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

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Osoon outohemoterapie beskerm teen Ketamien hidrochloried® geïnduseerde lewer en-spierskade in bobbejane

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Soli Deo Gloria

Hoe wonderlik is u gedagtes vir my, o God, hoe magtig hulle almal

Psalm 139:17

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Abstract

Ozone therapy has been used as a form of alternative medicine but has encountered scepticism by orthodox medicine concerning its effectiveness and toxicity. We assessed the acute and chronic effect of O_3 autohaemotherapy (AHT) on liver and muscle damage in baboons. During the acute effect three groups of baboons were used. The first group (n=11) were treated with an O_2/O_3 gas mixture containing different ozone concentrations i.e. 20, 40 and 80 µg/ml O_3. The second group (n=5) were treated with pure O_2 and the third group (n=3) received no treatment and were used to assess the effect of ketamine anaesthesia. O_2 and O_3-AHT was performed on the baboons by using 5% of their total blood volume. Blood samples were collected in heparin before each treatment and again at 4, 24 and 48 hours. Ketamine anaesthesia caused both liver and muscle damage. Aspartate aminotransferase (AST), alanine aminotransferase (ALT) and creatine kinase (CK) levels increased markedly. O_2-AHT had no marked effect on liver and muscle damage and O_3-AHT had a protective effect since the increase in AST, ALT and CK levels was not as dramatic as when ketamine alone was used. During the chronic effect O_3-AHT were done on 6 baboons, using a O_2/O_3 gas mixture containing 40 µg/ml O_3. Blood were collected before treatment and again at 4, 24, 28, 48, 52, 72 and 96 hours after the first treatment. ALT levels increased during the treatment period of 52 hours and remained elevated for 48 hours following treatment. AST levels increased during the four hours following each treatment and remained elevated for 48 hours following the last treatment. CK levels increased markedly during the four hours following each treatment, but after treatment was stopped the CK levels decreased dramatically. The magnitude of changes was small and does not support the view that in vitro ozonation of blood is toxic when the treated blood is reinjected back into the baboon.

In conclusion, the results do not prove or disprove that O_3-AHT caused severe cell damage in baboons. We used 40 µg/ml O_3 in the studies where the baboons were treated sequentially (chapter 3). This was because the results

obtained with dose of 80 µg/ml O_3 were not largely different from that with 40 µg/ml O_3 in the acute studies (chapter 2). It was proven in these two studies and also other studies. It was proven in these two studies and also in other studies. I also have no ready explanation of the mechanism through which the liver and muscle were protected by ozone. This has to be investigated.

Opsomming

Osoon terapie word gebruik as 'n vorm van alternatiewe mediese behandeling, maar daar bestaan egter kommer oor die effektiwiteit en toksisiteit van O_3-terapie. Ons het die akute en kroniese effek van O_3 outohemoterapie (AHT), op lewer en spierskade in bobbejane ondersoek. Gedurende die akute effek is drie groepe bobbejane gebruik. Die eerste groep (n=11) is behandel met 'n O_2/O_3 gas mengsel wat verskillende konsentrasies osoon bevat het bv. 20, 40 en 80 µg/ml O_3. Die tweede groep (n=5) is behandel met suiwer O_2 en die derde groep (n=3) het geen behandeling ontvang nie, maar is gebruik om die effek van ketamien hidrochloried wat as verdowingsmiddel gebruik is te bepaal. Vyf persent van 'n bobbejaan se geheparinieseerde bloed is vir 20 minute met O_2 en O_3 behandel. Die gas is verwyder en die bloed via die femorale arterie aan die dier terug gespuit. Bloedmonsters is versamel voor elke behandeling en dan weer na 4, 24 en 48 uur na behandeling. Ketamien hidrochloried het beide lewer en spier skade veroorsaak. Die plasma vlakke van aspartaat aminotransferase (AST), alanien aminotransferase (ALT) en creatien kinase (CK) het merkbaar toegeneem. O_2-AHT het geen effek op lewer en spierskade gehad nie aangesien die vlakke nie verskillend was as in die ketamien groep nie. O_3-AHT het 'n beskermde effek op lewer en spierskade gehad, aangesien die AST,ALT en CK vlakke nie so dramaties toegeneem het as in die geval van die ketamien groep. In die studie waar die kroniese effek van O_3-AHT ondersoek is, is 6 bobbejane met 'n O_2/O_3 gas mengsel wat 40 µg/ml O_3 bevat het, behandel. Drie opeenvolgende behandelings, 24 uur uitmekaar is gedoen. Geheparinieseerde bloedmonsters is voor elke behandeling versamel en dan weer 4, 24, 28, 48, 52, 72 en 96 uur na die eerste behandeling. ALT vlakke het tydens die behandelingsperiode van 52 uur toegeneem en het vir 48 uur na behandeling gestop is, hoog gebly. AST vlakke het tydens die vier uur na elke behandeling dramaties toegeneem, en ook hoog gebly vir 48 uur na die laaste behandeling gestop is. CK vlakke het drasties verhoog 4 uur na elke behandeling, maar nadat die behandeling gestop is het die CK vlakke vinnig afgeneem. Die mate van toename in beide

lewerensieme en CK was klein en ondersteun dus nie die bewering dat osonering van bloed toksies is as dit na in vitro behandeling aan die bobbejane terug gespuit word.

In samevatting, die resultate bevestig of weerlê nie die aanname dat O_3-AHT ernstige selskade veroorsaak in bobbejane nie. In die studies waar die bobbejane drie agtereenvolgende behandelings ontvang het, is 40 µg/ml O_3 gebruik (hoofstuk 3). Die rede hiervoor is dat die resultate wat met die dosis van 80 µg/ml verkry is nie grootliks verskil het van die wat met 40 µg/ml gedurende die akute studies (hoofstuk 2) verkry is nie (Lubuschagne et al., 2007). Dit is bewys gedurende die twee studies sowel as vorige studies. Ek het geen maklike verklaring vir die bevinding dat ozonering selskade as gevolg van ketamienbehandeling verminder nie. Dit behoort verder ondersoek word.

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Chapter 3: The chronic effect of autohaemotherapy on liver and muscle damage in baboons

List of abbreviations

4-HNE	4-hydroxy-2,3 transnonenal
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
ATP	Adenosine 5'-triphosphate
CAT	Catalase
CK	Creatine kinase
GPX	Glutathione peroxidase
H_2O_2	Hydrogen peroxide
HClO	Hypochlorous acid
LD	Lactate dehydrogenase
LOOH	Lipid hydroperoxide
LOP	Lipid oxidation product
MD	Malate dehydrogenase
MDA	Malonyldialdeyde
NAD^+	Nicotinamide adenine dinucleotide (Oxidized form)
NADH	Nicotinamide adenine dinucleotide (Reduced form)
NADPH	Nicotinamide adenine dinucleotide phosphate
NMDA	N-methyl-D aspartic acid
O_2	Oxygen
$O_2^{\bullet-}$	Superoxide anion
O_2-AHT	Oxygen Autohaemotherapy
O_3	Ozone Autohaemotherapy
O_3-AHT	Ozone Autohaemotherapy
$OH^{\bullet}$	Hydroxyl radical
$OLOO^{\bullet}$	Epoxyallylic peroxyl radicals
PCP	Phenylcyclohexylpiperidine
PHGPX	Phospholipids hydroperoxide glutathione peroxidase
$ROO^{\bullet}$	Peroxyl radicals
R-OOH	Hydroperoxides
ROS	Reactive oxygen species
SePX	Selenoperoxidase

SOD	Superoxide dismutase

Introduction

1. Background and motivation

The interest to use ozone as an alternative form of medicine is growing fast. The advantage of ozone as an alternative form of medicine is that it eliminates the use of medical immunomodulators, for example, in diseases such as HIV. Therefore it can decrease the drug-load to organs in the body and so lengthen the patient's lifespan with up to 5 years (Bocci, 1999). Similar claims have been made for ozone treated patients having certain types of cancer. Ozone can also be used in combination with other medicines to potentiate their actions (Bocci, 1999; Bocci, 2002).

When O_3 comes into contact with biological fluids it instantaneously dissolves in plasmatic water and generates the same ROS that are produced under normal physiological conditions. Because of the latter it is believed that, when ozone is administered at the right concentration, it has the capacity to protect the liver and muscle against damage that could be caused by other substances. It is further believed that O_3-therapy improves O_2 supply to tissues and that, it exerts an immunomodulating effect (Bocci, 2002).

There are different methods of applying O_3-therapy, but ozone autohaemotherapy (O_3-AHT) seems to be the method of choice. O_3-AHT has the advantage of rapidly distributing the ozonated blood through the body. It offers a meaningful and reproducible delivery system. O_3-AHT involves the ex vivo exposure of a certain volume of blood to an equal volume of O_3/O_2-gas mixture with a precise O_3 concentration, followed by reinfusion of the ozonated blood.

2. Aim

This dissertation forms part of a holistic study in which the effect of O_3-AHT on various biochemical parameters is assessed. In this part of the study we assessed the protective effect of O_3-AHT on liver and muscle damage caused by Ketamine hydrochloride® in baboons.

Chapter 1: Literature overview

1. Introduction

When working with Ozone, it is important to keep the following quote in mind. "Some amazing things happen when three atoms of oxygen dance together, like three little girls holding each other's hands in ring-around-the-rosie." Majid Ali, M.D.

Ozone was first discovered in 1840 by the German scientist, C.F. Sconbein. The name is derived from the Greek word "Ozein" which means to smell. Ozone is a triatomic allotrope of oxygen. Its chemical formula is O_3 and it is always present in trace quantities in the atmosphere of the earth (Bocci, 2002).

Ozone is produced when oxygen molecules (O_2) are split into two oxygen atoms (O_1). One of these oxygen atoms (O_1) then combines with molecular oxygen (O_2) to form ozone.

In nature, ozone is generated by short-wave solar ultra-violet radiation, and appears in the upper atmosphere (ozonosphere) in the form of a gas. Ozone can also be produced by passing an electrical discharge, such as lightning, through oxygen.

In the industry ozone is mainly used in water disinfection and purification because of its antiseptic properties. It is also used to neutralize sulphate containing draining gasses and in various organic syntheses (Bocci, 2002).

In medicine, ozone has a major disinfectant power, and as a result, it was used during the First World War (1915-18) to prevent and avoid the diffusion of gangrene in injuries to the limb (Bocci, 2002).

1.1 Properties of Ozone

Physical: O_3 is a colorless gas, and has a characteristic prickly smell that is detectable at concentrations as low as 0.01 PPM in air at ambient temperature. O_3 is about 1.5 times denser than O_2 (Haensler, 2000). It condenses to a dark blue liquid at -170° F. At -420° F ozone will freeze to a solid phase. When ozone is in either of these two phases it is very unstable, especially when present in high concentrations and in the presence of water. The decomposition velocity of ozone depends on the temperature. Therefore, ozone should be prepared only at the moment of use and preserved for short periods of time, especially when used in medicine.

