Die effek van osoon outohemoterapie op antioksidant status en DNA integriteit in bobbejane

G. van Helden, Hons. B.Sc.

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Soli Deo Gloria

"U het baie dinge geskep, Here, die aarde is vol van wat U gemaak het, en tog, U het alles in wysheid geskep."

Psalm 104:24

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Abstract

Ozone therapy forms part of a group of complementary and alternative medical treatments that are often preferred above conventional medical treatments and surgery. Although it seems as if O_3-therapy can be used to treat various medical conditions, there is some concern regarding the toxicity and effectiveness of O_3-therapy. The strong oxidative nature of O_3 gives it the ability to cause severe damage to lung tissue when it is inhaled and it may also cause oxidative DNA damage in cells when it is used in therapy. On the other hand, it is believed that O_3 may exert a stimulatory effect on the antioxidant defence and immune systems. Therefore, controlled clinical studies are necessary to assess the safety and effectiveness of O_3-therapy. We assessed the effect of O_3 autohaemotherapy (AHT) with relatively high O_3 concentrations on the oxidant/antioxidant status in baboons by evaluating serum hydroperoxides, glutathione redox status, antioxidant capacity and antioxidant enzyme activity. We also assessed the genotoxicity of O_3 by measuring DNA damage and DNA repair capacity in lymphocytes using the Comet assay. O_3-AHT was performed on the baboons by using 5% of their total blood volume. Blood samples were taken before reinfusion and again 0.5-48 hours following reinfusion of the ozonated blood. O_3-AHT caused an increase in the level of oxidative stress in baboons, but did not deplete cellular antioxidants such as GSH. Although catalase activity was not altered by O_3-AHT, SOD activity was slightly increased. Serum antioxidant capacity was elevated after O_3-AHT, however, we are not sure to what extent the ketamine hydrochloride that was used as anaesthetic influenced the results. DNA damage was induced 24 hours after O_3-AHT was performed but this was not significant and the effect was eliminated after 48 hours. DNA repair was up-regulated within four hours following O_3-AHT but returned to control levels after 24 hours. In general, it appears as if O_3-AHT may have a beneficial, though transient, effect without causing cellular damage.

Opsomming

Osoon terapie vorm deel van 'n groep komplementêre en alternatiewe mediese behandelings wat dikwels bo konvensionele mediese behandeling en chirurgie verkies word. Alhoewel dit voorkom asof O_3-terapie gebruik kan word vir behandeling van verskeie mediese toestande, bestaan daar kommer oor die toksisiteit en effektiwiteit van O_3-terapie. Die sterk oksidatiewe aard van O_3 gee aan dit die potensiaal om ernstige skade aan longweefsel aan te rig wanneer dit ingeasem word. Dit kan ook oksidatiewe DNA skade veroorsaak in selle wanneer dit tydens terapie gebruik word. Hierteenoor, word beweer dat O_3 'n stimulerende effek uitoefen op die antioksidant verdedigingstelsel asook die immuunstelsel. Dit is daarom nodig dat gekontroleerde kliniese studies uitgevoer moet word om die veiligheid en effektiwiteit van O_3-terapie te ondersoek. Ons het die effek van O_3 outohemoterapie (OHT), met relatiewe hoë O_3-konsentrasies, op die oksidant/antioksidant status in bobbejane ondersoek deur serum hidroperoksiede, glutatioon redoks status, antioksidantkapasiteit en antioksidant ensiemaktiwiteit te bepaal. Ons het ook die genotoksisiteit van O_3 ondersoek deur DNA skade en DNA herstelkapasiteit in limfosiete te meet m.b.v. die Komeetanalise. O_3-OHT is uitgevoer op die bobbejane deur 5% van hul totale bloedvolume te gebruik. Bloedmonsters is geneem voor- en weer 0.5-48 uur nadat die geosoneerde bloed teruggespuit is. O_3-OHT het die oksidatiewe stresvlak in die bobbejane verhoog, maar het nie sellulêre antioksidante, soos GSH, uitgeput nie. Alhoewel katalase aktiwiteit nie verander het nie, het SOD aktiwiteit effens toegeneem. Serum antioksidantkapasiteit was verhoog na O_3-OHT, maar ons is nie seker tot watter mate die ketamien hidrochloried wat as verdowingsmiddel gebruik is, die resultate beïnvloed het nie. DNA skade is geïnduseer 24 uur na O_3-OHT, maar dit was nie betekenisvol nie en die effek was opgehef na 48 uur. DNA herstel was opgereguleer binne vier uur na O_3-OHT, maar het teruggekeer na kontrole vlakke na 24 uur. In die algemeen wil dit voorkom asof O_3-OHT 'n voordelige, dog kortstondige, uitwerking het sonder dat dit selskade veroorsaak.

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List of abbreviations

6-HD	6-Hydroxydopamine
AAPH	2,2'-azobis(2-aminopropane)dihydrochloride
AODS	Antioxidant defence system
AP site	Apurinic/Apyrimidinic site
AP-1	Activator protein-1
BCA	Bicinchoninic acid
CAM	Complementary and alternative medical therapy
Carr U	Carratelli units
Cat	Catalase
CuZnSOD	Copper-zinc containing superoxide dismutase
DETAPAC	Diethylene-triamine-pentaacetic acid
DHA	Dehydroascorbate
DTNB	5,5' Dithiobis-2-nitrobenzoic acid
EDTA	Ethylenediamine tetra-acetic acid
FRAP	Feric Reducing/Antioxidant Power assay
GPx	Glutathione Peroxidase
GR	Glutathione Reductase
GSH	Glutathione (Reduced)
GSHt	Total Glutathione (Reduced)
GSSG	Glutathione (Oxidised)
H_2O_2	Hydrogen peroxide
Hb	Haemoglobin
HCT116	human colorectal carcinoma
HMPA	High melting point agar
$HO^{\bullet}$	Hydroxyl radical
k	Rate constant of a first-order reaction
LMPA	Low melting point agar
M2VP	1-methyl-2-vinylpyridinium trifluoromethane sulfonate

MMR	Mismatch repair
MnSOD	Manganese containing superoxide dismutase
NAAQS	National ambient air quality standard
NEIL	family of endonuclease VIII (Nei)
NER	Nucleotide excision repair
O_2 -AHT	Oxygen autohaemotherapy
$O_2^{\bullet-}$	Superoxide anion
O_3 -AHT	Ozonated Autohaemotherapy
ORAC	Oxygen Radical Absorbance Capacity
PBS	Phosphate buffered saline
PCA	Perchloric acid
RBC	Red blood cell
ROS	Reactive oxygen species
SCGE	Single cell gel electrophoresis
SDA	Semidehydroascorbate
SOD	Superoxide dismutase
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
TE	Trolox equivalents
TNF-α	Tumour necrosis factor alpha
TPTZ	Tripyridyltriazine
WBC	White blood cell

List of addenda

Introduction

1. Background and motivation

Ozone is well known for its antimicrobial action and has been used as disinfectant since the First World War. Ozone also plays an important role in industrial processes such as treatment of waste and drinking water. The interest to use O_3 as an alternative medicine is growing rapidly and claims are being made that O_3-therapy can be used to treat various medical conditions, such as retinitis pigmentosa (Copello *et al.*, 2003), diabetes mellitus (Al-Dalien *et al.*, 2001), ischemic disorders (Ajamieh *et al.*, 2001), open wounds and ulcerations (Van der Zee *et al.*, 2001), nosocomal infections (Matsumoto *et al.*, 2001) and malaria (Viebahn-Hänsler *et al.*, 2001). O_3-therapy is also being considered as a possible treatment for AIDS (Shallenberger, 1998).

In biological fluids, O_3 produces the same oxidants that are generated during normal aerobic respiration within a cell and does not produce any 'new' toxic oxidants (Bocci, 2002). With this in mind it has been suggested that ozone, at low concentrations, can stimulate the antioxidant defence system (AODS) without causing severe oxidative stress. O_3-therapy apparently lowers total plasma cholesterol and LDH (Hernández *et al.*, 1994), improves O_2 supply to tissues as well as the rheological properties of blood (Giunta *et al.*, 2001) and exerts an immunomodulating effect (Bocci, 2002).

Although there are different methods for ozone administration, ozone autohaemotherapy (O_3-AHT) appears to be the method of choice. It offers a meaningful and reproducible delivery system and has the advantage that the ozonated blood is rapidly distributed through the entire body. O_3-AHT involves

the *ex vivo* exposure of a volume of blood to an equal volume of O_3/O_2-gas mixture with a precise O_3 dose, followed by reinfusion of the ozonated blood.

Although it seems as if O_3-therapy can be used to treat various medical conditions, there is some concern regarding the toxicity and effectiveness of O_3-therapy. In an England study by Furnham (2000a) it was found that O_3-therapy is not considered to be very effective. In general it was found that the participants were not familiar with the therapy, less that 10% had tried it and only 4% knew how it worked. Marchetti and La Monaca (2000) also reported an unexpected death due to gas embolism during O_3-AHT. In addition, ozone may cause severe respiratory damage when it is inhaled (Mehlman & Borek, 1987; Cotgreave, 1996). Ozone therapy has therefore encountered scepticism by orthodox medicine and very little scientific and controlled clinical studies have been done to assess O_3-therapy. A well constructed and controlled study to assess the effects of O_3-AHT is therefore needed. The nature and extent of the changes (if any) need to be determined and the advantages of O_3-therapy must be weighed up against the harmful effects it may have.

2. Aim of this study

This dissertation forms an integral part of a holistic study in which the effect of O_3-AHT on various biochemical parameters, such as metabolism, antioxidant status and DNA integrity, as well as blood biochemistry and haematology is assessed. In this part of the study we assessed the effect of O_3-AHT with a high O_3-dose on oxidant/antioxidant status as well as DNA integrity and DNA repair capacity in baboons.

3. Structure of this dissertation

This dissertation was compiled in publication format and consists of two articles which are formatted according to the instructions for authors for the journal *Free Radical Biology and Medicine*. Chapter one gives a literature review on ozone, the antioxidant defence system of the body, oxidative stress and the medical use of O_3. In the first article (chapter two) the effects of O_3-autohaemotherapy (AHT) on the oxidant/antioxidant status was studied in baboons by evaluating total hydroperoxide levels, glutathione redox status, antioxidant capacity and antioxidant enzyme activity. The effect of O_3-AHT on DNA integrity and DNA repair capacity in lymphocytes was also evaluated in baboons and the findings presented in chapter three. In chapter four, a general discussion is given as well as conclusions and recommendations. References are listed at the end of this dissertation and the calculation of results, examples of the Comet assay images and instructions for authors are included as addenda to the dissertation.

Chapter 1: Literature review

1.1. Introduction

Complementary and alternative medical (CAM) therapies are gaining more and more interest worldwide and are often preferred above conventional medical treatments and surgery (Furnham, 2000). Ozone (O_3) therapy forms part of this group of CAM therapies and has been used to treat various medical conditions. O_3-therapy apparently lowers total plasma cholesterol and LDL (Hernández *et al.*, 1994), improves O_2 supply to tissues as well as rheological properties of blood (Giunta *et al.*, 2001) and exert an immunomodulating effect (Bocci, 1999). In contrast, ozone is a toxic gas and may cause severe damage to lung tissue when it is inhaled (Mehlman & Borek, 1987; Cotgreave, 1996). The question therefore arises: Can O_3 autohaemotherapy be safe and effective?

1.2. Ozone

The word ozone is derived from the Greek word *ozein* which means 'to smell'. Ozone (O_3) is a highly reactive, pale-blue gas with a distinctive pungent smell and plays an important role in the higher levels of the atmosphere by screening out solar radiation (Halliwell & Gutteridge, 2000). In nature, O_3 is generated during thunderstorms because of electrical discharge that catalyzes the formation of O_3 from atmospheric oxygen (O_2). In the laboratory O_3 is prepared by passing pure oxygen through a high voltage field. Energy derived from the electrical discharge leads to the decomposition of O_2-molecules to form O-atoms, which, in the presence of excess O_2, produces O_3. An O_3/O_2 gas mixture that may contain up to 5% O_3, is generated. The concentration of ozone depends on the flow rate of oxygen as well as the conditions of voltage and spacing of electrodes.

Ozone has a short half-life of approximately 1 hour in air during which it is rapidly converted to O_2. The degradation of O_3 in solution depends strongly on the pH of the solution. At pH >5, the rate of O_3-degradation is markedly increased. Degradation starts as soon as the first molecule of O_3 dissolves into the reaction medium (Hahn *et al.*, 2000) and leads to the formation of other reactive species, such as the superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($HO^{\bullet}$) (section 1.4).

1.3. Ozone toxicity

Ozone toxicity manifests in respiratory damage (Mehlman & Borek, 1987). As much as 90% of the ozone that is inhaled is absorbed and may cause severe damage to lung tissue (Cotgreave, 1996). The National Ambient Air Quality Standard (NAAQS) level for ozone in the USA and Europe is 0.12 parts per million (p.p.m.). O_3 levels of 0.5 p.p.m. (~0.001 μg/ml) can cause lung damage within a few hours of inhalation, especially through loss of ciliated cells and type I alveolar cells. Ozone toxicity may be due to direct oxidation of lung tissue by O_3 but also to the action of free-radicals. O_3 induces inflammation and causes activation of pulmonary macrophage and recruitment of neutrophils to the lungs. Reactive oxygen species (ROS) produced by these cells may provide an additional source for development of oxidative stress after exposure to O_3. Proteins and lipids in surfactant, secreted by type II alveolar cells to lower the surface tension of the fluid lining in the lungs, may also be attacked by O_3. Extensive damage to type I and II alveolar cells can lead to fibrosis, i.e. the laying down of inelastic fibrous material, and so permanently impair gas exchange (Halliwell & Gutteridge, 2000). Because of its high reactivity and toxicity, when inhaled, fast and precise handling of ozone is necessary when patients are being treated with ozone.

