THE EFFECT OF DIFFERENT OZONE CONCENTRATIONS ON WHITE BLOOD CELL ENERGY HOMEOSTASIS

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DIE EFFEK VAN VERSKILLENDE OSOON KONSENTRASIES OP WITBLOEDSELENERGIE HOMEOSTASE

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To my Family

Abstract

Ozone therapy is an alternative form of therapy that has gained attention in the last couple of years. It is believed that O_3 may exert a stimulatory effect on the antioxidant defense and immune systems and may therefore be effective in the treatment of ischemic disorders, diabetes mellitus, AIDS and other diseases. On the other hand, it is well known that O_3 is a reactive molecule that is toxic to the pulmonary system. Therefore, there remains skepticism regarding its use as a form of therapy. In order to shed some light on this, the effects of ozone autohemotherapy (O_3-AHT) on the energy homeostasis of white blood cells were investigated. The possible protective effects of the plasma antioxidant defense system during O_3-AHT, were also investigated.

Venous blood from six apparently healthy human donors was collected in heparin. In one aliquot a precise volume of blood was mixed with an equal volume of O_2/O_3 gas mixture containing 20 or 80 µg/ml O_3 for 20 minutes. In the other aliquot, the plasma was washed out and the cells resuspended in a buffered phosphate solution. The buffered blood cells were treated with the same concentrations of O_3. Control samples was either not treated or treated with a corresponding volume of O_2. Various biochemical analyses were done on the whole blood and buffered cells to determine the oxidant/antioxidant status, cell viability, apoptosis and mitochondrial function.

The higher concentration of O_3 increased oxidative stress and caused death of white blood cells. Antioxidant enzyme (catalase, glutathione reductase and glutathione peroxidase) activity and the plasma antioxidant capacity decreased, whereas superoxide dismutase levels increased slightly. Exposure to O_3 also increased caspase 3/7 activity. A decrease in mitochondrial function was measured by a decrease in ATP levels and an increase in $NADH/NAD^+$ ratio. Complex IV of the respiratory chain was almost completely inhibited by both O_3 concentrations. These results indicated that the death of white blood cells was probably through apoptosis. These effects were more evident in the absence of plasma antioxidants. Therefore, high concentrations of O_3 were damaging to

the cells, but this effect was lessened by antioxidants present in plasma. In view of the results, the use of O_3 as a therapy needs to be reconsidered.

Key words: O_3-AHT, alternative medicine, oxidative stress, mitochondrial function, apoptosis.

Opsomming

Osoon (O_3) terapie is 'n alternatiewe vorm van terapie wat aandag verwerf het in die laaste paar jare. Daar word geglo dat O_3 'n stimulerende effek op die antioksidant verdedigings stelsel en imuunstelsel het en daarom suksesvol gebruik kan word in die behandeling van diabetes mellitus, isgemiese siektes, VIGS en ander siektetoestande. Aan die ander kant is O_3 'n reaktiewe molekule wat toksies is vir die pulmonêre stelsel. Daar bestaan dus twyfel oor gebruik daarvan as 'n vorm van terapie. Om duidelikhied daaroor te kry is die effek van osoon-outohemoterapie (O_3-OHT) op die energie homeostase van witbloedselle ondersoek. Die moontlike beskermde effek van die plasma antioksidant sisteem tydens O_3-OHT is ook ondersoek.

Veneuse bloed van ses gesonde proefpersone is versamel in heparien. In een deel is 'n presiese volume bloed gemeng met 'n gelyke volume O_2/O_3 gas mengsel met 'n konsentrasie van 20 en 80 µg/ml O_3 vir 20 minute. In 'n ander deel is die plasma uitgewas en vervang met 'n fosfaatbuffer oplossing. Die gebufferde selle is ook met dieselfde O_3 konsentrasies behandel. Kontrole monsters het geen behandeling ontvang of is behandel met 'n ooreenstemmende volume O_2. Verskeie biochemiese analises is op heelbloed en die gebufferde selle uitgevoer om die oksidant/antioksidant status, seloorleweing, apoptose en mitochondriale funksie te ondersoek.

Die hoër konsentrasie van O_3 het verhoogde oksidatiewe stres en die dood van witbloedselle veroorsaak. Antioksidant ensiem (katalase, glutatioon reduktase, glutatioon peroksidase) aktiwiteit en die antioksidant kapasiteit is verminder, terwyl superoksiend dismutase aktiwiteit verhoog was. Blootstelling aan O_3 het ook verhoogde vlakke van kaspase 3/7 tot gevolg gehad. 'n Verlaging in mitochondrial funksie is ook waargeneem soos gemeet deur verlaagde ATP vlakke en 'n verhoging in die $NADH/NAD^+$ verhouding. Daar was ook amper volledige inhibisie van kompleks IV van die elektron transport ketting deur albei O_3 konsentrasies. Die resultate het daarop gedui dat seldood van wibloedselle waarskynlik deur apoptose gemedieer is. Hierdie effekte was meer

prominent in die afwesigheid van plasma antioksidante. Die hoë konsentrasies van O_3 het selskade veroorsaak, maar die skade is beperk deur antioksidante in die plasma. Na aanleiding van die resultate, moet die gebruik van O_3 as terapie heroorweeg word.

Sleutelwoorde: O_3-AHT, alternatiewe medisyne, oksidatiewe stres, mitochondriale funksie, apoptose.

Preface

The composition of this thesis was done according to the rules of the North-West Universities, Potchefstroom campus guidelines as stipulated in the manual for post graduate studies. This manual can be viewed online at http://www.nwu.ac.za/research.

Outline of the thesis

Chapter 1, the general introduction, gives some background, including the problem statement, objectives and the strategy.

Chapter 2 presents a detailed literature review on the relevant scientific literature. These include background on ozone (O_3), free radicals, the antioxidant defence systems, mitochondrial function and ozone therapy.

Chapter 3 describes the different materials and methods. This chapter also describes the statistical methods used to analyse the data.

Chapter 4 outlines the results, with an accompanying detailed discussion.

Chapter 5 includes a final conclusion and discussion. This chapter also focuses on recommendations and future perspectives.

The final part of the thesis consists of Appendixes and a reference list.

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Abbreviations and symbols

4-HNE	4-hydroxy-2,3 transnonenal
6-HD	6-hydroxydopamine
$\Delta\psi$	electrochemical gradient
α	alpha
Å	angstrom
ΔA	mean difference in absorbance
A1	first absorbance reading
A2	second absorbance reading
AAPH	2,2'azobis(2-amino-propane) dihydrochloride
ADH	alcohol dehydrogenase
ADP	adenosine diphosphate
AIDS	acquired immunity deficiency disorder
A-NH_2	N,N-diethyl-paraphenylendiamine (chromogenic substrate)
[A-NH_2]	coloured radical cation of chromogenic substrate
ANOVA	analysis of variance
ATP	adenosine triphosphate
β	beta
BCA	bicinchoninic acid
BSA	bovine serum albumin
°C	degrees Celsius
ca.	circa
Ca^{2+}	calcium
CARR U	carratelli units; 1 CARR = 0.08 mg. 100 ml^{-1} H_2O_2
CCl_4	carbon tetrachloride
CoA	coenzyme A
CoQ	coenzyme Q
$CoQH^{\cdot}$	semiquinone anion form of CoQ

$CoQH_2$	reduced coenzyme Q
CO	carbon monoxide
Cu	copper
Cu^{2+}	copper II ion
Cu_2SO_4	copper sulphate
Cyt	cytochrome
DETAPAC	diethylenetriaminepentaacetic acid
DHA	dehydroascorbic acid
DNA	deoxyribonucleic acid
DTNB	5,5'Dithiobis-2-nitrobenzoic acid
d-ROMs	Reactive oxygen metabolites assay
e^-	electron
EDTA	ethylenediamine tetra-acetic acid
FAD	flavin adenine dinucleotide
$FADH_2$	flavin adenine dinucleotide (reduced form)
Fe	iron
Fe^{2+}	ferrous iron
Fe^{3+}	ferric iron
Fe-S	iron-sulphur
FMN	flavin mononucleotide (oxidised)
$FMNH_2$	flavin mononucleotide (reduced)
FRAP	ferric reducing ability of plasma
γ	gamma
g	grams
GC-MS	gas chromatography-mass spectrometry
GM-CSF	granulocyte-monocyte colony stimulating factor
GPx	glutathione peroxidase
GR	glutathione reductase
GSH	glutathione (reduced)
GSSG	glutathione (oxidised)
H^+	hydrogen ion

HNO_2	nitrous acid
H_2O	water
H_2O_2	hydrogen peroxide
$HO_2^{\cdot}$	hydroperoxyl
HOCl	hypochlorous acid
HPLC	high performance liquid chromatography
IFNγ	interferon γ
IFNβ	interferon β
IκB	inhibitor of nuclear factor kappa B
IL2	interleukin 2
IL4	interleukin 4
IL6	interleukin 6
IL8	interleukin 8
IL10	interleukin 10
INT	iodonitrtetrazolium violet-formazan
KCl	potassium chloride
kDa	kilo Dalton
km	kilometer
K_2HPO_4	di-potassium hydrogen phosphate
LDH	lactate dehydrogenase
LMWA	low molecular weight antioxidants
LOPs	lipid ozonation products
µl	microlitres
µl/ml	microlitres/millilitres
µg/ml	micrograms/millilitres
M	molar
M2VP	1-methyl-2-vinyl-pyridinium trifluoromethane sulfonate
MDA	malonaldehyde
mg	milligrams
ml	millilitres
mM	millimolar

MPA	metaphosphoric acid
mRNA	messenger ribonucleic acid
MTT	3-[4,5-dimethylthiazol-2-yl]2,5-diphenyltetrazolium bromide
N	normality
NAD^+	nicotinamide adenine dinucleotide (oxidised form)
NADH	nicotinamide adenine dinucleotide (reduced form)
$NADP^+$	nicotinamide adenine dinucleotide phosphate (oxidised form)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
Na_2CO_3	di-sodium carbonate
$NaHCO_3$	sodium carbonate
NaH_2PO_4	di-sodium hydrogen phosphate
NaN_3	sodium azide
$NaPO_4$	sodium phosphate
NF κB	nuclear factor kappa B
ng	nanograms
nM	nanomolar
$NO^{\cdot}$	nitric oxide
$NO_2^{\cdot}$	nitrogen dioxide
N_2O_4	dinitrogen tetroxide
N_2O_3	dinitrogen trioxide
O_2	oxygen
$O_2^{\cdot-}$	superoxide
$^1O_2$	singlet oxygen
O_3	ozone
O_3-AHT	ozone autohemotherapy
OH	hydroxyl
$OH^{\cdot}$	hydroxyl radical
$ONOO^-$	peroxynitrite
ORAC	oxygen radical absorbance capacity
%	percentage
PBS	phosphate buffered saline

pCO_2	high pressure carbon dioxide
PES	N-ethyldibenzopyrazine
pH	measure of acidity: numerically equal to the negative logarithm of H^+ concentration expressed in molarity
pO_2	high pressure oxygen
ppb	parts per billion
ppm	parts per million
PUFAs	polyunsaturated fatty acids
RNS	reactive nitrogen species
ROS	reactive oxygen species
R-OOH	hydroperoxide
$RO_2^{\cdot}$	peroxyl radical
$R\text{-}O^{\cdot}$	alkoxyl radical of hydroperoxide
$R\text{-}OO^{\cdot}$	hydroperoxyl radical of hydroperoxide
Se	selenium
1SEM	1 standard error of mean
-SH groups	cystein groups
SOD	superoxide dismutase
TBARS	thiobarbituric acid-reactive substances
t-BHP	*tert*-butyl hydroperoxide
TCA cycle	tricarboxylic acid cycle of citric acid cycle
TE	trolox equivalents
TNF α	tumour necrosis factor α
TPTZ-Fe^{3+}	ferric-trpyridyltriazine complex
Tris-HCl	tris-hydrochloric acid
Trolox	6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxulic acid
U	units of enzyme activity
$UrH_2^{\cdot}$	urate radicals
UV	ultraviolet
v/v	volume per total volume
WBC	white blood cells

w/v	weight per volume
Zn	zinc

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1 Introduction

Cancer, cardiovascular disease, diabetes, chronic inflammatory disease and neurodegenerative disease are but some of the well known diseases of the 20th century. These diseases are usually age related. Unfortunately younger people are increasingly diagnosed with some of these diseases. It is important to keep in mind that free radical damage to biomolecules and oxidative stress plays an important role in the etiology of these diseases. The focus of research in these fields has also shifted to the development of new preventative and therapeutic strategies in order to limit oxidative stress and free radical damage (Halliwell and Gutteridge, 2000, Halliwell and Whiteman, 2004). Some of the research has also focussed on the use of alternative medicine. Alternative and complementary medicine are used when conventional medicine have failed and sometimes used in conjunction with conventional medicine. Ozone (O_3) therapy is only one of many alternative approaches that have gained attention in the last couple of years.

Claims are made that O_3 therapy can be used to treat various medical conditions, including diabetes mellitus (Al-Dalain *et al.*, 2001), ischaemic disorders (Ajamieh *et al.*, 2001), malaria (Viebahn-Hansler *et al.*, 2001), open wounds and ulcerations (Jordan *et al.*, 2002) and has even been proposed as a possible treatment for AIDS (Bocci, 1996a, Shallenberger, 1998). Ozone can be introduced into the body by various means. Ozone gas can be directly injected into arteries, veins or muscles, i.e. as intra-arterial, intravenous or intramuscular application. Topical application of O_3 or ozonated water is used to treat oral affections (Bocci, 2002). Intradiscal injection of an O_3 mixture has also been used to treat lumbar disk herniation (Lo Giudice *et al.*, 2004, Corea *et al.*, 2004). Since these methods are dangerous, it is not recommended (Bocci, 2002). Ozone autoheamotherapy (O_3-AHT) and rectal insufflation seems to be the methods of choice. With O_3-AHT, a specific volume of blood is drawn from the patient, mixed with a given volume of O_3/O_2 gas mixture having a predetermined O_3 concentration, and then returned to the patient. Once returned, ozonated blood is rapidly distributed to all tissues (Bocci, 2002).

Ozone can act as an oxidant either directly, when it dissolves in plasma and reacts with polyunsaturated fatty acids, antioxidants, cysteine-rich proteins and carbohydrates or indirectly, through the formation of reactive oxygen species (ROS), including hydrogen peroxide (H_2O_2) (Pryor *et al.*, 1995). The purpose of O_3-AHT is to use O_3 as a drug for a brief period to achieve certain biological effects that can block an infection, improve O_2 delivery to anoxic tissue and upregulate antioxidant system and so reverse chronic oxidative stress. This is done without directly exposing the patient to O_3 (Bocci, 2002). It has been shown that ROS, including H_2O_2 and lipid oxidation products (LOPs) generated by O_3-AHT, can enter the cells from the plasma and activate nuclear factor kappa B (NF-κB) to induce cytokine production in normal cells (Bocci, 1996b) and so enhance the immune response (Larini *et al.*, 2001, Larini and Bocci, 2005). Ozone also seems to stimulate and activate the enzymes involved in peroxide and free radical scavenging such as glutathione peroxidase, catalase and superoxide dismutase. However, these effects require that precise therapeutic concentrations of O_3 is used for a specified time. Thus, the concentration of O_3 needs to be carefully controlled. When it is too low, the oxidative effect will be quenched by the antioxidant system in the plasma. If it is too high, too much ROS will be generated with subsequent damage to tissues and cells (Larini and Bocci, 2005). In view of this, O_3-AHT may impose a potential cytotoxic hazard for patients, especially those with compromised antioxidant defence mechanisms.

Thus, although O_3 therapy appears to have certain positive therapeutic effects, there is concern regarding its toxicity and effectiveness. In addition, there is very little knowledge based on well controlled clinical studies. It is therefore important to determine the possible harmful effects of O_3-AHT in order to delineate advantages of therapy. So far only a few biochemical and pharmodynamic mechanisms of O_3 therapy have been elucidated. The extent of hemolysis of erythrocytes (Bialas *et al.*, 2001, Ballinger *et al.*, 2005), formation of plasma lipid peroxides (Bocci *et al.*, 1998), production of cytokines (Bocci, 1996b) and the level of intra-erythrocyte reduced glutathione (Ballinger *et al.*, 2005) have been evaluated. Therefore there is scope to investigate the effect of O_3 on the energy status of white blood cells.

1.1 Aims and objectives

Many studies have focused on the harmful effects of O_3 in the lung and have claimed that the toxic effects observed after inhalation of O_3 are due to the inefficient antioxidant capacity of the lungs. The antioxidant concentrations in the surfactant of the lung range between 10 - 50 μM ascorbate, 100 - 400 μM urate, 5 - 225 μM glutathione (GSH) and 5 - 500 μM α-tocopherol. In the plasma these concentrations are slightly higher at 20 - 65 μM ascorbate, 100 - 500 μM urate, 0.45 - 0.80 μM total –SH groups (cystein), 5 - 25 μM α-Tocopherol. (Plasma GSH levels are usually below 5 μM, and are given as total - SH groups) (Mudway and Kelly, 2000). It is clear that the surfactant, the first compartment that comes in contact with O_3 when inhaled, is well equipped (under normal circumstances) with antioxidants to counter the harmful effects. But O_3 remains toxic and inflammatory when it is inhaled, although it appears to be non-carcinogenic (Witschi *et al.*, 1999). Therefore the question should be asked whether O_3 can also be toxic to blood in spite of the fact that the antioxidant defence system in the blood is supposedly more than adequate to deal with the ROS generated by O_3.

This study forms part of a bigger project. Its main objective was to determine whether O_3-AHT could be used as an effective form of alternative medicine, or if it is toxic. The specific hypothesis of this study was to evaluate whether the plasma provides an effective antioxidant defence system that can protect against the harmful effects of O_3 to white blood cells. It was also important to evaluate whether low concentrations give a placebo effect or high concentrations a toxic effect. The last objective was to evaluate if the effects obtained with O_3-AHT could be measured when using O_2, i.e. a positive control. The effect of O_3 on the energy homeostasis and mitochondrial function has to my knowledge not been published in the literature.

The strategy was to use a model where the effects of O_3 could be determined on white blood cells in an intact antioxidant defence system (whole blood, including plasma

antioxidants) and on white blood cells were the antioxidant system was removed (without plasma antioxidants). In the latter case the plasma, and therefore the antioxidant defence system, was washed out and replaced with phosphate buffered saline (buffered cells). These two groups were then exposed to O_2 and different O_3 concentrations to evaluate possible positive and/or negative effects to attain the objectives of the study.

The effect of O_3 on the following biochemical parameters were measured:

1. The antioxidant status. This was achieved by measuring oxidative stress and antioxidant capacity biomarkers in plasma.
2. White blood cell cytotoxicity by using the trypan blue exclusion assay. Necrosis was assessed by the lactate dehydrogenase (LDH) leakage assay.
3. Apoptosis in white blood cells, was determined by using the caspase 3/7 assay.
4. Mitochondrial function in white blood cells, by measuring ATP levels and the $NADH/NAD^+$ ratio.
5. Mitochondrial function in isolated rat liver preparations, by measuring respiration (O_2 consumption) and the activity of respiratory chain complexes.

2 Literature review

2.1 Introduction

Ozone (O_3) has become a controversial gas, not only because of its effect on the environment, but also of its use in humans as an alternative treatment against disease. Ozone is well known as a pollutant gas. When inhaled it is harmful and causes an inflammatory responses that cause lung damage (Klestadt *et al.*, 2005, Johnston *et al.*, 2000, Johnston *et al.*, 1999b), but it appears to be non-carcinogenic (Witschi *et al.*, 1999). Recently, the discovery that human neutrophils catalize the formation of O_3 *in vivo* opened new controversy. The generated O_3 may participate in an amplification of the inflammatory cascade through membrane disruption of invading micro-organisms and production of tumor necrosis factor α (TNF-α) and interleukin 8 (IL-8). The O_3 generated could also be harmful because it can react with hydrogen peroxide (H_2O_2) to generate even more toxic chemicals (Babior *et al.*, 2003). Ozone can also be formed in atherosclerotic plaques to contribute to atherosclerogenesis and the pathogenesis of the disease (Wentworth *et al.*, 2003a). Thus the discovery of new reaction pathways in which O_3 may play a critical role in normal and pathological situations, opens a new field of research (Wentworth *et al.*, 2003a, Wentworth *et al.*, 2003b).

Ozone is also advocated as an alternative form of medical therapy and has gained attention in the last couple of years. Two different schools of thought exist. The first believes that O_3 is a toxic gas and should not be used in any medical therapy. The other is of the opinion that O_3 could be used to treat a variety of pathological conditions (Bocci, 2002). This literature overview will focus on the general properties of O_3, the molecular reactivity of O_3 *in vivo*, the toxicity of O_3 and the use of O_3 as a therapeutic agent.

2.2 Properties of O_3

Ozone is a colorless to bluish gas with a characteristic pungent odor perceptible at a concentration of 0.005-0.01 parts per million (ppm). The molecule is composed of three oxygen atoms and has a molecular weight of 48.0 g/mol. It has a cyclic structure with 1.26 Å between O_2 atoms. The solubility of O_3 in water is 0.1 g/100 ml at 0°C. It is far more soluble in water than O_2. The half life of O_3 in water is approximately 25 minutes at 20°C, which is much longer than that of O_2. Ozone is very reactive and a powerful oxidizer with a redox potential of +2.07V (NIOSH; Bocci, 2002).

