the multiple biochemical reactions that follow after treatment with O_3 (Bocci, 2005). There are various routes to treat with O_3. They include topical application, vaginal, rectal, bladder or intraperitoneal insufflations. O_3-AHT appears to be the preferred method. This approach offers the advantage that the ozonated blood is rapidly distributed throughout the body (Sunnen, 1998). For O_3-AHT to be effective, it is crucial to use the correct dose.

Liver enzymes leak into the blood when liver cells are damaged (Widmaier et al., 2004). ALT is present in high concentrations in the liver and is regarded as a specific marker of hepatocellular damage (Marshall and Bangert,

*Corresponding author. E-mail: BCHLG@puknet.puk.ac.za. Tel: +2718 299 4196. Fax: +2718 299 2316.

Abbreviations: O_3-AHT, Ozone autoheamotherapy; **O_2-AHT**, oxygen autoheamotherapy; **ROS**, reactive oxygen species; **LOP**, lipid oxidation products; **NADHP**, nicotinamide adenine dinucleotide phosphate; **NADH**, nicotinamide adenine dinucleotide (reduced form); **NAD^+**, nicotinamide adenine dinucleotide (oxidized form); **ALT**, alanine aminotransferase; **AST**, aspartate aminotransferase; **CK**, creatine kinase; **MD**, malate dehydrogenase; **LD**, lactate dehydrogenase.

2004). Acute liver damage therefore causes high blood levels of ALT. ALT is also present in the heart and muscles but in much lower concentrations (Giannini et al., 2005). AST, on the other hand, is present in liver, heart, kidneys, skeletal muscle and red blood cells. AST levels are increased in shock and are less specific for liver damage (Giannini et al., 2005). Aminotransferase alterations as an indication of cell damage are classified into three groups i.e. “mild” (<5 times the upper reference limit), “moderate” (5-10 times the upper reference limit) and “marked” (>10 times the upper reference limit) (Giannini et al., 2005). CK is present in high concentrations in skeletal muscle, cardiac muscle, thyroid, prostate and the brain. It is present only in small amounts in the liver, kidney, lung and other tissues. Hence, an increase in serum CK activity is primarily due to damage of striated muscle (skeletal or cardiac) and, in rare cases, to the brain (Lang, 1981). The CK molecule is a dimmer and consists of two subunits M and B. M predominates in skeletal muscle and B in the brain. There are three isoenzymes: CK-BB, which has two B chains, CK-MM, with two M chains and CK-MB, with one B and one M chain. It is the CK-MM isoenzyme that is predominantly found in skeletal muscle (Lang, 1981).

METHODS

Treatment protocol for the acute phase

The study was approved by the Ethics Committee of the North-West University according to 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). Ozone was prepared as described in detail (Du Plessis et al., 2007). Sixteen healthy baboons, weighing between 16 and 25 kg, were used. The baboons were anesthetized with intramuscular ketamine hydrochloride (±10 mg/ml) to enable handling and blood sampling. Five percent of the blood volume of a baboon was treated (Kotzé et al., 1985). Eleven baboons were treated in random order with an O_2/O_3 gas mixture containing 20, 40 or 80 µg/ml O_3. Five other baboons were treated with ultrapure O_2. Briefly, blood was drawn in heparin in polypropylene syringes. It was then transferred to siliconised glass syringes and ozonated by adding an equal volume of an O_2/O_3 gas mixture. The blood was gently mixed for 20 min, the gas removed and the blood reinfused into the donor. Sham studies were done on three baboons, i.e. blood was withdrawn but not treated with a gas. Blood samples were collected before each AHT in vacutest tubes containing heparin and again after 4, 24 and 48 h.

Treatment protocol for the chronic phase

Six baboons were used. Five percent of the blood volume was treated with an O_2/O_3 gas mixture containing 40 µg/ml O_3, similar to that described for the acute phase. Three consecutive treatments, 24 h apart, were done. Blood samples were collected before O_3-AHT treatment and again at 4, 24, 28 and 48, 52, 72 and 96 h after the first treatment.

Assessment of liver damage

The Dimension® clinical chemistry system (Dade Behring, South Africa) was used to determine the levels of ALT and AST as an indication of liver damage.

ALT

35 µL serum was used in a diluent volume of 215 µL. The assay was done at 37˚C. The rate of formation of pyruvate was determined by coupling the ALT reaction with that of LD which converts pyruvate to lactate. The decreased absorbance at 340 nm was measured with a spectrophotometer (Dade Behring, Dimension xpand, South Africa) during the oxidation of NADH to NAD^+. ALT activity was indirect proportional to the decrease in absorbance (Bergmeyer, 1983).