Chemical: Ozone is a strong oxidizing agent that is much more reactive than oxygen. It has an oxidation potential of 2.07 V in alkaline solution. Ozone reacts with the double bonds in organic substrates where it determines the division. This reaction is called ozone-lysis (Haensler, 2000). The reaction with the double bond forms an unstable primary ozone-ide. It degrades rapidly and gives rise to a carbonyl and a zwitterion. The zwitterions are very reactive. In the absence of substances to react with, ozone-ides form. If water and substrates are present, peroxides form.

Biochemical properties of ozone: Ozone effects metabolism, because of its strong oxidizing capacity. Therefore, understanding its biochemical properties underlines the explanation of its therapeutic effect (Haensler, 2000). In addition the basis of the protection against the negative in vivo effects of ozone are the different affinities for different substrates.

On endocellular level, antioxidant mechanisms protect the membrane lipids from peroxidation and defend the nucleuc acids and proteins (Haensler, 2000). Non-toxic hydroperoxides are produced from antioxidant substances such as β-carotin, α-tocopherol and vitamin C.

The ozone oxidant effect on the co-enzymes, NADH and NADPH, forms part of many metabolic reactions. Ozone actions on co-enzymes and on organic

substrates occur in all the three fundamental metabolic lines: (Bocci et al, 1999)

In glucose metabolism, ozone increases glycolysis. The catabolism of glucose leads to the formation of energy in the form of ATP. This has a benefit in certain pathological situations, because some organs need a large quantity of ATP for proper function.

Ozone also plays a role in protein metabolism due to its affinity for sulfidrilic groups. Ozone reacts with the essential amino-acids or with the sulphur contained in amino acids. Their degradation may lead to the protection against oxidation of the glutathione and coenzymes NADH and NADPH (Haensler, 2000).

Ozone regulates activation of lipid metabolism and can so increase energy production.

1.2 Medical uses of O_3-therapy

Ozone is best known for its UV protective role in the atmosphere but also have unique biological properties. It was used as early as the First World War as a disinfectant because of its antimicrobial properties. Attempts to treat patients with ozone have been dampened because of technological difficulties especially in producing O_3. Fortunately, new generation ozone generators have been developed and refined to enable us to monitor and control the concentration of ozone accurately. This allowed the use of ozone as an alternative form of treatment in various medical fields.

There are various routes of treatment with O_3. Ozone Autoheamotherapy (O_3-AHT) appears to be the most effective (Sunnen, 1998). A given volume of blood is drawn from a patient. This volume of blood is then exposed ex vivo to an equal volume of ozone-oxygen gas mixture having a precise O_3

concentration. The gas is then removed after a given time of incubation and the treated blood reinfused. For O_3-AHT to be effective and atoxic, it is crucial to use the correct dose. The O_3 dose can be calculated by the following equation: (Bocci, 1999; Bocci, 2000).

$$O_3 \text{ dose } (\mu g) = \text{Gas volume (ml)} \times O_3 \text{ concentration } (\mu g/ml)$$

In order to activate biochemical and immunological pathways, the generation of H_2O_2 is crucial. The H_2O_2 concentration must reach a threshold. Below this threshold O_3-treatment will be uneffective. Above this threshold the O_3 may be toxic to the body. The production of H_2O_2 is proportional to the O_3 dose. Therefore it is of utmost importance that the O_3 concentration must be determined to ensure effective and safe use of O_3 concentrations (Bocci, 1999).

There are five major areas where O_3-AHT plays an important role. This includes, infectious diseases, vascular disorders, immune depression, degenerative disease and orthopedic pathology (Bocci, 1999).

1.3 Ozone toxicity

Although ozone therapy has been used as a form of alternative medicine it has encountered skepticism by orthodox medicine. Its effectiveness and toxicity are both in question and is a matter of concern. In 1995 the Office of Alternative Medicine of the National Institutes of Health (NIH, MD, USA) included ozone therapy as part of its pharmacological and biological approach.

Ozone and oxygen dissolve partitially in plasmatic water. While oxygen is relative stable, O_3 is unstable and immediately reacts with a range of

substrates, including polyunsaturated fatty acids (PUFAs), antioxidant compounds and carbohydrates (Cataldo et al., 2005).

Three important points that must be taken into account when blood is ozonated are:

1. H_2O_2 is generated and have a relatively long half life.

2. The level of H_2O_2 depends on the concentration of O_3 and results from a dynamic equilibrium between its formation, its diffusion into intracellular fluid and its degradation.

3. Intracytoplasmic H_2O_2 have the ability to activate biochemical and immunological pathways (Bocci, 1999).

It is important to remember that endogenous ROS are constantly produced in several different cell types during mitochondrial electron transport, metabolism of peroxisomal fatty acids, cytochrome P450 reactions in the presence of xenobiotics and in respiratory burst activity of phagocytes (Bocci, 2002). It is also proven that ROS from O_3 are generated and quenched in the plasma (du Plessis et al., 2007). This reduces the danger of the exogenous oxidant and makes it not as dangerous as endogenous ROS.

The toxicity of O_3 is potentiated by compounds such as CO, NO_2 and H_2SO_4, exposure to these compounds is harmfull to lungs, because of the inability of the fluid lining the respiratory tract to neutralize the acidic pH. This leads to the obstruction of oxidants and therefore leads to cell damage (Bocci, 1999). However blood is a fluid tissue and the components of blood are in a highly dynamic state. Both plasma and blood cells contain very powerful defense systems of both hydrophilic and lipophilic antioxidants and protein metal chelators, which limits ROS production (Bocci, 1999).

1.4 Mechanism of Ozone participation in chemical reactions

1.4.1 Production of reactive species

Ozone is a very strong oxidizing agent. When it comes in contact with plasma, it reacts immediately with a number of molecules. It includes carbohydrates, antioxidants, proteins and preferentially, polyunsaturated fatty acids (PUFAs) (Bocci, 2005). This is illusrated in Figure 1:

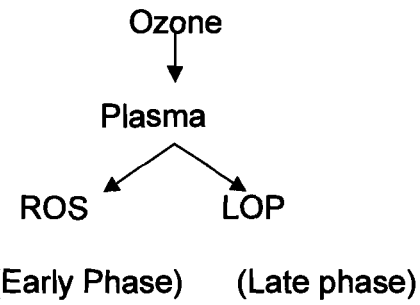

Figure 1. Ozone dissolved in plasmatic water. The products that are formed includes ROS and LOP

Ozone can apparently act through two mechanisms. The first, which is responsible for the production of ROS, is an O_3-olefin reaction where H_2O_2, aldehydes and peroxides are produced via the reaction between O_3 and the double bonds in organic substrates. (Pryor, 1994; Pryor et al, 1995., Bocci, 2005). The second mechanism is the O_3-electron donor reaction that involves an O_3-radical that are formed and that reacts with a proton to produce a hydroxyl-radical. (Pryor, 1994; Bocci, 2002). The major products of these reactions are cytotoxic superoxide radicals ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hypochlorous acid (HClO) and hydroxyl radicals ($OH^{\bullet}$). All of these are potentially cytotoxic, but the have a very short half-life, of normally a fraction of a second. In addition both the plasma and the cells have antioxidant systems that are capable of neutralizing them. It must be noted that if their concentrations overwhelm the antioxidant capacity oxidative stress follows (Bocci, 2005).

When ozone reacts with the polyunsaturated fatty acids, lipid oxidation products (LOPs) are formed. They include peroxyl radicals ($ROO^{\bullet}$), a variety of hydroperoxides (R-OOH), alkenals, amongst which 4-hydroxy-2,3

transnonenal (4-HNE) is the most cytotoxic, and a complex mixture of aldehydic end products which includes malonyldialdeyde (MDA) (Bocci, 2005).

It is important to remember that ROS are only produced during the short time that O_3 is present in the glass syringe, ex vivo, and they yield early biological effects on blood. The LOPs have a longer half-life and during reinfusion of ozonated blood, they reach the vascular system and therefore practically all organs (Bocci, 2005). As soon as ozone comes in contact with plasma water it dissolves and reacts with PUFAs. This reaction increases the hydrogen peroxide concentration, which almost instantaneously decreases because hydrogen peroxide is an unionized molecule capable of diffusing into erythrocytes, leucocytes and platelets. In the cell it triggers a variety of biochemical pathways (Bocci, 2005).

H_2O_2 is considered to be the most important ROS. It dissolves to form $OH^{\cdot}$ which is one of the most destructive radicals that adversely affect enzymes and DNA.To be effective the momentary increase in intracytoplasmic H_2O_2 needs two considerations.

1. There must be enough ozone to produce sufficient H_2O_2 to activate transducer molecules and to counteract its simultaneous degradation.
2. The H_2O_2 must reach a critical threshold. Below this no stimulation of cells will occur. If the concentration is excessive, oxidative damage may result. It is therefore crucial to identify a therapeutic window.

Based upon these considerations, the quick increase in hydrogen peroxide will not be toxic for the cell. It undergoes almost simultaneous reduction to water, because of the powerful antioxidant enzymes (Bocci, 2005). The antioxidant status varies considerably, i.e. in the European working population; the mean total antioxidant status varies between 1.28 and 2.83 mmol/L plasma (Rice-Evans et al., 1994). Because of this variation, effective ozone concentrations to achieve the H_2O_2 threshold will range between 20-80 µg/ml per gram of blood.