Ozone is present in the stratosphere surrounding the earth about 20-30 km from the surface as an ozone-layer, with a maximum concentration of 10 ppm. The O_3 is continuously formed by the action of short wavelength solar radiation (<242 nm) on molecular O_2. The ozone-layer absorbs most of the ultra violet (UV) radiation (<290 nm) emitted by the sun, including band A (316-400 nm) and bands B and C (from 100 up to 315 nm). Since the UV radiation is mutagenic, the ozone-layer has an important protective function (Mudway and Kelly, 2000, Bocci, 2002).

Ozone is also present in the troposphere (ground level to 20 km). It is present at exceptionally high levels in large cities, sometimes exceeding 100 ppb. The O_3 in the troposphere is almost entirely a secondary air pollutant, formed through a complex photochemical reaction sequence requiring reactive hydrocarbons, nitrogen oxide and sunlight. The photochemical smog that is formed has become the main toxicant affecting the respiratory tract and the eyes, nose and to a lesser extent the skin (Mudway and Kelly, 2000, Bocci, 2002). Ozone is therefore both a source of protection and of risk for all species (Manning and Tiedemann, 1995).

When introduced to an organism, O_3 interacts with biological fluids in different ways. Thus, to understand its molecular reactivity, an overview of free radicals, oxidative stress and the antioxidant defence system follows.

2.3 Free radicals and reactive oxygen species

Most biological molecules have two electrons that spin in the external orbital to make it stable. Free radicals, on the other hand contain one or more unpaired electron which makes them unstable and reactive (Bland, 1995, Curtin *et al.*, 2002, Halliwell and Gutteridge, 2000). They are formed by the loss or gain of a single electron from a non-radical. Free radicals can react with nearby atoms or molecules in different ways. A free radical can react with a non-radical molecule to form a new radical to start a chain reaction where the new radical acts as a reducing or oxidizing agent. A radical can also react with another free radical to combine their unpaired electrons to form a stable covalent bond (Bocci, 2002, Halliwell and Gutteridge, 2000).

Table 2.1. Different types of ROS, RNS and important radical molecules (adapted from Halliwell and Gutteridge, 2000, Curtin *et al.*, 2002).

	ROS		RNS	
	Name	**Symbol**	**Name**	**Symbol**
Radicals	Hydroxyl	$OH^{\cdot}$	Nitric oxide	$NO^{\cdot}$
	Peroxyl	$RO_2^{\cdot}$	Nitrogen dioxide	$NO_2^{\cdot}$
	Alkoxyl	$RO^{\cdot}$		
	Hydroperoxyl	$HO_2^{\cdot}$		
	Superoxide	$O_2^{\cdot-}$		
Non-radicals	Hypochlorous acid	HOCl	Nitrous acid	HNO_2
	Ozone	O_3	Dinitrogen tetroxide	N_2O_4
	Singlet oxygen	$^1O_2$	Dinitrogen trioxide	N_2O_3
	Hydrogen peroxide	H_2O_2	Peroxynitrite	$ONOO^-$

Different types of free radicals include the hydrogen atom, most transition metals, oxides of nitrogen and the singlet O_2 molecule (Halliwell and Gutteridge, 1984). Reactive oxygen species (ROS) is a collective term that describes radical and non-radical derivatives of O_2. Reactive nitrogen species (RNS) includes species that are derived from

nitrogen (Halliwell and Gutteridge, 2000). The different types of ROS and RNS and a few important radical molecules are summarized in Table 2.1. ROS includes O_2 radicals such as $O_2^{\cdot-}$, $OH^{\cdot}$, $RO_2^{\cdot}$ and $RO^{\cdot}$ and certain nonradicals such as HOCl, O_3, $ONOO^-$, singlet oxygen ($^1O_2$) and H_2O_2 which are either oxidizing agents or easily converted into radicals (Guetens *et al.*, 2002). The term reactive is not always appropriate, because not all of the radical and non-radical species are equally reactive. The most reactive and biological important free radical is $OH^{\cdot}$, which reacts quickly with most molecules (Halliwell and Whiteman, 2004).

Cells are exposed to ROS derived from both exogenous and endogenous sources. Exogenous sources include UV radiation, γ-radiation, ultrasound, pollutants and toxins (Kohen and Nyska, 2002). The major endogenous sources include the mitochondrion, endoplasmic reticulum, plasma membrane and cytosol (Curtin *et al.*, 2002). ROS are generated by macrophages and neutrophils, which phagocytose microbes (Brenneisen *et al.*, 2005). Certain diseases, such as ischeamic disorders and metal imbalances can also lead to the generation of ROS (Kohen and Nyska, 2002). Mitochondria use O_2 to produce adenosine triphosphate (ATP) via mitochondrial electron transport. The mitochondria are also a major source of free radicals. Under physiological conditions, electrons that are carried by the electron transport chain can leak out of the pathway and pass directly to O_2 to generate $O_2^{\cdot-}$ (Raha and Robinson, 2000, Halliwell, 1994). Other sources of $O_2^{\cdot-}$ include enzymes such as cytochrome P450 in the endoplasmic reticulum, lipoxygenases, cyclooxygenases, xanthine oxidase and NADPH oxidase. The dismutation of $O_2^{\cdot-}$ results in the generation of H_2O_2 which can react with iron (Fe^{2+}) via the Fenton reaction to form $OH^{\cdot}$ (Cross *et al.*, 1987, Halliwell and Gutteridge, 2000). Excess $O_2^{\cdot-}$ can also be converted to $OH^{\cdot}$. The reaction is catalyzed by transition metals, i.e Fe^{2+} via the Haber-Weiss reaction (Table 2.2) (Halliwell, 1978, Yamazaki and Piette, 1990, Bland, 1995, Cross *et al.*, 1987, Halliwell and Gutteridge, 2000).

Table 2.2. Generation of ROS by the Haber-Weiss and Fenton reactions (adapted from Halliwell, 1978).

Fenton reaction	$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\bullet} + OH^-$
Haber-Weiss reaction	$O_2^{\cdot-} + H_2O_2 \xrightarrow{\text{Metal catalyst}} O_2 + {}^{\cdot}OH + {}^{\cdot}OH$

Hydroxyl radicals can generate further ROS and organic radicals by interaction with biological macromolecules. Lipid peroxyl radicals ($RO_2^{\cdot}$) and lipid hydroperoxides (ROOH) are produced during lipid peroxidation of biological membranes (Curtin *et al.*, 2002). ROS can react with deoxyribonucleic acid (DNA), proteins, carbohydrates and lipids in a destructive way. As a result, ROS has been implicated in more than 50 human diseases including cancer, diabetes and neurodegenerative disorders (Guetens *et al.*, 2002, Curtin *et al.*, 2002).

On the other hand free radicals, including ROS and RNS, are important in a variety of physiological functions. These include regulation of the vascular tone via $NO^{\cdot}$ (Klein, 2002, Griendling *et al.*, 2000), sensing of O_2 tension and subsequent regulation of functions that are controlled by the O_2 concentration, enhancement of signal transduction from various membrane receptors and oxidative stress responses that ensures the maintenance of redox homeostasis ($O_2^{\cdot-}$ and related ROS) (Droge, 2002). Hydrogen peroxide has been implicated as a second messenger in the start and amplification of signalling of the antigen receptors of lymphocytes (Reth, 2002, Soberman, 2003).

2.4 The antioxidant defense system

In response to damage by ROS, cells have developed defence mechanisms (Figure 2.1). It includes repair mechanisms, adaptation and physical defences. Repair mechanisms repair the damage caused by free radicals and include lipase, protease and DNA repair enzymes (Guetens *et al.*, 2002). Adaptation means an increased syntheses of antioxidant

enzymes (Halliwell and Gutteridge, 2000). Physical defences include stabilization of biological membranes by vitamin E and other substances, including phospholipids (Kohen and Nyska, 2002). The antioxidant defence system is very important and consists of radical scavenging antioxidants and preventative antioxidants (Halliwell and Gutteridge, 2000).

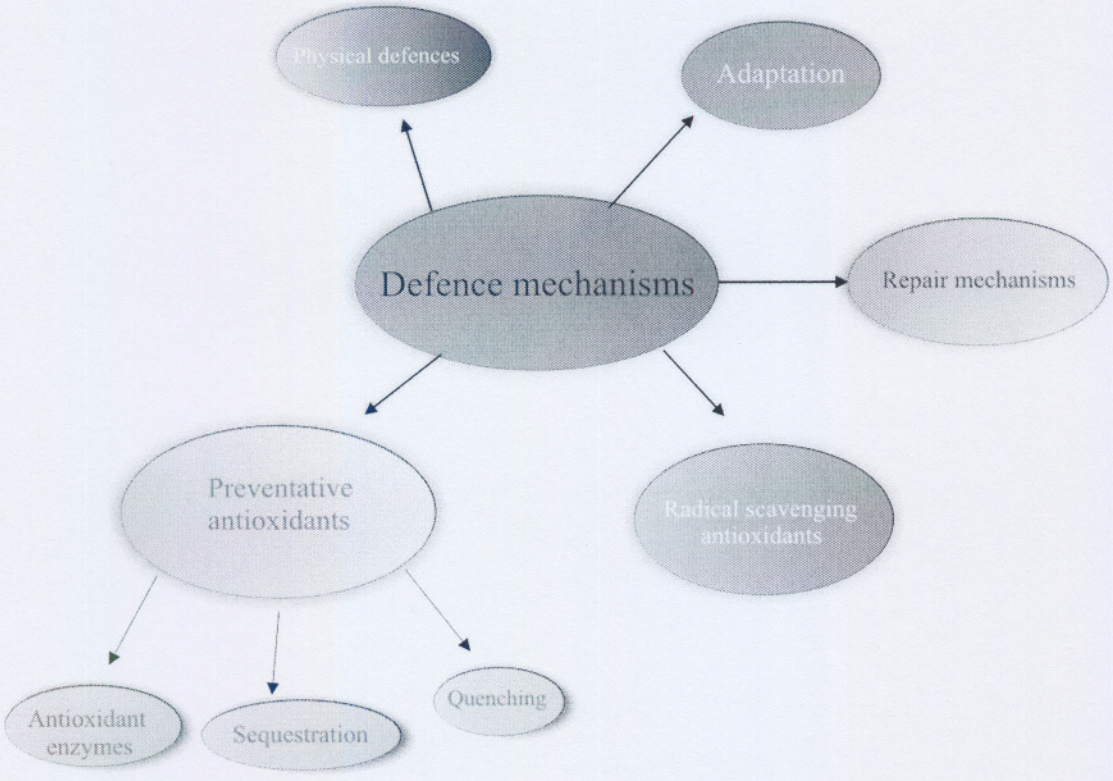

Figure 2.1. The different defence mechanisms against oxidative damage (modified from Rimbach *et al.*, 1999, Halliwell and Gutteridge, 2000).

Antioxidants are substances that can, at relatively low concentrations, compete with other oxidizable substances to significantly delay or inhibit the oxidation of these substrates (Halliwell and Gutteridge, 2000). Under normal circumstances, antioxidant systems minimize the damage that is caused by ROS. Antioxidants apparently plays an important role in longevity and anti-aging (Mecocci *et al.*, 2000, Szeto *et al.*, 2002, Salgo and Pryor, 1996).

Radical scavenging antioxidants scavenge radicals by reacting directly with the radical molecule to remove it by donating an electron to the reactive species. These include low molecular weight antioxidants (LMWA) such as vitamins C and E and carotenoids derived from the diet. Others include substances synthesized by the cell and include glutathione (GSH), uric acid, billirubin and albumin (Halliwell and Gutteridge, 2000). The interest in the role of micronutrient elements such as selenium (Se) and zinc (Zn) that are integral parts of protective enzymes via special amino acids (selenocysteine, selenomethionine) or structural components (Zn-metallothionein) are growing (Brenneisen *et al.*, 2005).

Vitamin C (ascorbic acid) is a hydrophilic antioxidant that is present in blood plasma at a concentration of 30-100 μM. When it reacts with ROS, it is transformed into an ascorbyl radical and then into dehydroascorbic acid (DHA). DHA may either be taken up by erythrocytes, neutrophils or other cells to be converted back into ascorbate, or it may undergo rapid non-enzymatic breakdown to produce oxidation products (Halliwell and Gutteridge, 2000). Vitamin E (α-tocopherol) is a lypophylic antioxidant found in the inner membranes and in lipoproteins. It is a chain breaking antioxidant and is important to protect lipoproteins against oxidation and lipid peroxidation. Vitamin E can interrupt the chain reaction by forming an α-tocopherol radical that is fairly stable due to the delocalization of the unpaired electron (Kohen and Nyska, 2002). Normal plasma levels of tocopherol vary between 6 and 14 μg/ml (Berman and Brodaty, 2004). Uric acid is produced by the oxidation of hypoxanthine and xanthine by xanthine oxidase and xanthnie dehydrogenase respectively. Its plasma concentration varies between 200-400 μM (Kohen and Nyska, 2002). Uric acid is a good scavenger of $OH^{\cdot}$, $O_2^{\cdot-}$, $ROO^{\cdot}$ and $ONNO^{\cdot-}$. The reaction between urate, $OH^{\cdot}$ and organic peroxyl radicals give rise to urate radicals ($UrH_2^{\cdot-}$), which in turn can be recycled through a reaction with vitamin C (Halliwell and Gutteridge, 2000).

Table 2.3. The different LMWA that act as scavenging antioxidants (adapted from Halliwell and Gutteridge, 2000).

LMWA	Scavenging mechanism
Vitamin C	Ascorbic acid ↔ Ascorbic$^{\cdot}$ ↔ DHA DHA → non-enzymatic breakdown → oxidation products
Vitamin E	α-tocopherol + ROO$^{\cdot}$ → α-tocopherol$^{\cdot}$ + ROOH
Uric acid	$RO_2^{\cdot} + UrH_2^{\cdot} \rightarrow ROOH + UrH^{\cdot -} + H^+$ $UrH^{\cdot -} + AscH^{\cdot} \rightarrow UrH_2\text{-} + Asc^{\cdot -}$

GSH is a thiol-containing tripeptide that can react with OH$^{\cdot}$, HOCl, peroxynitrite, RO$^{\cdot}$, $RO_2^{\cdot}$, and $O_2^{\cdot}$. The tripeptide (γ-Glu-Cys-Gly) not only acts as a ROS scavenger but also regulates the intracellular redox state (Figure 2.2). The levels of GSH in mammalian cells is in the millimolar range (0.5 – 10 mM), whereas in plasma it is usually in the micromolar range (Pastore *et al.*, 2003). The glutathione redox system consists of GSH, glutahione peroxidase (GPx) and glutathione reductase (GR) (Figure 2.2). GPx reduces H_2O_2 that is produced by superoxide dismutase (SOD) by dismutation of $O_2^{\cdot -}$, to form H_2O and so converts GSH to reduced glutathione (GSSG). The GSSG is then reduced back to GSH by GR at the expense of NADPH. Under normal conditions more than 95% of the GSH in a cell is reduced, meaning that the intracellular environment is highly reducing (Curtin *et al.*, 2002). However, depletion of GSH levels lowers the reducing capacity of the cell and can so induce oxidative stress (Curtin *et al.*, 2002).

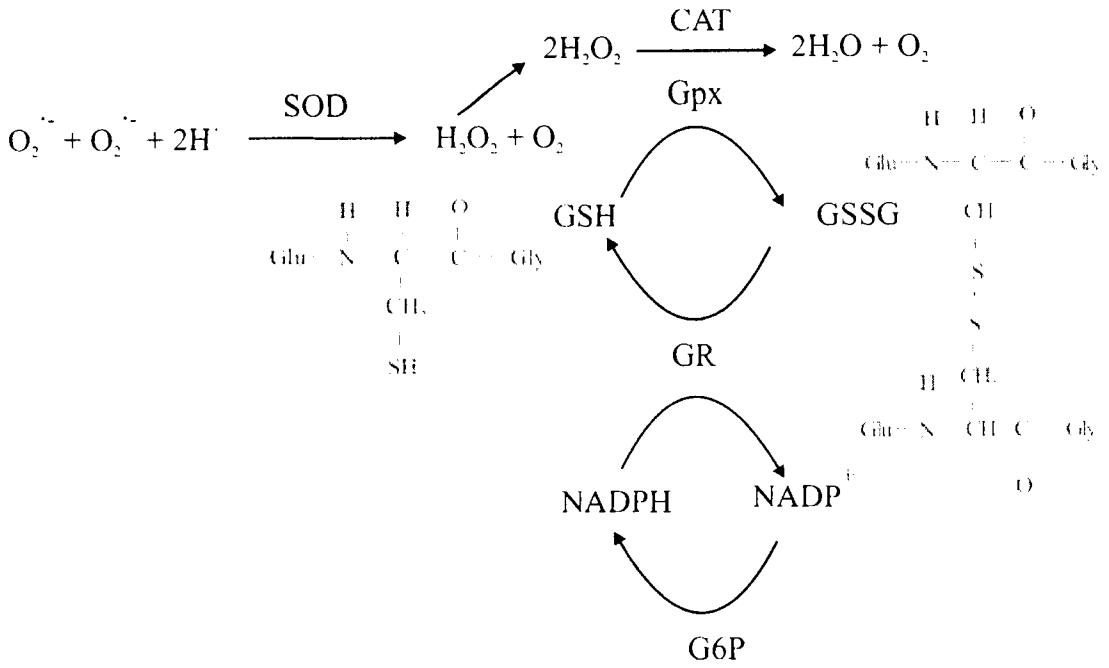

Figure 2.2 A schematic representation of the glutathione redox cycle (modified from Curtin *et al.*, 2002, Halliwell and Gutteridge, 2000).

The preventative antioxidants suppress the formation of free radicals and can be subdivided into two groups (Figure 2.1; Rimbach *et al.*, 1999, Benzie, 2003). The first sequestrates metals through chelation and include transferring-lactoferrin, haptoglobin-hemopexin and albumin. These proteins bind to iron, free haemoglobin and copper respectively and prevent these ions to participate in the Haber-Weiss or Fenton reactions (Cross *et al.*, 1987). The metallothioneins are sulphur rich proteins in the cytosol of eukaryotic cells. They remove metals including zinc, silver, copper, cadmium and mercury through binding of the metals to cysteine (-SH) groups. The high content of cysteine groups in the metallothioneins also makes them excellent radical scavengers (Nath *et al.*, 2000).

The second group is the antioxidant enzyme system that consists of SOD, GPx, catalase and GR. To date four classes of SOD have been identified. They include manganese (Mn-SOD), copper (Cu-SOD), zinc (Zn-SOD) and extracellular SOD (Curtin *et al.*, 2002, Raha and Robinson, 2000). In animal cells, SOD are located in the cytosol, lysosomes,

nucleus, and the space between the inner and outer mitochondria membrane (Comhair and Erzurum, 2002). SOD catalyses the dismutation of $O_2^{\cdot-}$ (Figure 2.2). Dismutation of $O_2^{\cdot-}$ leads to the formation of H_2O_2 which is broken down to H_2O and O_2 by catalase. In animals and humans catalase is present in all major organs, but mainly in the liver (Halliwell and Gutteridge, 2000).

2.5 Oxidative stress

Oxidative stress was initially defined as an imbalance between pro- and antioxidants that leads to irreversible damage (Halliwell and Gutteridge, 2000). The imbalance can result from a lack of antioxidant capacity, due to disturbed production, distribution or excess of ROS from exogenous sources. It can also be from environmental stressors. Oxidative damage was defined as the damage caused by the direct attack of reactive species during oxidative stress. Consequences include either adaptation of the cell or organism by upregulating the antioxidant defence systems or, cell injury (Halliwell and Whiteman, 2004, Comhair and Erzurum, 2002). This concept was revised by Cutler *et al.* (2005) which proposed that oxidative stress status represents the base level oxidative injury in a given cell, where any oxidatively mediated damage represents a stress on the cell. Oxidative damage can cause cell injury through damage to lipids, proteins, nuclei acids, DNA and carbohydrates that can ultimately lead to cell death (Cross *et al.*, 1987, Cutler *et al.*, 2005). The damage can lead to depletion of NADH, GSH and ATP and increases in cytocolic calcium ions (Ca^{2+}) which can also cause cell damage (Halliwell and Gutteridge, 2000).

It is now well established that ROS can cause cell death by either necrosis or apoptosis (Bland, 1995). Necrosis usually occurs in response to severe trauma/injury to the cell and is morphologically characterised by cytoplasmic and mitochondrial swelling, rupturing of the plasma membrane and release of the cellular contents into the extracellular space. Inflammatory response, which can cause further injury to neighbouring cells, follows. Apoptosis also known as programmed cell death is a highly regulated form of cell death in which a cell effectively contribute to its own demise. Morphological and biochemical

changes of apoptosis include mitochondrial depolarisation and alterations of phospholipid asymmetry, chromatin condensation, nuclear fragmentation, membrane blebbing, cell shrinkage and the formation of membrane bound vesicles, termed apoptotic bodies (Kiechle and Zhang, 2002, Halliwell and Gutteridge, 1999). Oxidative stress has been implicated in aging and in a number of human diseases such as cardiovascular and neurodegenerative diseases and cancer (Brenneisen *et al.*, 2005, Cross *et al.*, 1987, Curtin *et al.*, 2002).