AST

40 µL serum was used in a diluent volume of 235 µL. The test was performed at 37˚C. The reaction of AST was coupled to that of malate dehydrogenase (MD) where the oxaloacetate is reduced to malate while NADH is simultaneously oxidized to NAD^+. The decrease in absorbance at 340 nm was measured. AST activity was indirect proportional to the decrease in absorbance (Bergmeyer, 1983).

Assessment of muscle damage

Serum plasma levels of CK were determined to assess both heart and skeletal muscle damage. It was done by using the Dimension® clinical chemistry system (Dade Behring, South Africa). A sample of 14 µL serum was used in a diluent volume of 255 µL. The assay was done at 37˚C. The activity of CK was followed by measuring the ATP produced from creatine phosphate to form glucose-6-phosphate. The glucose-6-phosphate was then dehydrogenated and the rate of formation of NADPH was measured spectrophotometrically (Dade Behring, Dimension xpand, South Africa) at 340 nm. CK activity was directly proportional to the increase in absorbance (Bergmeyer, 1983).

Statistical analysis

The values are expressed as the fold change ± 1 standard error of the mean (1 SEM). Fold change was calculated as 1 – (Value Ti/value T0) where T = time and i the measurement at time i, i.e during the acute phase at 4, 24 and 48 h and during the chronic phase at 4, 24, 48, 52, 72 or 96 h. The data were compared using the Student’s t-test for paired and unpaired samples, and were regarded significant when $p < 0.05$.

RESULTS

Acute effects

ALT levels increased markedly at all time points following treatment with ketamine hydrochloride, O_2 and 20 and 40 µg/ml ozone which reached a maximum after 24 h (Figure 1). The most prominent increase of 229% was 24 h after treatment with ketamine hydrochloride, and it remained high for the next 24 h. It is of interest to note that the increase 4 h after treatment with 80 µg/ml ozone was significantly less than in the other cases and that it remained low for up to 24 h following treatment.

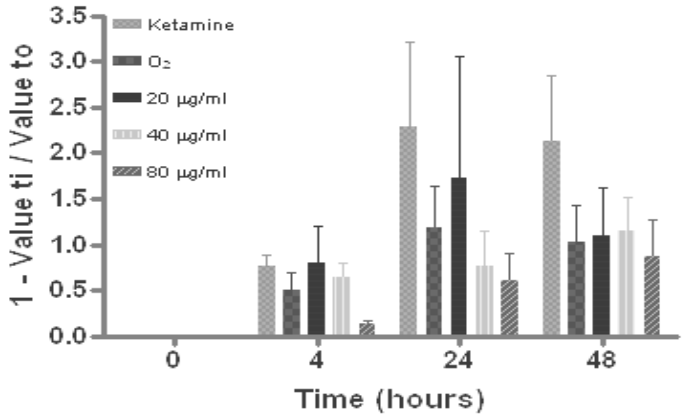

Figure 1. Acute effect of O_3-AHT treatment on ALT. Values are given as fold change ± 1 SEM.

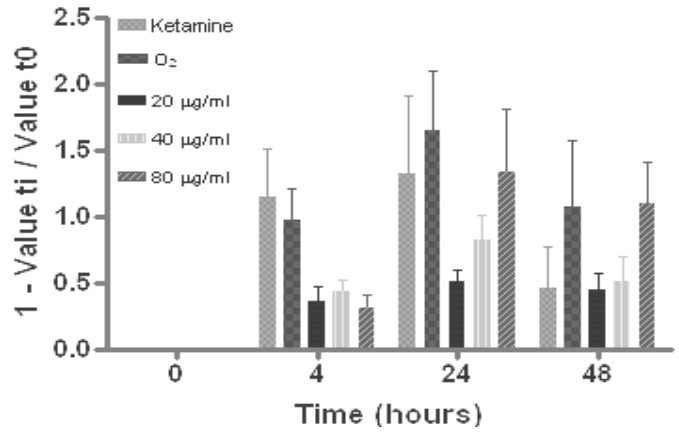

Figure 2. Acute effect of O_3-AHT treatment on AST. Values are given as fold change ± 1 SEM.

AST levels increased markedly during all treatment regimens, and reached a maximum 24 h following treatment. The largest increase was observed in the ketamine hydrochloride group, approximately 132% after 24 h. After ozonation, the increase in AST levels was significantly smaller than in the ketamine hydrochloride group The increase in the AST levels following oxygenation was similar to that in the ketamine hydrochloride group at all time points (Figure 2). At 24 and 48 h AST levels following treatment with 20 and 40 µg/ml O_3 were still significantly less than the ketamine hydrochloride group. AST levels after treatment with 80 µg/ml O_3, on the other hand, was unchanged.