1.4.2. Biological effects of Ozone generated reactive species

Lipid peroxidation is the oxidative deterioration of unsaturated lipids. Peroxidative modifications can be triggered by free radical species. For example, hydroxyl radicals can form from iron-mediated reduction of hydrogen peroxide or, it can be triggered by non-radical species such as ozone (Girotti., 1998). It affects cell membranes and other lipid containing structures. Unsaturated phospholipids, glycolipids and cholesterol in cell membranes are well-known targets of oxidant attack. During peroxidation, the reactive species remove a hydrogen atom from a methylene group ($-CH_2-$) resulting in an unpaired electron on the carbon i.e. (Halliwel et al 2000).

$$-CH_2- + OH^{\bullet} \rightarrow -CH^{\bullet}- + H_2O$$

This attack on the hydrogen atom generates free radicals from polyunsaturated fatty acids (PUFA). The double bond in the fatty acid weakens the C-H bond on the carbon atom adjacent to it and so makes H removal easier. The carbon radical that forms can either collide with and bind to another carbon radical, or combine with O_2 to produce a peroxyl radical ($ROO^{\bullet}$). Lipid peroxidation can be continued through a chain reaction in which the peroxyl radicals remove a hydrogen atom from another lipid molecule to produce a lipid hydroperoxide (LOOH) and an additional carbon radical. The fates of these LOOHs are explained in Figure 2: (Girotti, 1998)

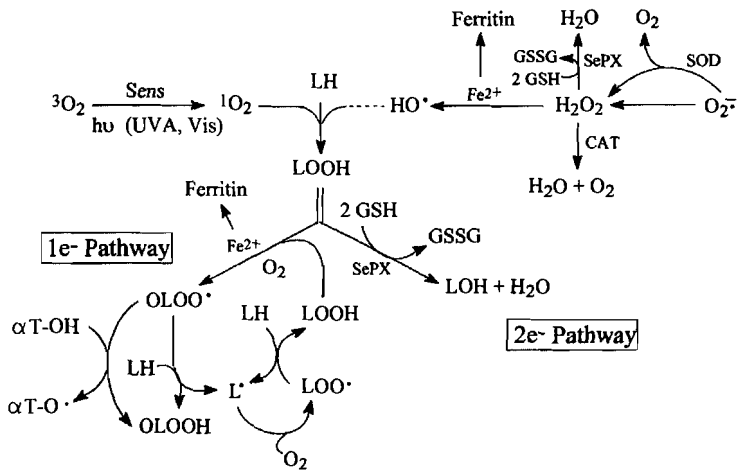

Figure 2. Important routes of lipid hydroperoxide (LOOHs) formation and turnover in biological systems.

Reactive Oxygen Species also produces LOOHs. These LOOHs may follow one of two routes. The first is when LOOHs undergoes iron-mediated single-electron reduction and oxygenation to form epoxyallylic peroxyl radicals ($LOO^{\bullet}$), which triggers free radical mediated lipid peroxidation (Girotti, 1998). The second route is a two-electron reduction to redox-inert alcohols (LOHs). The latter reaction is catalyzed by GSH-dependant selenoperoxidase (SePX), most prominently phospholipids hydroperoxide glutathione peroxidase (PHGPX). This represents a secondary level of cytoprotection (Girotti, 1998). The antioxidants that protect at primary level include glutathione peroxidase (GPX), catalase (CAT) and members of the superoxide dismutase (SOD) family. Agents such as α-tocopherol (α-TOH) and ferritin can suppress LOOH formation by protecting at both primary and secondary stages of lipid peroxidation (Girotti, 1998).

The role of ozone in lipid peroxidation is as follows: In the plasma environment, one mole of olefin reacts with one mole of ozone produces two moles of aldehydes and one mole of hydrogen peroxide. The hydrogen peroxide forms part of the ROS family and the aldehydes, better known as lipid oxidation products (LOPs) (Halliwel et al., 2000). It is important to note that it is not ozone but the ROS and LOPs that are responsible for the subsequent successive and multiple biochemical reactions in all cells in the body. Thus ozone is a powerful non-radical oxidant, and can produce free radicals that provokes membrane lipid peroxidation, enzyme inactivation, and cytodamage (Girotti, 1998). Ozone is also capable of directly oxidizing lipids to give rise to ozonides which can be catabolised to form aldehydes (LOPs). On the other hand O_3 induces ozone cytoprotective antioxidant enzymes in cellular and animal models (Girotti, 1998).

1.5 The liver and the role of alanine aminotransferase (ALT) and aspartate aminotransferase (AST)

The liver plays a pivotal role in intermediary metabolism and is vitally important to detoxify and eliminate toxic substances (Marshall et al., 2004).

This takes place in the liver acinus. It is the territory that is supplied by each terminal branch of the hepatic artery, the main oxygen supplier. Two-thirds of the blood supply of the liver comes from the hepatic portal vein, which drains from the gut and supplies most of the absorbed nutrients to the liver (Giannini et al., 2005). The liver sinusoidal cells are responcible to clear, for example aminotransferases from the liver (Marshall et al., 2004), The acinus is divided into 3 zones on the basis of the distance from the supplying vessel. The acinus is illustrated in Figure 3: (Curtis et al, 2003)

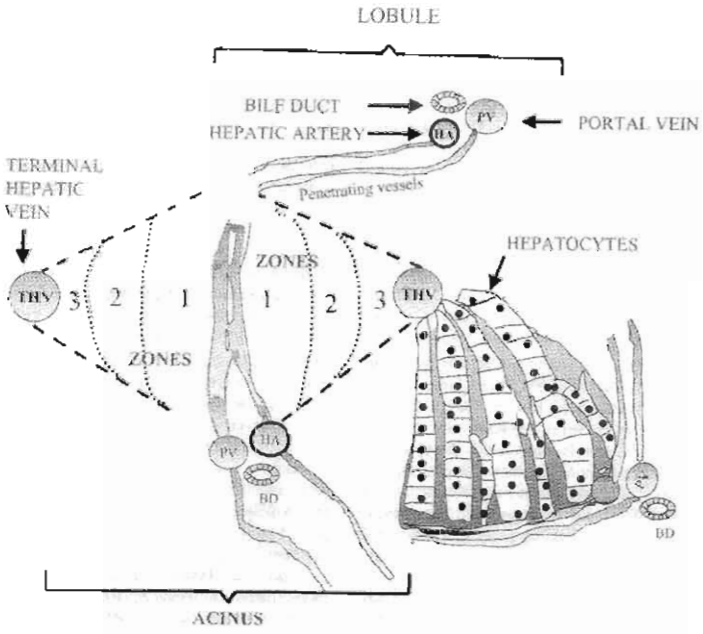

Figure 3. Schematic of liver operational units: the acinus.

Damage to the liver may not affect its activity since the liver is large and has considerable functional reserve. Therefore, simple tests of liver damage can be used as indicators of liver damage and liver disease (Marshall et al., 2004).

An abnormal level is a value higer than the upper reference limit. Since there is no clinical significance to low levels of biochemical markers, with serum albumin as an exception (Giannini et al., 2005). As many as 2.5% of normal patients have "abnormal" aminotransferase levels, a finding that is important to consider when interpreting the results is that strenuous exercise can also increase ALT and AST levels (Giannini et al., 2005).

Acute or chronic injury to the liver ultimately results in an increase in serum concentrations of the aminotransferases (Giannini et al., 2005). ALT and AST are enzymes that catalyze the transfer of α-amino groups from alanine and aspartate to a α-keto group of ketoglutatic acid. During this process it generates pyruvic and oxalacetic acid. Both products contribute a great deal to the citric acid cycle (Bergmeyer, 1983). In order to carry out these reactions, both ALT and AST requires pyridoxal – 5' – Phosphate (Vitamin B6). The effect of Vitamin B6 is greater on ALT than on AST (Giannini et al., 2005). Both aminotransferases are present in high concentrations in the liver. ALT is however more liver specific and is localized solely in the cellular cytoplasm. It has a half-life time in the circulation of ± 47 hours and is also present in lower concentrations in other tissues, including cardiac and skeletal muscle (Giannini et al., 2005)). An increase in ALT activity is an indication of various intracellular hepatic disorders.

AST is found in both cytosol (20% of total activity) and mitochondria (80% of total activity) (Giannini et al., 2005). It has a half- life time of approximately 17 hours in circulation and approximately 87 hours in the mitochondria. It is present in practically every tissue in the body (Giannini et al., 2005). An increased activity of AST is an indication of intracellular liver damage (hepatitis, cirrhosis, hepatotoxins), but it can also be increased after myocardial infarction or a renal infarct. Congestive heart failure is also associated with an increase in AST because of the hepatic ischaemia and anoxia that are present. A decreased activity of AST has no clinical significance.

The magnitude of aminotransferase alterations can be classified as "mild" (<5 times the upper reference limit), "moderate" (5-10 times the upper reference limit) or "marked" (> 10 times the upper reference limit). The full assessment of enzyme alterations involves careful evaluation that includes the following: First, the predominant pattern of enzyme alteration, second the magnitude of enzyme alteration (mild, moderate or marked), third the rate of change and fourth the nature of the course of alteration (Giannini et al., 2005).

According to previous studies. Dr. G. Amato, one of the most reliable Italian ozonetherapist, showed in1999 that three O_3-AHT per week for three weeks followed by one O_3-AHT every month for a period of one year resulted in a decreased ALT and AST activity and they were in normal range (Bocci, 2005). A clinical trail in 2004 combined Rectal insufflation (RI) with AHT including 16 patients with chronic hepatitis C virus infections. The results showed a marked decrease of transaminase plasma levels without any reported side effects. The preliminary conclusion was that O_3-therapy was effective to treat chronic hepatitis C virus infections (Bocci, 2005).

1.6 The cardiovascular system and the role of CK

CK is present in high concentrations in skeletal muscle, cardiac muscle, thyroid, prostate and 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. CK levels is usually higher in men than in woman because of the greater muscle mass in men. A decreased activity of CK has no clinical significance (Lang, 1981)

CK catalyses the transfer of phosphate from adenosine triphosphate (ATP) to creatine as shown in the following reaction.

$$\text{Creatine} + \text{ATP} \xrightarrow{CK} \text{Creatine phosphate} + \text{ADP}$$

Creatine phosphate is,a much more stable high energy phosphate. This reaction makes the storage of an high-energy phosphate in a more stable form than ATP possible.

The enzymatically active CK molecule is a dimer that 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.

CK-MB is the important isoenzyme and located in the heart muscle. To make CK-MB more specific for heart muscle, the relative index can be calculated as follows:

$$\frac{\text{CK-MB mass}}{\text{Total CK}} \times 100$$

Increases of plasma CK activity are usually the result of skeletal or cardiac muscle damage. The CK in skeletal muscle is MM, whereas in cardiac muscle, up to 30 % is the MB isoenzyme. When plasma CK activity is increased, the demonstration that more than 5% of the total CK is due to the MB isoenzyme is highly suggestive of its being cardiac in origin. Isoenzyme electrophoresis was not done during this study because it is technically demanding and our laboratory did not have the facilities to do this determination (Lang, 1981).

Ketamine hydrochloride® is largely catabolic in nature (Zhang et al., 1997). Furthermore, ketamine causes increases in plasma levels of CK (Gonzales et al., 2002).