2.6 Mitochondria and ROS

Mitochondria are responsible for the synthesis of ATP from ADP and inorganic phosphate through the process of oxidative phosphorylation. Mitochondria uses over 80% of inhaled oxygen to oxidize hydrogen rich molecules present in food to produce over 90% of the ATP that the cells use (Perkins and Frey, 2000). The mitochondrion is either a spherical or cylindrical organelle which consists of an outer membrane and a folded inner membrane. The proteins mediating electron transport and oxidative phosphorylation are bound to the inner membrane. The mitochondrial respiratory chain consists of four complexes that function through the transfer of electrons from the NADH/$FADH_2$ reducing equivalent to molecular oxygen. Electron transport along the respiratory chain generates mitochondrial potential as protons are pumped out of the matrix across the inner membrane space (Munnich and Rustin, 2001, Wallace, 1999). This electrochemical gradient ($\Delta\psi$) is essential for ATP-synthase to operate in the oxidative phosphorylative pathway (Figure 2.3).

The reduced equivalents produced by the tricarboxylic acid (TCA) cycle in the form of NADH is passed onto ubiquinone by the action of complex I (NADH:ubiquinone oxidoreductase), which contains flavin mononucleotide (FMN) and seven iron-sulphur clusters (FeS). Complex II (succinate ubiquinone oxidoreductase) carries the reduced equivalents also to ubiquinol (UQH_2) resulting in ubiquinone (UQ). The electrons are then passed onto cytochrome c by complex III (ubiquinol: cytochrome c oxidoreductase), resulting in pumping of four electrons across the inner membrane. Cytochrome c then

transfers electrons to complex IV (ferricytochrome:oxygen oxidoreductase or cytochrome c oxidase). Complex IV transfers the electrons to oxygen as the final electron acceptor oxygen to produce water. The energy released by the electron transfer is used to pump protons across the membrane: four via complex I, none via complex II, four via complex III and two via complex IV. The resulting electrochemical gradient enables complex V (ATP synthase) to form ATP from ADP and inorganic phosphate, by the reverse flow of electrons back into the matrix (Wallace, 1999, Munnich and Rustin, 2001).

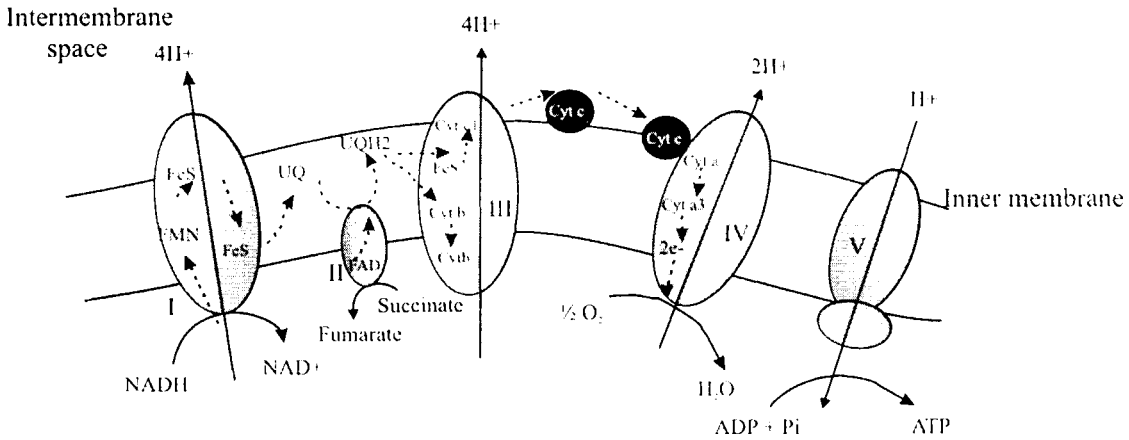

Figure 2.3 Schematic representation of the process of oxidative phosphorylation. (Adapted from MITOMAP, 2006). FMN=flavin mononucleotide; FeS = iron-sulphur clusters; FAD = flavin adenine dinucleotide; UQ = ubiquinone; NADH = reduced nicotinamide dinucleotide; NAD^+ = oxidised nicotinamide dinucleotide; UQH_2 = ubiquinol; Cyt *a, b, c* = cytochrome *a, b, c*; H^+ = hydrogen; e^- = electron; I, II, III, IV and V = respiratory chain complexes I, II, III, IV and V. Broken arrows indicate electron flow.

Mitochondrial energy production is important for maintaining the proper redox potential of cells. Disruption in the mitochondrial electron transport chain will result in diminished ATP production and decreased cell viability (Bota and Davies, 2001, Bras *et al.*, 2005). There is a much greater susceptibility to alterations inside the mitochondrion than in the rest of the cell. Much of the current research is therefore focused on the relationship between oxidative phosphorylation and oxidative stress related disorders (Bland, 1995). The production of ROS occurs at both complexes I and III of the electron transport chain.

These two sites are prime sites for superoxide production because the flavin mononucleotide (FMN) of complex I and ubiquinone in complex III can exist in semiquinone anion form. They therefore contain an unpaired electron that can be donated to molecular oxygen to form the superoxide anion (Bailey and Cunningham, 2002). Mitochondria are also pivotal in controlling apoptosis by initiating the process. The mechanisms involved is the release of cytochrome c that trigger the activation of caspases, disruption of electron transport, oxidative phophorylation and ATP production and changes in the cellular redox balance. Increased oxidative stress and depletion of antioxidant defence systems activate these mechanisms (Perkins and Frey, 2000).

2.7 Molecular reactivity of O_3

It is important to note that the majority of the effects that will be discussed here pertain to the *in vitro* effect of ozonation and that references to cell function were inferred from these results. Ozone is an extremely reactive oxidant. Its toxicity is complex because of the large number of biological systems it can affect and the variety of effects that can result from interactions with cellular components. Ozone damage is in part produced by free radical mechanisms including peroxidation of polyunsaturated fatty acids and oxidation of thiols, amines and proteins (Mehlman and Borek, 1987). Although O_3 is not a radical molecule, most of its actions are mediated through free radical reactions (Cotgreave, 1996). When O_3 comes into contact with biological fluids such as the surfactant of the lung or blood, a series of chemical and physical processes occur. The O_3 dissolves in the plasma and saturates the haemoglobin in red blood cells to form oxyheamoglobin. The pO_2 increases to levels higher than the physiological level, while the pCO_2 remains constant (Bocci, 1996b). Chemically it then acts in two ways: 1) by directly oxidizing molecules to give reactive species or 2) by driving radical-dependent production of cytotoxic, non-radical species such as aldehydes and ozonides (Mudway and Kelly, 2000). The first 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 (Table 2.4, reaction number I and II; Pryor, 1994; Bocci, 2002).

Table 2.4. The mechanisms of O_3 -action (adapted from Bocci, 2002).

Reaction	Number
$GSH + O_3 \rightarrow GSH^{\bullet+} + O_3^{\bullet-}$	I
$O_3^{\bullet-} + H^+ \rightarrow OH^{\bullet} + O_2$	II
O_3 + unsaturated fatty acid $\rightarrow$ aldehyde + H_2O_2	III

The second mechanism is an O_3-olefin-reaction where O_3 reacts with the double bonds in organic molecules, such as fatty acids, and produces H_2O_2, aldehydes and other peroxides (Table 2.4, reaction number III; Pryor, 1994, Pryor *et al.*, 1995). Free radicals are mainly formed by O_3 in a medium with a pH higher than 8. At pH less than 7.5, the ozonolysis mechanism prevails, mainly leading to the formation of peroxides (Andreula *et al.*, 2003).

2.7.1 Antioxidant reactions with O_3

Ozone that dissolves in the plasma can react with several substrates. One of them is GSH, which gives rise to an anion O_3 radical that is unstable and form $OH^{\cdot}$ after protonation (Bocci, 2002). Ozonation of GSH causes rapid oxidation of the molecule. Acute exposure of animals to O_3 increases the levels and/or activities of various components of the GSH dependent antioxidant defences. Ozone also reacts with ascorbate and vitamin E, which together with GSH forms a first line of defence against O_3 (Van der Vliet *et al.*, 1995, Cotgreave, 1996). GSH levels in erythrocytes initially decrease following exposure to O_3. Disulfide levels increase during O_3 exposure as a result of thiol oxidation by O_3. Thiols are essential parts of the active sites of many enzymes and oxidation of these thiols to disulfides can damage the enzymes (Mehlman and Borek, 1987). Ozone also reacts with uric acid and during ozonation the uric acid is oxidized to allantoin. Uric acid plays an important role as a scavenger during ozone autohemotherapy (O_3-AHT).

It is important to note that ozonation decreases the antioxidant reservoir by 70-80% (Bocci, 2002).

2.7.2 Lipid peroxidation

Ozone reacts with all hydrocarbons, but especially with those present in polyunsaturated fatty acids (PUFAs). PUFAs, particularly arachidonic acid, are present in membrane phospholipids, triglycerides, lipoproteins and chylomicrons. In general, the more unsaturated, the more a fatty acid is attacked by O_3 during lipid peroxidation (Mudway and Kelly, 2000). The initial reaction with a PUFA in a lypophylic environment is the addition of O_3 to the carbon-carbon double bond to produce a trioxygen intermediate, 1,2,3-trioxalane. This molecule rearranges to form the 1,2,4-trioxalane that is also called the Griegee ozonide (Halliwell and Gutteridge, 2000, Mudway and Kelly, 2000). In a hydrophilic environment such as plasma, H_2O_2 and hydroperoxides, rather than the Criegee ozonide, is formed. The H_2O_2 can begin lipid peroxidation directly or indirectly via the formation of $OH^{\cdot}$ (Pryor *et al.*, 1995).

During lipid peroxidation, free radicals ($OH^{\cdot}$) can attract hydrogen atoms (H^+) from a methylene group ($-CH_2-$) of a PUFA. That leaves an unpaired electron on the carbon (-CH-). The remaining carbon-centred radical undergoes molecular rearrangement to form a conjugated diene. This compound can combine with O_2 to form a peroxyl radical which can remove a H^+ from an adjacent PUFA. This can start a chain reaction that terminates only when there is a lack of substrate, or more likely by chain-breaking antioxidants such as liposoluble α-tocopherol. The lipid peroxide (-C-O-OH) that forms is a fairly stable compound. However, traces of Fe^{2+} of Cu^+ can catalyse its decomposition to form alkoxyl ($RO^{\cdot}$) and alkoperoxyl ($ROO^{\cdot}$) radicals. These can induce further peroxidation. Eventually a complex mixture of low molecular weight aldehyde end products, such as malonyldialdehyde (MDA), n-alkanals, 2-alkenals, 4-hydroxy-2,3 transnonenal (4-HNE) and other 4-hydroxy-2,3-alkenals of variable lengths may be formed (Halliwell and Gutteridge, 2000, Bocci, 2002, Cotgreave, 1996). Numerous studies have shown that O_3 reacts specifically with the polyunsaturated fatty acids and proteins in the membranes (Uppu *et al.*, 1995, Mudd *et al.*, 1997). Lipid peroxidation

therefore may result in changes in membrane fluidity, alterations in the ion transport mechanisms with distortion of signal transduction, increased permeability and possibly also membrane rupture (Pryor *et al.*, 1976, Berman and Brodaty, 2004).

2.7.3 Protein oxidation

Damage to proteins can be caused directly by free radicals or indirectly by end products of lipid peroxidation, including toxic aldehydes (Hamilton *et al.*, 1998, Halliwell and Gutteridge, 2000). Several amino acids such as cysteine, methionine, tryptophan and tyrosine are targets for direct reactions with O_3. Ozone oxidizes the sulphur and nitrogen atoms in proteins. Proteins that are modified by reaction with the products of ozonolysis may be recognized as foreign and be removed through immune reactions (Lerner and Eschenmoser, 2003). Oxidation of the amino acids can lead to detrimental consequences for the cell. Enzymes, receptors and transport proteins can be targets of oxidative damage by O_3. This can have important negative consequences for cell functions such as metabolism, regulation of fluid balance and communication (Halliwell and Gutteridge, 2000).

2.7.4 Oxidative DNA damage

Ozone can react directly with DNA to degrade the purine and pyrimidine bases. Ozonation of double stranded DNA *in vitro* such as bacterial plasmids, can cause single and double stranded breaks with loss of structural integrity of the plasmid by linearization which causes loss of transforming action. Other effects include sugar peroxidation, base hydroxylation and protein-base cross linking (Guetens *et al.*, 2002). ROS can cause structural alterations to DNA via base pair mutations, rearrangements, deletions, insertions, sequence amplification, gross chromosomal alterations and point mutations. It can also affect cytoplasmic and nuclear signal transduction pathways and modulate the activity of proteins and genes that respond to stress and also influence cell prolifieration, differentiation and apoptosis (Marnett, 2000). Oxidative DNA damage by free radicals has been implicated in mutagenesis, carcinogenesis, reproductive cell death and aging. ROS can attack mitochondrial DNA as can the intermediate radicals that form during lipid peroxidation. The latter can also attack DNA (Cooke *et al.*, 2003).

If the DNA damage is not repaired, it can lead to accumulation of modified nucleotides which can negatively affect the integrity of the genome. In order to counter this, organisms have defence mechanisms that can effectively repair oxidative DNA damage. The repair occurs primarily through base excision repair and double strand break repair, although nucleotide excision may also be involved (Cooke *et al.*, 2003, Guetens *et al.*, 2002). During base excision repair, single strand breaks and singly modified bases, such as 8-hydroxy-guanine, are repaired through the action of glucosylase enzymes. Double strand breaks are complicated to repair and usually involves two processes. The first is homogenous recombinant repair, where a homologous sequence forms a template for accurate genetic exchange. During the genetic exchange between two homologous chromatids, the original DNA sequence is restored. The second is by non-homologous end joining, where the ends of the double stranded breaks are modified by adding or deleting nucleotides (Mohrenweiser *et al.*, 2003).

2.8 O_3 toxicity

Since the lungs are the first organ to be exposed to O_3 in the atmosphere, it is also the organ most studied to assess the toxic effect of O_3. Breathing slightly elevated concentrations of O_3 can result in a range of respiratory symptoms. Acute exposures, lasting from five minutes to six hours causes changes in lung capacity, flow resistance, epithelial permeability and reactivity of lung tissue. These changes are observed within the first few hours after exposure has started and may persist for many hours or even days after the exposure was stopped (Lippmann, 1998). Chronic exposures cause alterations in baseline lung function and structure which can result from cumulative damage and/or from the side effects of adaptive responses to repetitive daily or intermittent exposures. The National Ambient Air Quality Standard level for daily eight hours O_3 exposure, revised in 1997, in the United States is 80 ppb (160 µg/ml, see Appendix B for conversion; Lippmann, 1998).

Ozone toxicity may be caused by the action of free radicals or by the direct oxidation of lung tissue. Ozone first comes in contact with the surfactant, where it reacts with substrates such as protein and lipids (Valacchi *et al.*, 2004). Secondary oxidation products form which can transmit toxic signals to the underlying pulmonary epithelium to initiate a number of cellular responses (Kafoury *et al.*, 1998, Ballinger *et al.*, 2005, Mudway and Kelly, 2000). The responses include inflammation which causes activation of pulmonary macrophages and recruitment of neutrophils to the lungs (Corradi *et al.*, 2002). ROS produced by these cells may provide an additional source of oxidative stress. Lung permeability is also increased and oedema develops (Mudway and Kelly, 2000). The toxicity of O_3, when inhaled by test animals, is well known (Dormans *et al.*, 1999, Finlayson-Pitts *et al.*, 1998, Johnston *et al.*, 1999a). The same applies to studies in controlled clinical trials in humans (Trenga *et al.*, 2001).

Various studies have been done in an attempt to prove that O_3 is toxic to blood. Extensive research has been done by Cataldo on the reactions of O_3 with bovine blood (Cataldo, 2004, Cataldo, 2005, Cataldo and Gentilini, 2005a, Cataldo and Gentilini, 2005b). Ozone reacts especially with haemoglobin in red blood cells. It is selectively absorbed by the Fe^{2+} atoms of the haeme prosthetic groups in haemoglobin. The binding implies oxidation of the central Fe^{2+} atom of the haeme groups with formation of methaemoglobin similar to binding of carbon monoxide (CO) (Cataldo and Gentilini, 2005a). Ozone destroys the heame prosthetic groups of methaemoglobin and hemoglobin (Cataldo, 2004); (Cataldo and Gentilini, 2005b). It also reacts with cholesterol and fatty acids in the blood (Cataldo and Gentilini, 2005b).

Results of the carcinogenicity of O_3 in long term studies are inconclusive (Boorman *et al.*, 1995) with no significant increase found in tumor multiplicity or incidence of rats exposed to concentrations up to 1 ppm (Witschi *et al.*, 1999). Increased hemolysis was observed when purified red blood cells was exposed to an O_3 atmosphere (Bialas *et al.*, 2001) or when red blood cells were suspended in phosphate buffered saline (Fukunaga *et al.*, 1999). Ozone treatment of A549 cells in culture (human lung carcinoma cell line) (Cheng *et al.*, 2003) and isolated human peripheral blood leukocytes (Diaz-Llera *et al.*,

2002) caused a decrease in cell viability and resulted in oxidative DNA damage, including 8-oxoguanine and DNA single-strand breaks. Ozone has a strong mutagenic effect on cell cultures when compared to ionizing radiation, singlet oxygen, $OH^{\cdot}$ and metals (Jorge *et al.*, 2002). Exposure of white blood cells of arterosclerotic patients to therapeutic doses of O_3 caused a significant increase of 8-oxoguanine in the cells' DNA (Foksinski *et al.*, 1999).

Unfortunately the majority of these studies investigated the effects of O_3 on cells either in antioxidant poor media (in the case of cell cultures) or *in vitro* on blood cells not removed from plasma. Thus, the results cannot be extrapolated to whole blood because of the different antioxidant capacities in the study media. The antioxidant defence mechanisms are also slightly lower in the lung surfactant compared to plasma. This may explain many of the toxic effects of O_3 in the respiratory tract i.e. that one measures experimental artefacts and not real life situations (Bocci, 2002).

2.9 O_3 and medicine

Initially O_3 use was based on its powerful bactericidal effect on anaerobic bacteria in waste water treatment (Hoigne and Bader, 1979, Kogelschatz *et al.*, 1988, Glaze *et al.*, 1987). With time, its objectives expanded and it was later used as a form of alternative medicine. In 1995 the office of Alternative Medicine of the National institutes of health (NIH, MD, USA) included ozone therapy among its pharmacological approaches and it is commonly used in Europe (Bocci, 1996). There is a growing interest to use non-conventional, complementary and alternative medicine all over the world to treat certain diseases (Furnham, 2000). Ozone therapy is classified as one of over sixty such complementary medical approaches (Furnham, 2000). The medical use of O_3 was made possible when O_3-generators were developed. Pure medical O_2 is passed through the generator and the energy from an electrical discharge causes the breakdown of the O_2 molecule into atoms, which then recombine to form an O_2/O_3 gas mixture containing various O_3 levels depending on the conditions of the generator. The concentration can be calculated precisely by using UV-absorption photometry (Masschelein, 1998). It is

important that the O_3 concentration is known when it is used as a medicine, because high concentration measured incorrectly can be toxic (Bocci, 2002).

Unfortunately, most of the uses of O_3 as a therapeutic agent have only been reported in journals that are not accredited (or peer reviewed) or have only been postulated. Very few controlled clinical trials to evaluate the effectiveness of the therapy have been done. It is therefore not surprising that the use of O_3 as alternative form of medicine has met scepticism from the medical fraternity.

2.9.1 Routes of administration of medical O_3

Ozone can be administrated to patients in a variety of ways in order to treat a variety of diseases. These include topical application of ozonated distilled water or olive oil, rectal insufflation of an O_2/O_3 gas mixture, infusion of an ozonated saline solution via parenteral routes, including intravenous, intramuscular or intradiscal injection and ozonated autohemothaerapy (O_3-AHT; Bocci, 2002). Topical application of ozonated oil or water (Viebahn, 2002, Bocci, 2002), vapour close to the skin (Jordan *et al.*, 2002) or aerosol baths (Tafil-Klawe *et al.*, 2002) have been used successfully in the treatment of dental lesions and ulcers, wound healing and lower limb ischaemia respectively. Rectal insufflation of O_2/O_3 gas have been used in the treatment of retinitis pigmentosa (Copello *et al.*, 2002) and seems to be useful to treat hypoxia and ischemia in patients with advanced head and neck tumors (Clavo *et al.*, 2004c). The intradiscal or periganglionic injection of an O_2/O_3 gas mixture containing 27 µg/ml O_3 is used as a minimally invasive treatment for lower back pain caused by lumbar disk herniation (Andreula *et al.*, 2003). The intravenous infusion of O_2/O_3 gas mixture has however been banned by the European society for O_2/O_3 therapy because of the possible dangers such as secondary air embolisms, which can result because of the intravenous injection of large amounts of O_2/O_3 gas too quickly (Corea *et al.*, 2004). Bilateral intra-ocular haemorrhage (Lo Giudice *et al.*, 2004) and vertebrobasilar stroke were reported after transcutaneous intradiscal and periganglionic infusion of O_3 (Corea *et al.*, 2004).