1.4. Mechanism of O_3 action

1.4.1. Production of reactive species

In biological fluids, O_3 apparently acts through two mechanisms. The first is an O_3-olefin-reaction where O_3 reacts with the double bonds in organic substances, such as fatty acids, and produces H_2O_2, aldehydes and other peroxides (Pryor, 1994; Pryor *et al.*, 1995). The second mechanism is an O_3-electron donor reaction where an O_3-radical is formed that reacts with a proton to produce a hydroxyl-radical (Pryor, 1994; Bocci, 2002):

$$GSH + O_3 \rightarrow GSH^{\bullet+} + O_3^{\bullet-}$$

$$O_3^{\bullet-} + H^+ \rightarrow HO^{\bullet} + O_2$$

Since the pH of interstitial fluids (e.g. in the lungs) is 7.35 and that of blood ranges between 7.35 and 7.4 (Guyton & Hall, 2000), one can assume that O_3 will undergo rapid decomposition as it dissolves into these fluids. It is therefore more likely that the majority of the reactions that occur in lung fluids and blood are due to the reactive species derived from O_3-decomposition. Although O_3 is far more reactive than O_2, it produces the same reactive oxygen species that are produced via biochemical processes in the body (Bocci, 2002), e.g., mitochondrial electron transport (Mathews *et al.*, 2000). The major products of O_3 decomposition in solution are cytotoxic superoxide radicals ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($HO^{\bullet}$). Although less reactive than $HO^{\bullet}$, $O_2^{\bullet-}$ can cause direct damage through inhibition of antioxidant enzymes such as catalase and glutathione peroxidase (section 1.5) but also through superoxide-derived species such as H_2O_2 from the dismutation of $O_2^{\bullet-}$ (section 1.5) and $HO^{\bullet}$ through the Haber-Weiss reaction (Halliwell & Gutteridge, 2000):

$$H_2O_2 + O_2^{\bullet-} \rightarrow O_2 + HO^{\bullet} + HO^-$$

In vivo, H_2O_2 is also produced from other oxidase reactions such as xanthine, urate, and D-amino oxidase reactions (Halliwell & Gutteridge, 2000). This product

can be converted to $HO^{\bullet}$ in the presence of ferrous or cuprous ions through the Fenton reaction where an organic compound is oxidised in a free-radical reaction catalysed by transition metals (Halliwell & Gutteridge, 2000):

$$Fe^{2+} + H_2O_2 \rightarrow \text{intermediate complexes} \rightarrow Fe(III) + HO^{\bullet} + HO^{-}$$

The hydroxyl radical ($OH^{\bullet}$) is the most potent and most abundant oxidant produced by O_3. It triggers a radical chain reaction and can, together with other ROS, oxidise lipids, inactivate proteins and cause strand breaks in DNA.

1.4.2. Biological effects of reactive species

Lipid peroxidation

Lipid peroxidation is the oxidative deterioration of polyunsaturated lipids (Halliwell & Gutteridge, 2000). Polyunsaturated fatty acid (PUFA) side chains of membrane lipids are especially prone to peroxidation. During peroxidation the reactive specie abstracts a hydrogen atom from a methylene ($-CH_2-$) group in the carbon chain and an unpaired electron is left on the carbon ($-^{\bullet}CH-$). Double bonds adjacent to a methylene group weaken the binding of hydrogen atoms that are present on the methylene group. This causes that a hydrogen atom is easily removed during peroxidation. The resulting carbon radical can either collide with and bind to another carbon radical, or combine with O_2 to produce a peroxyl radical ($ROO^{\bullet}$). The process of lipid peroxidation can be continued through a chain reaction in which the peroxyl radical subtracts a hydrogen atom from another lipid molecule to produce a lipid hydroperoxide (LOOH) and another carbon radical (Halliwell & Gutteridge, 2000). Membrane proteins as well as cholesterol molecules in membranes and lipoproteins can be oxidised during lipid peroxidation. In addition, O_3 can directly oxidise lipids to ozonides which can decompose to form aldehydes. Fatty acid peroxides can be removed from membranes by phospholipase enzymes and reduced by glutathione peroxidase. If not removed or repaired, peroxides can have toxic effects by causing fatty liver development and damage to heart tissue (Halliwell & Gutteridge, 2000).

Protein oxidation

Oxidative damage to proteins *in vivo* affects receptors, enzymes, signal transduction pathways and enzymes that maintain low intracellular free Ca^{2+} levels. Damage to proteins become visible when amino acid residues close to or within the active sites are affected. Proteins can be oxidised by ROS or by secondary products of oxidation, for example from lipid peroxidation. Thiol (-SH) groups are easily oxidised by ROS and may result in formation of disulphides or other oxidation products such as sulphonates (Halliwell & Gutteridge, 2000). Oxidised proteins may be repaired. Glutathione and thioredoxin can re-reduce disulphides and peptide methionine sulphoxide reductase can reduce methionine sulphoxide back to methionine. Proteins in which amino acid residues other than cysteine or methionine are oxidised, seem to be irreversibly damaged and are removed. Proteolytic systems that recognise these proteins are present in both the cytosol and mitochondria of mammalian cells. Multi catalytic protease complexes (proteasomes) are mainly responsible for recognition and degradation of 'unwanted' proteins. Proteins that are damaged by ROS *in vivo* are often 'marked' with a 76 amino acid protein called ubiquitin (Mathews *et al.*,2000). These proteins are recognised by the 26S proteasome and proteolytically degraded. In this way protein turnover is increased in cells that are exposed to oxidative stress (Halliwell & Gutteridge, 2000).

Oxidative DNA damage and repair of DNA damage

Of all the ROS, only H_2O_2 has the ability to easily cross cell membranes (Duell *et al.*, 1995). When H_2O_2 enters the cell, it may cause DNA damage, although it is not in itself the ultimate DNA-attacking agent. Instead, H_2O_2 reacts with endogenous superoxide anions ($O_2^{\bullet-}$) and transition metal ions (Fe^{2+} and Cu^{2+}) via the Fenton reaction and produces the highly reactive hydroxyl radical ($HO^{\bullet}$). The hydroxyl radical ($HO^{\bullet}$) is indiscriminant in its reaction with DNA, meaning that it has no marked sequence preference (Halliwell & Gutteridge, 2000). Oxidative DNA damage caused by $HO^{\bullet}$ includes single-strand breaks, double-

strand breaks, alkali-labile regions and oxidized purines and pyrimidines (Collins *et al.*, 1995). It may also cause sugar modifications as well as damage to nuclear proteins (Halliwell & Gutteridge, 2000). If DNA damage is not repaired it can lead to accumulation of modified nucleotides (Mohrenweiser *et al.*, 2003) and negatively affect the integrity of the genome. For this reason organisms have defence mechanisms to repair damaged DNA to its original state.

DNA repair processes are divided into five pathways (Mohrenweiser *et al.*, 2003) of which base excision repair and double strand break repair are involved in repair of oxidative DNA damage. In the base excision repair process, single strand breaks and singly modified DNA bases, such as 8OH-guanine, are repaired through the action of the glycosylase enzymes. These enzymes recognize specific base modifications and catalyse the hydrolysis of the N-glycosydic bond between the base and the sugar-phosphate backbone. A non-coding abasic site (AP-site) is generated. AP endonuclease cleaves the DNA backbone at the 5' position of the AP (apurinic/apyrimidinic) site and induces a single strand break. DNA polymerase β fills the single nucleotide gap before the 5' abasic fragment is excised. Ligase then closes nicks in the DNA strand. Repair of double strand breaks is more challenging and involves two processes. The first process is homologous recombination repair, which relies on extensive nucleotide sequence complementarity between the intact chromatid and the damaged chromatid (or homologous region) as the basis for strand exchange and repair. The second process is non-homologous end joining, and requires little or no sequence homology and involves direct DNA end-joining (Mohrenweiser *et al.*, 2003).

1.5. The antioxidant defence system

An antioxidant can be defined as "any substance that, when present at low concentrations compared with those of an oxidisable substrate, significantly

delays or prevents oxidation of that substrate" (Halliwell & Gutteridge, 2000). When an organism is exposed to ROS, antioxidant defences are induced in order to prevent or limit oxidative stress (section 1.6). Antioxidant defence mechanisms differ from tissue to tissue and from cell-type to cell-type. Extracellular fluids have different protective mechanisms from the intracellular environment (Halliwell & Gutteridge, 2000).

The antioxidant defence system (AODS) mainly consists of two classes of antioxidants, namely preventive and scavenger antioxidants (Psotova *et al.*, 2001; Bocci, 2002). Apart from these two groups, the AODS also comprise proteins that minimise the availability of pro-oxidants such as iron ions, copper ions and haem, e.g. transferrins, haptoglobins, haemopexin and metallothionein, and proteins that protect biomolecules against damage by other mechanisms, e.g. heat shock proteins (Halliwell & Gutteridge, 2000).

Preventive antioxidants

These include the antioxidant enzymes superoxide dismutase, catalase, glutathione reductase and glutathione peroxidase that play an important role in the prevention of oxidative stress.

Superoxide dismutase (SOD) is an ubiquitous metalloprotein that catalyzes the dismutation of the superoxide radical ($O_2^{\bullet -}$) to hydrogen peroxide (H_2O_2) and molecular oxygen (O_2). Dismutation is a reaction in which two identical substrate molecules have different fates. Here, one molecule of SOD is oxidised and one is reduced (Mathews *et al.*, 2000):

$$2O_2^{\bullet -} + 2H^+ \rightarrow H_2O_2 + O_2$$

SOD thus plays an important role in the protection of living cells (Ukeda *et al.*, 1997). Animals have two types of SOD. Copper-zinc containing SODs (CuZnSOD) are mostly localised in the cytosol but are also present in the

lysosome, nucleus and the intermembrane space of mitochondria. Manganese containing SODs (MnSOD) are largely present in mitochondria. Because erythrocytes do not contain mitochondria, MnSODs are not found in these cells (Halliwell & Gutteridge, 2000).

Catalase is present in red blood cell (RBC), kidney and especially liver peroxisomes. Except for heart muscle, mitochondria do not contain catalase. This enzyme catalyzes the dismutation of H_2O_2 to water and oxygen:

$$2\ H_2O_2 \rightarrow 2\ H_2O + O_2$$

Catalase is especially important in RBC to prevent haemolysis due to H_2O_2. Catalase is also integrated with SOD since it is needed to remove H_2O_2 to prevent inhibition of SOD (Bocci, 2002).

Glutathione reductase (GR) is an ubiquitous enzyme that catalyzes the reduction of oxidized glutathione (GSSG) to reduced glutathione (GSH). For this reaction to take place, GR relies on sufficient NADPH supply (Figure 1.2). The pentose phosphate pathway is the most important source of NADPH for the regeneration of GSH (Dringen & Gutterer, 2002).

Glutathione peroxidase (GPx) is widely distributed in the cytoplasm and mitochondria of cells. It protects cells against free radicals that form as a result of peroxide decomposition. GPx uses GSH as reducing agent and catalyzes the reduction of H_2O_2 and organic peroxides (ROOH) to water and the corresponding stable alcohol (Halliwell & Gutteridge, 2000; Smith & Lavender, 2002):

$$H_2O_2 + 2GSH \rightarrow GSSG + 2\ H_2O$$

$$ROOH + 2GSH \rightarrow ROH + GSSG + H_2O$$

Scavenger antioxidants

The second group of antioxidants in the AODS consists of a number of low molecular mass antioxidants, such as glutathione, bilirubin, uric acid (urate), and

vitamins C and E. Glutathione (GSH) is an ubiquitous molecule that acts as a water soluble antioxidant in most aerobic tissue. It is involved in the reduction of hydrogen peroxide and lipid hydroperoxides to produce water. GSSG is the inactive oxidized form of glutathione and is generated in two ways (Dringen & Gutterer, 2002): through a non-enzymatic reaction of radicals with GSH, or through Glutathione peroxidase reactions where GSH acts as electron donor for the reduction of peroxides. A GSSG molecule consists of two molecules of GSH, joined together by a disulphide bridge resulting from oxidation of the –SH groups (Figure 1.1).

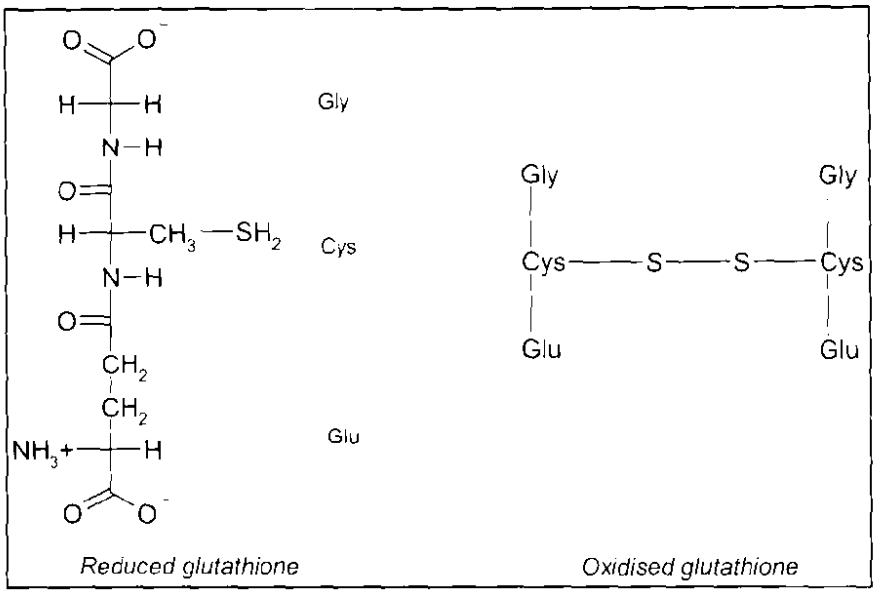

Figure 1.1. The structures of reduced (GSH) and oxidised (GSSG) glutathione.

In order for the cell to maintain a high GSH:GSSG ratio, GSSG is reduced to GSH by glutathione reductase (GR) and NADPH (Figure 1.2). Adequate cellular levels of GSH are essential because GSH prevents apoptosis, and plays an important role in signal transduction and gene expression (Bocci, 2002). It is also involved in the metabolism of ascorbic acid and plays a role in protein folding (Halliwell & Gutteridge, 2000). The GSH:GSSG ratio gives an indication of oxidative stress in the body – when cells are exposed to oxidative stress, the ratio will decrease as a consequence of GSSG accumulation.

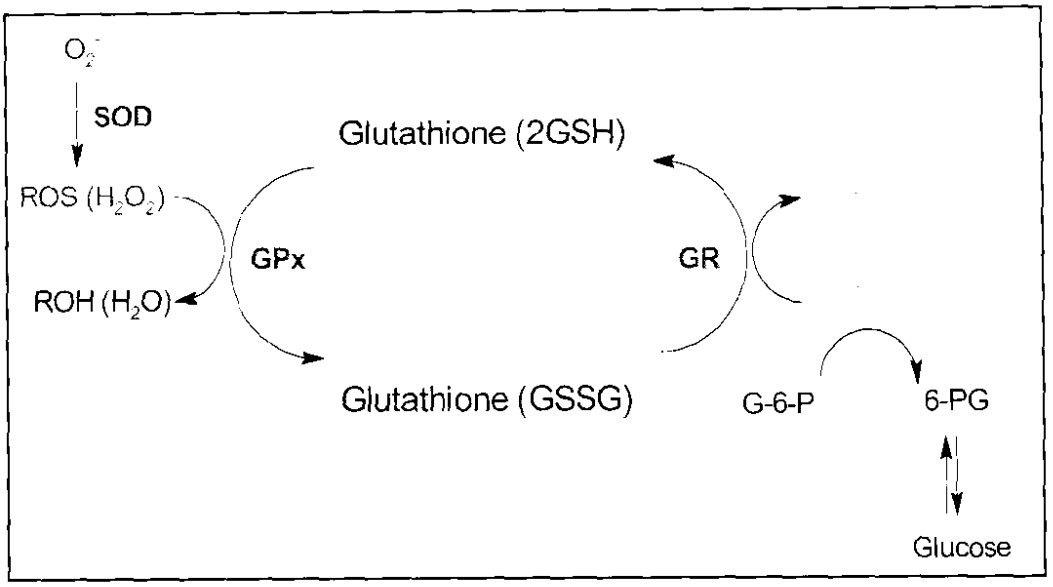

Figure 1.2. A schematic representation of the glutathione redox cycle showing the relationship between antioxidant enzymes and glutathione.