1.7 The mechanism of action of Ketamine® hydrochloride

Ketamine is a rapid acting anesthetic that produces an anesthetic state known as dissociative anesthesia, i.e. it uncouples sensory, motor, integrative memory and emotional activities in the brain (Hirota et al., 1996) . Ketamine is clinically used as a racemic mixture of optical isomers that differ in their analgesic properties and psychomimetic effects. Ketamine has a S(+) isomer and R(-) isomer (Kharasch et al., 1992). Its enantiomers also differ with respect to their hepatic clearance and duration of anaesthetic effect. S(+) ketamine has a quiker clearance and faster anaeshtetic recovery time when compared to R(-) ketamine (Kharasch et al., 1992). Ketamine is extensively metabolised by liver microsomes, primarily via N-demethylation to norketamine. At the concentration at which anesthesia is achieved, the rate

of S(+) ketamine demethylation are 20 % greater than that of R(-) ketamine (Kharasch et al., 1992).

N-methyl-D-aspartate receptors are ionotropic receptors, that are responcible to transfer electrical signals between neurons in the brain and spinal column. They form part of a class of receptors that are abundant in all parts of the brain (Kandal, 2001). NMDA receptors are special because it is able to transmit Ca^{2+} ions as well as sodium and chloride ions across the cell membrane. Since Ca^{2+} are second messenger molecules, intracellular calcium can activate Ca^{2+}/Calmodulin kinase and protein kinase C. Both enzymes play central parts in long-term potentiation or long-term depression of neuron signals (Kandal, 2001).

The NMDA receptor has four binding sites. The first is the ligand site for L-glutamate, which also binds NMDA and Aspartate. The second is a site for glycine, which facilitates the primary ligand-binding site. Glycine allosterically activates the glutamate site (Hirota et al., 1996). The third site is a magnesium ion-binding site. The fourth site is the PCP binding site. Ketamine is chemically analogous to PCP, and binds to the PCP site to block the NMDA receptor channel (Hirota et al., 1996) . Studies suggest that the blocking action of Ketamine requires the binding of glutamate. It is proposed that, when glutamate binds to the receptor, a conformation change in the receptor allows the ketamine to bind to block the ion channel (Monaghan, 1989). This is illustrated in Figure 4: (Hirote et al, 1996).

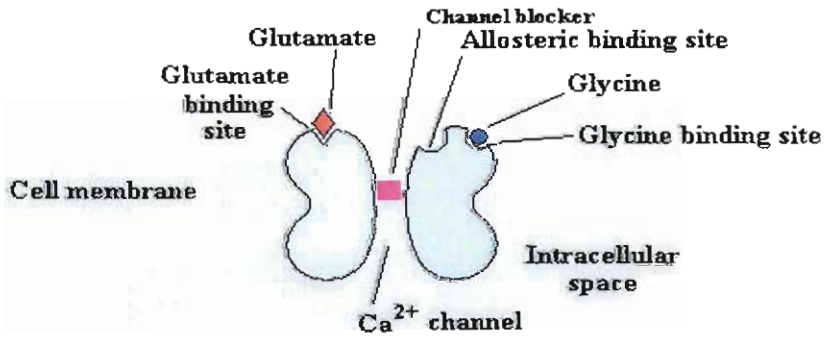

Figure 4. Mechanism of action of NMDAR blockers.

The NMDA receptor antagonists are responsible for dissociative anesthetia, which is caracterized by catalepsy, amnesia and analgesia. Frequent administration of NMDA receptor antagonists lead to resistance because the liver eliminate the antagonist more quickly from the bloodstream (Livingston et al., 1978).

Chapter 2

The acute effect of autohaemotherapy with different ozone concentrations on liver and muscle damage in baboons

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Abstract

Ozone therapy has been used as a form of alternative medicine but has encountered scepticism by orthodox medicine concerning its effectiveness and toxicity. In order to shed some light on the controversy we assessed the acute effect of O_3 – autoheamotherapy (O_3-AHT) on liver and muscle damage in baboons. The baboons were anaesthetized with intramuscular ketamine hydrochloride to enable handling and blood sampling. Three groups of baboons were used. The first group (n=11) were treated with an O_2/O_3 gas mixture containing different ozone concentrations 20, 40 and 80µg/ml O_3. The second and third groups were control groups. The one group (n=5) was treated with pure oxygen to balance for the O_2 present in the O_2/O_3 mixture. The third group (n=3) received no treatment and was used to assess the effects of ketamine anaesthesia. Blood samples were collected in heparin before each treatment and again after 4, 24 and 48 hours following treatment. Our results indicated that ketamine anaesthesia cause liver and muscle damage. The plasma levels of aspartate aminotransferase and alanine aminotransferase as markers of liver damage and creatine kinase as marker for muscle damage increased markedly. O_2-AHT had no marked effect on liver and muscle damage. O_3-AHT had a protective effect since the increase in the levels of the markers was not as dramatic as when ketamine alone was used. Our results strongly suggest that O_3-treatment protect against liver and muscle damage caused by ketamine, through an unknown mechanism. Our results does not support the notion that ozonation of blood can be harmfull.

Keywords: Ozone, Acute effect, Autoheamotherapy, liver and muscle damage

1. Introduction

Ozone is a strong oxidant and much more reactive than oxygen [1]. 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 [1, 2]. It is these LOPs and ROS, and not the ozone, that are responsible for the multiple biochemical reactions that follows [3].

Liver enzymes leak into the blood when liver cells are damaged [4, 5]. Alanine aminotransferase is present in high concentrations in the liver and considered as a specific marker of hepatocellular damage [6]. Acute liver damage therefore causes high blood levels of ALT. ALT is also present in the heart and muscles but in much lower concentrations [7]. Aspartate aminotransferase, 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 [8]. Aminotransferase alterations can be 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) [7]. 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 [9]. 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 are predominantly found in skeletal muscle.

In this study we assessed the acute effect of O_3-AHT on liver and muscle damage in baboons. Three O_3 concentrations were used. An oxygen-treated group and a non-treated sham group were used as controls. Liver enzymes (AST and ALT) were used to determine liver damage and the creatine kinase

levels were determined as a marker of muscle damage over a period of 48 hours following O_3-AHT.

2. Materials and methods

2.1. O_3-AHT treatment of baboons

Ozone was prepared as described in detail [10].The Ethics Committee of the North-West University approved the study 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). Healthy baboons weighing between 16 and 25 kg were used. The baboons were maintained at the animal facility of the Potchefstroom Campus, North-West University, on standard laboratory chow. The baboons were anesthetized with ketamine hydrochloride (±10mg/kg) to enable handling and blood sampling.

Autoheamotherapy was done on 16 baboons. Five percent of the blood volume of a baboon was treated. A baboon has 65 ml blood per kg [11]. Eleven baboons were treated in a random order with an O_2/O_3 gas mixture containing 20, 40 and 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, i.e. containing 20, 40 or 80µg/ml O_3 or pure O_2. The blood was gently mixed for 20 minutes, 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 hours.

2.2 Laboratory assays

2.2.1 Liver damage

The Dimension® clinical chemistry system supplied by Dade Behring was used to determine the levels of ALT and AST as an indication of liver damage.

ALT: 35µL 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 is measured with a spectrophotometer (Dade Behring,Dimension xpand) while NADH is oxidized to NAD^+. ALT activity was indirectly proportional to the absorbance [8].

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) in which 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 indirectly proportional to the absorbance [8].

2.2.2 Muscle damage

Serum plasma levels of CK was determined to assess both heart and skeletal muscle damage. It was done by using the Dimension® clinical chemistry system. 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) at 340nm. CK activity was directly proportional to the increase in absorbance [8].

2.3 Statistical analysis

The values are expressed as the fold change ± 1 SEM. Fold change were calculated as (Ti – Tc) / Tc where T = time, c is the measurement in the control sample and i the measurement at 4, 24, or 48 hours. The data were compared using the Student's t-test for paired and unpaired samples, and were regarded significant when $p < 0.05$.

3. Results

3.1 Acute effect on alanine aminotransferase (ALT)

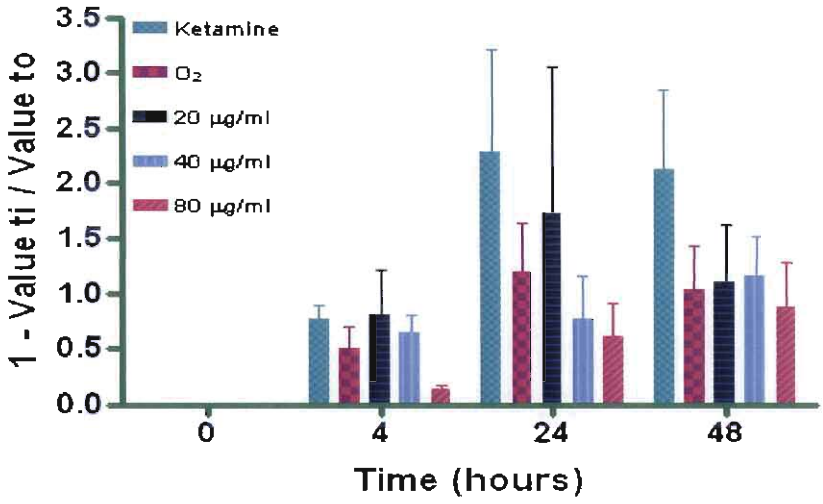

Figure1 Acute effect of treatment on ALT. Values are given as fold change ± 1 SEM

There were marked increases in ALT levels at all time points, following treatment ketamine, O_2, 20 and 40 µg/ml which reached a maximum after 24 hours. The most prominent increase of 229% was 24 hours after treatment with ketamine, and it remained high for a further 24 hours. It is of interest to note that the increase 4 hours after treatment with 80 µg/ml ozone was significant less than in the other cases and that it remained low for up to 24 hours following treatment.