2.9.2 O_3 autohemotherapy (O_3-AHT)

Compared to other applications, O_3-AHT appears to remain the method of choice. It entails the *ex vivo* exposure of a precise volume of blood or serum to an equal volume of an O_2/O_3 gas mixture at a precisely determined O_3 concentration (from 5 – 100 µg/ml, see Appendix B for calculation of dose), followed by reinfusion of the blood (Bocci, 2002). When blood is exposed to O_2/O_3, both gases dissolves into plasma and the O_3 react immediately with organic molecules. A great deal of O_3 is also neutralized by the antioxidant defence system in blood. Nonetheless, a variety of new products are formed. These include ozonides, H_2O_2, hydroperoxides, free radicals and aldehydes. These compounds are reinfused into the patient and are responsible for the effects observed after therapy (Bocci *et al.*, 1998, Bocci, 2002, Bocci, 2004) (see section 2.5). The therapeutic response is generated by ROS and LOPs, which causes temporary oxidative stress in the organism. The oxidative stress upregulates the antioxidant defence system, which in turn can reverse the chronic oxidative stress that is observed in the patient (Leon *et al.*, 1998, Bocci, 2002). The efficiency of O_3-therapy relies on the use of an accurate O_3 dose, which have to be high enough to elicit a response, but not so high that it is toxic (Bocci *et al.*, 1998, Larini *et al.*, 2001, Larini *et al.*, 2003).

The effectiveness of medical O_3 has been studied in terms of the interaction of O_3 with human blood, the effects of O_3 on human white blood cells and other immunocompetent cells and on certain biological models where O_3 preconditioning protects against injury. Ozone–AHT has also been investigated in certain disease states in humans, in animals and in cell culture models. Ozone reacts with all the different blood components including red blood cells, white blood cells, platelets and plasma (Bocci *et al.*, 2001).

Ozonation of red blood cells causes activation of the pentose phosphate pathway which in turn activates glycolysis. The hypothesis that O_3 restores normoxia is based on the accelerated glycolysis of red blood cells *in vitro*. This will then lead to an increase in ATP levels. These reinfused red blood cells enhance O_2 delivery to ischeamic tissues because of a shift of the O_2-hemoglobin dissociation curve to the left. This takes place as a result of a decrease of the intracellular pH and or increase of 2,3-diphosphoglycerate levels. This can lead to a control of neoplastic growth in cancer tissues, which usually

thrive in hypoxic conditions (Clavo *et al.*, 2004b, Bocci *et al.*, 2005, Martinez-Sanchez *et al.*, 2005). Results suggest that O_3-AHT stimulated erythropoiesis and the newly formed erythrocytes have improved metabolic characteristics which are able to correct hypoxia in vascular disease (Bocci, 2004). Hemolysis does take place, but it is negligible because the hematocrit does not change (Bocci, 2002). Bocci *et al.* also reported platelet aggregation when heperanized blood is treated with O_3 concentrations of 40 and 80 µg/ml(Bocci *et al.*, 1999).

One of the earliest discoveries was that O_3-AHT induces cytokine production. These include interferon (IFN) γ, IFNβ, TNFα, IL-2, IL-4, IL-6, IL-8, IL-10, transforming growth factor (TGF) β and granulocyte-monocyte colony stimulating factor (GM-CSF) (Bocci, 2002, Bocci, 2004, Bocci *et al.*, 1998, Larini and Bocci, 2005, Valacchi and Bocci, 1999). Following ozonation, H_2O_2 freely diffuses into the white blood cell cytoplasm where it activates specific protein kinases which, through phosphorylating IκB (inhibitor of nuclar factor kappa B) bind to the nuclear factor-κB (NF- κB) to allow the migration of the transcription heterodimer p50-p65 into the nucleus where it activates cytokine gene expression (Bocci, 2004). This in turn is responsible for switching on the synthesis of several types of proteins, including the cytokines (Larini and Bocci, 2005). Also, O_3 dose dependently increased the levels of antioxidant enzymes, including SOD, GR and GPx, in cultured Jurkat T cells (Larini *et al.*, 2003).

Several studies focused on the protective effects of rectal insufflation and O_3-AHT in various animal models. Controlled O_3 administration promoted an oxidative preconditioning or adaptation to oxidative stress, thereby preventing damage induced by ROS. This mechanism apparently protects against liver ischeamia-repurfusion injury (Ajamieh *et al.*, 2001, Ajamieh *et al.*, 2002, Peralta *et al.*, 2000), oxidative damage by CCl_4 (Leon *et al.*, 1998) and renal dysfunction (Barber *et al.*, 1999, Borrego *et al.*, 2004). Low doses of O_3 increases antioxidant systems and so provide an antioxidant-pro-oxidant balance that favors preservation of the cell redox balance. It also ensures that toxic aldehydes are not formed (Ajamieh *et al.*, 2002, Ajamieh *et al.*, 2001). The xanthine/xanthine oxidase pathway for ROS generation is also blocked during

preconditioning that leads to lower levels of H_2O_2. This leads to O_3 being an effective treatment on the injury associated with ischemia repurfusion (Peralta *et al.*, 1999).

The effects of O_3-AHT as a treatment for ischaemic disorders have also been studied and compared to that of topical application (Section 2.5) (de Monte *et al.*, 2005, Gracer and Bocci, 2005). Ozone increases oxygenation in resting muscles, particularly in muscles that are hypoxic (Clavo *et al.*, 2003). It also increases blood flow in the common carotid and middle carotid arteries, which can explain its effectiveness in the treatment of ischaemic disorders (Clavo *et al.*, 2004a). Ozone also enhances the release of $NO^{\cdot}$ from endothelial cells, which may induce vasodilatation in ischaemic areas and so reduce hypoxia (Valacchi and Bocci, 2000). It was proposed that enhancement of the antioxidant defence system and O_2 flow to tissues play an important role in the treatment of conditions where orthodox medicine have failed. It includes conditions such as diabetes (Al-Dalain *et al.*, 2001), retinits pigmentosa (Copello *et al.*, 2002), asthma (Hernandez *et al.*, 2001), high cholesterol (Hernandez *et al.*, 1995) and HIV infection (Bocci, 1996a, Shallenberger, 1998).

3 Materials and methods

This project forms part of a broader O_3 project where various aspects of O_3-AHT were assessed. These include biochemical, genotoxic and metabolic effects. The broader project has been approved by the Ethics Committee of the North-West University (Potchefstroom campus), under the title *"In vitro effects of treatment of blood with different O_3 concentrations on cell integrity, plasma antioxidant status and plasma organic and amino acids"* with the reference number 05M07. This study did not include the measurement of plasma organic and amino acids. All biochemical analysis were performed in duplicate, unless stated otherwise.

3.1 O_3 generation and measurement

Ozone was generated from ultrapure O_2 (>99.9% BOC special products, Afrox) using electrical corona discharge by an O_3 generator developed and build by the School of Physics, Potchefstroom Campus of the North-West University (USA patent 09/914,199). The O_3 concentration was determined by a UV/Vis spectrophotometer (Pharmacia Biotech Ultraspec 3000) by measuring the gas absorbance at 254 nm (see Appendix B, Table B3). The O_3 concentration was monitored during exposures, using a specially designed quarts cell containing an inlet, where gas entered from the O_3 generator and an outlet, where the gas left the cell and entered the syringe containing the blood.

3.2 Study design

Blood samples were collected from six apparently healthy human donors who gave informed consent (Appendix D). Exclusion criteria included any type of medication or antioxidants, including vitamin supplements and any current diseases or infections. Each test subject served as his/her own control. The blood was divided into two aliquots. In intervention group one the effect of ozonation of whole blood were assessed. In

intervention group two the effect of ozonation of blood cells in the absence of plasma were assessed. The treatment protocol is summarised in Figure 3.1.

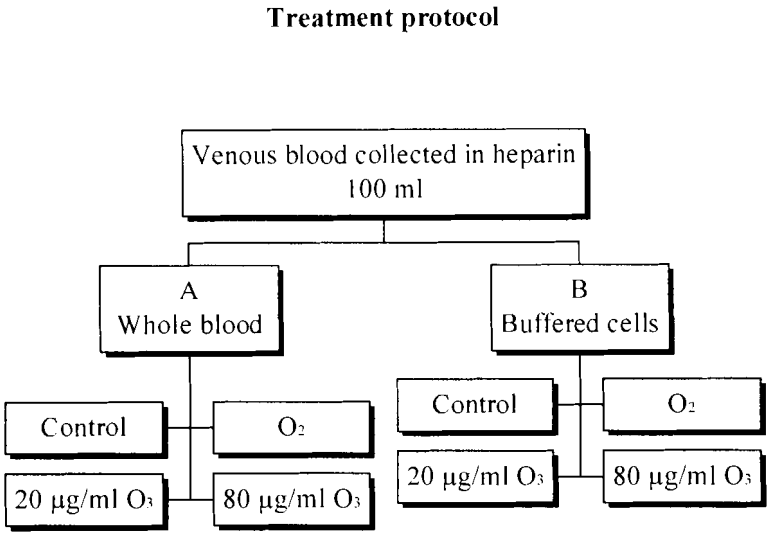

Figure 3.1 Summary of the treatment protocol showing the two intervention groups A and B. The O_2 treatment consisted of > 99.9% O_2.

One hundred ml of venous blood was collected from each volunteer. It was collected from the anticubitul vein without stasis into polypropylene syringes containing heparin (10 ml) as anticoagulant. The blood was then divided into two aliquots of 70 and 30 ml for Groups A and B respectively. The blood of Group A was divided in five 14 ml aliquots in siliconised glass syringes. The first aliquot was incubated at room temperature and served as the control sample. The second aliquot was exposed to an equal amount of ultra pure O_2 (> 99.9%) and the other aliquots were treated with an O_2/O_3 gas mixture containing either 20 or 80 µg O_3/ml gas.

In the second group the plasma was removed using centrifugation (centrifugation had no marked effect on cell viability, see section 4.1.2). Briefly, the blood was centrifuged for 15 minutes at 180 x g. The plasma was then carefully removed without disturbing the plasma/cell interface and replaced with the same volume of phosphate buffered saline (PBS). This wash procedure was repeated twice to give a total of three washes. The PBS

suspended blood cells were then divided in four aliquots of 7.5 ml each. The aliquots included a control sample, a treatment with O_2 and treatment with 20 and 80 µg/ml O_3 in the O_2/O_3 gas mixture. The whole blood and the buffered cell samples were gently and continuously mixed with the gas for 20 minutes. Thereafter the gas was carefully removed and the blood dispensed into test tubes for biochemical analyses or storage.

Table 3.1 A summary of the different biochemical parameters that were analysed in the two intervention groups.

Parameter	Biochemical assay
Cell viability	White blood cell count
	Cell viability (Trypan blue assay)
	Lactate dehydrogenase (LDH) assay
Oxidative stress	Hydroperoxides
	GSH/GSSG ratio analysis
	Oxygen radical absorbance capacity (ORAC) assay
	Ferric reducing antioxidant power (FRAP) assay
Antioxidant enzymes	Catalase, SOD, GPx, GR
Apoptosis	Caspase 3/7
Impairment of mitochondrial function	NADH/NAD^+ ratio analysis
	ATP levels
	Polagraphic measurement of oxygen consumption (respiration)
Respiratory chain enzymes	Complex I (NADH:ubiquinone oxidoreductase)
	Complex II + III (Succinate-cytochrome c reductase)
	Complex I + III (NADH:cytochrome c reductase)
	Complex IV (Cytochrome c oxidase)

ATP = adenosine triphosphate; NADH = reduced nicotinamide adenine dinucleotide; NAD^+ = oxidised nicotinamide adenine dinucleotide.

A small volume of blood was also collected in ethylenediamine tertra-acetic acid (EDTA) tubes to determine the white blood cell count according to standard laboratory techniques (Coulter counter). The redox status, oxidative stress, antioxidant capacity, levels of antioxidant enzymes, cell viability and apoptosis (Table 3.1) was also determined. Impairment of mitochondrial function was also investigated. All the reagents utilised in the biochemical investigations were purchased from Sigma Aldrich unless stated otherwise.

3.3 White blood cell viability

3.3.1 Lactate dehydrogenase enzyme (LDH) analysis

Cell viability most often refers to the integrity of the cell membrane and is most commonly measured by observing the exclusion of trypan blue or other dyes. Measurement of leakage of components from the cytoplasm into the surrounding fluid is widely accepted as a valid method to estimate the number of non-viable cells. In this case, the LDH was measured in the blood plasma to determine if cells were damaged. The analysis is based on the reaction shown in Figure 3.2. In this reaction, β-nicotinamide adenine dinucleotide (NAD) is reduced to NADH through conversion of lactate to pyruvate by LDH. The NADH then reduces tetrazolium dyes to formazan dyes in the presence of electron coupling agents or diaphorase (Decker and Lohman-Matthes, 1988). The resultant formazan absorbs at 492 nm, which was spectrophotometrically measured.

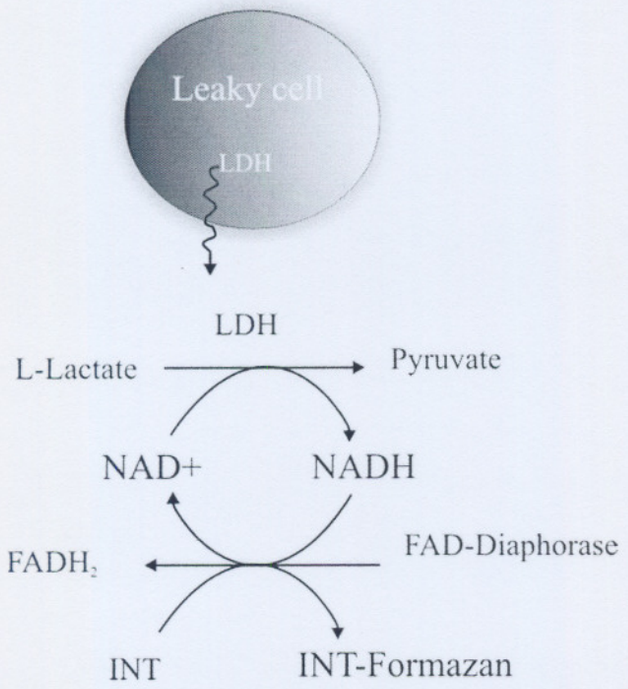

Figure 3.2 The principle of the LDH enzyme analysis (adapted from Decker and Lohman-Matthes, 1988). FAD – Flavine adenine dinucleotide; $FADH_2$ – Flavine adenine dinucleotide (reduced form)

Method

Plasma was prepared by centrifugation different aliquots of blood at 4000 x g for 30 minutes. The assay mixture consisted of a substrate buffer mixture, tetrazolium salts and an electron carrier, diaphorase, which was mixed with the plasma. The substrate buffer consisted of $NaPO_4$ (60 mmol/l), 0.15%, (v/v) Triton-X 100, 0.1%, (w/v) sodium azide and l-Lactate (48 mmol/l). The tetrazolim salt was iodonitetrazolium violet-formazan (INT) at a concentration of 0.6 mg/ml. The reaction was started by a solution containing diaphorase (40 U/ml in potassium phosphate buffer, pH 6.5), 6.25 mg/ml NAD^+, bovine serum albumin (BSA, 2mg/ml), sucrose (8 mg/ml) and sodium azide (NaN_3, 1 mg/ml). For the assay, 166 µl substrate buffer, 13 µl INT and 13 µl diaphorase-solution was mixed in a microtitre plate and pre read in a BIO-TEK microplate reader at 492 nm for 3 minutes. The plasma sample (8 µl) was added and the plate read for at least 20 minutes. The absorbance values of the samples were subtracted from the blank (containing only

distilled water) and expressed per µg protein (as determined by the BCA method described in Section 3.6.1). The lysis of the cells was calculated using Equation 3.1.

Equation 3.1 Calculation of the % lysis of the cells

$$\% \text{ specific lysis} = [\Delta A \text{ (exp)} / \Delta A \text{ (contr)}] \times 100$$

Δ A (exp) = absorbance at 492 nm of experimental group; ΔA (contr) = absorbance of the control

3.3.2 Isolation of white blood cells

White blood cells were isolated from the heparinized blood and from the buffered cells. The whole blood or buffered cells (2 ml) were added to 2 ml of Histopaque in a 15 ml polypropylene tube and centrifuged at 50 x g for 30 minutes. The cells (buffy coat layer) were removed, resuspended in 250 µl PBS and centrifuged at 50 x g for 30 minutes. The pellet was suspended 300µl PBS and again centrifuged. After removing the supernatant, the pellet was resuspended in 500 µl PBS or RPMI1640 culture medium. Fifty µl of this cell suspension was used to determine the cell viability (Section 3.3.3).

3.3.3 Trypan blue exclusion assay

The principle of the assay is that viable cells do not take up Trypan blue, whereas non-viable cells do. A cell suspension (50 µl) was added to 250 µl 0.4% (w/v) Trypan blue solution (Biowhitaker™[1]) and 150 µl PBS. The cells were incubated at room temperature for 10 minutes. With the cover slip in place on a heamocytometer, 7.5 µl of the cell suspension was pipetted onto the cover slip so that the fluid filled the chamber by capillary force. The cells were counted in the five 1 mm squares, where the cells on top and left touching the middle lines of the perimeter of each square were counted. Each square of the heamacytometer, with a cover slip in place, represents a total volume of 10^{-4} cm^3. Since one cm^3 is equivalent to approximately 1 ml, the subsequent cell concentration per ml was determined using the calculation in Equation 3.2. The viable (unstained) and non-viable (stained) cells were counted to determine cell viability according to Equation 3.3.

[1] Biowhittaker ™ is a trademark of Cambrex Bio Science Inc, Walkersville, MD, U.S.S.

Equation 3.2 Determination of the cell concentration

Cells/ml = average count per square x dilution factor x 10^4

Total cells = cells/ml x original fluid from which cell sample was removed

Equation 3.3 Determination of cell viability

Cell viability (%) = (Total viable cells / Total cells) x 100

3.4 Oxidative stress

3.4.1 Hydroperoxides

The hydroperoxides (R-OOH) in plasma were measured with a commercially available kit (the d-ROMs test) from DIACRON International (Grosseto, Italy). The test is based on the concept that the organic hydroperoxides present in plasma react with transition metals via the Fenton reaction. The plasma sample is mixed with an acidic buffer, which facilitate the release of Fe^{2+} ions initially bound to the plasma proteins. The hydroperoxides react with the Fe^{2+} ions to form alkoxyl and peroxy radicals (Table 3.2).

Table 3.2 The reactions of the reactive metabolites involved in the d-ROMs test

Reaction number	Reactions
1	$R\text{-}OOH + Fe^{2+} \rightarrow R\text{-}O^{\cdot} + Fe^{3+} + OH^{-}$
2	$R\text{-}O^{\cdot} + A\text{-}NH_2 \rightarrow R\text{-}O^{-} + [A\text{-}NH_2]$
3	$R\text{-}OOH + Fe^{3+} \rightarrow R\text{-}OO^{\cdot} + Fe^{2+} + H^{+}$
4	$R\text{-}OO^{\cdot} + A\text{-}NH_2 \rightarrow R\text{-}OO^{-} + [A\text{-}NH_2^{\cdot}]^{+}$

R-OOH = hydroperoxide; R-O· = alkoxyl radical of hydroperoxide; R-OO· hydroperoxyl radical of hydroperoxide; A-NH_2 = N,N-diethyl-paraphenylendiamine; [A-NH_2]$^+$ coloured radical cation of chromogenic substrate, note that the R- represents a lipid group.

These newly formed radicals are able to oxidize the chromogenic reagent (N,N – diethylparaphenylen-diamine) to change its colour which can be measured using spectrophotometry. It is important to note that hydroperoxides in biological fluids is considered a marker of oxidative stress and should not be confused with hydrogen peroxide (H_2O_2).

Method

Plasma was prepared by centrifugation of the blood at 4000 x g for 30 minutes. The plasma was frozen at -80°C until the dROMs test was done. Two microlitres (µl) of the chromogenic reagent and 200 µl of acetate buffer (pH 4.8) was mixed with the plasma in a microtitre plate. The change in absorbance was monitored at 485 nm for 15 minutes at 37 °C using a BIO-TEK (FL 600) microplate reader. The results were expressed as Carratelli units (CARR U) where 1 CARR U corresponds to 0.08 mg/100 ml H_2O_2 (Iorio, 2002). A blank was prepared by replacing the plasma with water. A standard with a concentration of 300 CARR U which was provided with the kit, was also prepared and included with the blank for each series of assays. The absorbance of the blank was subtracted from those of the plasma samples and the standard to calculate the hydroperoxide concentration according to Equation 3.4 (Lubrano *et al.*, 2002).

Equation 3.4 Determination of the concentration of plasma hydroperoxides

$$\text{CARR U} = (\Delta A/t \text{ of sample}) / (\Delta A/t \text{ of Standard}) \times 300$$

ΔA = absorbance; t = time; 300 = standard concentration.

3.4.2 Glutathione redox status

GSH protects against oxidative stress. The glutathione redox status, i.e the GSH/GSSG ratio is a good indicator of oxidative stress. The GSH/GSSG ratio was measured as described by Asensi *et al* (1999), with slight modifications. As GSSG is only present in minimal amounts when compared to GSH, auto-oxidation of GSH could result in a false GSSG representation. Accurate determination requires the prevention of GSH auto-oxidation during sample preparation. This can be achieved by using a GSH scavenger

like 1-methyl-2-vinyl-pyridinium trifluoromethane sulfonate (M2VP; 30 mM in 0.1 N hydrochloric acid (HCl). The method of analysis is based on the recycling process of GSH by the enzyme GR (see Section 2.3).