GSH is in a constant state of metabolic turnover. The half-life of GSH in human erythrocytes is estimated to be 4 days. GSSG is secreted into plasma from the red blood cells and GSH is mainly secreted into plasma by the liver cells. Plasma GSH and GSSG is rapidly degraded by the high levels of γ-glutamyltranspeptidase present in the plasma membranes of kidney cells (Halliwell & Gutteridge, 2000).

Uric acid is probably the antioxidant involved when body fluids are exposed to O_3. It is produced through the oxidation of hypoxanthine and xanthine by xanthine oxidase and dehydrogenase enzymes. In humans and primates, uric acid is not converted to urea. Instead, it accumulates in blood plasma and is eventually excreted in urine. Strong oxidants, such as $OH^{\bullet}$, react with urate and produce urate radicals. The urate radical ($UrH^{\bullet-}$) is resonance-stabilised because of the delocalisation of the unpaired electron over the purine ring (Halliwell & Gutteridge, 2000). Reactions between urate and organic peroxyl radicals also give rise to urate radicals:

$$RO_2^{\bullet} + UrH_2^- \rightarrow ROOH + UrH^{\bullet-} + H^+$$

Urate may be recycled through a reaction between ascorbic acid (Asc) and the urate radical (Halliwell & Gutteridge, 2000):

$$UrH^{\bullet-} + AscH^{-} \rightarrow UrH_2^{-} + Asc^{\bullet-}$$

Vitamin C (ascorbic acid) is a low-molecular-mass antioxidant derived from the diet. In reaction with ROS, extracellular ascorbate is oxidised to the relatively unreactive semidehydroascorbate (SDA) or ascorbyl radical. SDA undergoes a disproportionation reaction and gives rise to ascorbate and dehydroascorbate (DHA). DHA may either be taken up from body fluids by erythrocytes, neutrophils and other cells to be converted back to intracellular ascorbate at the expense of GSH or NADPH, or it may undergo rapid non-enzymatic breakdown to produce oxalate, threonate and other oxidation products (Halliwell & Gutteridge, 2000).

Vitamin E (also known as α-tocopherol) is a fat soluble antioxidant found in the interior of membranes and in lipoproteins (Halliwell & Gutteridge, 2000:215). It is involved in the scavenging of peroxyl radicals and regarded as the most important inhibitor of free-radical chain reactions of lipidperoxidation. Tocopherols scavenge lipid peroxyl radicals ($LO_2^{\bullet}$) much faster than these radicals can react with adjacent fatty acid side-chains of membrane proteins:

$$\alpha\text{-TocH} + LO_2^{\bullet} \rightarrow \alpha\text{-Toc}^{\bullet} + LO_2H$$

The α-tocopherol radical can react with another peroxyl radical to yield a non-radical product:

$$LO_2^{\bullet} + \alpha\text{-Toc}^{\bullet} \rightarrow \alpha\text{-TocOOL}$$

One molecule of α-tocopherol can therefore terminate two peroxidation chains. α-Tocopherol also reacts with $O_2^{\bullet-}$ and $OH^{\bullet}$. In a reaction with ascorbate, α-tocopherol can be recycled while ascorbate itself is oxidised to the ascorbyl radical (Halliwell & Gutteridge, 2000).

The antioxidant system present in plasma and blood cells is an extremely powerful defence system and oxidative processes can eventually be blocked. If, however, ROS production exceeds the capacity of the antioxidant defence system (AODS), it will result in oxidative stress.

1.6. Oxidative stress

An imbalance between pro- and antioxidants, where pro-oxidant levels exceed that of antioxidants, causes oxidative stress and may lead to cell damage. In cells that are exposed to oxidative stress, Ca^{2+} metabolism may be disregulated because of mitochondrial damage that leads to the release of Ca^{2+}. Elevated levels of Ca^{2+} in the mitochondrial matrix can lead to swelling of the matrix and subsequent rupture of the outer mitochondrial membrane. This is known as the mitochondrial permeability transition (MPT). Oxidative stress induces MPT and therefore affects ATP production (Nicholls & Ferguson, 2002). Large rises in intracellular Ca^{2+}-levels may affect the cytoskeleton of the cell and also cause DNA fragmentation by activating Ca^{2+}-dependent nuclear endonucleases (Halliwell & Gutteridge, 2000). Intracellular communication through gap junctions may also be affected. For example, H_2O_2 can activate signal transduction pathways by oxidising –SH groups or by increasing Ca^{2+}-levels. Proliferating cells usually seize division in response to oxidative stress until repair (of at least DNA) is complete (Halliwell & Gutteridge, 2000). Oxidative stress either results in adaptation of the cell to new conditions or cellular injury.

Adaptation

Mild exposure of an organism to oxidative stress often results in increased synthesis of cellular antioxidant defence systems. This happens in order to restore the oxidant/antioxidant balance, to protect the cell against attack by oxidants and to prepare the cell for stronger oxidative attack that may follow

(Halliwell & Gutteridge, 2000; Finkel, 2003). Regulation of cellular defence systems usually occurs through regulation of transcription. NF-κβ and activator protein-1 (AP-1) are two transcription factors involved in redox regulation in mammalian cells. NF-κβ is a complex of proteins that activates the transcription of various genes in response to multiple stimuli. It is ubiquitously expressed and responds immediately to oxidative stress. ROS possibly act as second messengers in the activation of NF-κβ. Most genes that are activated by NF-κβ are involved in what seems to be an early defence network against the development of pathological conditions. AP-1 responds to the intracellular redox state and can be affected by both ROS and antioxidants. It binds to target sequences in many promoters like that of NADP(H)-quinone oxidoreductase and a subunit of glutathione S-transferase. DNA repair in which AP endonuclease is involved, e.g. base excision repair discussed earlier, is closely coupled to activation of transcription by AP-1 (Halliwell & Gutteridge, 2000).

Cell-cell interaction makes it possible for any cell under oxidative stress to cause an upregulation of defences in other cells and tissues. Hormones are responsible for communication between distant cells while cytokines are secreted for local communication between cells. Cytokines are polypeptides or glycoproteins involved in local regulation of cell growth and differentiation, e.g. tumour necrosis factor-α (TNF-α, which activates NF-κβ) and interleukins. They also serve as signals for inflammatory/immune responses and chemotactic agents (Halliwell & Gutteridge, 2000).

Cell injury

Cell injury is a 'result of a chemical or physical stimulus, in excess or deficiency, that transiently or permanently alters the homeostasis of the cell' (Halliwell & Gutteridge, 2000). When the cell is irreversibly injured, cell death occurs. This can be either through necrosis, which involves swelling and rupture of the cell,

affecting other cells by releasing its contents into the surrounding area, or apoptosis, where cell death is regulated and does not affect surrounding cells.

1.7. Medical use of Ozone: O_3-therapy

The principle of O_3-therapy is based on the hypothesis that the reaction of ozone with biological molecules triggers certain biological processes in the organism and causes an upregulation of the AODS and immune pathways involved. In biological fluids, O_3 produces the same oxidants that are generated during normal aerobic respiration within a cell and does not produce any 'new' toxic oxidants. With this in mind it has been suggested that ozone, at low concentrations, can stimulate the AODS without causing severe oxidative stress.

Different methods for administrating ozone during O_3-therapy have been used. These include rectal insufflation of an O_3/O_2 -gas mixture, local application of ozonated distilled water, infusion of an ozonated saline solution, intravenous infusion or intradiscal injection of an O_3/O_2-gas mixture and ozone autohaemotherapy (O_3-AHT). Endovenous injection of ozone has been prohibited since 1986 by the European Society of Oxygen-ozone therapy (Marchetti & La Monaca, 2000) because of the risk of gas embolism that may occur. Intradiscal injection of ozone is also a technique not accepted or standardised by the medical community (Corea *et al.*, 2004). A case of vertebrobasilar stroke during ozone intradiscal injection was reported by Corea *et al.* (2004) and another case of vitreo-retinal hemorrhage development after intradiscal injection was reported by Giudice *et al.* (2004). Compared to other methods, O_3-AHT appears to be the method of choice. It offers a meaningful and reproducible delivery system and has the advantage that the ozonated blood is rapidly distributed through the entire body.

O_3-AHT involves the *ex vivo* exposure of a volume of blood to an equal volume of O_3/O_2-gas mixture with a precise O_3 dose, followed by reinfusion of the blood. Since O_3 reacts very rapidly at room temperature, it is only the products from the reaction of O_3 with the blood that enter the organism during reinfusion. These products are believed to stimulate various biochemical systems, such as the AODS.

For O_3-therapy to be effective and not toxic, it is critical that the correct O_3 dose is used. The O_3 dose can be calculated as follows (Bocci, 1999; Bocci, 2000):

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

Generation of H_2O_2 is proportional to the O_3 dose. The O_3 dose in the O_3/O_2-gas mixture must be adequate to allow sufficient H_2O_2 generation. Hydrogen peroxide (H_2O_2) that is generated can activate biochemical and immunological pathways. The H_2O_2 concentration must reach a critical threshold: below this no stimulation of the antioxidant system will occur, but, if the concentration is excessive, oxidative damage may occur (Bocci, 1999). Therefore it is important to determine what O_3 concentration is most effective and, at the same time, safe to use.

Chapter 2

The effect of ozone autohaemotherapy on the oxidant/antioxidant status in baboons

Gerda van Helden, Francois H. van der Westhuizen, Piet J. Pretorius, Herculaas F. Kotzé*

Division of Biochemistry, School for Chemistry and Biochemistry, North-West University, Potchefstroom Campus, Private Bag X6001, Potchefstroom 2520, South Africa

*Corresponding author. *Fax:* +27 18 299 2316. *E-mail address:* bchhfk@puk.ac.za

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Abstract

Ozone (O_3) therapy forms part of a group of complementary and alternative medical therapies and is gaining more and more interest worldwide. There is, however, some concern regarding O_3-toxicity and uncertainty about the effectiveness of O_3-therapy. We assessed the effect of ozone autohaemotherapy (AHT) on the oxidant/antioxidant status in six baboons by evaluating selected biochemical parameters in blood that are associated with the oxidant/antioxidant status. Autohaemotherapy with oxygen-treated blood was performed to serve as control in a separate group of baboons. O_3-AHT with 75-84µg/ml O_3 caused a slight increase in the oxidative stress level in baboons, but did not deplete cellular antioxidants such as GSH. Although catalase activity was not changed by O_3-AHT, SOD activity increased slightly. Serum antioxidant capacity was elevated after O_3-AHT, although we are not sure to what extent the ketamine hydrochloride that was used as anaesthetic influenced the results. The changes following O_2-AHT were of the same nature but not as marked as in the case of O_3-AHT. The effect of O_3-AHT was transient and repeated sessions of O_3-AHT may therefore be necessary to make it therapeutically effective.

Keywords: Ozone, autohaemotherapy, oxidative stress, antioxidant capacity

1. Introduction

Ozone (O_3) is a highly reactive, pale-blue gas with a distinctive pungent smell and plays an important role in the higher levels of the atmosphere by screening out solar radiation [1]. In contrast, ozone may cause severe damage to lung tissue when it is inhaled [2,3] and therefore O_3-pollution is of greatest concern to environmentalists. The interest to use ozone as a form of complementary and alternative medical treatment (CAM) is growing rapidly and claims are being made that O_3-therapy can be used to treat various medical conditions [4-10]. The rationale behind O_3-therapy is based on the hypothesis that the reaction of ozone with biological molecules triggers certain biological processes in the patient and causes an up-regulation of the immune and/or antioxidant defence pathways involved [11]. There is, however, concern regarding O_3-toxicity and uncertainty about the effectiveness of O_3-therapy [12,13]. Although different methods are used for application of ozone during O_3-therapy, ozone autohaemotherapy (O_3-AHT) appears to be the method of choice and was also the method we investigated.

In biological fluids, O_3 apparently acts through two mechanisms. The first is an O_3-olefin-reaction where O_3 reacts with the double bonds in organic substances such as fatty acids and produces H_2O_2, aldehydes and other peroxides [14,15]. The second mechanism is an O_3-electron donor reaction where an O_3-radical is formed that reacts with a proton to produce a hydroxyl-radical [11,14].

In solution, the degradation of O_3 to produce other reactive species depends strongly on the pH of the solution [16]. At pH >5, the rate of O_3-degradation is markedly increased, therefore O_3 will undergo rapid decomposition as it dissolves into blood. It is thus more likely that the majority of the reactions that occur in blood are due to the reactive species derived from O_3-decomposition. Although O_3 is far more reactive than O_2, it produces the same reactive oxygen species (ROS) that are produced via biochemical processes in the body [11], for example

via mitochondrial electron transport [17]. The major products of O_3 decomposition in solution are cytotoxic superoxide radicals ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($HO^{\bullet}$). Although less reactive than $HO^{\bullet}$, $O_2^{\bullet-}$ can cause direct damage through inhibition of antioxidant enzymes such as catalase and glutathione peroxidase and also through superoxide-derived species such as H_2O_2 from the dismutation of $O_2^{\bullet-}$ and $HO^{\bullet}$ through the Haber-Weiss reaction. The hydroxyl ion ($OH^{\bullet}$) is the most potent and abundant oxidant produced by O_3. It triggers a radical chain reaction and can, together with other ROS, oxidise lipids, inactivate proteins and cause strand breaks in DNA [1].

When an organism is exposed to ROS, antioxidant defences are induced in order to prevent or limit oxidative stress. The antioxidant defence system (AODS) mainly consists of two classes of antioxidants, namely preventive antioxidants (e.g. antioxidant enzymes like superoxide dismutase and catalase) and scavenger antioxidants (e.g. low molecular mass antioxidants, such as glutathione, bilirubin, urate, and vitamins C and E) [11].

We assessed the effect of O_3-AHT with high O_3-dose on oxidant/antioxidant status in baboons by evaluating selected biochemical parameters in blood that are associated with the oxidant/antioxidant status. These included evaluation of antioxidant capacity, glutathione redox state, antioxidant enzyme activities and total hydroperoxide levels. Our aim was to investigate whether O_3-AHT caused oxidative stress in baboons and if so, whether it had damaging or beneficial consequences.

2. Materials and methods

2.1. Reagents

NaH_2PO_4, Na_2HPO_4, K_2HPO_4, KH_2PO_4, 2,4,6-tripyridyl-s-triazine (TPTZ), and $FeCl_3.6H_2O$ were obtained from Merck (Darmstadt, Germany). Reduced glutathione (GSH) and 5,5'Dithiobis-2-nitrobenzoic acid (DTNB) were obtained from Boehringer Mannheim, Germany. All other reagents were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA).