3.2 Acute effect on aspartate aminotransferase (AST)

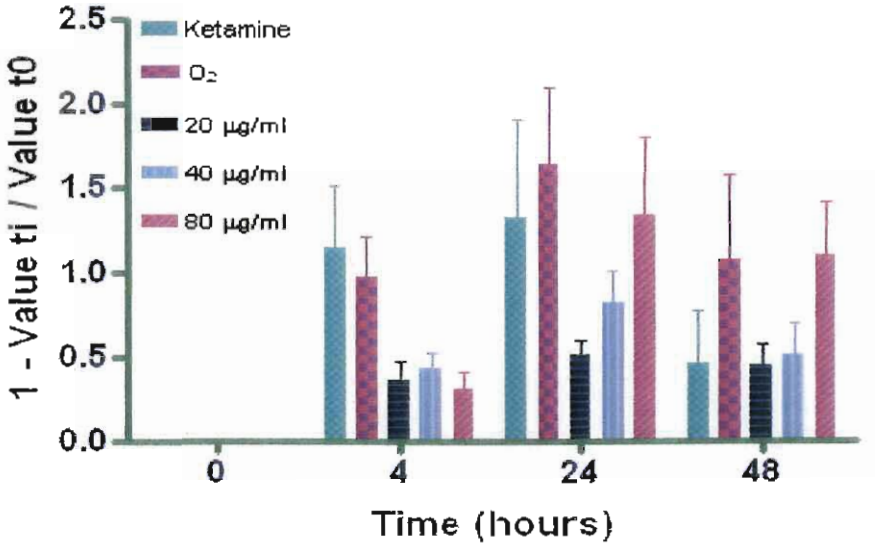

Figure 2 Acute effect of treatment on AST. Values are given as fold change ± 1 SEM

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

3.3 Acute effect on creatine kinase (CK)

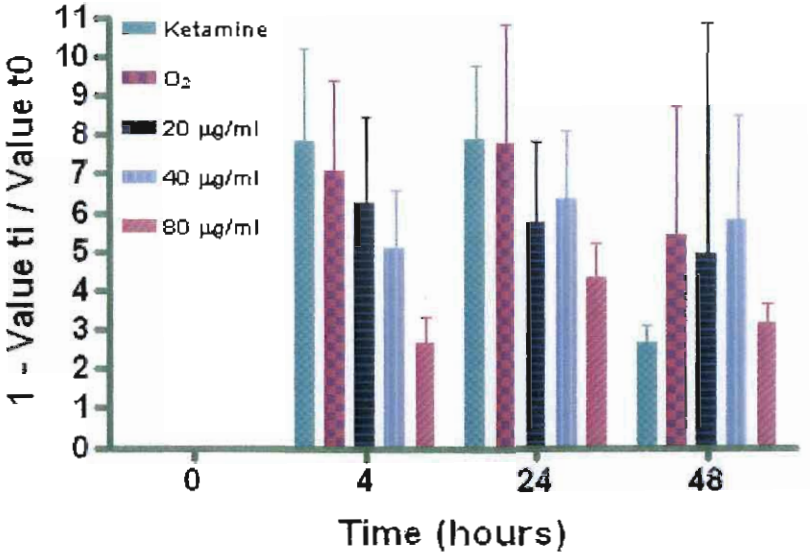

Figure 3 Acute effect of treatment on CK. Values are given as fold change ± 1 SEM

The CK levels increased markedly during the first four hours following all treatments and remained elevated for at least 48 hours. The results showed a dose-dependant increase in CK levels 4 hours after treatment, with the highest increase in the ketamine group and the lowest in the 80 µg/ml O_3 group. After 24 hours a similar pattern of decreases were observed, but not in such an ordered fashion as at four hours. There were no significant difference between CK levels after treatment with O_2 and the untreated (ketamine) group.

4. 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 LOP's and ROS that forms [2, 3].

Ketamine cause considerable damage to liver cells. ALT levels increased with more than 200% over the first 24 hours. It seems as if the effect was cumulative (Figure 1). These increases, in terms of a diagnostic marker, suggests that damage was not even mild i.e increase of ≈ 5 times [7]. It is well known that ketamine hydrochloride is catabolyzed in the liver. It is thus not surprising that it damages liver cells as is suggested by the increase in ALT [12]. Oxygenation or ozonation with 20 and 40 µg/ml O_3 resulted in similar increases in ALT levels after the first four hours after treatment. Of particular interest is the fact that the ALT levels only increased by approximately 15% following treatment with 80 µg/ml O_3. We have no ready explanation for this finding (Figure 1). The changes in the AST levels following ozonation (Figure 2) relative to that as a result of ketamine followed the same trend as was observed with ALT. It was however more variable, especially at 24 and 48 hours. This is understandable since AST is also present in heart, kidneys, skeletal muscle and red blood cells [8], while ALT which is mainly present in hepatocytes [8]. Thus the source of AST is more widespread and damage to muscle, as suggested by CK levels (Figure 3), may contribute markedly to the variation in results.

CK levels in plasma increased by approximately 750% at 4 hours and remained high for at least 24 hours (Figure 3). We did not measure CK isomers to distinguish between heart or skeletal muscle damage [8]. Since the ketamine was injected intramuscularly, it is highly likely that the increase in CK is mainly due to the increase in muscular CK – MM. Treatment with oxygenated blood had no inhibitory effect on the increase in CK, this is at all time points following treatment. At four hours ozone treatment inhibited the release of CK in a dose-dependant manner. This was also observed after 24 hours, although dose dependency was not so clear cut. Of interest are the findings that, at 48 hours, the release of CK following oxygenation and ozonation with 20 an 40 µg/ml O_3 was more pronounced than in the ketamine group. We have no ready explanation for this, especially

if one take into account that the treated injectate was probably not circulating any more. In conclusion, ketamine caused mild liver and most likely skeletal muscle damage in baboons. The liver damage was most likely due to the fact that the liver detoxifies ketamine hydrochloride. Muscle damage is due to intramuscular administration of the ketamine. Ozonation of blood, but not oxygenation, protected against the liver and muscle damage caused by ketamine, especially when one takes the effect in the first four hours after treatment into account. This protection was most prominent with 80 µg/ml O_3. It is difficult to explain our results, because there is no apparent link between ketamine detoxification and muscle damage to the in vitro ozonation of blood and intravenous infusion of the ozonation products such as ROS and LOP's. One possible explanation may be that the ROS and LOP's can bind to ketamine in the circulation. This will decrease the amount of ketamine that have to be detoxified by the liver. This will have to be investigated. We have no explanation in the case of muscle damage other than that ROS and LOP's may improve the repair capacity of the muscle cells that were mechanically damaged. Ozone treatment of open wounds accelerate wound healing [2, 3]. It is important to note that it is likely that the ROS and LOP's provoke membrane lipid peroxidation. This can affect membrane integrity that can result in less fluid membranes with consequent leaking of AST, ALT and CK into the intravascular fluid [13, 14].

We are not sure what the 24 and 48 hour results mean, and if the ROS and LOP's will remain in the circulation for up to 48 hours following injection of ozonated blood. The antioxidant systems are more than sufficient to quench ROS [15, 16] and the highly active LOP's that will react quickly with appropriate substrates [7, 14]. It is possible that the in vivo reactions of ROS and LOP's may begin an as yet unknown chain of reactions that may operate some time following their injection.

5. Acknowledgements

We would like to thank the National Research Foundation of South Africa for funding this project.

6. List of abbreviations

ALT	Alanine aminotransferase
ATP	Adenosine 5'-triphosphate
AST	Aspartate aminotransferase
CK	Creatine Kinase
H_2O_2	Hydrogen peroxide
LD	Lactate dehydrogenase
LOP	Lipid oxidation products
MD	Malate dehydrogenase
NAD^+	Nicotinamide adenine dinucleotide (Oxidized form)
NADH	Nicotinamide adenine dinucleotide (Reduced form)
NADPH	Nicotinamide adenine dinucleotide Phosphate
O_2	Oxygen
$O_2^{\bullet-}$	Superoxide anion
O_2-AHT	Oxygen Autoheamotherapy
O_3	Ozone
O_3-AHT	Ozone Autoheamotherapy
$OH^{\bullet}$	Hydroxyl radical
ROS	Reactive oxygen species

7. References

1. Bocci, V. 1999. Biological and clinical effects of ozone. Has ozone therapy a future in medicine? *British Journal of biomedical Science*, 56:270-279.
2. Bocci, V. 2002. Oxygen-Ozone therapy: a critical evaluation. The Netherlands: Kluwer academic publishers.
3. Bocci, V. 2005. OZONE: A new medical drug. Springer, Dordrecht, p19-111.
4. Guyton, A.C. & Hall, J.E. 2000. Textbook of Medical Physiology. 10th ed. Philidelphia: Saunders. 399-429 p.
5. Widmaier, E.P., Raff, H. & Strang, K.T. 2004. Human Physiology: The Mechanisms of Body Function. 9th ed. Mcgraw Hill. 696-729p.
6. Marshall, W.J., Bangert, S.K. 2004. Clinical Chemistry. 5th ed. Elsevier: Mosby. 85-103 p.
7. Giannini, E.G., Testa, R. & Savarino, V. 2005. Liver enzyme alterations: a guide for clinicians. *CMAJ.* 172: 367-377.
8. Bergmeyer, J. Ed. 1983. Methods of Enzymatic Analysis. 3rd ed. , Weinheim:VCH Publishers. Vol III, pp 418_1-424_1, 445_2-450_2, 510_3-517_3 .
9. Lang, H. 1981. Creatine Kinase Isoenzymes: Pathophysiology and Clinical Application. New York: Springer. 1-9pp
10. Du Plessis, L., Van der Westhuizen, F.H. & Kotzé, H.F. 2007. The effect of blood ozonation on mitochondrial function and apoptosis of peripheral blood mononuclear cells in the absence of plasma antioxidants. *African Journal of Biotechnology.* 6: 1763-1769.
11. Kotzé, H.F., Lötter, M.G., Badenhorst, P.N. & Heyns, A.duP. Kinetics of In-111-platelets in baboons: Isolation and labelling of a viable and representative platelet population. *Thrombosis and Haemostasis; 53: 404-407.*
12. Dundee, J.W., Free, J.P., Moore, J., Mellroy, P.D. & Wilson, D.B. 1980. Changes in serum enzyme levels following ketamine infusion. *Anaesthesia*, 35: 12-18.

13. Gonzales, G.A., Illera, J.C., Silvan, G., Lorenzo, P.L. & Illera, M. 2002. Changes in hepatic and renal enzyme concentrations and heart and respiratory rates in new Zealand white rabbits after anesthetic treatments. *Contemp Top Lab Animal Science*, 41: 30-32.
14. Girotti, A.W. 1998. Lipid hydroperoxide generation, turnover, and effector action in biological systems. *The Journal of Lipid Research*. 39: 1529-1542.
15. Rice-Evans C, Miller, N.J. Total antioxidant status in plasma and body fluids. In: Methods in Enzymology. New York: Acedemic press, Inc, 1994: 279- 293.
16. Haensler, R.V. 2000. Ozone therapy: Method. [Web:] http://www.medicasrl.com/002USA2000.htm [Date of use: 23 August 2006]

Chapter 3

The chronic effect of Ozone autohaemotherapy on liver and muscle damage in baboons

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Abstract

Ozone therapy has been used as a form of alternative medicine, but has encountered scepticism by orthodox medicine concerning its effectiveness and toxicity. We assessed the toxicity of O_3-AHT on liver and muscle damage by measuring changes in the plasma levels of ALT, AST and CK following three sequential O_3-AHT of baboons (n=6) with an O_2/O_3 gas mixture containing 40 µg/ml O_3. Blood was collected before treatment (0) and at 4, 24, 28, 48, 52, 72 and 96 hours after the first treatment. There was no marked increase in ALT levels during the four hours following each sequential treatment. It did increase by approximately 120-130% during the treatment period of 52 hours, and remained elevated during the 48 hours following the last treatment. AST levels increased during the four hours following each treatment and also during the chronic phase. It remained elevated for 48 hours after treatment was stopped. CK increased dramatically during the four hours after each treatment and also during the chronic phase. Following treatment, CK decreased dramatically. Three consecutive O_3-AHT with 40 µg/ml O_3 24 hours apart seems to cause both liver and muscle damage, but the damage was not severe. Muscle damage was more pronounced, especially during the treatment period. However the magnitude of changes was small and does not support the view that Ozonation of the blood can be toxic.