Sample preparation for GSSG analysis

Hundred µl of blood was aliquoted into a microcentrifuge tube containing 10 µl of 30 mM M2VP and frozen at -80 °C until assayed. Assays were done within 30 days. The samples were thawed, mixed and incubated at room temperature for 10 minutes. Five percent (v/v) metaphosphoric acid (MPA) (290 µl) was added to each sample. Samples were vortexed for 15-20 seconds and centrifuged at 10 000 x g for ten minutes at 4 °C. The acidic supernatant was carefully removed and diluted with 350 µl phosphate buffer (500 mM sodium phosphate ($NaPO_4$), pH of 7.5 and 1 mM EDTA).

Sample preparation for the total GSH (tGSH) analysis

For the GSH analysis, 50 µl blood was aliquoted into a separate microcentrifuge tube. The samples were stored at -80°C until assayed. MPA (350 µl) was added to the thawed samples to precipitate the proteins. The samples were vortexed and centrifuged at 10 000 x g for ten minutes at 4 °C. Subsequently, 5 µl of the MPA extract was diluted in a microtitre plate with 300 µl of the same buffer as for the GSSG analysis.

Method

A standard series was prepared in a 96 well micotitre plate (Nuncleon™[1]) from a GSH solution (3 µM) with final concentrations of 0, 0.5, 1, 1.5, 2, 2.5 and 3 µM respectively, that was made up to a final volume of 50 µl with phosphate buffer. Fifty µl 5,5' Dithiobis-(2-nitrobenzoic acid) (DTNB, 0.3 mM) was added to each well. After addition of 50 µl GR (0.02U/µl), the plate was incubated for 5 minutes at 25 °C in a preheated FL600 BIO-TEK microplate reader. Fifty µl of NADPH (1 mM) was added to each well to start the reaction and the absorbance measured kinetically at 412 nm for 5 minutes. The concentration of GSH and GSSG were calculated using the standard series and linear

[1] Nuncleon™ is a registered trademark of Nalge Nunc international, Rochester, NY, USA.

regression analysis. The equation used to calculate GSH and GSSG ratio is given in Equation 3.5.

Equation 3.5 Calculation of the GSH:GSSG ratio

GSHt[#] = µM x dilution factor
GSSG = µM x dilution factor
Ratio = (GSHt - 2GSSG) / GSSG

[#] GSHt = GSH + GSSG (Asensi *et al.*, 1999). For the GSHt the dilution factor was 488 and for GSSG 60.

3.5 Antioxidant capacity

3.5.1 Oxygen radical absorbance capacity (ORAC)

The method is based on the inhibition of the peroxyl-radical induced oxidation of fluorescein, initiated by thermal decomposition of the azo-compounds such as 2,2'azobis (2-amino-propane) dihydrochloride (AAPH). The inhibition of free radical damage to fluorescein is mediated by an antioxidant in the samples for the ORAC assay. The decrease in fluorescence of fluorescein is measured and reflects the capacity of antioxidants to directly quench free radicals (Cao and Prior, 1999). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxulic acid), which is a water soluble analogue of α-tocopherol is used a standard.

Method

The plasma samples prepared in Section 3.3.1 were pre-treated with perchloric acid (PCA, 0.25 M) and centrifuged at 10 000 x g to precipitate the proteins. The resulting supernatant was used in the ORAC assay. The sample supernatant was diluted 100-200 fold with 75 mM phosphate buffer (1M K_2HPO_4 and 1 M NaH_2PO_4, pH 7.4). A trolox standard series was prepared in opaque microtitre plates (Nuncleon™). The reaction contained 500 µM stock solution in a 75 mM phosphate buffer, (1M K_2HPO_4 and 1 M NaH_2PO_4 at pH 7.4). The final concentrations of the standards were 0, 2.5, 5, 10, 15 and 20 µM. The individual samples were diluted in the phosphate buffer and triplicate

aliquots (20 µl) added to the wells. Hundred and sixty µl of fluorescien (56 nM in phosphate buffer) was added to each well and incubated at 37 °C for 15 minutes. The reaction was started by adding 20 µl 2,2 Azobis (2-amidnopropane) dihydrochloride (AAPH, 240 mM in phosphate buffer). The decrease in fluorescence of fluorescein (excitation 485 nm, emission 520 nm) was measured every five minutes for three hours in a BIO-TEK (FL600) microplate reader, until the final reading was circa (ca.) 5% of the initial reading.

The difference in the decrease of fluorescence for various concentrations of Trolox is illustrated in Figure 3.3. The decrease due to damage to fluorescein does not follow first-order kinetics. Calculation of the antioxidant capacity should therefore not assume first-order kinetics. The area-under the curve method was used to calculate the antioxidant capacity of each sample in Trolox equivalents. To do this, a standard curve was constructed (Figure 3.4). The ORAC values of the samples were within the same range as those of the standards.

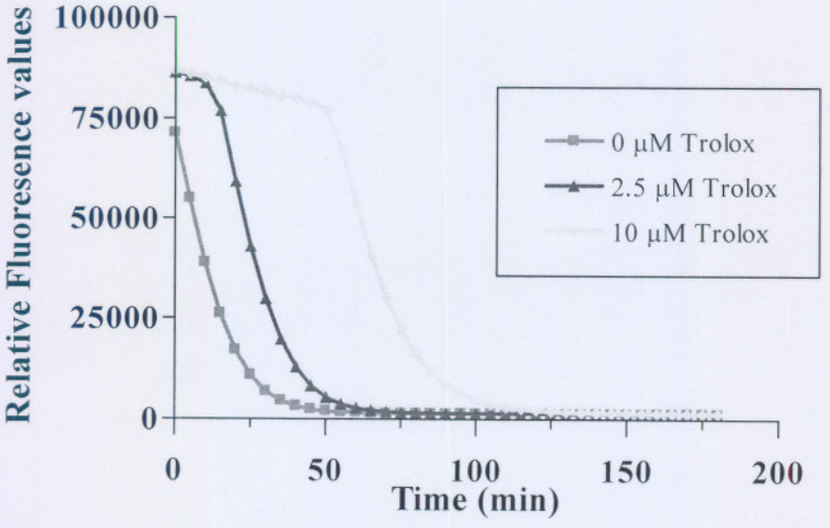

Figure 3.3 Representation of results generated with the Trolox standards by using the ORAC assay. The relative fluorescence values are plotted against time for 0, 2.5 and 10 µM Trolox.

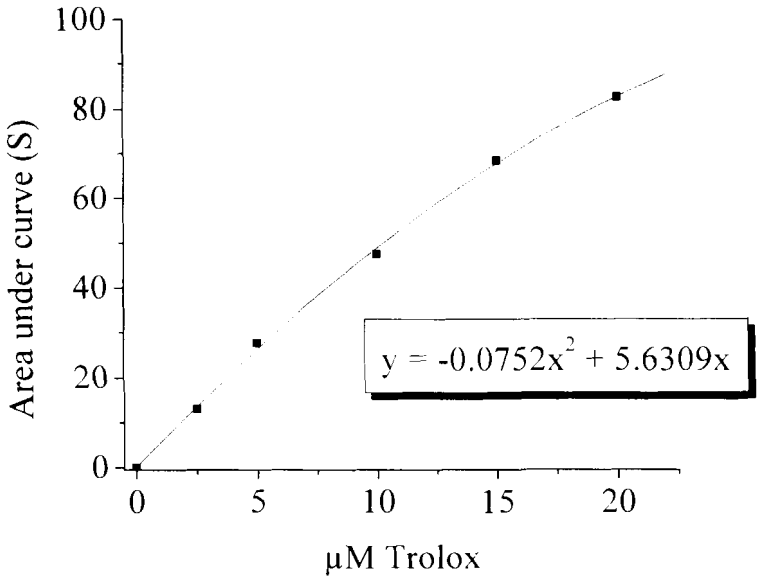

Figure 3.4 Standard curve of Trolox standards at varying concentrations

The area under the curve was calculated according to Equation 3.6 to quantify the antioxidant capacity. This was used, together with the concentrations of the standards used in the assay. A 2nd order polynomial function $y = ax^2 + bx + c$ (Figure 3.4) was used to determine the antioxidant capacity (ORAC value) which were calculated by using Equation 3.7 and expressed as µM Trolox equivalents (TE).

Equation 3.6 Determination of the area under the curve

$$S = (0.5 + F5/F0 + F10/F0 + F15/F0 + \ldots + Fi/F0) \times 5$$

S = area under the curve; F0 = initial fluorescence at 0 min; Fi = fluorescence measurement at time i.

Equation 3.7 Determination of the ORAC value

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

Where $y = (S_{sample} - S_{blank})$

3.5.2 Ferric reducing/antioxidant power assay (FRAP)

The FRAP assay measures the ability of plasma to reduce the colourless ferric-tripyridyltriazine complex (TPTZ-Fe^{3+}) to its ferrous, coloured form (Fe^{2+}) (Benzie and Strain, 1999). The absorbance of the change in the intense blue colour can be monitored spectophotometrically at 593 nm. The change in absorbance directly relates to the total reducing power of the antioxidants in the plasma.

Method

Plasma samples were prepared as discussed in Section 3.3.1. An acetate buffer (300 mM sodium acetate, pH 3.6 in glacial acetic acid, 6% (v/v)). A standard series was prepared from a 1 mM ascorbic acid stock solutions to final concentrations of 0, 25, 50, 100, 250 and 500 µM in a volume of 100 µl. Plasma (20 µl) and acetate buffer (30µl) were added to the wells of a microtitre plate. A FRAP reagent (acetate buffer, 10mM TPTZ and 20 mM $FeCl_3.6H_2O$; v/v 10:1:1) was added to standards and samples. The plate was incubated for 15 minutes at room temperature and read in a BIO-TEK (FL 600) microplate reader at 593 nm. Linear regression of data from the standards was used to calculate the antioxidant capacity. Because direct reaction of Fe^{2+} gives a change in absorbance half that of an equivalent concentration of ascorbic acid (Benzie & Strain, 1999), FRAP values was calculated by using Equation 3.8.

Equation 3.8 Determination of the FRAP value

$$\mu M \text{ of antioxidant power as FRAP} = 2 \times \mu M \text{ Ascorbic acid}$$

3.6 Antioxidant enzymes

The antioxidant enzyme analyses were done on isolated white blood cells (see Section 3.3.2). The results were expressed relative to the protein content.

3.6.1 Protein concentration

The standard bicinchoninic acid (BCA) method for protein determination was done using 1 $\mu g.\mu l^{-1}$ bovine serum albumine (BSA) as a standard (Smith *et al.*, 1985). Under alkaline conditions, divalent copper ions (Cu^{2+}) form a complex with peptide bonds in which it is reduced to a monovalent ion (Cu^{+}). The Cu^{+} ions and the radical groups of tyrosine, tryptophan and cysteine react with BCA to form a purple colour reaction.

Method

The sample supernatant (2µl) was mixed with 200 µl of a solution of BCA and $CuSO_4.5H_2O$ to a ratio of 50:1 in a microtitre plate. The samples were then incubated for 20 minutes at 37°C and the absorbance read at 560nm in a BIO-TEK microplate fluorescence plate reader. The production of Cu^{+} is proportional to the protein content. The protein content of the samples was inferred from the known concentration of the BSA standard using linear regression.

3.6.2 Catalase activity

When an organism is exposed to ROS, antioxidant defenses are upregulated to prevent or limit oxidative stress. One part of the antioxidant defense system is the antioxidant enzymes (Section 2.3). It was important to asses the antioxidant enzyme levels in the white blood cells. All antioxidant enzyme analyses were done at room temperature and analysed according to the methods of Ellerby and Bredesen (2000), but with slight modifications. The enzyme activity (catalase, GR and GPx) was calculated by using the following equation and expressed as U/µg of protein.

Equation 3.9 Calculation of the activity of the antioxidant enzymes

$$\text{Enzyme activity (U/µg protein)} = [(A1-A2)/\varepsilon] \times 0.5/\ \mu g \text{ protein}$$

A1= initial absorbance ; A2 = reaction absorbance ; ε = the extinction coefficient for the reaction; 0.5 = volume correction factor.

The isolated white blood cells were resuspended in 400 µl of phosphate buffer (50 mM $NaPO_4$, pH7.5). The cells were sonicated for two five second bursts on ice and centrifuged for 10 minutes at 15 000 x g and 4 °C. The resultant supernatant was carefully aspirated and transferred to a microcentrifuge tube and frozen at -80°C until assayed. The protein determination was done using the BCA method as described (Section 3.5.2). For the catalase assay, 10 µl of a 0.1 µg/µl homogenate in catalase assay buffer (50 mM KPO_4, pH 7.0) was used.

Method

Catalase assay buffer (50 mM $NaPO_4$, pH 7.0) and H_2O_2 stock solution (30% (v/v) H_2O_2 in catalase assay buffer) was added in a disposable UV cuvette to a volume of 500 µl. The absorbance was read at 240nm in a BIO-TEK UVIKON UV double beam spectrophotometer, to obtain a value of approximately 0.45 before the reaction was started. Ten µl of homogenate was then added, mixed well and the linear decrease recorded at 15 second intervals for at least 1 minute (at 25 °C). This was determined for the blank (A2) and the samples (A1). The activity was calculated by using the Equation 3.9. The extinction coefficient for catalase was $\varepsilon_{240} = 0.00394\ mM^{-1}.cm^{-1}$.

3.6.3 Superoxide dismutase (SOD) activity

The samples were prepared as described (Section 3.6.2). They were diluted to contain exactly 0.1 µg/µl in the SOD assay buffer (50 mM $NaPO_4$, pH 7.4). 6-Hydroxydopamine (6-HD) was prepared as follows: double distilled water containing concentrated perchloric acid ($HClO_4$) was nitrogen purged for 15 minutes and 6-HD was added to a final concentration of 1.6 mM. The reaction mixture was protected from light.

Method

The kintetics of auto-oxidation of 6-HD as follows: the SOD mediated prevention of auto-oxidation was monitored at 490 nm for approximately four minutes in a BIO-TEK microplate reader. Four different sample volumes were added ranging between 0 and 18 µl in duplicate to a microtitre plate. Hundred and seventy µl of DETAPAC solution (diethylenetriaminepentaacetic acid, 0.1 mm in SOD assay buffer) was added to this and

filled to a final volume of 185 µl with SOD assay buffer. To start the reaction, 15 µl of stock solution of 6-HD was added and the auto oxidation recorded. The linear increase was determined and the amount of protein that resulted in 50% inhibition of auto oxidation of 6-HD calculated. A decrease in 6-HD auto-oxidation therefore reflects an increase in the actual enzyme activity and vice versa.

3.6.4 Glutathione reductase (GR) activity

The cells were prepared as described (Section 3.6.2). For the GR assay, 10 µl of a 5 – 10 µg/µl protein supernatant was used.

Method

The GR activity was measured by placing GR assay buffer (50 mM Tris.HCl, 1 mM EDTA, pH 8.0) and GSSG solution (8 mM) in a disposable cuvette to a final volume of 500 µl. The cuvette was blanked and 5 µl NADPH solution (15 mM in 0.1% (w/v) $NaHCO_3$) was added. Ten µl of the sample supernatant was added and the linear decrease in absorbance recorded at 30 second intervals for five minutes at 340 nm using a Bio-Tek UVIKON UV-Vis double beam spectrophotometer. The GR activity was determined for the blank (A2) and for the samples (A1). The activity was determined by using Equation 3.9. The extinction coefficient for NADPH was $\varepsilon_{340} = 6.22\ mM^{-1}.cm^{-1}$.

3.6.5 Glutathione peroxidase (GPx) activity

The cells for the GPx assay was prepared as described (Section 3.6.2) and for the assay 10 µl of a 5-10 µg/µl supernatant was used.

Method

The assay buffer (50 mM K_2HPO_4, pH 7.0 and 1 mM EDTA), 5 µl of the GSH solution (30.7 mg/ml in water, Boehringer Mannheim™), 5 µl GR (187 U/ml in assay buffer) and 10 µl sample or buffer (blank) was added in a disposable cuvette. The cuvette was blanked and 5 µl of nicotineamide adenine dinucleotide phosphate (NADPH, 15 mM in 0.1% (w/v) $NaHCO_3$) was added. The *tert*-butyl hydroperoxide (*t*-BHP) independent NADPH oxidation was recorded at 340 nm for 3 minutes at 30 second intervals for the

samples (A1) and blank (A1b). The reaction was started with 50 µl *t*-BHP solution (7.7 M) and the hydroperoxide-dependent linear NADPH oxidation was recorded for 3 minutes in 30 second intervals for samples (A2) and blank (A2b). The activity of the enzyme was calculated by using Equation 3.9. The extinction coefficient for NADPH was $\varepsilon = 6.22\ mM^{-1}.cm^{-1}$.

3.7 Apoptosis

3.7.1 Caspase 3/7 activity

The measurement of cysteine aspartic acid-specific protease activities of caspases 3 and 7 (combined) were done using the Apo-ONE® Homogenous Caspase-3/7 assay kit. Active caspases participate in a cascade of cleavage events that disables repair enzymes and induce apoptosis. The assay is based on the cleavage of the caspase 3/7 substrate (N-CBZ-L-aspartyl-L-glutamyl-L-valyl-L-aspartic acid amide, Z-DEVD-R110) by caspases to create the fluorescent Rhodamine 110. The fluorescence was measured at excitation 485 and emission 521 nm, with a BIO-TEK fluorescence (FL 600) microplate reader.

Method

The isolated WBC were counted and 25 000 cells per well were seeded into microtitre plates (final volume of 100 µl in PBS). The positive control cells were incubated with 1 µg/ml staurosporine in the media for 2 hours. The Apo-ONE® Caspase 3/7 reagent was added to the cells and the cells allowed to incubate for two hours. Fluorescence was measured at 30 minute intervals at excitation 485 and emission 530 nm for at least three hours.

3.8 Mitochondrial function

3.8.1 NADH/NAD^+ assay

NADH and NAD^+ was determined as described by Zerez *et al* (1987). The favoured reduction of pyruvate to lactate under defective oxidative phosphorylation results in the

production of a high concentration of NAD^+. The reaction is a cycling reaction since alcohol dehydrogenase (ADH) reduces NAD^+ to NADH, which is oxidised by N-ethyldibenzopyrazine (PES) and then reoxidised by 3-[4,5-dimethylthiazol-2-yl]2,5-diphenyltetrazolium bromide (MTT) resulting in a colour change reaction that is measured at 570 nm. In order to avoid degradation the samples were not allowed to remain in the cycling buffer for longer than 15 minutes.

Method

Blood samples were collected in fluoride tubes. The fluoride treated blood was mixed with 1980 µl of a buffer solution (10 mM nicotinamide, 20 mM $NaHCO_3$ and 100 mM Na_2CO_3 pH 7.5) at 4 °C. The mixture was snap frozen in liquid nitrogen, thawed at room temperature in a water bath and promptly chilled to 0 °C. To destroy the oxidized forms of NAD^+, 1000µl of this mixture was incubated at 60 °C for 30 minutes in a dry heating block. The samples were stored at -80 °C until assayed. The following was added to 80 µl of the sample supernatant: 25 µl Tris-HCl (1 M, pH 8), 5 µl PES (100mM), 12.5 µl MTT (10mM), 7 µl ADH (29.4mg/ml) and 101.5 µl distilled water. Twenty µl ethanol (33% v/v) was added to start the reaction and the absorbance (A) was read at 570 nm in a BIO-TEK microplate fluorescence (FL 600) plate reader for 4 minutes (t). A standard curve with NADH ranging between 0 and 1000 nM was generated and used to quantify the concentration of NADH and NAD^+ in each sample (Zerez *et al.*, 1987). The absorbance values were used to calculate the NADH/NAD^+ ratio (Equation 3.10).

Equation 3.10 Determination of the NADH/NAD^+ ratio

$$NADH/NAD^+ = (A_{NADH}/t)/([A_{NADH}/t) - (A_{NADH}/t)]$$

NADHt = samples kept on ice (NADH /NAD^+); NADH = samples heated at 60°C (NADH); t = minutes.

3.8.2 ATP analysis

The ATP bioluminescent somatic cell assay kit was used to determine the ATP release from a suspension of viable white blood cells. The number of viable cells was selectively counted because when a cell dies, its ATP is rapidly degraded.

Method

Cells were prepared in a microtitre plate containing 20 000 cells/well. Cell protein content was determined as described before (Section 3.6.1). An ATP releasing agent was added and the mixture left at room temperature for 5 minutes. The cells were then transferred to an opaque microtitre plate which contained the ATP assay mix. The luminescence was determined with a BIO-TEK microplate fluorescence (FL 600) plate reader. An ATP standard curve was used to quantify the amount of ATP present in the samples.

3.8.3 Oxygen consumption measurements

Respiration rate, measured as oxygen consumption, is used in a wide variety of applications and is commonly associated with mitochondrial function. The respiration was measured by using closed cell respirometry in which a decrease in oxygen with time is measured. Isolated rat liver mitochondria were used in the assay, because of certain limitations of white blood cells. Ethical approval was obtained for the experimentation with rats (approval number 06D04). The liver sample was dissected, cut into pieces and suspended in medium A (210 mM mannitol, 70 mM sucrose, 50 mM Tris-HCl, 10 mM EDTA, pH 7.4 at 4°C) to a end concentration of 10% (w/v). The tissue was homogenized with a Potter-Elvehjam homogenizer set at 200 rpm for 15 strokes. The homogenate was transferred to a microcentrifuge tube and centrifuged (1000 x g) for 5 minutes at 4°C. The resulting supernatant was centrifuged (7000 x g) for 10 minutes at 4°C. The supernatant was discarded at the mitochondrial-enriched pellet suspended in Medium B (225 mM mannitol, 75 mM sucrose, 10 mM Tris-HCl, 0.1 mM EDTA, pH 7.4 at 4°C) and the centrifuge steps repeated. The final mitochondrial pellet was suspended in 50-100 µl medium B and kept on ice during assays. The protein concentration of the mitochondria was determined as described (Section 3.6.1).