The CK levels increased markedly during the first four hours following all treatments and remained elevated for at least 48 h. The results showed a dose-dependant increase in CK levels 4 h after treatment, with the highest increase in the ketamine hydrochloride group and the lowest in the 80 µg/ml O_3 group (Figure 3). After 24 h a similar pattern were observed, but not in such an ordered

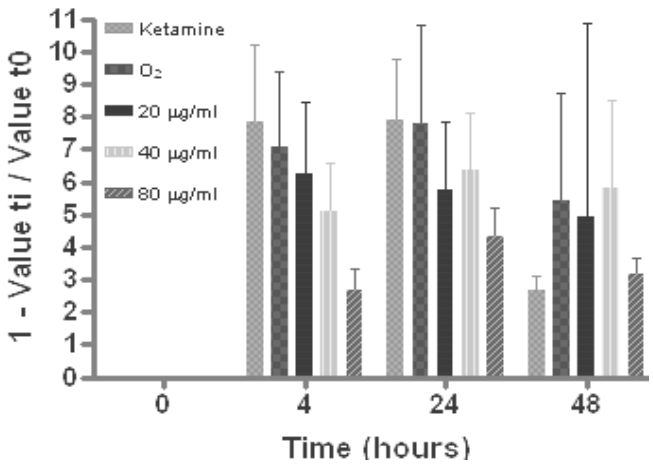

Figure 3. Acute effect of O_3-AHT treatment on CK. Values are given as fold change ± 1 SEM.

fashion as at four hours. There were no significant difference between CK levels after treatment with O_2 and the untreated (ketamine hydrochloride) group.

Chronic effects

Following O_3-AHT, no marked increase in ALT levels was observed from 0 to 4, 24 to 28 and 48 to 52 h, i.e. the acute effect of each treatment. After 24 h the pretreatment value increased by approximately 82% and by approximately 120% at 48 h (Figure 4). Thus, ALT levels increased during the chronic but not acute phase. It remained elevated after treatment was stopped at 48 h.

AST levels increased during the four hours following each treatment with the largest increase observed at 4 and 28 h (Figure 5). AST levels increased markedly on a 24 h basis and reached a maximum at 72 h, i.e. 24 h following the last O_3-AHT. It was still markedly increased at 96 h.

The CK levels increased dramatically from 0 to 4, 24 to 28 and 48 to 52 h and decreased in the 48 h following the last treatment. Of interest is the fact that CK levels increased from 4 to 24 h but decreased from 28 to 48 h (Figure 6). CK levels reached a maximum 4 h following the third treatment by approximately 1644%. After treatment was stopped, CK levels decreased markedly.

DISCUSSION

When interpreting the results, it is important to keep in mind that the blood was ozonated or oxygenated *ex vivo*. The animals were therefore not directly exposed to either oxygen or ozone. The changes that we have seen must therefore be attributed to the products that formed when exposing the blood to the gas, particularly the LOPs and ROS that form (Bocci, 2002; Bocci, 2005).

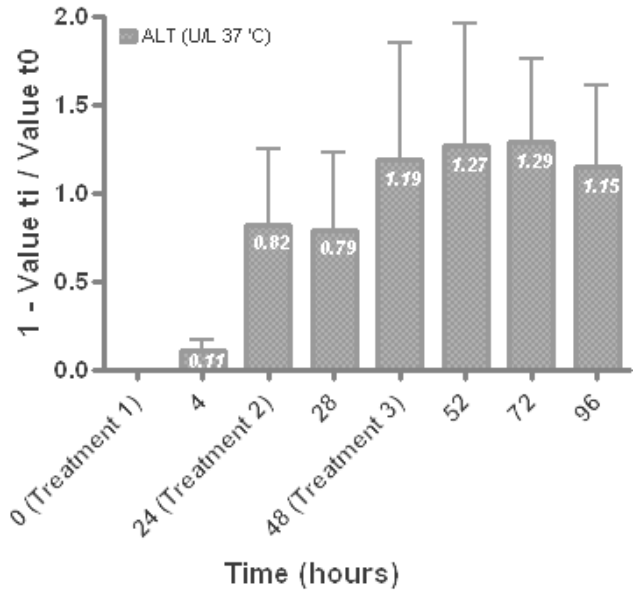

Figure 4. The chronic effect of three consecutive O_3-AHT treatments 24 hours apart on ALT (fold change ± 1 SEM).