2.2. Preparation of ozone

The ozone was prepared by passing pure oxygen (>99.5% O_2, BOC Special products) through a dry glass-isolated alternating current corona discharge ozoniser, developed and built by the School for Physics of the North-West University (USA patent 09/914,199). A UV/Vis spectrophotometer (Pharmacia Biotech Ultrospec 3000) was used to determine the O_3-concentration by measuring the absorbance of the gas mixture at 254 nm. A specially designed quartz cell was used to monitor the O_3-concentration of the O_3/O_2-gas mixture in real time while it was added to the blood. The cell was provided with an inlet, where the gas entered the cell from the ozoniser, and an outlet, where the gas left the cell and directly entered the glass syringe containing the blood. Ozone concentrations varying between 75 and 84 μg/ml (3.5-4% O_3) were used.

2.3. Treatment of baboons

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 male and female baboons, weighing between 8 and 25 kg, were used. The baboons were maintained in the animal facility of the North-West University, Potchefstroom Campus, on standard laboratory chow. Water was available ad lib. The baboons were individually caged in a room at constant room temperature and with automated day-night cycles. The baboons were anaesthetised with ketamine hydrochloride (±10mg/kg) to allow handling. The therapeutic window range of O_3 concentrations for AHT is 20-80 µg/ml per gram of blood [18]. In order to ensure that a definite effect (good or bad) will be seen, we decided to use high O_3 concentrations of 75-84 µg/ml.

Blood (5% of total blood volume) from six baboons was drawn in heparin in polypropylene syringes. The blood was then transferred to siliconised glass syringes and ozonated by adding an equal volume of an O_2/O_3 gas mixture, containing 3.5-4% (75-84 µg/ml) O_3. After gently mixing the blood for 20 minutes, the gas was carefully removed from the syringe and a blood sample was taken to assess the effect of ozonation. The remainder of the treated blood was then reinfused into the donor. Blood samples were collected in Vacutest tubes containing heparin, EDTA or no anticoagulant directly before reinfusion of the ozonated blood (control), and again at 0.5, 1.0, 4.0, 24 and 48 hours following reinfusion. The blood was stored at 4°C for no longer than six hours until analysis or until appropriate sample preparation for storage was performed. Autohaemotherapy with oxygen-treated blood (O_2-control) was performed to serve as control in five separate baboons.

2.4. Oxidative stress

2.4.1. Hydroperoxide assay

Total hydroperoxides present in serum were measured using an assay kit (d-ROMs test) from DIACRON International (Grosseto, Italy). Reagents were used as supplied in the d-ROMs test kit and the test was performed as described in

the test kit protocol. Absorbance changes were measured kinetically at 485 nm every two minutes for 15 min using a Bio-Tek (FL 600) microplate reader. The concentration of hydroperoxides in the sample was expressed in Carratelli (CARR) units (Iorio, 2002).

2.4.2. Glutathione redox status

Glutathione redox status was evaluated in whole blood treated with EDTA according to the OXIS BIOXYTECH® assay kit (GSH/GSSG-412™) with minor modifications. Samples were immediately prepared for total GSH determination and stored at -70°C. DTNB was added to convert GSH into a spectrophotometrically detectable product followed by glutathione reductase and NADPH. GSH (3 μM) was used as standard. The absorbance was measured at 412 nm every minute for 5 min using a Bio-Tek microplate reader (FL 600).

2.5. Antioxidant capacity

2.5.1. Oxygen radical absorbance capacity (ORAC)

Oxygen radical absorbance capacities were measured in the non-protein fractions of serum essentially as described by Cao and Prior [19]. Trolox was used as standard series in the assay and serum antioxidant capacity was expressed as Trolox equivalents (TE). Fluorescein (56 nM) was used as fluorescent decay marker in the assay and the reaction started by adding 240 mM AAPH (2,2'-azobis(2-aminopropane)dihydrochloride). The fluorescence (excitation 485nm, emission 520nm) was recorded with a Bio-Tek (FL 600) microplate reader every 5 minutes for 2 hours or until the last reading has declined to less than 5% of the first reading. Polynomial regression analysis was used to determine antioxidant capacity.

2.6. Antioxidant enzyme activity

2.6.1. Catalase activity

Diluted erythrocyte hemolysate was incubated with a 30 mM H_2O_2 substrate solution and the decrease in H_2O_2 absorption at 240 nm was monitored kinetically every 15 seconds for 1 minute as described by Aebi [21] using a Beckman UV/Visible spectrophotometer. The first order rate constant (k) was calculated and related to the haemoglobin content (k/g Hb) as a measure of the specific activity of erythrocyte catalase. Haemoglobin content was determined by standard Haematology laboratory procedures using an automatic analyzer (Abbot Cell-Dyn-3500).

2.6.2. Superoxide dismutase activity

The ability of SOD to inhibit 6HD (6-Hydroxydopamine) auto-oxidation was measured spectrophotometrically as described by Ellerby [22]. Diluted erythrocyte precipitate was treated with an ethanol/chloroform extraction medium (62.5:37.5, v/v) and the protein content of the supernatant was determined. Supernatant containing ~0.1 $\mu g/\mu l$ protein was used in the SOD activity assay. Diethylene-triamine-pentaacetic acid (DETAPAC, 0.1 mM) solution was added to the samples followed by 1.6 mM 6-HD stock solution. The auto oxidation of 6-HD was recorded at 490 nm for 4 minutes in 1 min intervals by using a Bio-Tek (FL 600) microplate reader. The linear slope of the increase in absorbance was calculated and the amount of protein that resulted in 50% inhibition of 6-HD was determined.

2.7. Protein assay

Protein concentrations were determined by using the BCA method with bovine serum albumin as standard [23].

2.8. Statistical analysis

All statistical analyses were performed using the repeated measure analysis of variance (ANOVA). Differences in mean values within a group were considered significant when $P < 0.05$. All values are expressed as means $\pm$ 1 standard deviation. The baseline (control) values of the two groups of baboons differed markedly in some cases and therefore the results were presented as the fold change relative to the control samples. We have no ready explanation for the differences between the groups. The fold change was calculated as $(X_{ti} - X_c)/X_c$, where, X_c is the measurement in the control sample and X_{ti} the measurement at time 0.5, 1.0, 4.0, 24 or 48 hours.

3. Results

3.1. The in vitro effect of ozone

Serum hydroperoxide concentrations were not markedly altered after either ozonation or oxygenation of the blood. After ozonation, GSHt decreased significantly by approximately 15%, although GSHt levels did not change after treating the blood with oxygen (Table 1). There appeared to be a marked increase in the serum ORAC of the ozonated as well as the oxygenated blood (Table 1).

Table 1. Hydroperoxide, total glutathione (GSH) and ORAC values measured in the control blood and in the O_3- and O_2-treated blood.

	O_3 - treated group		**O_2 -control group**	
	Control	O_3	Control	O_2
Hydroperoxides (Carr U)	333 ± 60	327 ± 122	487 ± 114	435 ± 72
Total GSH (µM)	1016 ± 206	*862 ± 122	634 ± 98	650 ± 50
ORAC (µM TE)	500 ± 330	*830 ± 152	1035 ± 406	1429 ± 536

O_3-treated group: n = 6; O_2-treated group: n = 5). Values are given as means ± 1 SD. (*Significant at $p < 0.05$)

3.2. The effect of autohaemotherapy

3.2.1. Oxidative stress

- *Hydroperoxides*

Throughout the 48 hour time period hydroperoxide levels for the O_2-control group varied between 400-500 Carr U while that for the O_3-treated subjects were closer to the normal range of 300-400 Carr U. Although there was a 15% increase in serum hydroperoxide levels 1 hour after reinfusion in the O_3-treated group, it was

not significant and after 24 hours the hydroperoxide concentrations returned back to control levels (Figure 1). Although hydroperoxides for the O_2-control group were 7% lower at 48 hours following reinfusion, this group did not show marked changes.

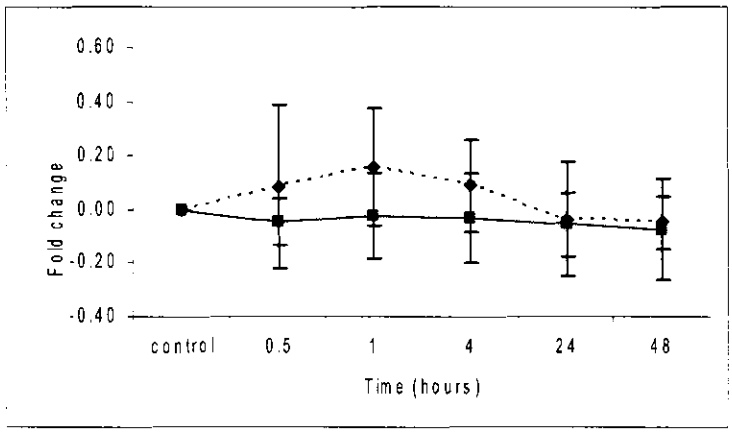

Figure 1. Changes in hydroperoxide levels in the O_3-treated group (--♦--; n = 6) and the O_2-control group (—■—; n = 5). Hydroperoxides were determined in the blood drawn directly before reinfusion (control) and again 0.5 to 48 hours following reinfusion of the treated blood. Values are given as mean change ± SD.

- *Glutathione redox state in vivo*

Total plasma GSH concentrations are not age- and sex-dependent [24] and are often measured to evaluate the redox state of an individual [25, 26]. In Figure 2 the fold change in total GSH during the 48 hours after reinfusion is presented for both groups. Following O_3-AHT, total GSH levels declined and at 48 hours GSHt levels were approximately 36% lower than before reinfusion in the O_3-treated group. Although GSHt levels did not change after treating the blood with oxygen (table 1), a gradual decrease was observed after reinfusion in the O_2-control group. After 1 hour GSHt was significantly decreased but only by approximately 15 % and after 48 hours it was still only 15% lower than before reinfusion.

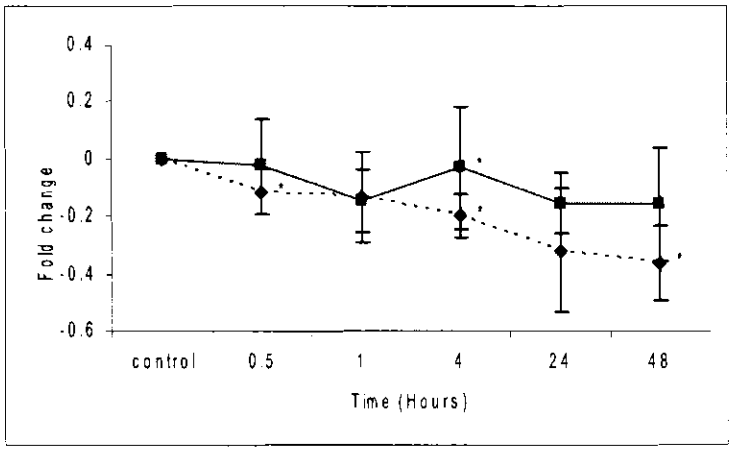

Figure 2. Changes in total blood glutathione in the O_3-treated group (--♦--; n = 6) and the O_2-control group (—■—, n = 5). GSHt was measured in the blood drawn directly before reinfusion (control) and after reinfusion (0.5-48 hours) of the treated blood. Values are given as mean change ± SD. (*Significant at $p < 0.05$)

3.2.2. Antioxidant capacity

- *Oxygen radical absorbance capacity*

Control ORAC values for the O_3-group were 500.29 ± 330.19 µM TE and for the O_2-control group 1034.97 ± 406.1 µM TE. After O_3-AHT the ORAC was higher than before reinfusion (control) but the increase was not significant until 4 hours following reinfusion. A maximum serum ORAC was observed for the O_3-group at 24 hours following reinfusion but after 48 hours the increase was reversed and the ORAC approached control levels. The results of the O_2-control group followed the same pattern, although the changes were not as marked as after O_3-AHT. The serum ORAC of the O_3-subjects increased by approximately 193% after 24 hours while that of the O_2-control group increased by approximately 112%.

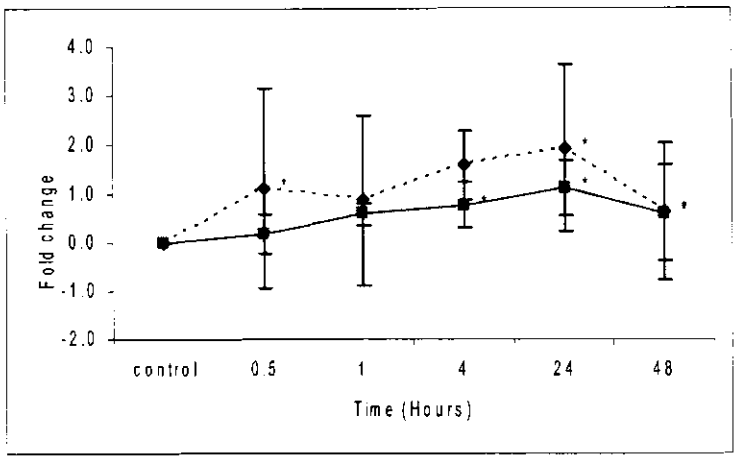

Figure 3. Changes in serum ORAC in the O_3-treated group (--♦--; n = 6) and the O_2-control group (—■—; n = 5). ORAC was measured in the blood drawn directly before reinfusion (control) and again 0.5 to 48 hours following reinfusion of the treated blood. Values are given as mean change ± SD. (*Significant at $p < 0.05$)

3.2.3. Antioxidant enzyme activity

- *Catalase activity*

Catalase activity in the control samples of the O_3-group were 0.0158 ± 0.004 k/g Hb and of the O_2-control group 0.0212 ± 0.014 k/g Hb. Catalase activity in both the O_3- and O_2-groups did not change significantly after AHT (Figure 4). The O_2-control group showed more marked, albeit statistically insignificant, changes in catalase activity than the O_3-group. During the first hour following O_2-AHT the activity declined to approximately 80% of the control value. After four hours the activity was restored to control level but this was followed by another decrease and after 48 hours catalase activity was reduced to approximately 72% of the control value.

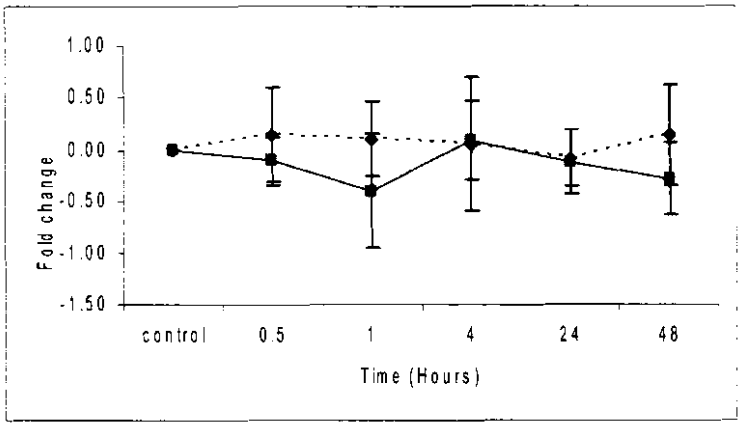

Figure 4. Changes in catalase activity in the O_3-treated group (--♦--; n = 6) and the O_2-control group (—■— n = 5). Activity was determined for both groups in blood drawn before reinfusion (control) and after reinfusion (0.5-48 hours). Values are given as mean change ± SD.