Keywords: Ozone, ozone-autoheamotherapy, liver damage, muscle damage.

1. Introduction

Ozone was first discovered by the German scientist C.F. Sconbein in 1840, and the first report that ozone was therapeutically used to purify blood was published in 1870 [1]. Ozone is best known for its UV protective role in the atmosphere, but also has unique biological properties. It was used as early as the First World War as a disinfectant because of its antimicrobial properties [1, 2]. There are claims that O_3-therapy can be used to treat various medical conditions such as diabetes mellitus [3], ischemic disorders [4], malaria [5] and open wounds and ulcerations [6].

There are various routes of treatment with O_3, which includes topical application, vaginal, rectal, bladder or intraperitoneal insufflations [7]. O_3-AHT appears to be the preferred method. [7]. This approach offers the advantage that the ozonated blood is rapidly distributed throughout the body [7]. For O_3-AHT to be effective it is crucial to use the correct dose.

Ozone apparently acts through two mechanisms. The first is responsible for producing ROS via the reaction between ozone and the double bonds in organic substrates [8 - 10]. The second mechanism results in the production of hydroxyl-radicals [1, 9]. The major products of these reactions include superoxide radicals ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hypochlorous acid (HClO) and hydroxyl radicals ($OH^{\bullet}$) [1], all of which are potentially toxic. Fortunately, both the plasma and cells have antioxidant systems with capacities sufficient to quench these reactive species. If the antioxidant capacity is not sufficient enough oxidative stress will occur [8, 11, 12]. When ozone reacts with polyunsaturated fatty acids, lipid oxidation products (LOPs) are formed which include peroxyl radicals ($ROO^{\bullet}$), a variety of hydroperoxides (R-OOH), alkenals and a mixture of aldehydic end products [8]. All of these products triggers a variety of biochemical pathways.

In this study we assessed the chronic effect of O_3-AHT, i.e. three consecutive ozone autoheamotherapy treatments 24 hours apart, on liver and muscle damage in baboons. The ozone concentration used was 40 µg/ml O_3 in a O_2/O_3 gas mixture. Liver function tests were done to determine the liver damage and the creatine kinase levels to determine the muscle damage during the three days of treatment and for 48 hours following the last treatment.

2. Materials and methods

O_3-AHT treatment of baboons

Ozone was prepared as described in detail [12].The Ethics Committee of the North-West University approved the study 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). Healthy baboons weighing between 16 and 25 kg, were used. The baboons were maintained at the animal facility of the Potchefstroom Campus, North-West University, on standard laboratory chow and water at lib. The baboons were anesthetized with ketamine hydrochloride (±10mg/kg) to enable handling and blood sampling.

Autoheamotherapy was done on 6 baboons. Five percent of the blood volume were treated. A baboon has 65 ml blood per kg [13]. The baboons were treated in a with an O_2/O_3 gas mixture containing 40 µg/ml O_3. Briefly the blood was drawn into 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, containing 40 µg/ml O_3. The blood was gently mixed for 20 minutes, the gas removed and reinfused into the donor. Blood samples were collected before each AHT treatment, at 4, 24, 28 and 48, 52, 72 and 96 hours after the first treatment .

2.2 Laboratory assays

2.2.1Liver damage

The Dimension® clinical chemistry system supplied by Dade Behring was used to determine the levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) as an indication of liver

damage. ALT and AST activity is indirect proportional to the absorbance measured.

ALT: 35µL 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 into lactate. The decreased absorbance at 340 nm was measured with spectrophotometer (Dade Behring,Dimension xpand) as NADH that is oxidized to NAD^+ [14].

AST: 40 µL serum was used in a diluent volume of 235 µL. The test was done at 37°C This reaction of AST was coupled to that of malate dehydrogenase (MD) in which the oxaloacetate was reduced to malate while NADH is simultaneously oxidized to NAD^+. The decrease in absorbance at 340 nm was measured [14].

2.2.2 Muscle damage

Serum plasma levels of creatine kinase (CK) was determined to assess muscle damage, by using the Dimension® clinical chemistry system. A sample size of 14 µL was used in a diluent volume of 255 µL. The assay was done at 37°C. The activity of CK is followed by using the ATP production from creatine phosphate to form glucose-6-phosphate, the glucose-6-phosphate are then dehydrogenated and the rate of formation of NADPH is measured spectrophotometrically (Dade Behring,Dimension xpand) at 340nm. CK activity is direct proportional to the increase in absorbance [14].

2.3 Statistical analysis

The values are expressed as the fold change ± 1 SEM. Fold change was calculated as (Ti – Tc) / Tc where T = time, c is the measurement in the control sample and i the measurement at 4, 24, 48, 52, 72 or 96 hours.

The data were compared using the Student's t-test for paired and unpaired samples, and were regarded significant when $p < 0.05$.

3. Results

3.1 Chronic effect on alanine aminotransferase (ALT)

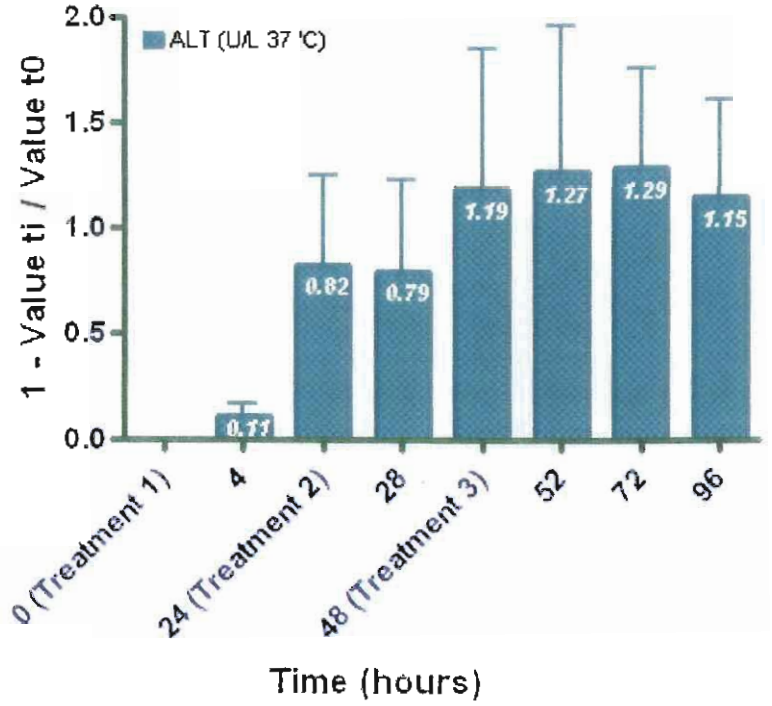

Figure 1. The chronic effect of three consecutive O_3-AHT treatments 24 hours apart on ALT (Fold change ± 1 SEM).

No marked increase in ALT levels were observed from 0 to 4, 24 to 28 and 48 to 52 hours, i.e. the acute effect of each treatment. After 24 hours the pretreatment value increased by approximately 82% and by approximately 120% at 48 hours. Thus ALT levels increased during the chronic phase. It remained elevated after treatment was stopped, at 48 hours.

3.2. Chronic effect on aspartate aminotransferase (AST)

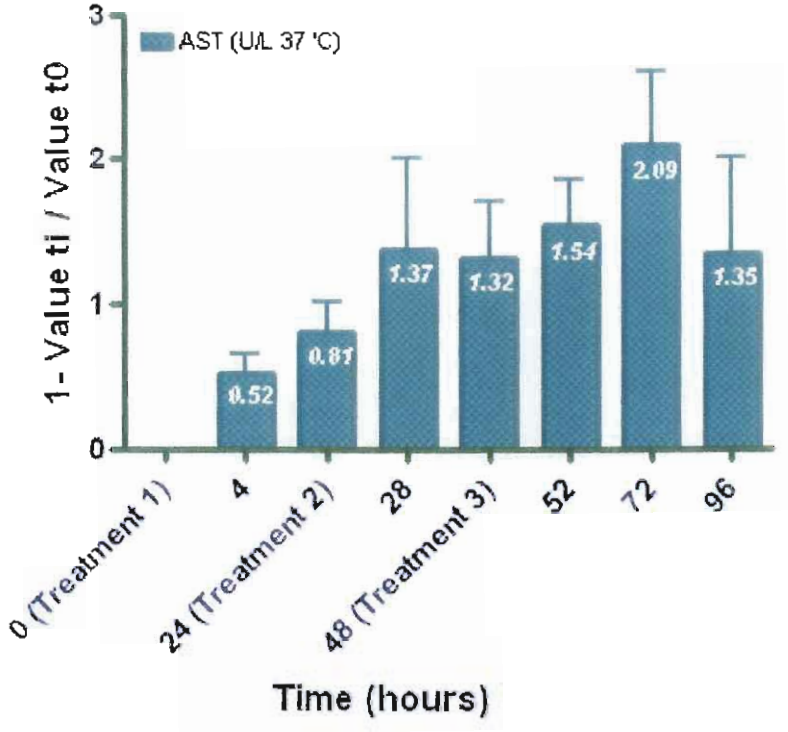

Figure 2. The chronic effect of three consecutive O_3-AHT treatments 24 hours apart on AST (Fold change ± 1 SEM).

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

3.3 Chronic effect on creatine kinase (CK)

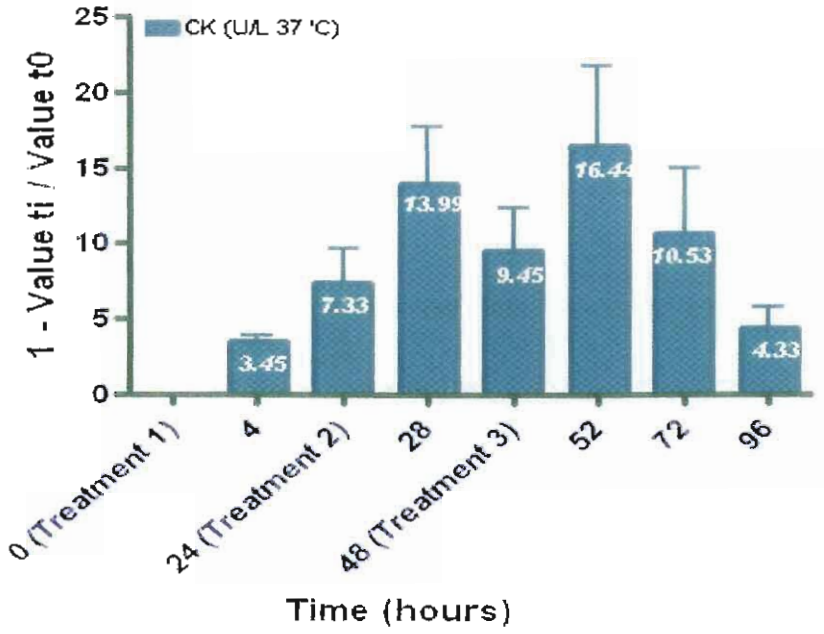

Figure 3. The chronic effect of three consecutive O3-AHT treatments 24 hours apart on CK (Fold change ± 1 SEM).