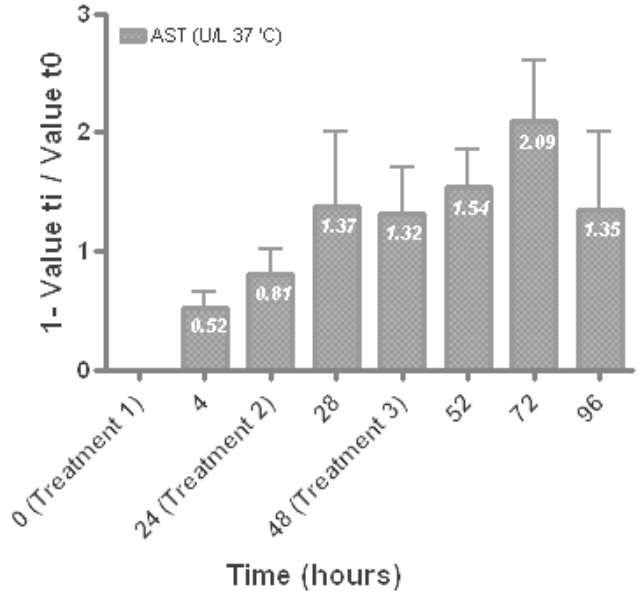

Figure 5. The chronic effect of three consecutive O_3-AHT treatments 24 hours apart on AST (fold change ± 1 SEM).

When considering the relationship between ozone and liver damage it is clear that O_3-therapy protected against liver damage caused by ketamine hydrochloride. Ketamine hydrochloride is catabolyzed in the liver (Dundee et al., 1980). It is therefore not unexpected that it damages the hepatocytes. During the acute phase, oxygenation or ozonation with 20 or 40 µg/ml O_3 increased the ALT levels in plasma after the first four hours following treatment. It is of interest to note that ozonation with 80

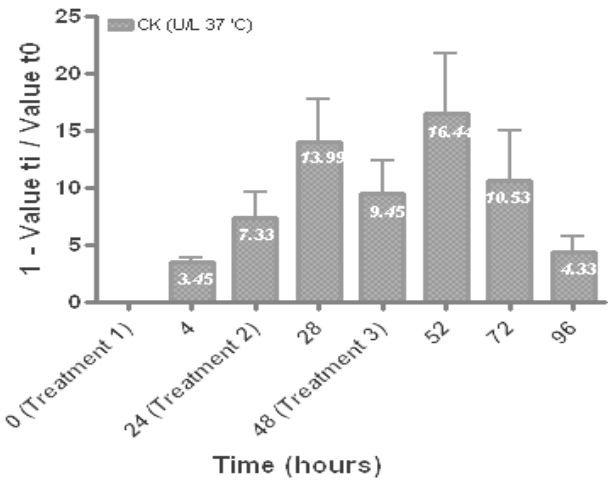

Figure 6. The chronic effect of three consecutive O_3-AHT treatments 24 hours apart on CK (Fold change ± 1 SEM).

µg/ml O_3 only caused a slight increase in ALT levels at 4 and 24 h (Figure 1). The AST levels during the acute phase (Figure 2) followed the same pattern than that of ALT during the acute phase (Figure 1). We are not sure what the 24 and 48 h results mean, and if the ROS and LOPs will remain in the circulation for up to 48 h following injection of ozonated blood. The antioxidant systems are more than sufficient to quench ROS (Rice-Evans and Miller, 1994; Haensler, 2000) and the highly active LOPs that will react quickly with appropriate substrates (Giannini et al., 2005). During the chronic phase both ALT and AST levels increased. ALT levels increased by approximately 130%, while AST levels increased by approximately 210% during the treatment period i.e. the first 48 h. These larger variations in the AST levels during the acute and chronic phases are expected because AST is present in the heart, red blood cells, kidneys and skeletal muscle, while ALT is mainly present in hepatocytes (Marshall and Bangert, 2004, Giannini et al, 2005). Fortunately, the increases in plasma levels of ALT and AST during the acute and chronic phases could not even be classified as mild damage (Giannini et al., 2005). Thus, the damage was not severe and possibly not of clinical relevance. One possible explanation could be that the ROS and LOPs that are formed during *ex vivo* ozonation bind to the ketamine hydrochloride present in the circulation and this decreased the load of ketamine hydrochloride that has to be detoxified by the liver. The remainder of the liver damage can be explained by the fact that ketamine hydrochloride is metabolized in the liver and it is therefore not unexpected that it damaged the hepatocytes. Another possible explanation could be that, when ozone came into contact with the plasma during AHT, ROS and LOPs were produced. These were responsible for membrane lipid peroxidation and the release of these enzymes into the blood, because the integrity of cell membranes were compromised.