- *Superoxide dismutase activity*

Samples were diluted to contain 0.1 µg/µl protein and SOD activity was determined in terms of the amount of protein needed to cause 50% inhibition of 6-HD auto-oxidation. Control values for the O_3-group were 569.65 ± 121.58 ng protein and for the O_2-control group 539.271 ± 85.72 ng protein. Figure 5 illustrates the changes that occurred after reinfusion of the treated blood. In both groups there was an increase in SOD activity within the first hours, but it was not statistically significant. In the O_3-treated group, the SOD activity declined again after four hours and control levels was again reached. This was, however, followed by another slight increase in SOD activity and after 48 hours it was approximately 10% higher than before reinfusion. The O_2-control group followed the same pattern, although the increase after 0.5 hours was less than in the O_3-group and after 48 hours SOD activity was approximately 33% higher than before reinfusion.

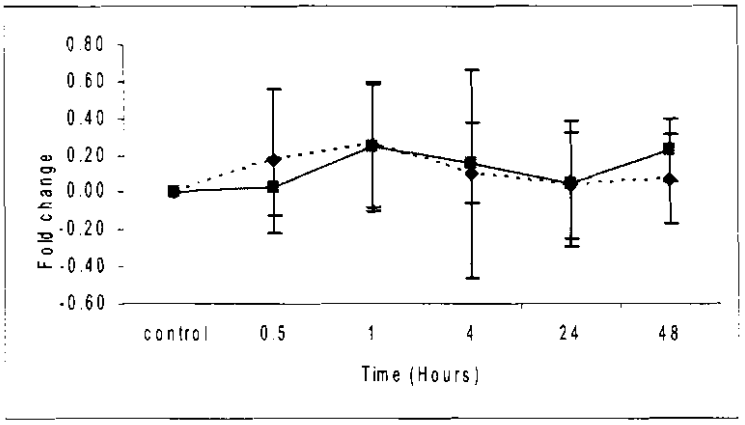

Figure 5. Changes in SOD activity in the O_3-treated group (--♦--; n = 6) and the O_2-control group (—■—; n = 5). Activity was determined for both groups in blood drawn before reinfusion (control) and after reinfusion (0.5-48 hours) of the treated blood. Values are given as mean change ± SD.

4. Discussion

There is no universal 'marker' for oxidative stress. According to Halliwell & Gutteridge [1] "often, the only evidence that oxidative stress has occurred *in vivo* may be the up-regulation of antioxidant defence systems". It is also important to note that depletion of antioxidants does not necessarily indicate that oxidative damage has occurred. It may only mean that the defence mechanisms have fulfilled their normal function [1]. Oxidative stress can therefore lead to either adaptation of the cell or cellular damage. Cells can resist mild oxidative stress, which will result in up-regulation of the AODS to restore the balance between pro- and antioxidants. If, on the other hand, oxidative damage is excessive and the homeostasis of the cell is transiently or permanently altered, it will lead to cellular injury [1].

Treatment of the blood with ozone did not increase serum hydroperoxides while total glutathione concentrations were significantly reduced by 15% after ozone treatment (Table 1). It is therefore likely that GSH acted as sacrificial substrate in

order to terminate the free radical action triggered by ozone in the treated blood and so prevented the production of hydroperoxides. Our results support that of Bocci who also reported a 15% decrease in GSH after blood was exposed to 80 µg/ml O_3 [18]. Furthermore, our results indicate that the antioxidant capacity in deproteinated serum was already increased in the ozonated blood of all six baboons. This was also found in four of the five baboons treated with oxygenated blood (Table 1). This was a rather surprising finding, because O_3 is a very reactive oxidant and gives rise to other ROS the moment it dissolves into solution [16]. In addition, Bocci [18] reported a 20% decrease in total plasma antioxidant status after O_3 exposure of whole blood. It is therefore unlikely that ozone was responsible for this increase in antioxidant capacity. Reinke *et al.* [29] suggested that ketamine may have antioxidative features. Under oxidative conditions, ketamine can be converted into other radical intermediaries such as hydroxylamine [29]. Under these conditions, hydroxylamine can react with other oxidants to produce nitroxide, which is a relative stable radical and not likely to initiate secondary free radical cascade reactions. We therefore suggest that the anaesthetic that was used could have contributed to the rise in antioxidant capacity observed in the treated blood of the two groups.

After O_3-AHT, hydroperoxides, as measured by using the d-Rom test, were elevated within 1 hour following reinfusion. Although this increase was not statistically significant, the hydroperoxide levels that were reached were within the range associated with low to mild (321-400 Carr U) levels of oxidative stress [27]. Glutathione (GSH) is a ubiquitous molecule that acts as a water-soluble antioxidant in most aerobic tissue. It is a sacrificial substrate involved in the reduction of hydrogen peroxide and lipid hydroperoxides to produce water and may well have been responsible for reducing the hydroperoxide levels back to control level after O_3-AHT. This would explain the 34% decrease in GSHt 48 hours after O_3-AHT (Figure 2). In the O_2-control group there was no increase in hydroperoxide levels and after 48 hours total GSH decreased by only 15% which is less than half the change observed in the O_3-treated group. This suggests that

O_2-AHT did not induce an increased state of oxidative stress as was observed after O_3-AHT.

Antioxidant defences are induced in an organism in order to prevent or limit oxidative stress following exposure to ROS. The increase in serum antioxidant capacity (ORAC) observed after O_3-AHT was therefore not an unexpected finding. After reinfusion serum ORAC increased significantly in both groups. O_3-AHT, however, had a more pronounced effect on the antioxidant capacity than O_2-AHT. The serum ORAC of the O_3-subjects increased by approximately 193% after 24 hours while that of the O_2-control group increased by approximately 112%. This statistically significant difference between the groups can be attributed to the higher reactivity of O_3 as an oxidant, which may in turn elicit a more pronounced antioxidant defence reaction. While hydroperoxide levels reached a maximum within one hour following O_3-AHT, serum ORAC continued to increase for up to 24 hours. This indicates that the rise in antioxidant capacity was not induced only to quench ROS that resulted from O_3, but also to prepare the cells for sequential free radical attack that may follow. It is not clear to what extent ketamine with its antioxidative properties may have influenced the results and the results may therefore be slightly compromised. However, the effect of ketamine will be equal in both groups. Therefore, the fact that more pronounced changes were observed after O_3-AHT than after O_2-AHT suggests that the antioxidant capacity was improved *in vivo* after O_3-AHT in spite of ketamine.

Antioxidant enzyme activity does not attribute to serum antioxidant capacity as measured by the ORAC assay because the deproteinated fraction of the serum is used to perform this assay. Therefore, antioxidant enzyme activity was also determined. Catalase catalyzes the dismutation of H_2O_2 (a product of O_3 decomposition) to water and oxygen. This enzyme is present in red blood cell (RBC), kidney and especially liver peroxisomes [11]. In RBC it is especially important to prevent haemolysis due to the action of H_2O_2. Catalase is also integrated with SOD since it is needed to remove H_2O_2 to prevent inhibition of

SOD [11]. Lee *et al.* [30] reported an inhibitory effect of aqueous ozone on catalase activity *in vitro* and that GSH protects this enzyme against O_3-inhibition. In the present study, however, no significant changes were observed in catalase activity after ozonation of the blood (Figure 4). The presence of plasma antioxidants (of which GSH is only one) seems to have been sufficient to quench this inhibitory effect of O_3 (or its derivatives) on RBC catalase. Catalase activity was also not significantly enhanced or reduced after O_3-AHT. In contrast, there was some reduction in catalase activity after O_2-AHT. One can speculate that O_2 is a far more stable compound than O_3 and that generation of ROS due to O_2 degradation is therefore minimal. Because O_2 is one of the products derived from H_2O_2 dismutation, it is possible that catalase was inhibited by the increased O_2 concentrations in the blood after O_2-AHT. Another ROS derived from O_3 decomposition that could have attributed to the increased level of oxidative stress after O_3-AHT, is the superoxide radical ($O_2^{\bullet-}$). Superoxide dismutase (SOD) is a ubiquitous metalloprotein that catalyzes the dismutation of $O_2^{\bullet-}$ to H_2O_2 and molecular oxygen (O_2). SOD thus plays an important role in the protection of living cells [31]. Within the first hour following O_3-AHT, SOD activity was markedly up-regulated. The same effect was observed after O_2-AHT, but to a lesser extent. In both groups a biphasic response was observed that may comprise an initial stimulation of enzyme activity to protect the cells against oxidative stress, and a secondary effect where the cells seem to have up-regulated their metabolic activities in order to adapt or prepare for sequential exposure to oxidative stress that may follow.

In conclusion, O_3-AHT with relatively high O_3 concentrations caused a slight increase in the oxidative stress level in baboons, but did not deplete cellular antioxidants such as GSH. Although catalase activity was not altered by O_3-AHT, SOD activity was slightly increased. Total serum antioxidant capacity was also increased, although it is not clear to what extent the anaesthetic could have influenced the results. The changes following O_2-AHT were of the same nature but not as marked as in the case of O_3-AHT. Nonetheless, the changes due to

O_3-AHT appear to be within physiological boundaries and may therefore be beneficial through stimulation of the AODS, but without causing severe oxidative stress or depleting cellular antioxidants. The effect of O_3-AHT was, however, transient and repeated sessions of O_3-AHT may therefore be necessary to induce a more prolonged effect and/or to precondition the cells and organs for future exposure to oxidative stress.

5. Acknowledgements

We would like to thank the Dean of the Faculty of Natural Sciences and the National Research Foundation of South Africa for funding this project.

6. List of abbreviations

6-HD	6-Hydroxydopamine
AAPH	2,2'-azobis(2-aminopropane)dihydrochloride
AODS	Antioxidant defence system
BCA	Bicinchoninic acid solution
CAM	Complimentary and alternative medical therapy
Carr U	Carratelli units
Cat	Catalase
DETAPAC	Diethylene-triamine-pentaacetic acid
DTNB	5,5' Dithiobis-2-nitrobenzoic acid
GSH	Glutathione (Reduced)
GSHt	Total Glutathione (Reduced)
GSSG	Glutathione (Oxidised)
Hb	Haemoglobin
O_2-AHT	Oxygen autohaemotherapy
O_3-AHT	Ozonated Autohaemotherapy
ORAC	Oxygen Radical Absorbance Capacity
PCA	Perchloric acid
RBC	Red blood cell
ROS	Reactive oxygen species
SOD	Superoxide dismutase
TE	Trolox equivalents
TPTZ	Tripyridyltriazine
WBC	White blood cell

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Chapter 3

The effect of ozone autohaemotherapy on DNA integrity and DNA repair capacity of lymphocytes in baboons

Gerda van Helden, Piet J. Pretorius, Herculaas F. Kotzé*

Division of Biochemistry, School for Chemistry and Biochemistry, North-West University, Potchefstroom Campus, Private Bag X6001, Potchefstroom 2520, South Africa

*Corresponding author. *Fax:* +27 18 299 2316. *E-mail address:* bchhfk@puk.ac.za

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Abstract

The interest in ozone as an alternative form of medical treatment is growing rapidly but there is some concern regarding the effectiveness and safety of O_3-therapy. *In vivo* studies have been performed to evaluate the effect of environmental O_3-exposures in humans but the genotoxic effect of O_3-autohaemotherapy (AHT) has not yet been intensively studied *in vivo.* We assessed the effect of O_3-AHT on DNA integrity and DNA repair capacity in five baboons. Autohaemotherapy with oxygen-treated blood and also untreated blood were performed to serve as controls in separate groups of baboons. The animals were anaesthetised with Ketamine hydrochloride to allow handling. The Comet assay was used to measure DNA damage in isolated lymphocytes. Our results indicate that Ketamine has genotoxic properties but that the DNA damage it causes is rapidly repaired. We suggest that O_3-treatment and, to a lesser extent, O_2-treatment protect against the DNA damaging effects of Ketamine hydrochloride. Oxygen therapy did not cause damage to lymphocyte DNA. DNA damage was induced 24 hours after O_3-AHT was performed but this was not significant and the effect was eliminated after 48 hours. DNA repair was up-regulated within four hours following O_3-AHT but returned to control levels after 24 hours.

Keywords: Ozone, autohaemotherapy, DNA damage, DNA repair

1. Introduction

Complementary and alternative medical (CAM) therapies are gaining more and more interest worldwide and are often preferred above conventional medical treatments and surgery [1]. Ozone (O_3) therapy forms part of this group of CAM therapies. The principle of O_3-therapy is based on the hypothesis that the reaction of ozone with biological molecules triggers certain biological processes in the patient and causes an up-regulation of the immune and/or antioxidant defence pathways involved [2]. Methods for application of ozone during O_3-therapy include rectal insufflation, local application or intravenous infusion of an O_3/O_2-gas mixture or ozonated saline solution, and ozonated autohaemotherapy (O_3-AHT). We investigated O_3-AHT in baboons. Claims are being made that O_3-therapy can be used to treat various medical conditions [3-9]. There is, however, some uncertainty about the effectiveness of O_3-therapy [1,10] and also some concern regarding O_3-toxicity, mainly because of the lack of proper control studies.

Ozone (O_3) is a strong oxidant and may cause severe damage to lung tissue when it is inhaled [11]. It is highly reactive and undergoes rapid degradation to produce oxygen and other reactive species, such as the superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($HO^{\bullet}$). In solution (e.g. blood), degradation starts as soon as the first molecule of O_3 dissolves into the reaction medium [12]. Ozone also reacts with the double bonds in organic substances, such as fatty acids [13,14], and acts as an electron donor [13,2] to produce reactive oxygen species (ROS).

Ozone has a genotoxic effect that can be attributed to the reaction of ROS, that arises from the degradation of O_3, with DNA. Hydrogen peroxide (H_2O_2) has the ability to easily cross cell membranes [15]. It reacts with endogenous $O_2^{\bullet-}$ and transition metal ions (Fe^{2+} and Cu^{2+}) via the Fenton reaction and produces highly reactive $HO^{\bullet}$. The hydroxyl radical ($HO^{\bullet}$) is indiscriminant in its reaction with

DNA, meaning that it has no marked sequence preference [16]. Oxidative DNA damage caused by $HO^{\bullet}$ includes single-strand breaks, double-strand breaks, alkali-labile regions and oxidized purines and pyrimidines [17]. It may also cause sugar modifications as well as damage to nuclear proteins [16]. Díaz-Llera *et al.* [18] studied the genotoxic effects of O_3 on human peripheral blood leukocytes *in vitro* and found that O_3 induced DNA damage in these cells. *In vivo* studies have been performed to evaluate environmental O_3-exposures in humans but the genotoxic effect of O_3-AHT has not yet been intensively studied *in vivo.*

If DNA damage is not repaired it can lead to accumulation of modified nucleotides and negatively affect the integrity of the genome. For this reason organisms have defence mechanisms to repair damaged DNA to its original state. DNA repair processes in mammalian cells are divided into five pathways [19] of which base excision repair and double strand break repair are involved in repair of oxidative DNA damage. In the base excision repair process single strand breaks and singly modified DNA bases, such as 8OH-guanine, are repaired through the action of the glycosylase enzymes.