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

4. Discussion

Blood was ozonated in vitro. The animals were therefore not directly exposed to the ozone. The changes that were measured were therefore due to the ROS and LOPs that were injected following in vitro blood ozonation [8]. This must be kept in mind when interpreting the results.

The liver plays an important role in metabolism, detoxification and elimination of toxic substances from the body [15]. Damage to the liver cells causes the

release of ALT and AST into the bloodstream. Chronic injury cause elevated serum concentrations of both [15, 16]. Both ALT and AST is present in high concentrations in the liver, but ALT is the more liver specific aminotransferase [17]. Increased plasma levels of ALT are therefore an indication of various intracellular hepatic disorders. An increase in plasma AST levels, on the other hand, is an indication of intracellular liver damage caused by hepatitis, cirrhosis or hepatotoxins [17].

Reinfusion of the ozonated blood increased both ALT and AST levels in plasma markedly (Figures 1 and 2) over the 72 hours of the study. Some liver damage took place. The increases were up to 130% for ALT and 210% for AST. The liver damage adjudged by increased levels of ALT and AST in plasma, can be mild, moderate or marked [17]. Fortunately the increase of up to 210 % was such that it could not even be classified as mild. It is therefore reasonable to conclude that although some damage took place, it is probably of no health consequence. One must also bear in mind that ozonation, but not oxygenation protected the baboons against ketamine hydrochloride induced liver damage [18]. The remainder of the liver damage may possibly be due to oxidative deterioration of unsaturated lipids. When ozone comes into contact with plasma it rapidly dissolves and produces ROS and LOPs [2, 3]. The LOPs can be responsible for membrane lipid peroxidation and ultimately the release of the enzymes into the blood because membrane integrity is compromised. It should be noted that the control samples in the study were drawn from the animals immediately before reinfusion of the treated blood i.e. after the baboons were anaesthetized and 5% of their total blood volume was drawn. Physical and emotional stress could therefore also have contributed to the increased levels of ALT and AST.

The fact that the increase in AST was much more than the ALT is most likely because AST is also present in other cells types, that were also affected by ketamine hydrochloride and ozonation. The increase in CK (Figure 3) supports this. It is evident that the short term (<4 hours) effects were more pronounced than the long term (>4 hours) effects (Figure 1 and 2). For example, the increase in ALT from 0 to 4 hours was 11% from 24 to 28 hours

it was 79% and from 48 to 52 hours 119%. The increases between 4 and 24, 28 and 48 and 52 and 72 hours were not so marked.

The short term increases can be explained by the fact that the ROS and LOPs that were injected had a short half-life and the effect would not have been observed after four hours. What is of interest is that repeated treatments apparently had a cumulative effect on the liver (ALT) and most likely also on the other cell types that contain AST. The mechanism of damage is not certain, especially when one takes into account that only 5% of blood was ozonated and that is was mainly the ROS and LOPs that were injected [1, 2]. One possibility is that the LOPs could have reacted with unsaturated fatty acids in cell membranes and so compromised the integrity of cell health. This will also apply to the increase in CK levels in plasma (Figure 3). We have shown [18] that ketamine anaesthesia cause both liver and muscle damage, and that ozonation protected, to some extent, against this damage.

CK levels increased dramatically following each O_3-AHT, reaching a maximum of 1644% after the third treatment (Figure 3). Furthermore, a prominent decrease were observed 48 hours after treatment was stopped. The effect of ketamine was similar to that of AST, also suggesting a protective effect against muscle damage caused by ketamine [18]. We are not sure if this is a result of heart muscle damage or of intramuscular injection of the anaesthetic. The different isoenzymes must be determined in order to determine the origin of this enzyme. It is however important to remember that the baboons were anesthetized before each treatment as well as before each blood sampling. It is therefore safe to conclude that the dramatic increases were due to the intramuscular injection before each treatment and blood sampling. Since ketamine was injected intramuscularly, it is highly likely that there were a marked increase in CK-MM and thus increased CK levels. Furthermore previous studies have shown that ketamine is greatly catabolic and when ketamine is used as an anesthetic, increased levels of CK is observed [19].

In conclusion, we have no supporting evidence that O_3-AHT was damaging to the baboons and that it caused severe liver and muscle damage. We rather believe that O_3-AHT protected against damage to the liver and muscle cells caused by the ketamine hydrochloride.

5. Acknowledgements

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6. List of abbreviations

ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
ATP	Adenosine 5'-triphosphate
CK	Creatine Kinase
H_2O_2	Hydrogen peroxide
HClO	Hypoclorous acid
LD	Lactate dehydrogenase
LOP	Lipid oxidation products
MD	Malate dehydrogenase
NAD+	Nicotinamide adenine dinucleotide (Oxidized form)
NADH	Nicotinamide adenine dinucleotide (Reduced form)
NADPH	Nicotinamide adenine dinucleotide Phosphate
O_2	Oxygen
$O_2^{\bullet-}$	Superoxide anion
O_2-AHT	Oxygen Autoheamotherapy
O_3	Ozone
O_3-AHT	Ozone Autoheamotherapy
$OH^{\bullet}$	Hydroxyl radical
$ROO^{\bullet}$	Peroxyl radicals
R-OOH	Hydroperoxides
ROS	Reactive oxygen species

7. References

1. Bocci, V. 2002. Oxygen-Ozone therapy: a critical evaluation. The Netherlands: Kluwer academic publishers.
2. Bocci, V. 1999. Biological and clinical effects of ozone. Has ozone therapy a future in medicine? *British Journal of biomedical Science*, 56:270-279.
3. Al-Dalien, S.M., Menéndez, S., Martinez, G., Fernández-Montequín, J.I., Candelario, e.J. & León, O.S. Ozone therapy effects in the oxidative stress associated to diabetes mellitus. . Proceedings – 15th World Congress of the International Ozone Association organized by Imperial College, London, U.K. 11th -15th September 2001.
4. Ajamieh, H.H., Menéndez, S., Merino, N., Sam, S. & León, O.S. Ischemic and ozone oxidative preconditioning in the protection against hepatics ischemic-reperfusion injury. Proceedings – 15th World Congress of the International Ozone Association organized by Imperial College, London, U.K. 11th -15th September 2001.
5. Viebahn-Hänsler, R., Lell, B. & Kremsner, P.G. The effect of ozone on Plasmodium falciparum-infected red blood cells. Proceedings – 15th World Congress of the International Ozone Assosiation organized by Imperial Colledge, London, U.K. 11th-15th September 2001.
6. Van der Zee, H. & De Monte, A. Ozone auto-haemotherapy in lower limb ulcerations. International Ozone Associations. Proceedings – 15th World Congress of the International Ozone Association organized by Imperial College, London, U.K. 11th -15th September 2001.
7. Sunnen, G.V. 1998. Ozone in medicine: Overview and Future Directions. http://www.triroc.com/sunnen/index.htm [Date of use: 15 July 2006].
8. Bocci, V. 2005. OZONE: A new medical drug. Springer, Dordrecht, p19-111.

9. Pryor, W.A. 1994. Mechanisms of radical formation from reactions of ozone with target molecules in the lung. Free radical Biology and Medicine, Volume 17, issue 5. p 451-465. [Web:] [Date of use: 20 August 2006].
10. Pryor, W.A., Squadrito, G.L. & Friedman, M. 1995. The cascade mechanism to explain ozone toxicity: The role of lipid ozonation products. Free radical Biology and Medicine Volume 19, Issue 6, p 935-941. [Web:] [Date of use: 20 August 2006]
11. Halliwell, B. & Gutteridge, J.M.C. Free radicals in biology and medicine. 3rd ed. Oxford : Oxford university press; 2000.
12. Du Plessis, L.H., van der Westhuizen, F.H., Kotzé, H.F. 2007. The effect of blood ozonation on mitochondrial function and apoptosis of peripheral blood mononuclear cells in the presence and absence of plasma antioxidants. *African Journal of Biotechnology*. 6: 1763-1769.
13. Kotzé, H.F., Lötter, M.G., Badenhorst, P.N. & Heyns, A.duP. Kinetics of In-111-platelets in baboons: Isolation and labelling of a viable and representative platelet population. *Thrombosis and Haemostasis; 53: 404-407.*
14. Bergmeyer, J. Ed. 1983. Methods of Enzymatic Analysis. 3rd ed. , Weinheim:VCH Publishers. Vol III, pp 418_1-424_1, 445_2-450_2, 510_3-517_3 .
15. Marshall, W.J., Bangert, S.K. 2004. Clinical Chemistry. 5th ed. Elsevier: Mosby. 85-103 p.
16. Dundee, J.W., Free, J.P., Moore, J., Mellroy, P.D. & Wilson, D.B. 1980. Changes in serum enzyme levels following ketamine infusion. *Anaesthesia*, 35: 12-18.
17. Giannini, E.G., Testa, R. & Savarino, V. 2005. Liver enzyme alterations: a guide for clinicians. *CMAJ*. 172: 367-377.
18. Gibhard, L., Labuschagne, C.F. & Kotzé, H.F. The acute effect of autoheamotherapy with different ozone concentrations on liver and muscle damage in baboons, (In preparation)
19. Zhang, X., Cortiella, J., Doyle, Jr. & Wolf, R.R. 1997. Ketamine anesthesia caused greater muscle catabolism in rabbits than does

propofol, Metabolism Unit, Shriners Burns Institute and Departments of Surgery and Anesthesiology, 8: 133-139.

Chapter 4: General discussion

"Dysoxygenosis is a term for dysfunctional oxygen metabolism. In the cell membrane, it causes leaky membrane dysfunction, so that what is inside the cell hemorrhages out and what is outside floods the cell innards. Thus, the cell becomes dehydrated, shrunken, and loaded with toxins. Such a cell cannot function well." Majid Ali, M.D (Ali, 1987). There is alot of controversy regarding the effectiveness of ozone, but it is a proven fact that ozone improved oxygen metabolism in cells (Bocci, 2005).