Method

Oxygen consumption was measured with a Mitocell oxygen electrode (model MT 200: Strathkelvin Instruments LTD.). Recordings were done by an oxymeter (model 787: Strathkelvin Instruments LTD.) using Oxygen System software (Strathkelvin 782,

version 3.0). The apparatus was set up and operated according to instructions from the manufacturer. Before the start of experiments, the electrode was calibrated by using a low point calibration (distilled water with Na_2SO_3) and a high point calibration (distilled water saturated with air). All calibrations and reactions were done at 30 °C. The reaction medium consisted of 150 mM mannitol, 250 mM sucrose, 200 mM KCl, 20 mM Tris-phosphate, 20 mM Tris-HCl, 0.1mM EDTA, pH 7.4 in a reaction volume of 70 µl. The reaction medium together with 0.25 µg/µl mitochondria were placed in the respiration chamber and the measurement started. The reaction was initiated for 2 minutes (state 1 respiration), after which either 10 mM glutamate + 10 mM malate or 25 mM succinate + 3 mM rotenone was added and the recording (state 2 respiration) continued for another 2 minutes before adding 2 mM ADP (state 3 respiration). The state 3 respiration was recorded until the ADP was exhausted (state 4 respiration). Oxygen consumption was calculated using the slopes of the various respiration states using the software (Oxygen System) and the amount of protein used in the reaction.

3.9 Respiratory chain enzyme analysis

3.9.1 Citrate synthase activity

The complexity of respiratory chain defects stresses the necessity to measure the activity of at least one mitochondrial enzyme that is not involved in the respiratory chain (e.g. citrate synthase). This is to distinguish between a low count or general activity of mitochondria per gram tissue, and a disturbance restricted to the enzyme activities of the respiratory chain. Citrate synthase is located in the matrix of the mitochondria and catalyses the conversion of oxaloacetate to citrate by acetyl-coenzyme A . The citrate enters the Krebs cycle (Ruitenbeeck *et al.*, 1989). The assay measures the reduction of DTNB (5,5'Dithiobis-2-nitrobenzoic acid; Ellmans reagent) to TNB at 412nm (extinction coefficient $13.6mM^{-1}$), coupled to the reduction of coenzyme A (CoA) by the citrate synthase in the presence of oxaloacetate.

Method

Citrate synthase activity was measured at 30°C by placing 408µl H_2O, 50µl DTNB

(0.4mg/ml in 1M Tris-HCl, pH 8.0), 2µl Triton X100 (10%), 10µl mitochondrial preparation and 5µl acetyl-CoA (6mM) in a disposable cuvette. The absorbance (ΔA1) was recorded at 412nm on a Biotek UVIKON UV-Vis double beam spectrophotometer for two minutes (Δt1). Oxaloacetate (1.32mg/ml in H_2O) was then added and absorbance (ΔA2) measured for another three minutes (Δt2) (Pitkänen *et al.*, 1996b). Activity was calculated in µmole/min according Equation 3.11.

Equation 3.11 Citrate synthase activity calculation

$$\text{CS activity} = ((\Delta A2/\Delta t2) - (\Delta A1/\Delta t1)) \times (V/v \times 1000) / 13.6$$

V = total volume (µl) in cuvette; v = volume (µl) mitochondrial preparation; t = min; A = absorbance; extinction coefficient for TNB = $13.6 mM^{-1}.cm^{-1}$.

3.9.2 Complex I activity

The activity of complex I can be measured spectrophotometrically by either measuring the oxidation of NADH or the reduction of ubiquinone analogues or homologues. In this study, the oxidation of NADH was monitored at 340 nm. Liver mitochondrial preparations were prepared described (Section 3.8.3).

Method

Respiratory chain complex I activity was assayed on the mitochondria-enriched pellet of liver homogenates according to the method described by Rahman *et al.* (1996). To measure complex I activity, 10µl of mitochondrial preparation was mixed with 395 µl H_2O, 2.5 µl coenzyme Q1 (10 mM), 5 µl ethanol and 87.5 µl of an assay reagent containing 0.5 M K_2HPO_4 (pH 7.4), 2 mM NADH, 50 mM KCN, 0.5 mM antimycin and 10% (w/v) bovine serum albumine (BSA, fatty acid free) in an UV disposable cuvette at 30°C. The final reaction volume was 500 µl. The linear decrease in absorbance (A1) was measured for 1 minute at 340nm in a Biotek UVIKON UV-Vis double beam spectrophotometer. NADH oxidaiton was also measured in a separate reaction in the presence of 0.75 nM rotenone (A2). The difference in absorption with or without rotenone approximates the rotenone-sensitive activity of complex I. Activity was

calculated in µmole/min and normalized to citrate synthase activity (see Section 3.9.1) using Equation 3.12.

Equation 3.12 Complex I activity calculation

Complex I activity = [(A1 –A2)/ 6.81]/[µl protein used/1000) x mg/ml protein] x 0.5
Complex I activity per units citrate synthase (µmole/min/UCS) = Complex I activity / CS activity

A = absorbance at 340 nm; extinction coefficient for NADH = 6.81mM^{-1}.cm^{-1} and not the recommended 6.22 in order to compensate for the contribution of coenzyme Q1.

3.9.3 Complex I + III activity

The combined activity of complexes I+III can be measured as rotenone-sensitive NADH-dependent cytochrome c reduction (Rahman *et al.*, 1996). It is measured in the presence of a natural, instead of an artificial electron acceptor.

Method

The mitochondrial-enriched pellet was prepared as in Section 3.8.3. To measure the combined activity of complex I+III, 2.5 µl of the mitochondrial preparation was mixed with 402.5 µl H_2O in a disposable cuvette. Ethanol (5 µl) was added to the cuvette and blanked. An assay reagent containing 0.5 M K_2HPO_4 (pH 7.4), 2 mM NADH, 50 mM KCN and 10% (w/v) BSA was added. Finally, 12.5 µl cytochrome c (2 mM) was added and the linear decrease in absorbance (A1) recorded at 550 nm for 3 minutes at 30°C in a Biotek UVIKON UV-Vis double beam spectrophotometer. The final reaction volume was 500 µl. Rotenone-sensitive cytochrome c reduction was measured in the presence of 0.75 mM rotenone (A2). The activity was calculated using Equation 3.13.

Equation 3.13 Calculation of complex I+III activity

Complex I+III activity = [(A1-A2)/29.5]/[µl protein used/1000)x mg/ml protein] x 0.5
Complex I+III activity per units citrate synthase (µmole/min/UCS) = Complex I+III activity/ CS activity

3.9.4 Complex II + III activity

An estimation of complex II activity was measured as the combined complex I+III activity. The rate of succinate dependent reduction was measured in the presence of rotenone (complex I inhibitor) and KCN (complex IV inhibitor).

Method

The mitochondrial-enriched pellet (2.5 µl, as prepared in section 3.8.3) was mixed with 356 µl H_2O and 124 µl of an assay reagent containing 0.5 M K_2HPO_4 (pH 7.4), 0.1 M sodium succinate, 50 mM KCN, 10% (w/v) EDTA (pH 7), 10% (w/v) BSA and 0.25 mM rotenone in a disposable cuvette. Five µl ATP (0.2 M) was added and the reaction started by adding 12.5 µl cytochrome c (2 mM). The final reaction volume was 500 µl. The linear decrease in absorbance (A1) was recorded at 550 nm for 3 minutes at 30°C in a Biotek UVIKON UV-Vis double beam spectrophotometer. For the blank (A2) the reduced cytochrome was replaced with H_2O. The activity was calculated by using Equation 3.13.

3.9.5 Complex IV activity

Complex IV activity can be measured by isolating the activity from the rest of the respiratory chain with rotenone and antimycin A. In this study, the complex IV activity was measured spectrophotometrically at 550 nm using cytochorme c as substrate.

Method

The mitochondrial-enriched pellet (1 µl, as prepared in Section 3.8.3) was mixed with 90 µ K_2HPO_4 (0.5 M, pH7.4) and 389 µl H_2O. A calculated volume of reduced cytocrome c to give an end concentration of 20 µM was added. The final reaction volume was 500 µl. The linear decrease in absorbance (A1) was recorded at 550 nm for 1 minute at 30°C in a Biotek UVIKON UV-Vis double beam spectrophotometer. For the blank (A2) the reduced cytochrome was replaced with H_2O. The activity was calculated by using Equation 3.13.

3.10 Statistical analysis

All statistical analyses was done using Graphpad Prism®[1], version 4 or Statistica©[2] version 7 software packages. Results were expressed as the mean ± 1 standard error of the mean (1SEM). The results are presented either in tabular or graphical form. In order to measure the level of statistical difference, the distribution of the data was determined with normal probability plots (see Appendix B). To determine the variation between the means values of each of the groups, an analysis of variance (ANOVA) was done. The repeated measure ANOVA was used because different treatments were given to the same individual and each individual served as his/her own control. In cases where significant differences were measured, the Bonferroni post-hoc test was used to determine the significance between the different groups. Differences in mean values were considered significant when $p < 0.05$. Data of the buffered cell group where compared to control of the whole blood group to determine protective effects of plasma. Data of the O_3 treated samples were compared to the O_2 treated samples to determine whether similar effects could be observed with O_2. Statistical significant differences were indicated with an asterisk (*) when compared to the control of the whole blood group and with a number sign (#) when compared to the oxygentated samples of the whole blood group.

[1] Graphpad® Prism is a registrated trademark of GraphPad Software Inc., San Diego, CA,USA.
[2] Statistica © is a copyright of Statsoft Inc., Tulsa, OK, USA.

4 Results and discussion

The specific hypothesis of this study was to evaluate if plasma provides an effective antioxidant defence system that can protect against the possible harmful effects of O_3. The interaction of O_3 with blood, more specifically red blood cells, has been studied in detail. Ozone has also received much interest because it induces production of cytokines and so upregulates immunity (Bocci, 2002). Relatively few studies have focused on the viability of white blood cells. Study of the effects of O_3 on cell cultures presents problems because it gives an inaccurate picture due to the poor antioxidant capacity of media and serum (Leist *et al.*, 1996). In this study the effects of O_3 on white blood cells under conditions similar to that during O_3-AHT, was evaluated. The designed treatment protocol served this purpose. The treatment protocol also included an O_2 treated group, which was used to determine whether similar effects could be measured when using only O_2.

Different approaches were followed to evaluate possible therapeutic and/or toxic effects of O_3. The first was to determine the effect of O_3 on the white blood cell count. The effect on the viability, apoptosis and mitochondrial function of white blood cells was also determined. The mitochondrial function was further determined in rat liver preparations. Various techniques were used to determine the ROS levels and redox status in the two intervention groups. The results of the two intervention groups will be discussed by referring to whole blood and buffered cells. The term whole blood is indicative of the protective role of the antioxidants and buffered cells, of the removal of the antioxidants (see Figure 3.1).

4.1 White blood cell viability

4.1.1 White blood cell count

The white blood cell count provided information on how the different blood components were affected by O_3 and it allowed comparisons on how the observed results related to some of the biochemical analysis (Table 4.1).

White blood cells is a heterogeneous cell population composed of lymphocytes (20-25%), monocytes (5%) and three types of granulocytes of which neutrophils are most abundant (70-72%). Changes in the neutrophil population therefore contribute more to changes in the white blood cell count than the other populations (Benjamini *et al.*, 2000).

Table 4.1 The effect of exposure to O_2 and O_3 (20 and 80 µg/ml) on the white blood cell count.

Whole blood	Control	O_2	O_3 [20]	O_3 [80]
WBC x 10^9/ℓ	5.82±0.04	4.30±0.58	4.46±0.65	4.20±0.51
Neutrophils x 10^9/ℓ	3.14±0.29	2.45±0.30	2.59±0.41	2.33±0.29
Lymphocytes x 10^9/ℓ	1.93±0.10	1.40±0.24	1.46±0.22	1.61±0.36
Monocytes x 10^9/ℓ	0.60±0.08	0.45±0.09	0.41±0.07	0.41±0.10
Buffered cells	**Control**	**O_2**	**O_3 [20]**	**O_3 [80]**
WBC x 10^9/ℓ	4.92±0.26	3.46±0.56*	3.54±0.64*	3.66±0.54*
Neutrophils x 10^9/ℓ	2.64±0.34	2.13±0.37*	2.18±0.38*	2.31±0.36*
Lymphocytes x 10^9/ℓ	1.43±0.15	1.04±0.19	1.05±0.23	1.11±0.15
Monocytes x 10^9/ℓ	0.55±0.07	0.29±0.06	0.31±0.08	0.24±0.09

Results are given as mean ± 1SEM, significance (*$p<0.05$, compared to control) determined with Bonferroni test (n= 5). WBC = Differential white blood cell count

The white blood cell count in whole blood decreased by approximately 25% as a result of treatment with O_2, 20 µg/ml and 80 µg/ml O_3 (Table 4.1). Similar decreases were

observed in the neutrophil, lymphocyte and monocyte counts. Washing the whole blood to get rid of the plasma resulted in a loss of approximately 16% of the white blood cells. Again, this loss was similar for the neutrophils, lymphocytes and monocytes. Treatment of the buffered cells with O_2 and the two concentrations of O_3 also resulted in a loss of approximately 25% of the white blood cells. Again, the decreases in neutrophils, lymphocytes and monocyte counts were similar. It is, however, important to note that the decreases in white blood cell count in the buffer were significant, whereas it was not so for the cells treated in plasma.

Numerous studies have reported that white blood cells are sensitive to oxidative stress (Prasad *et al.*, 2005, Saraymen *et al.*, 2003, Vural *et al.*, 2005) and increases in ROS production following O_2 and O_3 treatment probably caused the decrease in white blood cells. The decreases were more pronounced in the buffered white blood cells. This suggests that these cells may have been more sensitive to the ROS generated by O_2 and O_3. The loss of white blood cells was random and the neutrophils, leukocytes or monocytes were equally sensitive to the different treatment regimes.

4.1.2 Cell viability

Cytotoxicity studies provide useful information for understanding the pathogenesis of certain diseases and for developing therapeutic drugs. A number of *in vitro* approaches were developed to evaluate cytotoxicity or cell viability. The trypan blue exclusion assay and the LDH release assay are two of the most frequently used approaches to study cytotoxicity. The effect of treatment with O_2 and O_3 on cell viability as measured with the trypan blue exclusion assay is summarised in Figure 4.1.

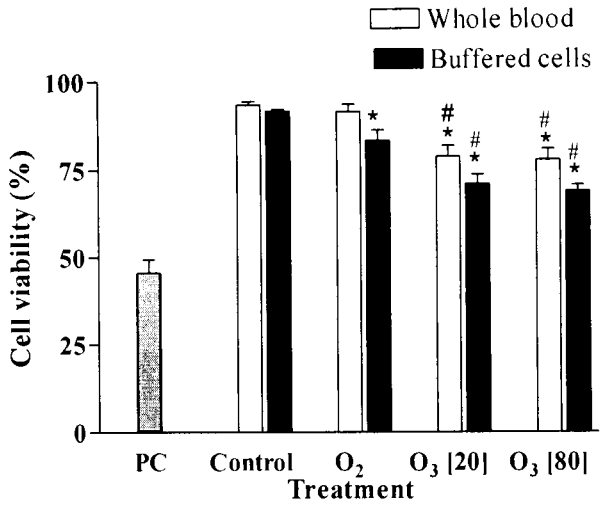

Figure 4.1 The viability of white blood cells measured in the two intervention groups. Results are given as mean ± 1SEM (n=6) cell viability (%) for the positive control (PC), whole blood and the buffered cells at baseline and after exposure to O_2 and 20 and 80 μg/ml O_3. $p<0.05$ (Bonferroni test) relative to the control (*) and O_2 (#) of the whole blood. In the PC, the cells were treated with 6% acetic acid.

Exposure of whole blood to O_2 had no significant effect. Following exposure to 20 and 80 μg/ml O_3 cell viability decreased significantly by 16 and 17% respectively. Viability of buffered cells decreased slightly due to removal of plasma. This is important since removal of the plasma (by centrifugation) and resuspending the cells in PBS without further treatment did not markedly affect cell viability. Exposure to O_2 significantly decreased cell viability by approximately 11%. Ozone treatment caused even further damage to the cells since viability decreased by 24 and 26% after exposure of 20 and 80 μg/ml O_3 respectively. Although cell viability decreased, it remained significantly higher than in the positive control cells, which were treated with 6% acetic acid (Figure 4.1). The results indicate that O_3 is toxic to white blood cells, even at low concentrations and that this is even more pronounced when the antioxidant capacity is lowered by removal of plasma.

LDH release measures the amount of intracellular LDH that is released when the plasma membrane of cells are damaged. It is considered a sensitive assay to measure cell necrosis (Decker and Lohman-Matthes, 1988). The results of the treatment of blood and buffered cells are given in Figure 4.2. In the whole blood LDH release increased, but not significantly, after treatment with O_2 and 20 µg/ml O_3 by 49 and 50% respectively. Treatment with 80 µg/ml O_3 increased the LDH release significantly by 79%. LDH release from untreated buffered cells did not differ significantly from that of whole blood. Treatment of buffered cells with O_2 and the different concentrations of O_3 appeared to have the similar levels of increase on LDH release as in whole blood. The values increased significantly by 23% after treatment with O_2. LDH release increased by 50 and 49% after treatment with 20 and 80 µg/ml O_3, respectively. This strongly suggested that O_3 caused necrosis in the white blood cells, even in the presence of plasma antioxidants.

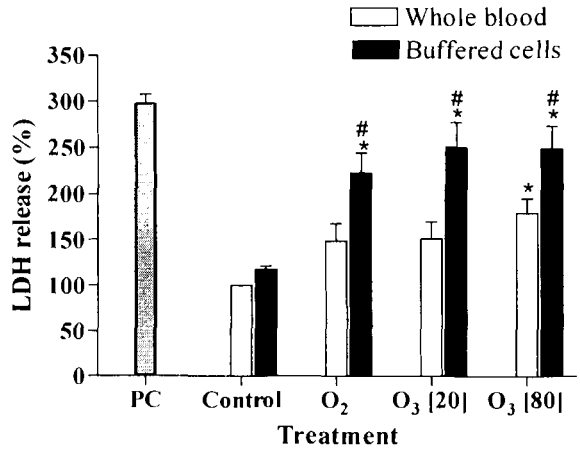

Figure 4.2 The LDH release from cells measured in the two intervention groups.
Results are given as mean ± 1SEM (n=6) LDH release (%) for the positive control (PC), whole blood and the buffered cells at baseline and after exposure to O_2 and 20 and 80 µg/ml O_3. $p<0.05$, relative to the control (*) and O_2 (#) of whole blood (Bonferroni test). The positive control (PC) was treated with 1% Triton X®.

It has been reported that ozone affects cell viability of cultured monocytic cells (Klestadt *et al.*, 2005). Exposure of white blood cells to O_3 also caused a dose dependent increase of DNA damage (Diaz-Llera *et al.*, 2002). *In vivo* studies with baboons showed that DNA

damage to white blood cells occur only during the first 24 hours after a single O_3-AHT treatment, but is not evident after 48 hours (Van Helden, 2006). Under conditions of oxidative stress, ROS can damage chromosomal DNA and other cellular components. This can results in DNA degradation, protein denaturation and lipid peroxidation (Higuchi, 2004). Thus, increased oxidative stress caused by O_3 is likely to be responsible for the increased cell death of white blood cells that we saw, since mechanical stress was not present. Ozone exposure increases the formation of H_2O_2 which can damage cells via the Fenton reaction. The O_3 also reacts with polyunsaturated fatty acids (PUFAs) in the membranes of the cells. This can also lead to the increased cell necrosis and can explain why LDH release increased. On the other hand, membrane damage due to O_3 binding to PUFAs can damage the cell membrane and so increase LDH release, without causing necrosis to the cells. We have no data to support this assumption, especially in view of the fact that treatment with O_3 did not affect white blood cell morphology adversely (Figure 4.3). The decreased antioxidant capacity in the buffered cells will most likely increase ROS and so increase cell death.

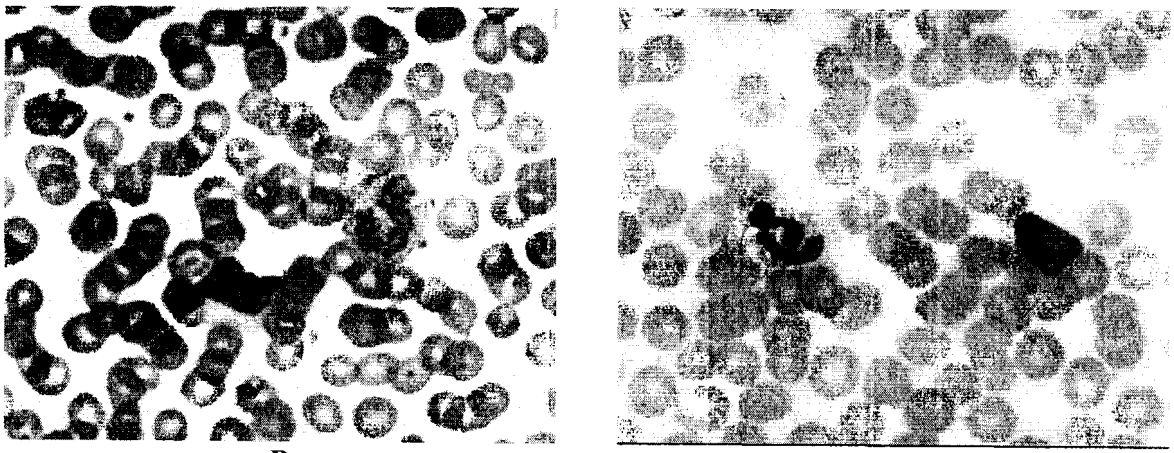

Figure 4.3 Blood smear of neutrophils before and after treatment with 80 µg/ml O_3. Pre = before ozonation, ozonation = 80 µg/ml O_3.