The aim of this study was to assess the effect of O_3-AHT on DNA integrity and DNA repair capacity of lymphocytes in baboons. The Comet assay (single cell gel electrophoresis) is a sensitive method for detecting oxidative DNA damage and was therefore used to assess DNA damage and repair.

2. Materials and methods

2.1. Preparation of ozone

Pure oxygen (>99.5% O_2, BOC Special products) was used. Ozone was prepared by passing the oxygen through a dry glass-isolated alternating current corona discharge ozoniser, developed and built by the local School for Physics of the North-West University (USA patent 09/914,199). A UV/Vis spectrophotometer (Pharmacia Biotech Ultrospec 3000) was used to determine the O_3-concentration by measuring the absorbance of the gas mixture at 254 nm. A specially designed quartz cell was used to monitor the O_3-concentration of the O_3/O_2-gas mixture in real time while it was added to the blood. The cell was provided with an inlet, where the gas entered the cell from the ozoniser, and an outlet, where the gas left the cell and directly entered the glass syringe containing the blood. Ozone concentrations varying between 75 and 84 μg/ml (3.5-4.0% O_3) were used for AHT.

2.2. Treatment of baboons

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 male and female baboons, weighing between 8 and 25 kg, were used. The baboons were maintained in the animal facility of the North-West University, Potchefstroom Campus, on standard laboratory chow. Water was available ad lib. The baboons were individually caged in a room at constant room temperature and with automated day-night cycles. The baboons were anaesthetised with ketamine hydrochloride (±10mg/kg) each time before blood was drawn or reinfused. The therapeutic window range of O_3-concentrations for AHT is 20-80

µg/ml per gram of blood [20]. In order to ensure that a definite effect (good or bad) will be seen, we decided to use high O_3 concentrations of 75-84 µg/ml.

Blood (5% of total blood volume) from 5 baboons was drawn in heparin in polypropylene syringes. The blood was then transferred to siliconised glass syringes and ozonated by adding an equal volume of an O_2/O_3- gas mixture, containing 3.5-4.0% (75-84 µg/ml) O_3. After gently mixing the blood for 20 minutes, the gas was carefully removed from the syringe and a blood sample was taken to assess the effect of ozonation. The remainder of the treated blood was then reinfused into the donor. Blood samples were collected in Vacutest tubes containing heparin directly before reinfusion of the ozonated blood, and again at 0.5, 1.0, 4.0, 24 and 48 hours following reinfusion.

Autohaemotherapy with oxygen-treated blood (O_2-control; n = 5) and also untreated blood (Ketamine control; n = 3) was performed to serve as controls. These baboons were also anaesthetised with ketamine hydrochloride every time before blood was drawn or reinfused. Figure 1 is a schematic layout of the steps that were followed during the AHT procedure.

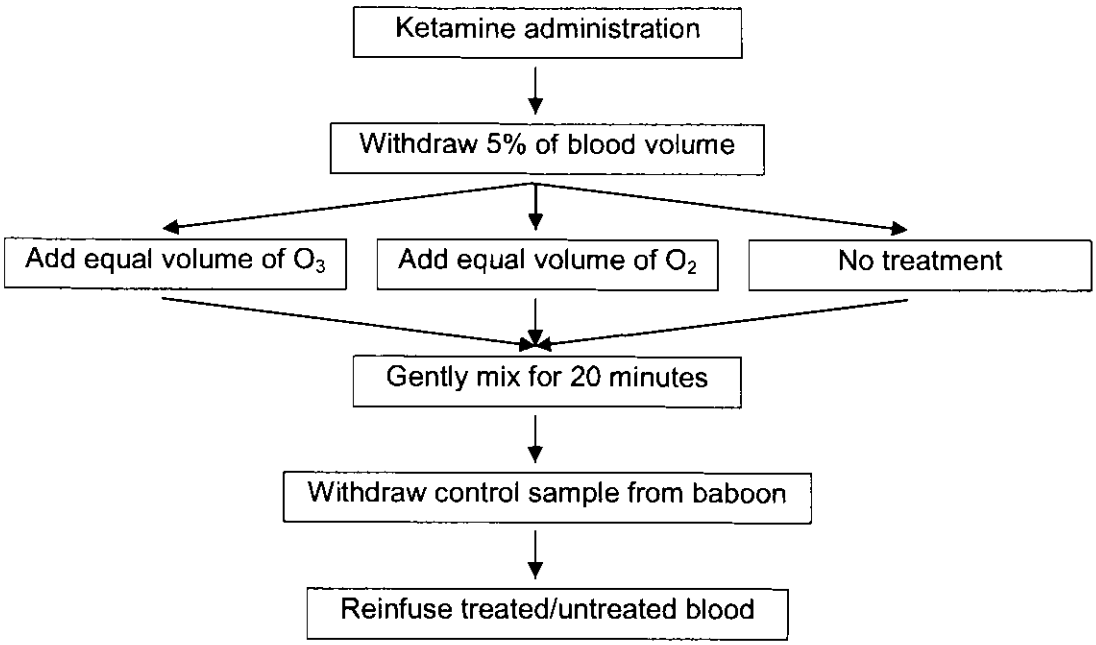

Figure 1. The protocol that was followed during AHT with O_3 (n = 5), O_2 (n = 5) and the Ketamine control (n = 3).

2.3. Comet assay

2.3.1. Materials

Hydrogen peroxide (H_2O_2), Na_2HPO_4 and KH_2PO_4 were obtained from Merck, South Africa. High melting point agarose (HMPA) was obtained from Techomp Ltd and low melting point agarose (LMPA) from Roche. All other reagents were obtained from Sigma-Aldrich Co., St. Louis, USA.

2.3.2. Preparation of cells

Lymphocytes were isolated from whole blood by layering 2ml of whole blood on top of 2ml Histopaque® in a polypropylene tube and centrifugation at 5000 x g for 30 minutes at room temperature. The cells (buffy coat layer) were removed, and washed twice in 300 µl PBS. The final cell pellet was suspended in 500 µl PBS. Of this cell suspension 40 µl was used to prepare the control slide. To assess the DNA repair capacity of the isolated cells, hydrogen peroxide (H_2O_2) was added to 400 µl cell suspension to a final concentration of 60 µM and incubated for 20 minutes at 37°C. The cells were then washed with PBS, suspended in 450 µl HAMS culture medium and incubated at 37°C. Subsequently, slides were prepared from this cell suspension after 10, 20 and 30 minutes of incubation.

2.3.3. Single cell gel electrophoresis (SCGE)

The SCGE assay was performed as described by Singh *et al.* [21], but with a few modifications to fit local laboratory conditions. Briefly, microscope slides were coated with 300 µl of 1% high melting agarose (HMPA). The slides were kept at 4°C to solidify the agarose. The PBS cell suspension (40 µl) was mixed with 150 µl of 0.5% low melting agarose (LMPA). Slides were prepared by spreading 130 µl of this cell/LMPA mixture on the pre-coated slides. The slides were kept at 4°C to solidify the agarose, whereafter they were immersed in lysis solution for at least 1 hour at 4°C. The slides were then placed in an electrophoresis chamber

containing 0.6 M NaOH and 0.05 M EDTA at 4°C for 30 minutes before undergoing electrophoresis (300 mA, 30 Volts, 40 min) in the same solution. The slides were rinsed in distilled water and then soaked in neutralising buffer for 15 minutes before staining with 5 μg/ml etidium bromide. The slides were examined under a fluorescence microscope (Olympus IX70) and images of 50 randomly selected cells were analysed.

When DNA strand breaks occur, the super coiling of the DNA is released and allows the DNA to extend toward the anode during electrophoresis, resembling a comet when observed under a fluorescent microscope. The length of the tail and the relative amount of DNA in the tail reflect the extent of DNA damage. The degree of DNA damage and repair in each cell was determined by measuring the Tail DNA% of the comets using the CASP (comet assay software project) programme.

2.4. Statistical analysis

All values are expressed as means ± standard deviation. Significant differences between measurements were assessed within a group using Student's t-test for paired measurements. Values for each time period were compared with that of the control samples taken before reinfusion. Changes were considered significant when $p < 0.05$.

3. Results

A typical result of the comet analysis of a single blood sample is given in figure 2. DNA damage was measured before H_2O_2 treatment of the cells (control), directly after H_2O_2 treatment and again after 10, 20 and 30 minutes of incubation in HAMS culture medium. The level of DNA damage in the control sample was low (tail DNA% of comets < 10%) and after treating the lymphocytes with H_2O_2 the tail DNA% increased to 70%. During the subsequent 30 minutes of incubation, DNA was repaired to almost the original level (~12% tail DNA). A similar pattern in DNA damage and repair was seen for all the animals.

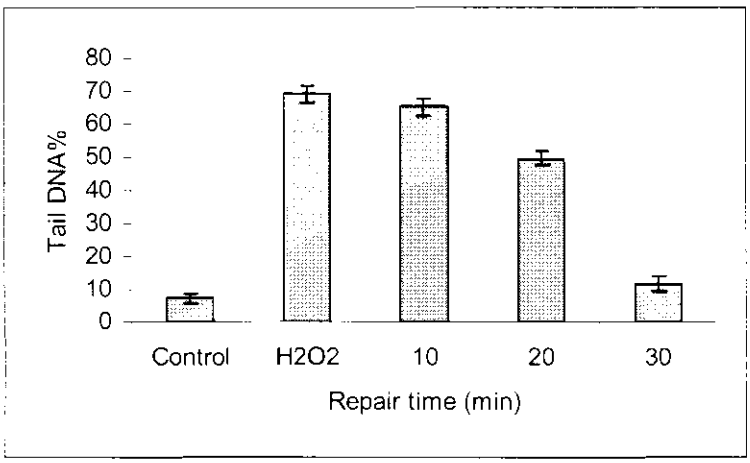

Figure 2. DNA damage and repair in lymphocytes of an individual baboon. Cells were analysed before (control) and after treatment of the sample with H_2O_2 and again after 10, 20 and 30 minutes of incubation to allow for DNA repair to take place.

In figure 3 linear regression analysis was done (using the last four data points in figure 2) to determine the rate of repair of the damaged DNA during the 30 minute incubation time. The slope of the linear function was calculated and related to the DNA repair capacity of the lymphocytes. A steeper slope represents a better DNA repair capacity. These manipulations allowed us not only to measure the effect of Ketamine and ozone on the integrity of DNA in single cells but also on the DNA repair capacity of the cells. After processing the results obtained for each baboon, the mean result was calculated in each case

for the O_3-treated group, the O_2-control group, and the Ketamine control group, respectively. Only the means of each group are presented.

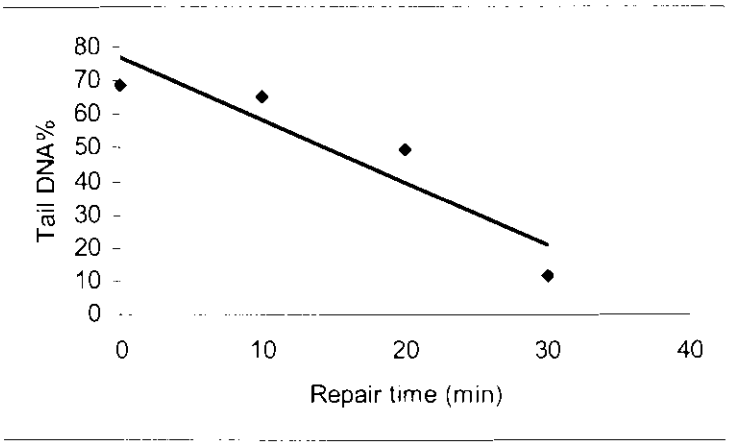

Figure 3. Linear regression analysis of Comet assay results to give an indication of the DNA repair capacity of the cells.

After treating the blood with ozone the DNA damage of the lymphocytes were less than that measured in the control blood (Figure 4). The opposite was true for the ketamine control group where DNA damage was significantly higher in the blood that was removed for reinfusion than in the control blood for this group. In the O_2-control group there was no marked difference in DNA damage levels of the control and treated blood.

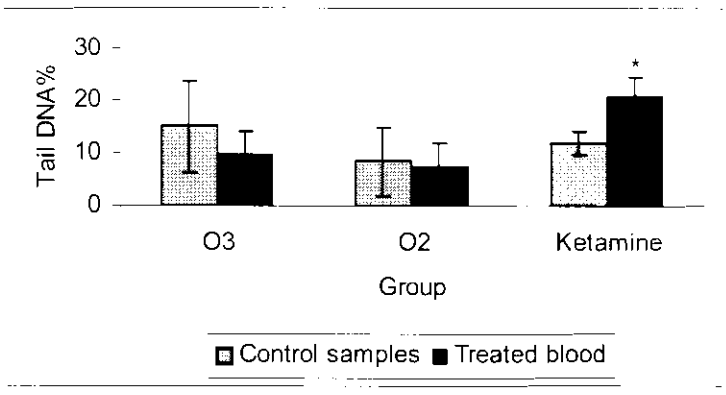

Figure 4. DNA damage measured in the control blood and the *in vitro* ozonated (O_3), oxygenated (O_2) or untreated (Ketamine) blood. Values are given as mean ± SD. (*Significant at $p < 0.05$)

Figure 5 shows the mean basal DNA damage in lymphocytes present in the blood of the baboons over the 48 hour time period following reinfusion. Although a slight decrease in basal DNA damage was observed after four hours, the O_3-treated group did not show major changes in basal DNA damage during the first 4 hours following reinfusion. This was followed by an increase in DNA damage reaching a maximum after 24 hours. This increase was, however, not significant and after 48 hours the basal DNA damage was the same as in the control sample. No significant changes were observed in the O_2-control group and, in general, the observed DNA damage in this group was less than in the O_3-treated group. Except for the 24 hour samples, higher levels of basal DNA damage was observed for the Ketamine control group than for both the O_3 - and the O_2-treated baboons. At 24 hours following reinfusion, DNA damage was drastically and significantly decreased in the lymphocytes of the Ketamine control group while, in contrast, a maximum was observed in the O_3 -treated group. At 48 hours following reinfusion this effect was reversed in both groups. Throughout the 48 hour period the changes occurring in the ketamine control group were opposite to that in the O_3-treated group, i.e. when the tail DNA% in the cells of the O_3-treated group was low, the tail DNA% in the control group was high.