When considering the relationship between ozone and liver damage it is clear that O_3-therapy protects against liver damage caused by ketamine hydrochloride. It is well known that ketamine hydrochloride is catabolyzed in the liver, 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 however of interest to note that ozonation with 80 µg/ml O_3 only caused a slight increase in ALT levels. The AST levels during the acute phase followed the same pattern than that of ALT during the acute phase. During the chronic phase an increase in both ALT and AST levels were observed. ALT levels increased with approximately 130%, while AST levels increased with 210% during the treatment period. These greater variations observed 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. Fortunately the increases in plasma levels of ALT and AST during the acute and chronic phase could not even be classified as mild. Thus damage was not severe. One possible explanation could be that the ROS and LOPs that are formed during ex vivo ozonation binds to the ketamine present in the circulation and this decreases the load of ketamine 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 damages hepatocytes. Another possible explaination could be that, when

ozone comes in contact with the plasma, it produces ROS and LOPs which are responcible for membrane lipid peroxidation and the release of these enzymes into the blood, because the integrity of cell membranes were comprimised.

During the acute phase the CK levels in the plasma increased by approximately 750% 4 hours following treatment. The CK levels remained elevated for at least 24 hours. During the chronic phase CK levels in plasma increased with approximately 345% after 4 hours and reached a maximum 4 hours following the last treatment. A prominent decreace were observed 48 hours after treatment was stopped. The results obtained for the CK levels 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. Once again, when the results that were obtained during the chronic and acute phase are compared to that of ketamine hydrochloride, it is clear that ozonation of the blood had a protective effect againts the damage caused by ketamine. 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. It is not untoward, since ozonation improves the oxygen metabolism in cells. One should bear in mind that frequant administration of NMDA receptor antagonists lead ta resistance because the liver eliminate the antagonist more quickly from the blood stream (Livingston et al, 1978). In future studies it may be necessary to asses the isoenzymes of CK, so that there can be closure whether the muscle damage observed are due to skeletal muscle or cardiac muscle damage. In conclusion the results does not prove or disprove that O_3-AHT caused severe cell damage in baboons. I used 40 µg/ml O_3 in the studies where the baboons were treated sequentially (Chapter 3). This was because the results obtained with the dose of 80 µg/ml O_3 was not largely different from that with 40 µg/ml in the acute studies (Chapter 2). It was proven in these two studies and also in other studies (Labuschagne et al, 2007)

References

- Ajamieh, H.H., Menéndez, S., Merino, N., Sam, S. & León, O.S. Ischemic and ozone oxidative preconditioning in the protection against hepatics ischemic-reperfusion injury. Proceedings – 15th World Congress of the International Ozone Association organized by Imperial College, London, U.K. 11th -15th September 2001.
- Al-Dalien, S.M., Menéndez, S., Martinez, G., Fernández-Montequín, J.I., Candelario, e.J. & León, O.S. Ozone therapy effects in the oxidative stress associated to diabetes mellitus. . Proceedings – 15th World Congress of the International Ozone Association organized by Imperial College, London, U.K. 11th -15th September 2001.
- Ali, M. Leaky Cell Membrane Dysfunction. Monograph. 1987. Teaneck, New Jersey.
- Bergmeyer, J. Ed. 1983. Methods of Enzymatic Analysis. 3rd ed. , Weinheim:VCH Publishers. Vol III, 418_1-424_1, 445_2-450_2, 510_3-517_3 p.
- Bocci, V. 1999. Biological and clinical effects of ozone. Has ozone therapy a future in medicine? *British Journal of biomedical Science*, 56:270-279.
- Bocci, V. 2002. Oxygen-Ozone therapy: a critical evaluation. The Netherlands: Kluwer academic publishers.
- Bocci, V. 2005. OZONE: A new medical drug. Springer, Dordrecht, 19 -111p.
- Bocci, V., Valacchi, G., Corradeschi, F., Fanetti, G., Borrelli, E. & Aldinucci, C. 2000. Ozone in medicine. Ozone science and engineering, 23: 207-217.
- Cataldo, F. & Gentilini, L. 2005. Chemical kinetics measurements on the reaction between blood and ozone. *International journal of biological Macromolecules*, 36: 61-65.
- Ceber M, Salihoglu, T. Ketamine may be the first choice for anesthesia in burn patients. *J Burn Care Res* 27 (5): 760-2.

- Chan, P. C., Kindya, R. J. & Kesner, L. 1977. Studies on the mechanism of ozone inactivation of erythrocyte membrane Na^+,K^+-ATPase. Journal of biological chemistry. 252:8537-8541.
- Changes in hepatic and renal enzyme concentrations and heart and respiratory rates in new Zealand white rabbits after anesthetic treatments. Contemp Top Lab Animal Science, 41: 30-32.
- Chen, HS; Lipton SA. "The chemical biology of clinically tolerated NMDA receptor antagonists". *Journal of Neurochemistry* 97 (6): 1611-1126.
- Curtis, D.K., Watkins, J.B. 2003. Essentials of toxicology. 1st ed. McGraw-Hill. 193-196p.
- Du Plessis, L.H., van der Westhuizen, F.H., Kotzé, H.F. 2007. The effect of blood ozonation on mitochondrial function and apoptosis of peripheral blood mononuclear cells in the presence and absence of plasma antioxidants. African Journal of Biotechnology. 6: 1763-1769.
- Dundee, J.W., Free, J.P., Moore, J., Mellroy, P.D. & Wilson, D.B. 1980. Changes in serum enzyme levels following ketamine infusion. Anaesthesia, 35: 12-18.
- Freeman, B. A., Sharman, M. C. & Mudd, J. B. 1979. Reaction of ozone with phospholipid vesicles and human erythrocyte ghosts. Archives of Biochemistry and Biophysics. 197:264-272.
- Giannini, E.G., Testa, R. & Savarino, V. 2005. Liver enzyme alterations: a guide for clinicians. CMAJ. 172: 367-377.
- Gibhard, L., Labuschagne, C.F. & Kotzé, H.F. The acute effect of autoheamotherapy with different ozone concentrations on liver and muscle damage in baboons, (In preparation)
- Girotti, A.W. 1998. Lipid hydroperoxide generation, turnover, and effector action in biological systems. The Journal of Lipid Research. 39: 1529-1542.
- Gonzales, G.A., Illera, J.C., Silvan, G., Lorenzo, P.L. & Illera, M. 2002.
- Guyton, A.C. & Hall, J.E. 2000. Textbook of Medical Physiology. 10th ed. Philidelphia: Saunders. 399-429 p.

- Haensler, R.V. 2000. Ozone therapy: Method. [Web:] http://www.medicasrl.com/002USA2000.htm [Date of use: 23 August 2006]
- Halliwell, B. & Gutteridge, J.M.C. Free radicals in biology and medicine. 3rd ed. Oxford : Oxford university press; 2000.
- Halliwell, B., Clement, M.V. & Long, L.H. 2000. Hydrogen peroxide in the human body. *FEBS Letters; 486: 10-13*
- Hirota, K., Lambert, D.G. 1996. Ketamine: its mechanism of action and unusual clinical uses, British Journal of Anaesthesia. 77: 441-444.
- Kharasch, E.D., Labroo, R. 1992. Metabolism of ketamine stereoisomers by human liver microsomes. *Anesthesiology* 77 (6): 1201-7.
- Kotzé, H.F., Lötter, M.G., Badenhorst, P.N. & Heyns, A.duP. Kinetics of In-111-platelets in baboons: Isolation and labelling of a viable and representative platelet population. *Thrombosis and Haemostasis; 53: 404-407.*
- Labuschagne, C.F., Van Helden, G., Kotzé, H.F. & van der Westhuizen, F.H., The effect of autoheamotherapy with different ozone concentrations on the antioxidant status of baboons. Proceedings – 47th Annual Congress of the Federation of South African Societies of Pathology, Warmbaths, July 2007.
- Lang, H. 1981. Creatine Kinase Isoenzymes: Pathophysiology and Clinical Application. New York: Springer. 1-9 p.
- Livingston, A., Waterman, A.E. 1978. The development of tolerance to ketamine in rats and the significance of hepatic metabolism. *British Journal of Pharmacoogy.* 64: 63-9.
- Marshall, W.J., Bangert, S.K. 2004. Clinical Chemistry. 5th ed. Elsevier: Mosby. 85-103 p.
- Mehlman, M.A. & Borek, C. 1987. Toxicity and biochemical mechanisms of ozone. *Environmental research*, 42: 36-53.
- Monaghan, D.T. 1989. The Excitatory Amino Acid Receptors: Their classes, pharmacology, and distinct properties in function of the central

nervous system. Annual Review Pharmacology and Toxicology, 29: 365-402.

- Pryor, W.A. 1994. Mechanisms of radical formation from reactions of ozone with target molecules in the lung. Free radical Biology and Medicine, Volume 17, issue 5. p 451-465. [Web:] [Date of use: 20 August 2006].
- Pryor, W.A., Squadrito, G.L. & Friedman, M. 1995. The cascade mechanism to explain ozone toxicity: The role of lipid ozonation products. Free radical Biology and Medicine Volume 19, Issue 6, p 935-941. [Web:] [Date of use: 20 August 2006]
- Rice-Evans C, Miller, N.J. Total antioxidant status in plasma and body fluids. In: Methods in Enzymology. New York: Acedemic press, Inc, 1994: 279- 293.
- Sunnen, G.V. 1998. Ozone in medicine: Overview and Future Directions. http://www.triroc.com/sunnen/index.htm [Date of use: 15 July 2006].
- Van der Zee, H. & De Monte, A. Ozone auto-haemotherapy in lower limb ulcerations. International Ozone Associations. Proceedings – 15th World Congress of the International Ozone Association organized by Imperial College, London, U.K. 11th -15th September 2001.
- Viebahn-Hänsler, R., Lell, B. & Kremsner, P.G. The effect of ozone on Plasmodium falciparum-infected red blood cells. Proceedings – 15th World Congress of the International Ozone Assosiation organized by Imperial Colledge, London, U.K. 11th-15th September 2001.
- Widmaier, E.P., Raff, H. & Strang, K.T. 2004. Human Physiology: The Mechanisms of Body Function. 9th ed. Mcgraw Hill. 696-729p.
- Zhang, X., Cortiella, J., Doyle, Jr., & Wolf, R.R. 1997. Ketamine anesthesia causes greater muscle catabolism in rabbits than does propofol, Metabolism Unit, Shriners Burns Institute and Departments of Surgery and Anesthesiology, 8: 133-139.