4.2 Determination of oxidative stress

Analysis of oxidative stress and its relationship to antioxidant needs have been approached by two different strategies. The first measures a common marker of oxidative damage to mainly lipids caused by free radicals, namely hydroperoxides. The second measures the antioxidant reserve through oxidative stress resistance and focuses on the redox dynamics/oxidative stress resistance. These are but only two of many methods and markers of oxidative stress and antioxidant capacity (Cutler *et al.*, 2005; Cutler, 2005). It is important to note that, in some cases, the control values differ from the normal published values. It is a well known fact that the total antioxidant status and oxidative stress are influenced by both *in vivo* and environmental factors (Halliwell & Gutteridge, 2000) and that differing values in a small cohort not necessarily mean that the subjects that were used are not normal. Moreover, the main objective of the study was to assess the effect of treatment of blood and buffered cells with O_2 and O_3 and not to evaluate normal distribution of these parameters. Each subject therefore served as his/her own control.

4.2.1 Hydroperoxides

A test kit (d-ROMs test, DIACRON International, Grosseto, Italy) was used to quantify hydroperoxides in the samples (Iorio, 2002). Values are expressed in CARR U (1 CARR U corresponds to 0.08 mg/100 ml H_2O_2). Reactive oxygen metabolites form derivatives that maintain good chemical reactivity, which can be accurately measured. Healthy individuals have CARR U values ranging between 250-300 (Iorio, 2002) (Table B1, Appendix B). In this study four of the five subjects had baseline values exceeding the normal values suggesting high to very high levels of oxidative stress in them (Table A1, Appendix A). One subject had normal levels.

Following exposure to O_2, hydroperoxide formation (mean values) in whole blood increased by approximately 4.3% (Figure 4.4). Following exposure to 80 µg/ml O_3 mean

values increased by approximately 5.8%. In the buffered cells, exposure to O_2 and both O_3 concentrations increased hydroperoxides significantly by approximately 13%.

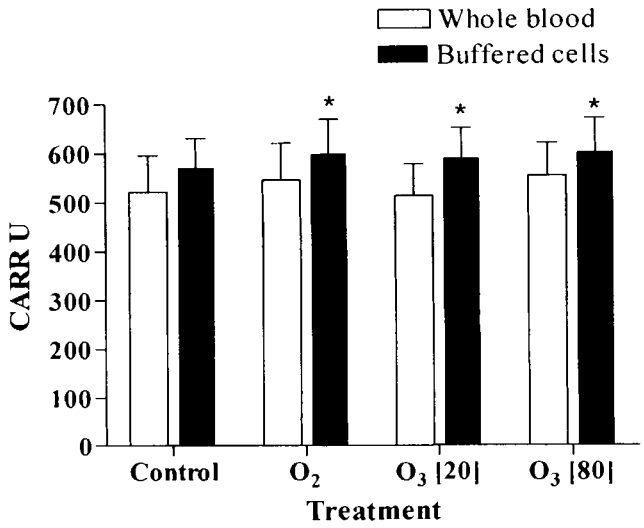

Figure 4.4 The hydroperoxides measured in the two intervention groups Results are given as mean ± 1SEM (n=5)CARR U for whole blood and the buffered cells at baseline and after exposure to O_2 and 20 and 80 μg/ml O_3. $p<0.05$ with respect to control (*) of the whole blood group (Bonferroni test).

Oxidative stress is common in several physiological and pathological conditions and a high levels of hydroperoxides strongly suggests an increase in the ROS or a decrease in antioxidant defences (Cornelli *et al.*, 2001). Plasma redox state indicates the sum of the reducing and oxidizing equivalents in plasma. This is dependent on many different factors, including protein present in plasma and certain metabolic processes such as oxidative phophorylation (Serafini and Del Rio, 2004). Bocci *et al* (1998) reported a four to five fold increase in hydroperoxides following exposure of blood to 70 μg/ml O_3. They used the thiobarbituric acid reactive substances (TBARS) assay that also measures hydroperoxides that form as a result of lipid peroxidation. This differs markedly from the results in whole blood, where no significant increases where found. Significant increases in the absence of antioxidants, i.e. in buffered cells, were seen however. The increase varied from 12-14%, also much less than that reported by Bocci *et al* (1998). The results in whole blood clearly indicate that, under the conditions of this study, there were enough

antioxidant capacity to quench the hydroperoxides. In the case of the buffered cells, the cells also contained enough oxidant capacity to largely quench hydroperoxides formed.

It is evident that the antioxidants present in plasma inhibited the formation of hydroperoxides to a certain extent. When the plasma antioxidants were removed, hydroperoxides production increased approximately threefold (Figure 4.4).

4.2.2 The glutathione redox status (GSH/GSSG ratio)

GSH is the major intracellular thiol antioxidant in virtually all mammalian tissues where it plays a crucial role in neutralizing free radicals. The glutathione redox status is a sensitive indicator of cell function, viability and the redox status. Low GSH, high GSSG and lower GSH/GSSG ratios in blood is associated with various pathological conditions where oxidative stress plays a role (Pastore *et al.*, 2003). Measurement of the GSH/GSSG ratio in blood is generally regarded as a sensitive index for whole-body glutathione status. When performing the method, care must be taken since the first step in the assay is the precipitation of proteins by acidification. During this step oxyhaemoglobin-derived oxidants are formed which oxidise GSH. This may lead to an overestimation of GSSG (Asensi *et al.*, 1999). Therefore, the accuracy of the assay is dependent on the prevention of auto-oxidation of GSH during sample preparation. To prevent GSH oxidation a GSH scavenger, M2VP, was added. M2VP is preferred because it rapidly reacts with GSH (Asensi *et al.*, 1999).

The reference values of GSH and GSSG in blood and plasma differ due to different sample processes, methods or patients selection. Taking this into account, GSH values range from as low as 2 µM to as high as 2525 µM (Pastore *et al.*, 2003). Reference values determined in healthy individuals without intervention was 349 for the GSH/GSSG ratio (GSH = 1359 µM and GSSG = 4 µM)(Giustrarini *et al.*, 2003). Our reference range for the GSH/GSSG ratio is between 525 and 712 (Janse van Rensburg *et al.*, 2005). The GSH/GSSG ratio in the six subjects ranged from 401 to 762 (Table A2, Appendix A). Based on these ratios it appears that the subjects had slightly elevated levels of oxidative stress, which corresponded with the levels of hydroperoxides found.

Treatment with O_2 caused a slight decrease (7%) in the mean GSH/GSSG ratio from 540 ± 54 in control to 501 ± 54 after treatment. This decrease was not statistically significant (Figure 4.5). After treatment with the different O_3 concentrations, the ratio decreased significantly by 27% (395 ± 47) and 63% (201 ± 20) from the control. The decreases in the ratio between O_2 and O_3 treated whole blood were also significant. The ratio in the control sample of the buffered cells (272 ± 22) was significantly lower than the control sample of the whole blood (Figure 4.5). The effects of O_3 was still observed. In the buffered cells, the ratio decreased by 69% (167 ± 31) after treatment with O_2 and by 73% (144 ± 21) and 76% (131 ± 15) after treatment with 20 and 80 μg/ml O_3 respectively. All the values were significantly less than that measured in the whole blood control and the O_2 treated sample.

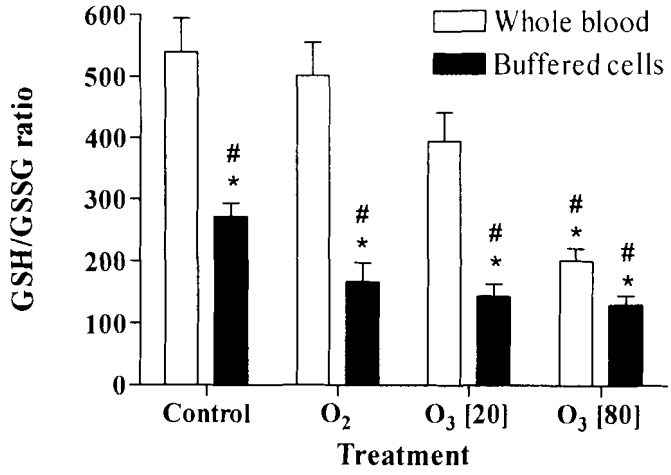

Figure 4.5 The GSH/GSSG ratio measured in the two intervention groups. Results are given as the mean ± 1SEM (n=6) GSH/GSSG ratio for whole blood and buffered cells at baseline and after exposure to O_2 and 20 and 80 μg/ml O_3. $p<0.05$ with respect to control (*) and O_2 (#) of the whole blood group (Bonferroni test).

The results confirms the results of Bocci (2002) who reported a 15% decrease in total GSH after exposure of blood to 80 μg/ml O_3. A lower GSH/GSSG ratio is determined by a decrease in GSH and/or an increase in GSSG, both of which is associated with

increased oxidative stress. GSH is a water soluble antioxidant which reduces hydroperoxides to water and thereby acts as a sacrificial antioxidant. Therefore, in this study some of the GSH may have reacted with the hydroperoxides and other radicals that formed after ozonation. Depletion of GSH has also been associated with accumulation of lipid hydroperoxides, which can cause cell death through either necrosis of apoptosis (Higuchi, 2004).

4.3 Antioxidant capacity

4.3.1 Oxygen radical absorbance capacity (ORAC) analysis

The variety of antioxidant defences in plasma (see Section 2.4) makes it difficult to measure the separate contribution of these mechanisms. This has lead to the approach to measure the total antioxidant capacity of plasma and other biological fluids (Cao and Prior, 1999). Thus, the total antioxidant capacity can be measured by determining the number of moles of a free radical that is scavenged by a test solution (Serafini and Del Rio, 2004). The ORAC is one of the many measurements of the total antioxidant capacity. It can be affected by dietary intake, oxidative reactions and the response of cells that increase their antioxidant defences in response to oxidative stress (Prior, 2004). A drawback is that lipid soluble antioxidants, which represents the main chain breaking antioxidants, cannot be measured directly. They are given as a total value of lipo- and hydrosoluble antioxidants (Prior, 2004). In addition, protein can interfere with the measurement. In this study the ORAC was done on deproteinated plasma samples to exclude the interference of protein. Rather than measuring the end product of free radical oxidation, this method evaluates the reserve of protection by means of oxidative challenge.

The total mean antioxidant capacity, as measured with the ORAC assay is ± 1800 μM for individuals between 60 and 71 years old and ± 1900 μM for individuals between 22 and 40 years (Prior, 2004). In this study the subjects tended to have total antioxidant capacity lower than the normal range with ORAC levels ranging from 1303 to 1916 μM TE

(Table A4, Appendix A). However, our reference values for the ORAC assay in serum is 829 μM TE (n = 88).

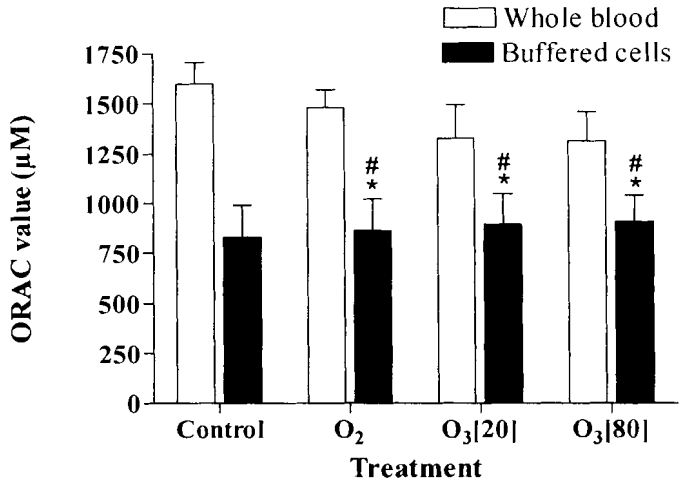

Figure 4.6 The ORAC values measured in the two intervention groups. Results are given as the mean ± 1SEM (n=6) ORAC value (μM) for whole blood and buffered cells at baseline and after exposure to O_2 and 20 and 80 μg/ml O_3. $p<0.05$ with respect to control (*) and O_2 (#) of the whole blood group (Bonferroni test). The ORAC was measured in plasma for the whole blood and cell homogenate for the buffered cells.

The mean baseline ORAC value was 1599 ± 109 μM TE (Figure 4.6). Following treatment with O_2 it decreased slightly by 7.4% to 1481 ± 89 μM TE. Treatment with different concentrations of O_3 decreased the ORAC by 17% (1328 ± 168 μM TE) and 18% (1315 ± 147 μM TE) respectively. The mean ORAC value of the control samples in the buffered cells was 829 ± 18 μM TE. In the buffered cells exposure to O_2 and O_3 (20 and 80 μg/ml) caused a slight increase, but not significant. The significant differences of the buffered cells from the whole blood were expected. It can also be ascribed to the removal of the plasma antioxidant capacity by removing the plasma. Bocci *et al* (Bocci *et al.*, 1998) found a decrease in the total antioxidant status after treatment with O_3. These results indicate that O_3 decreased the total antioxidant capacity.

Since the O_3 and O_2 results in the whole blood did not differ significantly, it is concluded that O_3 did not affect the total antioxidant capacity significantly. There was also no marked difference between the different O_3 concentrations.

4.3.2 Ferric reducing ability of plasma (FRAP assay)

The measurement of the total antioxidant capacity is a valuable tool to monitor the *in vivo* and *in vitro* redox status. However, the multivariate nature of the redox status requires the use of a combination of different assays to better evaluate the status. The FRAP assay measures the ability of plasma to reduce the colourless TPTZ-Fe^{3+} complex to its ferrous coloured form (TPTZ- Fe^{2+}). The ORAC assay measures the ability of the plasma to scavenge free radicals, whilst the FRAP measures the reducing ability of plasma.

The FRAP values for healthy individuals range between 600 and 1600 µM (Benzie and Strain, 1996). The baseline antioxidant capacity of the subjects in this study ranged between 208 and 326 µM FRAP units (Table A5, Appendix A), which is much lower than the published reference values. One must bear in mind that the total antioxidant capacity can be influenced by many variables such as redox potentials of the compounds present in the plasma, certain kinds of stress, nature of oxidizing substrate and types of antioxidants. It is possible that the antioxidant defences in the subjects could have been overwhelmed by external factors such as environmental stressors and dietary intake. The treatment with O_3 further compromised the antioxidant capacity, which probably lead to increased oxidative stress.

The mean FRAP values of the O_2 treated samples (258 ± 13 µM) did not differ much from the control values (262 ± 23 µM, Figure 4.7). The average FRAP values decreased significantly by 35% to 170 ± 9 µM and 43% to 151 ± 7 µM) with treatment with 20 µg/ml and 80 µg/ml O_3 respectively. Similar to the ORAC assay, the control values of the buffered cells (148 ± 6 µM) were much lower than in whole blood. The treated samples decreased even further and differed significantly from the whole blood control and O_2 treated samples (Figure 4.7).

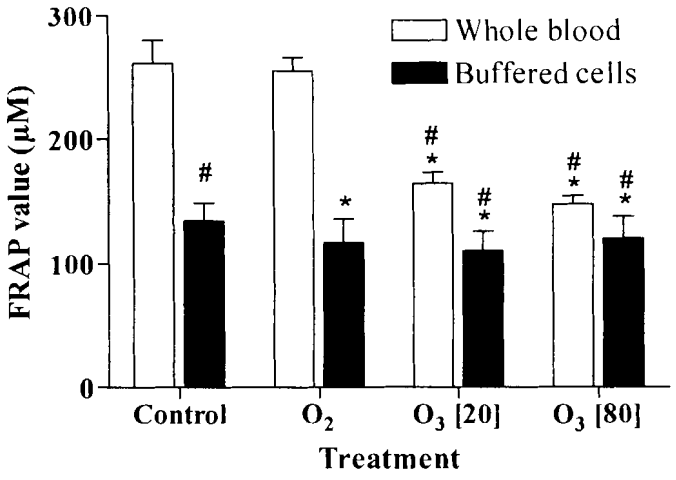

Figure 4.7 The FRAP values measured in the two intervention groups. Results are given as the mean ± 1SEM (n=6) FRAP value (µM) for whole blood and buffered cells at baseline and after exposure to O_2 and 20 and 80 µg/ml O_3. $p<0.05$ with respect to control (*) and O_2 (#) of the whole blood group (Bonferroni test). The FRAP was measured in plasma for the whole blood and cell homogenate for the buffered cells.

The antioxidant capacity decreases when human plasma is exposed to chemically produced peroxyl radicals or to other ROS. This is usually accompanied by a decrease in the onset of lipid peroxidation (Cross *et al.*, 1992). Similar decreases in antioxidant capacity has been found when plasma is exposed to O_3 (Cross *et al.*, 1992, Bocci *et al.*, 2001). In this case the water soluble antioxidants probably acts as sacrificial antioxidants and decrease as the O_3 increase. The most important scavenger of O_3 in human plasma seems to be uric acid, which is also most abundant in plasma (Cross *et al.*, 1992). The decreases in antioxidant capacity can contribute to a state of oxidative stress, which can cause oxidative damage to proteins, lipids and DNA. The results with the antioxidant capacity analysis support the levels of hydroperoxides, which ultimately shows that O_3 caused a possible state of increased oxidative stress. Therefore, O_3 exposure of patients with decreased antioxidant defences can be dangerous to the patient.

4.4 Antioxidant enzyme analyses

The ORAC or FRAP assay do not measure the contribution of the antioxidant enzyme defence mechansims to the antioxidant capacity. In order to better evaluate cellular responses to O_3 induced oxidative stress the levels of antioxidant enzymes were measured (Mates *et al.*, 1999).

Catalase is a tetrameric enzyme that breaks H_2O_2 down into H_2O and O_2. Its main function is to protect the cells from the H_2O_2 that they generate (Halliwell and Gutteridge, 2000). Reference values for enzyme activity in the samples that were used could not be obtained. The reference values quoted in the literature are also difficult to interpret due to the heterogenic population of white blood cells that were used to generate them. The individuals in this study had baseline values varying between 14.0 and 19.9 U/µg protein (Table A6, Appendix A), which corresponds with values we determined in our laboratory.

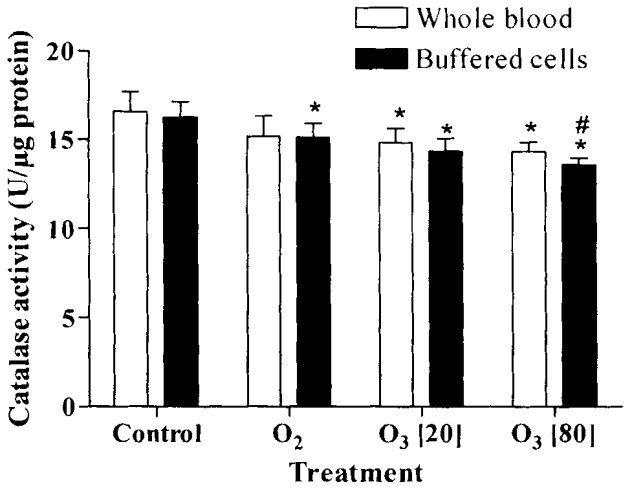

Figure 4.8 The catalase activity measured in the two intervention groups. Results are given as the mean ± 1SEM (n=6) catalase activity (U/µg protein) for whole blood and buffered cells at baseline and after exposure to O_2 and 20 and 80 µg/ml O_3. $p<0.05$ with respect to control (*) and O_2 (#) of the whole blood group (Bonferroni test).

The mean baseline catalase activity was 16.6 U/µg protein (Figure 4.8). It decreased by approximately 9% in the whole blood group after treatment with O_2 (15.2 U/µg protein). This was not statistically significant. After treatment with 20 and 80 µg/ml O_3, catalase activity decreased significantly by 11% (14.8 U/µg protein) and 14% (14.3 U/µg protein) respectively. The control of the buffered cells (16.2 U/µg protein) did not differ significantly from the whole blood control. After treatment with O_2 and the different concentrations of O_3, the levels of catalase decreased significantly by 9% (15.1 U/µg protein), 14% (14.3 U/µg protein) and 18% (13.6 U/µg protein). The activity in the buffered cells treated with 80 µg/ml O_3 also differed significantly from the O_2 treated samples of the whole blood group.