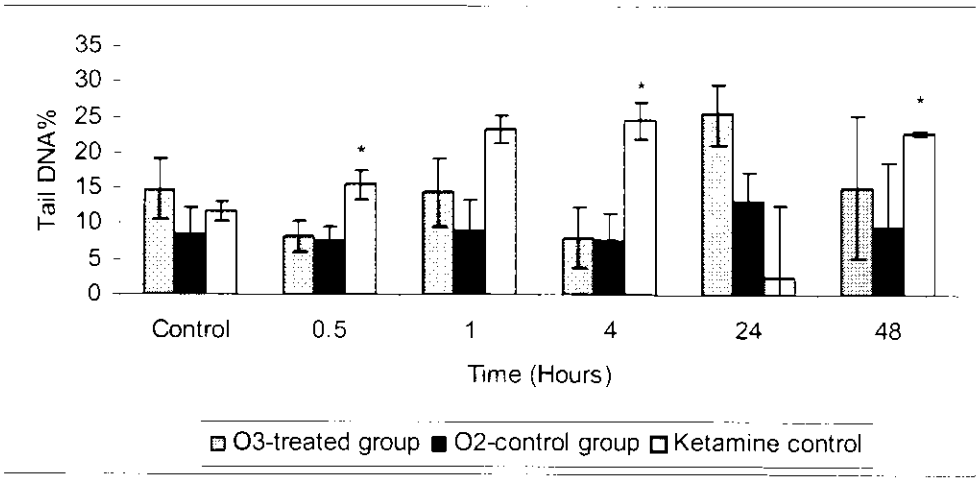

Figure 5. Basal DNA damage in lymphocytes. DNA damage was determined before reinfusion (control) and 0.5 to 48 hours after reinfusion. Values are given as mean ± SD. (*Significant at $p < 0.05$)

The DNA repair capacity was markedly reduced after O_3-treatment (Figure 6). In the O_2-control group the effect was less severe while the DNA repair capacity in the Ketamine control (untreated) group seems to have been unaffected.

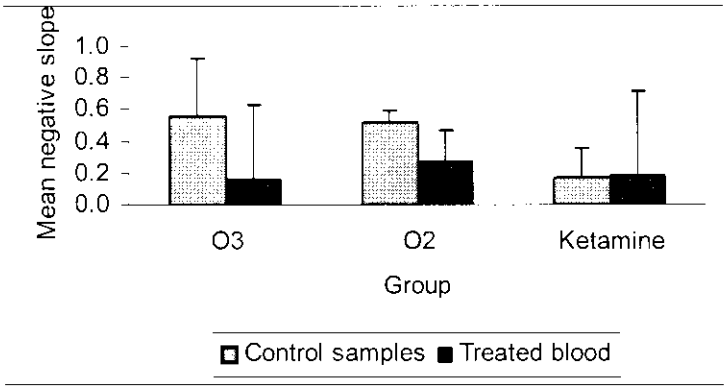

Figure 6. DNA repair capacities measured in the control blood and the *in vitro* ozonated (O_3), oxygenated (O_2) or untreated (Ketamine) blood. Values are given as mean ± SD.

In figure 7 the negative slopes of the linear functions were plotted. The O_3-group showed an increase in the ability of the cells to repair their DNA within the first 4 hours after reinfusion. After 24 hours the repair capacity normalised. A similar, but delayed effect was observed in the O_2-controls, where, one hour after reinfusion, DNA repair had reached a minimum level after which there was a gradual increase to a maximum at 48 hours. In the Ketamine control group, DNA repair was markedly decreased at 0.5 hours following reinfusion. After increasing and reaching a maximum after 24 hours, DNA repair was again dramatically reduced at 48 hours. Similar to the observations for DNA damage in figure 4, the changes observed in DNA repair capacity at 4 and 24 hours following reinfusion were exactly opposite in the O_3-treated group and the Ketamine control group.

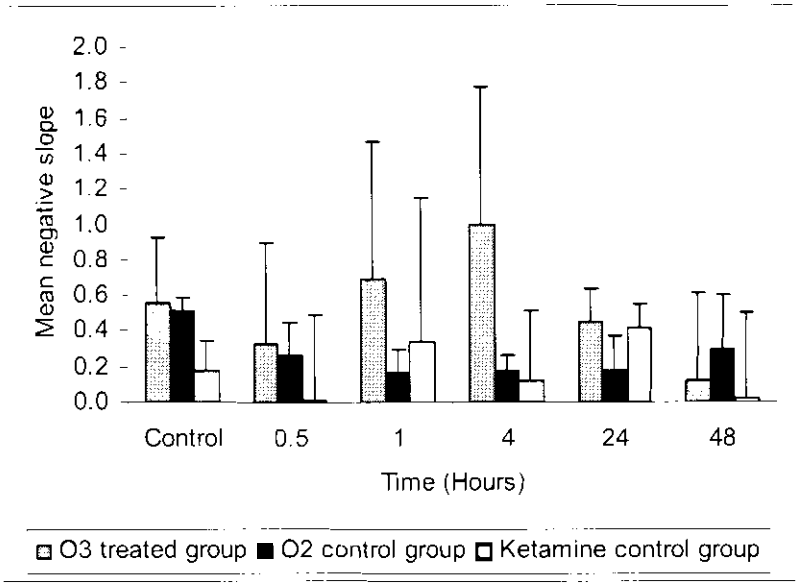

Figure 7. Mean negative slopes representing the DNA repair capacity of lymphocytes in the O_3-treated group, the O_2-treated group and the Ketamine control group. Repair capacities were determined before reinfusion (control) and 0.5 to 48 hours after reinfusion and are represented as the rate of DNA repair during the 30 minute incubation time. Values are given as mean slopes ± SD.

4. Discussion

DNA damage

Diaz-Llera *et al.* [18] reported that O_3 induces DNA damage in primary leukocytes in whole blood diluted with PBS. Ozone is a strong oxidant and gives rise to other ROS the moment it dissolves into blood, which makes it potentially genotoxic. As a result, we expected the levels of DNA damage in the ozonated blood to be higher than before ozonation (control blood). Surprisingly, the opposite was observed for the blood treated with O_3 (Figure 4). Blood plasma is rich in antioxidants such as GSH and urea and we suggest that the direct oxidative effect of O_3 and its derivatives was quenched by these antioxidants present in whole blood. In contrast, the results reported by Diaz-Llera *et al.* [18] can be attributed to insufficient protection by plasma antioxidants because the

blood was diluted in PBS. It should, however, be noted that the control samples in our study were drawn from the animals immediately before reinfusion of the treated blood, i.e. after the baboons were anaesthetised and 5% of their total blood volume was removed. Physical and emotional stress could therefore have caused the level of DNA damage in the control samples to be higher than in the ozonated blood. If this was true one would expect to see the same result for both the O_2 - and Ketamine control groups, but this is not the case. No marked changes occurred in the O_2-control group while DNA damage was significantly lower just before reinfusion in the Ketamine control group (Figure 4). Further investigation is necessary in order to explain the reduction in DNA damage observed in the ozonated blood.

If we keep in mind that the reinfused blood was drawn approximately 5 min after ketamine administration and 20 minutes before the control samples, we can assume that when the 5% blood volume was removed systemic ketamine levels were still relatively high, while, by the time the control blood was drawn (just before reinfusion), ketamine levels should have been less. From our results in figure 4 it therefore appears as if ketamine hydrochloride may have induced DNA damage. The damage to the DNA occurred within five minutes after Ketamine injection but it was also rapidly repaired because DNA damage in the control samples was significantly reduced. This seemingly fast genotoxic effect of Ketamine also explains the cumulative increase in DNA damage that was observed during the first 4 hours when Ketamine was sequentially used to keep the animals anaesthetised. The significant reduction in DNA damage at 24 hours following reinfusion was probably due to up-regulation of DNA repair mechanisms (Figure 7). The effect was, however, short-lived. This is evident in the marked reduction in DNA repair and increased levels of DNA damage at 48 hours following reinfusion. The level of DNA damage after 48 hours was comparable to that initially induced in the reinfused blood (~20%; Figure 5) and can therefore also be attributed to the action of systemic Ketamine. The strikingly opposite nature of the changes that occurred in the O_3 and Ketamine groups between the four and 24 hour data points could indicate that the mechanisms of

action of ozone and Ketamine to inflict DNA damage are different and consequently the DNA repair mechanisms that are involved will be different. Although it appears as if Ketamine causes DNA damage, it is not clear what the effect of Ketamine and its metabolites on DNA integrity is and we do not know what type of damage it induces or which repair mechanisms are involved. Therefore it is difficult to explain the changes observed for the Ketamine control group.

Both ozone and oxygen seem to have had a protective effect against the DNA damaging effect of Ketamine hydrochloride during the first four hours following reinfusion (Figure 5). In general, DNA damage in both the O_3- and O_2-treated groups was always less than in the Ketamine control group. The oxidative properties of ozone make it far more genotoxic than oxygen and therefore the differences that were observed between the O_2- and O_3-treated groups can be attributed to the higher reactivity of O_3. The effect that O_3-AHT had on DNA integrity was, however, limited. This is evident from our results in figure 5 where it is shown that the effect of O_3-AHT on DNA integrity was eliminated after a time period of 48 hours.

DNA repair capacity

DNA repair was markedly disrupted after treatment of the blood with O_3 (Figure 6). The generation of ROS seems to have exceeded the capacity of the cells to repair their DNA, possibly by oxidising DNA repair proteins and making them inactive. The increase in DNA repair after one hour (Figure 7) was most probably induced by ROS acting as second messengers in the activation of transcription factors such as NF-κβ and activator protein-1 (AP-1), causing an up-regulation in the expression of DNA repair enzymes to replace those that might have been inactivated by ozone. DNA repair in which AP endonuclease is involved, e.g. base excision repair, is closely coupled to activation of transcription by AP-1 [16]. Das *et al.* [22] have characterised and identified two glycosylase enzymes, NEIL1 and NEIL2, which are specifically involved in the base excision repair of active genes. The expression of human NEIL1 (hNEIL1) is activated by ROS and

the *hNEIL1* promoter contains a pair of CRE/AP-1 sequences that are involved in oxidative stress response. NEIL1 protein levels increase within 3 hours following ROS activation and maximum levels are reached within 6-9 hours before it declines to base line level after 20 hours. The same is true for activation of the hNEIL1 gene promoter [22]. This correlates with the increase in DNA repair in the O_3-treated group 4 hours after reinfusion and also the decrease observed after 24 hours. In addition, oxidative stress-inducing agents such as H_2O_2 cause an up-regulation in β-polymerase expression [23]. This enzyme is also involved in base excision repair and can therefore, together with activated glycosylases, account for the increase in DNA repair capacity following O_3-AHT.

Oxygen did not affect the DNA repair capacity of the lymphocytes in the reinfused blood (Figure 6), but after reinfusion a definite decrease in DNA repair capacity was observed (Figure 7). This was also observed for the ketamine control group and therefore it is very likely that the reduced DNA repair capacity was caused by the anaesthetic and not the oxygen. It seems as if the induction of the DNA repair processes after O_2-AHT was delayed and not as marked as in the case of O_3-AHT. Samples were not taken beyond 48 hours and we are therefore not sure to what extent DNA repair was up-regulated 48 hours after O_2-AHT or for how long this up-regulation was maintained.

In summary, our results indicate that Ketamine hydrochloride has genotoxic properties but that the DNA damage it causes is quickly and efficiently repaired. We were not able to explain the changes due to ketamine administration, but our results suggest that O_3-treatment and, to a lesser extent, O_2-treatment protected against the DNA damaging effects of ketamine hydrochloride. The clinical relevance of these findings is not clear and needs further study. Oxygen treatment did not cause damage to lymphocyte DNA. The DNA damage caused by O_3-AHT was not so severe that it could not be restored within 48 hours. Transcription of DNA repair enzymes like glycosylases was most probably activated by ROS arising from the decomposition of O_3 in blood. This caused an

up-regulation in DNA repair mechanisms within four hours following O_3-AHT, although the up-regulation was transient.

5. Acknowledgements

We would like to thank the Dean of the faculty of Natural Sciences and the National Research Foundation of South Africa for funding this project.

6. List of abbreviations

AP site	Apurinic/Apyrimidinic site
AP-1	Activator protein-1
CAM	Complementary and alternative medical therapy
CASP	Comet assay software project
EDTA	Ethylene diamine tetra acetic acid
H_2O_2	Hydrogen peroxide
HCT116	human colorectal carcinoma
HMPA	High melting point agar
$HO^{\bullet}$	Hydroxyl radical
LMPA	Low melting point agar
NEIL	family of endonuclease VIII (Nei)
O_2-AHT	Oxygen autohaemotherapy
$O_2^{\bullet-}$	Superoxide anion
O_3-AHT	Ozonated Autohaemotherapy
PBS	Phosphate buffered saline
ROS	Reactive oxygen species
SCGE	Single cell gel electrophoresis

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Chapter 4: General discussion and conclusions

4.1. Introduction

Ozone: is it good or is it bad? Major controversy exists concerning ozone. It plays a very important role in the stratosphere where the ozone layer serves as a filter to protect living organisms against the dangerous UV rays from the sun but on the other hand, tropospheric ozone is a major health hazard. Between these two extremities, lies the belief that ozone may be used as a form of alternative medical therapy to treat various medical conditions. The rationale is that O_3, as reactive oxidant, stimulates certain biological processes via generation of ROS and causes an upregulation in the AODS and immune pathways. There is, however, scepticism concerning the safety and effectiveness of O_3-therapy and not many well-controlled clinical studies have been done using O_3-therapy.

4.2. Approach to the study

The aim of the study was to assess O_3-AHT by determining the nature and extent of any changes in the oxidant/antioxidant status as well as DNA integrity and DNA repair capacity following O_3-AHT in baboons and to weigh the advantages up against the harmful effects it may have. In order to ensure that a definite effect (good or bad) will be seen, I decided to use high O_3 concentrations of 75-84 µg/ml to perform O_3-AHT. Serum hydroperoxides, total glutathione, antioxidant capacity, antioxidant enzyme activity and DNA damage and repair capacity was measured.

4.3. Major findings of the study

After evaluation of the results, I found that O_3-AHT induced a mild state of oxidative stress in baboons that resulted in oxidative DNA damage and stimulation of the AODS. In response to this, the DNA repair capacity as well as the antioxidant capacity of the cells were up-regulated. No significant increase was observed in DNA damage within the first four hours following O_3-AHT, although the increase in hydroperoxides indicates that oxidative stress was induced. Total blood glutathione concentrations were already significantly reduced after ozonation of the blood and were further reduced after reinfusion of the ozonated blood. Blood glutathione levels were, however, not depleted and the 34% decrease in GSHt following O_3-AHT indicates that GSH fulfilled its function as molecular antioxidant in order to reduce the level of ROS in the blood and offer protection against oxidative stress. Together with other scavenger antioxidants such as urea and bilirubin, GSH may therefore have contributed to limit the oxidative DNA damage during the first four hours following O_3-AHT. The results further suggest that transcription of certain enzymes was induced after O_3-AHT and most probably mediated by ROS derived from O_3 decomposition. Superoxide dismutase (SOD) activity was elevated after O_3-AHT and from the increase in DNA repair capacity observed after four hours, it appears as if transcription of DNA repair enzymes were also up-regulated.

The effect of O_3-AHT was transient since most of the parameters that were measured returned to normal within 24 hours. The changes due to O_3-AHT occurred within physiological boundaries. After 48 hours hydroperoxides, antioxidant capacity, SOD activity and DNA repair capacity returned to control levels and DNA integrity was restored. After O_3-AHT only a low to mild level of oxidative stress was induced, the oxidative DNA damage that occurred was not excessive and although total blood glutathione levels were not restored 48 hours after O_3-AHT, it was not completely depleted either. It therefore appears as if patients may indeed benefit from O_3-AHT as a form of complementary and

alternative medical treatment (CAM) in that the benefit of this treatment, analogous to moderate exercise, lies in the pre-conditioning of the body for future stresses (Finkel, 2003).