During the acute phase, the CK levels in the plasma increased by approximately 750% 4 h following treatment. The CK levels remained elevated for at least 24 h (Figure 3). During the chronic phase, CK levels in plasma increased by approximately 345% after 4 h and reached a maximum 4 h following the last treatment. A prominent decrease was observed 48 h after treatment was stopped. The result is difficult to explain because the isoenzymes were not measured. It is, however, important to keep in mind that the ketamine hydrochloride was administered intramuscular and the increase in CK levels were most likely because of the increase in CK-MM (Lang, 1981). Once again, when the results that were obtained during the chronic and acute phases are compared to that of ketamine hydrochloride, it is clear that ozonation of the blood had a protective effect against the damage caused by ketamine hydrochloride. One possible explanation could be that the ROS and LOPs that are formed during the *ex vivo* ozonation may improve the repair capacity of muscle cells that were damaged during the intramuscular administration of ketamine hydrochloride. It is not untoward, since ozonation improves the oxygen metabolism in cells (Bocci, 2005). In future studies it may be necessary to asses the isoenzymes of CK, so that there can be closure whether the observed muscle damage is due to damage to skeletal or cardiac muscle.

Conclusion

The result does not prove or disprove that O_3-AHT caused severe cell damage in baboons. Based on the result of the acute studies and those of Labuschagne et al. (2007), where there was no marked difference between treatment with 80 and 40 µg/ml, we decided to use the 40 µg/ml dose. This will inevitably decrease possible adverse effects of the high dose.

ACKNOWLEDGEMENTS

We appreciate the technical assistance of Cor Bester. This work is funded by the National Research Foundation (NRF) of South Africa.

REFERENCES

Bergmeyer J (1983). Methods of Enzymatic Analysis. 3rd ed. Vol III, VCH Publishers, Weinheim, pp 418_1-424_1; 445_2-450_2; 510_3-517_3.

Bocci V (1999). Biological and clinical effects of ozone. Has ozone therapy a future in medicine Br. J. Biomed. Sci. 56: 270-279.

Bocci V (2002). Oxygen-ozone therapy, a critical evaluation, Kluwer academic publishers, Netherlands.

Bocci V (2005). OZONE: A new medical drug, Springer, Dordrecht, pp 19-111.

Du Plessis L, Van der Westhuizen FH, Kotzé HF (2007). The effect of blood ozonation on mitochondrial function and apoptosis of peripheral blood mononuclear cells in the absence of plasma antioxidants. Afr. J. Biotechnol. 6: 1763-1769.

Dundee JW, Free JP, Moore J, Mellroy PD, Wilson DB (1980). Changes in serum enzyme levels following Ketamine hydrochloride infusion. Anaesthesia, 35: 12-18.

Giannini EG, Testa R, Savarino V (2005). Liver enzyme alterations: a guide for clinicians. CMAJ. 172: 367-377.

Haensler RV (2000). Ozone therapy: Method. [Web:] http://www.medicasrl.com/002USA2000.htm.

Kotzé HF, Lötter MG, Badenhorst PN, Heyns AduP (1985). Kinetics of In-111-platelets in the baboon: Isolation and labelling of a viable and representative platelet population. Thromb. Haemost. 53: 404-407.

Labuschagne CF, Van Helden G, Kotzé HF, van der Westhuizen FH (2007). The effect of autoheamotherapy with different ozone concentrations on the antioxidant status of baboons. Potchefstroom : NWU (Potchefstroom campus). (Dissertation – M.Sc.).

Lang H (1981). Creatine Kinase Isoenzymes: Pathophysiology and Clinical Application, Springer, New York, pp 1-9.

Marshall WJ, Bangert SK (2004). Clinical Chemistry. 5th ed, Elsevier, pp. 85-103.

Rice-Evans C, Miller NJ (1994). Total antioxidant status in plasma and body fluids. In: Methods in Enzymology. Acedemic press, New York, pp. 279-293.

Sunnen GV (1998). Ozone in medicine: Overview and Future Directions. http://www.triroc.com/sunnen/index.htm.

Widmaier EP, Raff H, Strang KT (2004). Human Physiology: The Mechanisms of Body Function. 9th ed. Mcgraw Hill, pp. 696-729.