4.4. Concluding remarks

The effect of repeated sessions of O_3-AHT was not studied. It may well be necessary to use repeated sessions of O_3-AHT to induce prolonged effects either to improve the current medical state of a patient, e.g. in the case of diabetes mellitus, or to precondition the cells and organs of the patient for future exposure to oxidative stress, e.g. during ischemia-reperfusion of the heart or liver. It is important to note that patients whose AODS is already under severe pressure may not benefit from O_3-AHT. This might worsen their condition instead of having therapeutic value. Evaluation of the antioxidant capacity and basal DNA integrity prior to O_3-AHT, is therefore of utmost importance. This, in turn, requires that medical doctors and therapists must be fully educated about ozone, its toxicity, its biological action, its therapeutic potential and the correct handling of ozone. They should also be trained to safely perform O_3-therapy. Only then will patients, in contrast to the findings of Furnham (2000), accept and trust O_3-therapy to be safe and effective.

4.5. Future directions

Ketamine hydrochloride was used as anaesthetic in this study to allow handling of the baboons. Ketamine is short acting and does not affect respiratory or vascular function and was therefore the anaesthetic of choice. In retrospect, it appeared not to be the best anaesthetic to use because ketamine hydrochloride may have antioxidative properties and could have influenced the results obtained from the ORAC assay by reacting with ROS to produce a relatively stable nitroxide ion and so improving the antioxidant capacity of the sample. It also became apparent that it has genotoxic properties, and therefore affected the results obtained from the Comet assay. In order to eliminate the problems

inherent to the anaesthetic, strapping the baboons in a specially designed 'operating chair' may be an alternative option. The baboons can then be kept conscious while blood is drawn or reinfused via a butterfly that remains fitted in the vain for the duration of the procedure.

The exact explanation for the biochemical changes that were observed lies beyond the scope of this study, namely on the level of signal transduction. The precise mechanism for induction of certain processes, such as DNA repair, by ROS as second messengers needs further study.

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Addenda

Addendum A

Calculation of results

- ***GSH redox state***

A linear standard curve was used to determine the GSH concentration in each sample. The total GSH concentration (GSHt) was calculated as follows:

GSHt = μM GSH x dilution factor (488)

- ***Oxygen radical absorbance capacity***

Results were calculated as follows, using Microsoft Excel:

$$S = \left(0.5 + \frac{f_5}{f_0} + \frac{f_{10}}{f_0} + \frac{f_{15}}{f_0} + \ldots\ldots + \frac{f_{65}}{f_0} + \frac{f_{70}}{f_0}\right) \times 5$$

where f_0 is the initial fluorescence at 0 minutes, f_i is the fluorescence measured at time i and S is the area under the curve (Figure 1).

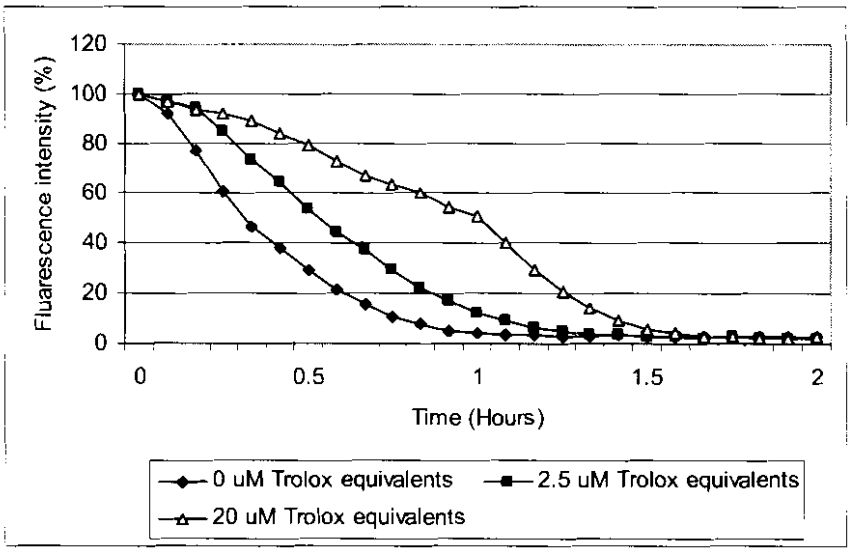

Figure 1. Principle of the ORAC assay. The figure illustrates the difference in inhibitory action of antioxidants of different strength.

A standard curve with 2nd order polynomial slope ($y = ax^2 + bx + c$) was drawn from the standard series. The final results (ORAC values, expressed using Trolox equivalents) for each sample were calculated by using the following equation:

$$\text{ORAC value } (\mu\text{M TE}) = x = \frac{-b + \sqrt{(b^2 - 4(c - y)a}}{2a} \times \text{dilution factor}$$

where $y = (S_{sample} - S_{blank})$.

- ***Catalase activity***

It is not possible to define international catalase units (U) according to IUB recommendations due to the abnormal kinetics of catalase. Use of the rate constant of a first-order reaction (k) is recommended.

For a time interval of 15 seconds the following relationship was used:

$$k = \left(\frac{2.3}{15}\right)\left(\log\frac{A_1}{A_2}\right)$$

where $A_1 = A_{240}$ at t = 0, and $A_2 = A_{240}$ at t = 15 sec. To calculate k/ml or k/g Hb the following formulas were used:

- k/ml = ka
- $\text{k/g Hb} = (\text{k/ml}) \times \frac{1000}{b} = \left(\frac{2.3}{15}\right)\left(\frac{a}{b}\right)\left(\log\frac{A_1}{A_2}\right)\ (\text{sec}^{-1})$

where a = dilution factor ([Hb] in blood or erythrocyte sediment/[Hb] in cuvette in mg Hb/ml), and b = Hb content of blood or erythrocyte sediment (grams/litre).

Addendum B

Comet images of DNA

The Comet assay is a sensitive method to assess DNA integrity and DNA repair capacity in isolated lymphocytes. Cells are embedded in agarose and lysed, electrophoresed, and fluorescently stained with etidium bromide. Breaks in the DNA release its supercoiling and allow DNA to extend toward the anode, resembling a comet when observed under a fluorescent microscope (Figure 2). The length of the tail and the relative amount of DNA in the tail reflect the extent of DNA damage.

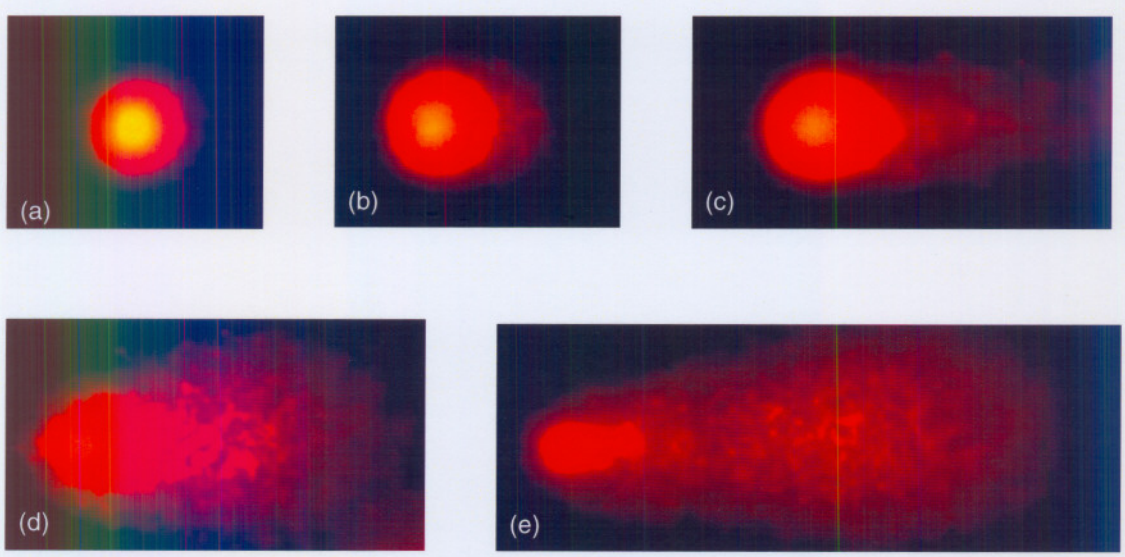

Figure 2. Comet assay images showing DNA comets with a) <6%, b) 6-17%, c) 17-35%, d) 35-60%, and d) 60-100% tail DNA.

Addendum C

Free Radical Biology & Medicine

Guide for Authors

Free Radical Biology & Medicine is an international, interdisciplinary publication encompassing chemical, biochemical, physiological, pathological, pharmacological, toxicological, and medical approaches to research on free radicals and oxidative biology. The journal welcomes original contributions dealing with all aspects of free radical and oxidant research including both in vitro and in vivo studies.

PREPARATION OF PAPERS

Full-Length Papers

The entire text, including figure and table legends and the reference list, should be double-spaced, leaving a left margin of approximately 3 cm (1 inch). All pages should be numbered consecutively and carry a running title, in the upper right corner, starting with the title page of the manuscript. Every new paragraph should be clearly indented.

Title Page

Page 1 should be concise, descriptive, and informative. It should include (1) the title of the article (80 spaces maximum); (2) the authors' full names (first name, middle initial(s), and surname); (3) affiliations (the name of department (if any), institution, city, and state or country where the work was done), indicating which authors are associated with which affiliations; (4) acknowledgments of grant support and of individuals who were of direct help in the preparation of the study; (5) the name, address, telephone and fax numbers, and e-mail address of the

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Abstract and Keywords

Page 2 should include the title of the article, followed by the abstract, which should have no more than 200 words. The abstract should be self-explanatory and intelligible without reference to the body of the paper and with an emphasis on the new aspects of the study. Because abstracts are increasingly used by abstracting services which will cut off after a fixed number of words, it is important not to exceed the maximum number of words and to avoid bibliographic references and nonstandard abbreviations. Following the abstract, list keywords for indexing. These keywords should cover precisely the contents of the submitted paper and should give readers sufficient information as to the relevance of the paper to their particular field.

Text

After the abstract, **Original Contributions** should be organized in the following format: Introduction, Materials and Methods (or Experimental Procedures), Results, Discussion, Acknowledgments, List of Abbreviations, References. Authors may insert a short Summary/Conclusions section following the Discussion section if they wish. In some cases, Results and Discussion sections may more appropriately be combined than separated (at the author's discretion). Every effort should be made to avoid jargon, to spell out all nonstandard abbreviations the first time they are mentioned, and to present the contents of the study as clearly and as concisely as possible.

References

Type references double-spaced and number them consecutively in the order in which they are first mentioned in the text, not alphabetically. Cite references in the text, tables, and legends in sequential, numerical order, placing the numbers in square brackets. References cited only in tables or figure legends should be

numbered in accordance with a sequence established by the first mention in the text of the particular table or figure. Journal titles are to be abbreviated according to the *List of Journals Indexed in Index Medicus* published by the U.S. Department of Health and Human Services. Examples of reference style are as follows:

Journal:

[1] Smith, M. A.; Casadesus, G.; Joseph, J. A.; Perry, G. Amyloid-β and τ serve antioxidant functions in the aging and Alzheimer brain. *Free Radic. Biol. Med.* **33**:1194-1199; 2002.

Book:

[2] Sen, C. K.; Packer, L.; Hänninen, O., eds. *Handbook of oxidants and antioxidants in exercise.* Amsterdam: Elsevier; 1999.

Chapter in edited book:

[3] Zuo, L.; Clanton, T. L. Detection of reactive oxygen and nitrogen species in tissues using redox-sensitive fluorescent probes. In: Sen, C. K.; Packer, L., eds. *Redox cell biology and genetics, part A. Methods in enzymology, volume 352.* San Diego: Academic Press; 2002: 307-325.

Abstract:

[4] Freeman, B.; Aslan, M. Tissue oxidation and nitration reactions in a mouse model and humans with sickle cell disease (abstract). *Free Radic. Biol. Med.* **33**:S298; 2002.

Manuscripts that have been accepted for publication may be cited as "in press" in the reference list using the estimated year of publication:
[5] Hoshino, N.; Kimura, T.; Yamaji, A.; Ando, T. Damage to the cytoplasmic membranes of Escherichia coli by catechin-copper (II) complexes. *Free Radic. Biol. Med.* In press; 1999.

Footnotes

Footnotes should be indicated by *, †,‡, etc., but typed at the end of the reference list and keyed to the appropriate manuscript page. Footnotes should be used for references to unpublished work (including work submitted for publication), personal communications, proprietary names of trademarked drugs, and other material not appropriately referred to in the text or in the numbered reference list. Because footnotes tend to interrupt the natural flow of ideas in a manuscript, they should be kept to a minimum.

Tables

Tables should be used sparingly: they should be used only when the data cannot be presented clearly in the text. Each table and every column should be provided with an explanatory heading, with units of measure clearly indicated. The same data should not be reproduced in both tables and figures. Footnotes to a table should be indicated by superscript, lowercase letters. Tables and illustrations (along with their footnotes or captions) should be completely intelligible without reference to the text.

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Figures should be used to illustrate experimental results clearly. Illustrations for reproduction should normally be about twice the final size required as figures are often reduced to a one-column width. Symbols, lettering and lines should be sufficiently large and clear to be legible after reduction. Photographs of tissues, cells, or subcellular components should be included only when they are essential.

Figure Legends

Legends should be collated, typed double-spaced, numbered with Arabic numerals corresponding to the illustrations, and submitted on a separate page. When symbols, arrows, numbers, or letters are used to identify parts of the

illustrations, each should be explained clearly in the legend. For photomicrographs, the internal scale markers should be defined and the method of staining should be given. The legends should permit the figures to be understood without reference to the text. If the figure has been previously published, a credit line should be included and a permission letter supplied by the author.

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Stylistic details must be kept constant. Formulas for radicals follow IUPAC recommendations and contain a superscripted (not centered) dot that precedes a charge, if any. In the text, names of radicals are preferred, rather than using formulas in the middle of sentences. For names of radicals, use alkoxyl, peroxyl, and hydroxyl and not alkoxy, peroxy, etc. (correct nomenclature requires the 'l' on the end of radicals, as in methyl, hydroxyl, etc.). Use *tert*, not t-, etc., for abbreviations.

Where possible, nomenclature and abbreviations should be in accordance with internationally agreed rules. When an enzyme or compound is first mentioned in the text, specification by its code number accompanied by its systematic name (as distinct from its trivial name) is requested by the Editors, but not checked for correctness.

Official names of drugs are preferred to trade names.

Standard three-letter codes for the common amino acids, nucleosides and nucleotides, carbohydrates and for purine and pyrimidine bases may be used freely and without definition. All other abbreviations should be defined when they first appear in the text. If an extensive list of abbreviations is used, please provide an alphabetical list with definitions followed by the references at the end of the article.