Superoxide dismutase (SOD) catalyses the conversion of $O_2^{\cdot-}$ to H_2O_2 and molecular O_2 and so prevents tissue damage by $O_2^{\cdot-}$ and its derivatives (Halliwell and Gutteridge, 2000). The SOD activity is measured as the amount of protein needed to cause 50% inhibition of 6-hydroxydopamine (6-HD) auto-oxidation. A decrease in 6-HD auto-oxidation therefore reflects an increase in the actual enzyme activity. The levels of SOD ranged between 441 and 570 ng for the different subjects (Table A7, Appendix A). The mean levels of SOD activity of the control was 483 ng (Figure 4.9) and this decreased after treatment with O_2 to 435 ng. This was not statistically significant. After treatment with 20 and 80 µg/ml the values increased significantly by approximately 17% in both cases (403 ng) and 17% (400 ng) respectively. The SOD activity of the control sample of the buffered cells (491 ng) was slightly lower than the control of the whole blood group. Treatment with O_2 and the different concentrations of O_3 caused a significant increase of 15% (410 ng), 20% (389 ng) and 17% (401 ng) respectively.

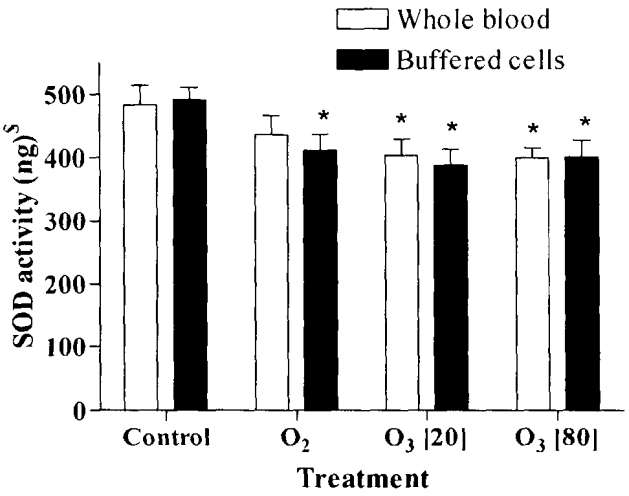

Figure 4.9 The SOD activity measured in the two intervention groups. Results are given as the mean ± 1SEM (n=6) SOD activity (ng) for whole blood and buffered cells at baseline and after exposure to O_2 and 20 and 80 µg/ml O_3. $p<0.05$ with respect to control (*) of the whole blood group (Bonferroni test). ^{S}ng = the amount of protein that results in 50% inhibition of 6-HD auto-oxidation.

Glutathione peroxidase (GPx) consists of four subunits, each containing a single selenocysteine that is essential for enzyme activity. It catalyses the reduction of hydroperoxides using GSH to protect cells against oxidative damage. Although GPx shares H_2O_2 as a substrate with catalase, it can also react with lipids and other organic hydroperoxides. As a result, GPx is the most important antioxidant enzyme at low levels of oxidative stress (Halliwell & Gutterdige, 2000).

The GPx activity of the different individuals in this study ranged between 0.02 and 0.07 U/µg protein (Table A9. Appendix A). The mean baseline GPx acitivty was 0.05 U/µg protein (Figure 4.10). Treatment of whole blood with O_2 and O_3 had no significant effect. In the buffered cells, exposure to 80 µg/ml O_3 caused a significant decrease by approximately 17% (0.04 U/µg protein). Treatment with O_2 and 20 µg/ml O_3 had no significant effect.

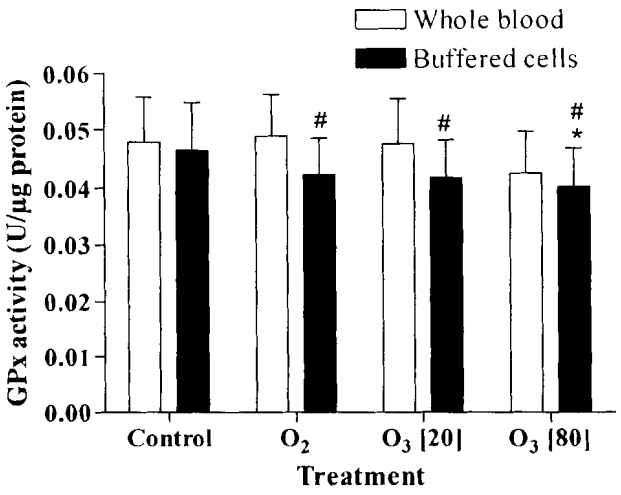

Figure 4.10 The GPx activity measured in the two intervention groups. Results are given as the mean ± 1SEM (n=6) GPx activity (U/µg protein) for whole blood and buffered cells at baseline and after exposure to O_2 and 20 and 80 µg/ml O_3. $p<0.05$ with respect to control (*) and O_2 (#) of the whole blood group (Bonferroni test).

Glutathione reductase (GR) is involved in the glutathione redox cycle and recycles the GSSG back to GSH (Halliwell and Gutteridge, 2000). The GR activity for the different individuals in this study ranged between 0.03 and 0.05 U/µg protein (Table A8, Appendix A). Mean baseline GR activity was 0.048 U/µg protein (Figure 4.11). There were no differences after treatment with O_2 and 20 µg/ml O_3 in both treatment groups. After treatment with 80µg/ml O_3, the level of GR decreased significantly by 15% (0.035 U/µg protein) in whole blood and 20% (0.033 U/µg protein) in buffered cells. The 80 µg/ml O_3 treated buffered cells also differed significantly from the oxygenated sample of the whole blood group.

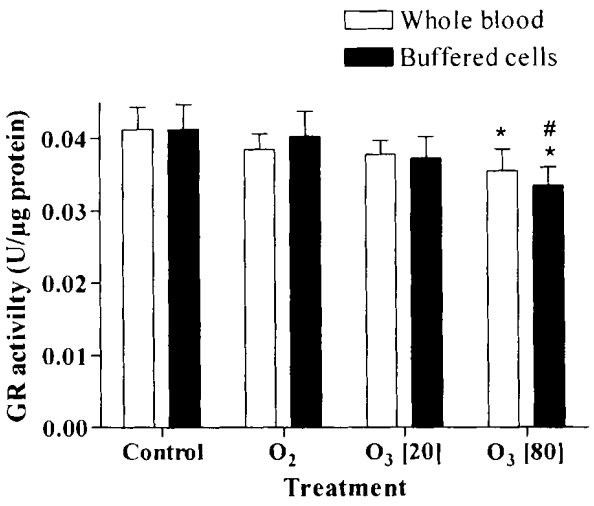

Figure 4.11 The GR activity measured in the two intervention groups. Results are given as the mean ± 1SEM (n=6) GR activity (U/µg protein) for whole blood and buffered cells at baseline and after exposure to O_2 and 20 and 80 µg/ml O_3. $p<0.05$ with respect to control (*) and O_2 (#) of the whole blood group (Bonferroni test).

In conclusion, it is well known that levels of antioxidant enzymes decreases when there is a state of acute oxidative stress. When cells are exposed to H_2O_2 over a long period there is a dose dependent increase in catalase, SOD and GPx mRNA and more enzymes are produced. Tissues are protected from ROS by expression of genes encoding antioxidant enzymes, but this usually occurs only after chronic oxidative stress (Mates *et al.*, 1999). Jurkat T cells treated with O_3 concentrations ranging from 12 to 72 µg/ml increased their levels of SOD, GPx and GR (Larini *et al.*, 2003). This upregulation of the enzyme synthesis was only observed after 24 hours, with even more pronounced effects after 72 hours. Results in baboons treated with a single dose of O_3 (80 µg/ml) indicated decreased activity of catalase with increases in SOD activity (Van Helden, 2006). Our results indicated a decrease in catalase, GPx and GR activity, but increases in SOD activity. Therefore, conditions of acute and transitory oxidative stress caused decreased levels of antioxidant enzymes as is suggested with the acute treatment in this study. Reduction of catalase, SOD and GPx can cause accumulation of H_2O_2 or $O_2^{\cdot-}$, which in turn can react with metal catalyst to form even more reactive species. This can ultimately lead to a state of increased oxidative stress, which can alter the redox state. The increase observed in the

SOD activity could indicate an initial stimulation of activity to protect the cells against oxidative stress.

4.5 Apoptosis

Apoptosis is programmed cell death and is most likely the mechanism by which most cells die both physiologically (in embryology) and pathologically. Many cell death events displays apoptotic and necrotic features, which can make it difficult to distinguish between the two forms of cell death. There are many defining features that distinguish apoptosis from necrosis, but all of them need not to be present at any given time. In necrosis there is a loss of cellular plasma membrane integrity accompanied by an inflammatory response, whereas in apoptosis the plasma membrane integrity is preserved. The normal physiological route to cell death is usually apoptosis. Only under extreme conditions when the cell cannot execute its apoptotic program, does the cell die through necrosis (LaCasse *et al.*, 2005).

4.5.1 Caspase activity

There are multiple pathways that triggers apoptosis and all of them culminate in the activation of a proteolytic cascade involving the caspases (cysteinyl-containing - aspartate-specific proteinase). The caspases are responsible for many of the effects associated with apoptosis. Increased levels are therefore associated with apoptosis.

The mean control levels of caspase 3/7 activity in the whole blood group was 1.04 AU/min/μg and after O_2 treatment this increased by approximately 16% to 1.24 AU/min/μg (Figure 4.12). In whole blood the caspase activity increased significantly after exposure to 20 and 80 μg/ml O_3 by approximately 56 and 68% respectively, when compared to the control. The O_3 treated groups also differed significantly from the oxygenated samples. The mean control caspase activity of the buffered cells was approximately 20% (1.30 AU/min/μg) higher than that of the whole blood. This was not significantly different. The oxygenated samples were also not significantly different. The

caspase activity of the O_3 treated buffered cells increased by 60 and 56% with 20 and 80 µg/ml O_3 respectively. Caspase activity in the positive control was significantly higher than in the treated and the control samples. One must however bear in mind that the measurement of apoptosis were made after 20 minutes of exposure. It is reasonable to assume that apoptotic events will only manifest at later stages. This however does not explain the threefold increase in caspase activity after treatment with 80 µg/ml O_3.

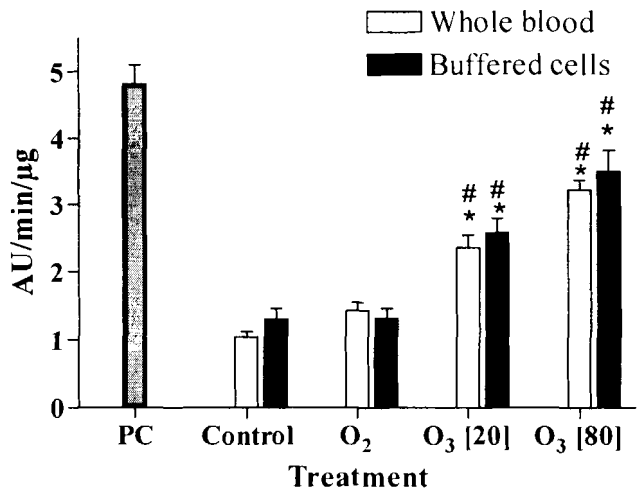

Figure 4.12 The caspase 3/7 activity measured in the two intervention groups. Results are given as the mean ± 1SEM (n=6) caspase 3/7 activity (AU/min/µg protein) for whole blood and buffered cells at baseline and after exposure to O_2 and 20 and 80 µg/ml O_3. $p<0.05$ with respect to control (*) and O_2 (#) of the whole blood group (Bonferroni test). The positive control (PC) was treated with 1 µg/ml staurosporine.

When O_3 dissolves in the plasma it react with various molecules, including PUFAs, generating lipid ozonation products (LOP). The LOP, particularly 4-hydroxynonenal (HNE), are able to activate biochemical an immunological pathways. Studies proved that HNE cause apoptosis and the release of caspase 3 (Larini *et al.*, 2004). This was also probably the cause of apoptosis in this study. In this case it would prove useful to measure the amount of LOP formed, in order to determine the relationship between the toxic aldehydes and caspase activity.

4.6 Inhibition of mitochondrial function

Mitochondria play an important role in generating energy in the form of ATP. It also plays a major part in apoptosis. Up till now, the effects of O_3 on mitochondrial function have not been evaluated. Several key parameters associated with mitochondrial function were evaluated in this study.

4.6.1 The NADH/NAD$^+$ ratio

NAD^+ plays an important role in several biosynthesis and catabolic reactions in the body. It participates in many redox reactions where it is converted to NADH, i.e. during glycolysis and in the citric acid cycle. In order to maintain a proper redox balance, NADH is reoxidized by complex I of the respiratory chain. Only a small fraction of the total cellular NADH is concentrated in the cytosol. The highest concentration is present in the mitochondria. The transfer of NAD^+ from the mitochondrial matrix to the cytosol is through the ethanol acetaldehyde shuttle. An increase in the mitochondrial NAD^+, will therefore lead to the production of acetaldehyde by mitochondrial alcohol dehydogenase (ADH). Acetaldehyde diffuses freely to the cytosol and is reduced to ethanol via the cytosolic ADH, resulting in as increase in cytosolic NAD^+. This process also forms the basis of the experimental method that is used to measure the NADH:NAD^+ ratio (Lin and Guarente, 2003).

Therefore, the NADH/NAD^+ ratio is a good measure of the redox state and the bioenergetic status of tissue. More important is that oxidative stress is mediated by changes in this ratio (Karp *et al.*, 1983). Various age related diseases and metabolical disorders associated with increased oxidative stress have been linked to an aberration in the NADH/NAD^+ ratio (Lin and Guarente, 2003).

The normal NADH/NAD^+ ratio in humans is not well established, but most likely range between 0.6 and 1.1 (Semete, 2004). In mammalian blood this ratio is 3.3 (10:3) (Swierezynski *et al.*, 2001). In this study the baseline NADH/NAD^+ ratios ranged between 0.6 and 1.9 (Appendix A, Table A3), confirming previous results from our

laboratory (Semete, 2004). After treatment with O_2 and 20 µg/ml O_3 the ratios in whole blood increased, but not significantly (Figure 4.13). With 80 µg/ml O_3 the ratio increased by 40%. In the buffered cells the ratios increased statistically significant following treatment with O_2 and 80 µg/ml O_3 with 10%. The increase in the $NADH/NAD^+$ ratio was brought about by both an increase in NAD^+ and a decrease in NADH. These results are also consistent with the changes measured in hydroperoxide levels and the GSH/GSSG ratio. Together they represent a state of increased oxidative stress after blood was exposed to O_3 and the effect was more pronounced in the buffered cells.

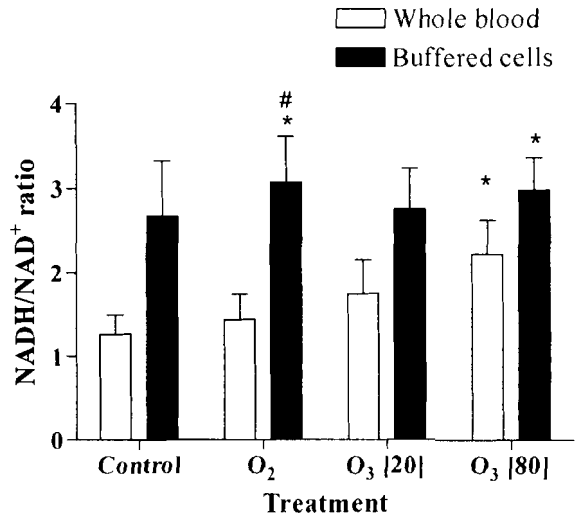

Figure 4.13 The $NADH/NAD^+$ ratio measured in the two intervention groups.
Results are given as the mean ± 1SEM (n=6) $NADH/NAD^+$ ratios for whole blood and buffered cells at baseline and after exposure to O_2 and 20 and 80 µg/ml O_3. $p<0.05$ with respect to control (*) and O_2 (#) of the whole blood group (Bonferroni test).

Under certain conditions of cellular activity NAD^+ can be released from mitochondria to the cytosol and so change the $NADH/NAD^+$ ratio. Decreases in cytosolic NADH can result in less reducing equivalents for the respiratory chain which in turn will decrease the electrochemical gradient ($\Delta\psi$) across the inner membrane. This will ultimately lead to decreased ATP production. Deficient flow of electrons through the respiratory chain may lead to a higher $NADH/NAD^+$ ratio as the oxidation of NADH by complex I will be less compared to when a free flow of electrons occurs. It is important to keep in mind that

oxidation of GSH and alterations in the $NADH/NAD^+$ ratio is commonly associated with apoptosis (Ellerby and Bredesen, 2000).

4.6.2 ATP levels

Mitochondrial oxidative phosphorylation is the major ATP synthetic pathway in eukaryotes. Electrons liberated from reducing substrates are delivered to O_2 via a chain of respiratory complexes (complex I-IV). The energy from the electrochemical gradient ($\Delta\psi$) across the inner mitochondrial membranes is used to drive ATP production by complex V (see Section 2.6). The measurement of ATP levels is therefore a good indicator of mitochondrial function. The ATP levels in white blood cells were measured to determine if the ROS generated by O_3 treatment affects mitochondrial function.

The ATP levels in the white blood cells in whole blood and in buffered cells decreased slightly, but significantly after treatment with O_2, 20 and 80 µg/ml O_3 (Figure 4.14). The treated samples in the buffered cells also contained significantly less ATP than the oxygenated sample in whole blood.

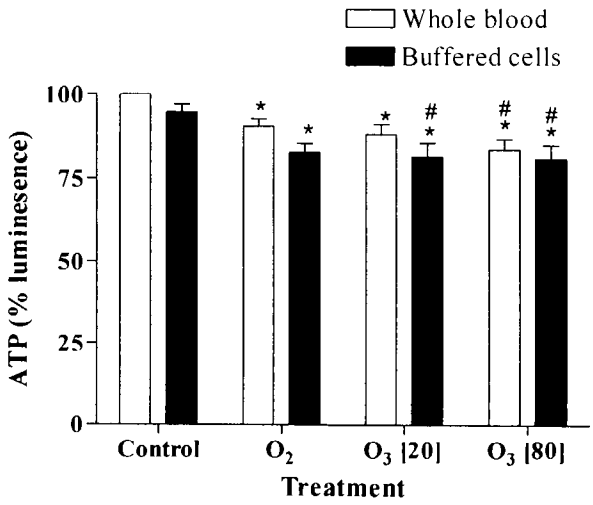

Figure 4.14 The ATP levels measured in the two intervention groups. Results are given as the mean ± 1SEM (n=6) ATP levels for whole blood and buffered cells at baseline and after exposure to O_2 and 20 and 80 µg/ml O_3. $p<0.05$ with respect to control (*) and O_2 (#) of the whole blood group (Bonferroni test).

The decrease in ATP levels clearly indicates that the increased formation of ROS during O_3 treatment plays a significant role in decreasing ATP production. There are at least three known mechanisms through which ROS can affect apoptosis and necrosis and so ATP production. They include the disruption of the mitochondrial membrane potential, oxidative phosphorylation and ATP production; the release of an unknown protein that triggers activation of the caspase family and alteration of the cellular reduction-oxidation (redox) potential (Higuchi, 2004).

4.6.3 Oxygen consumption

To further determine the effects of O_3 on mitochondrial function, O_2 consumption by isolated liver mitochondria was measured. The amount of mitochondria required for this and the respiratory chain complex analysis cannot be obtained from blood. Isolated liver mitochondria were used because various analyses can be performed, including spectral studies and polarography (Rustin *et al.*, 1994). Representative traces of O_2 consumption are shown in Figure 4.15 and Figure 4.16.

The traces in Figure 4.15 represent the oxidation of malate in the presence of glutamate and the various states of respiration, inlcuding state 3 respiration after the addition of ADP. The substrates malate and glutamate are NADH-generating substrates. The oxidation of these substrates stimulates respiration through complex I. The electron transport chain is coupled to ATP synthesis through a transmembrane electrochemical gradient ($\Delta\psi$). Mitochondrial oxygen consumption is therefore also regulated by $\Delta\psi$ and hence the matrix concentration of ADP. When ADP is added to the system, the $\Delta\psi$ decreases because ATP synthase uses the proton gradient to phosphorylate ADP to form ATP. Oxygen consumption then inreases as the electron transport chain reconstitutes the $\Delta\psi$. This is known as state 3 respiration (Wallace, 2001). An active oxidation of malate is observed in the control sample (Trace A) and after addition of ADP the mean state 3 respiration was 0.56±0.04. The rate of glutamate and malate oxidation increases slightly with O_2 treatment (Trace B), when compared to the control. The state 3 respiration increased by approximately 33%, but not significantly. With 80 μg/ml O_3 treatment

however the rate of malate oxidation decreased when compared to the control. The state 3 respiration also decreased significantly by 75%.

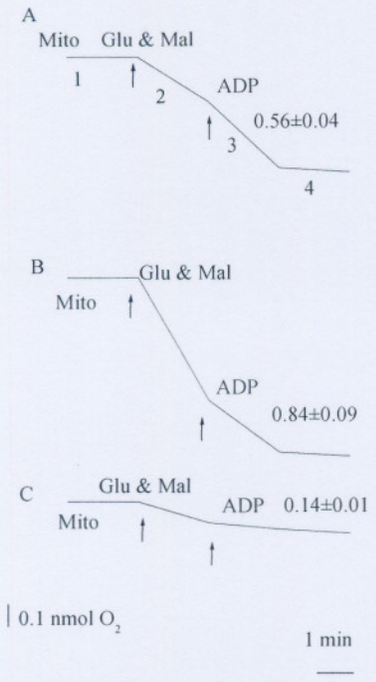

Figure 4.15 Representative traces showing the O_2 consumption from isolated liver mitochondria in the presence of glutamate and malate. Additions of 0.25 µg/µl mitochondria, 10 mM malate, 10 mM glutamate and 2 mM ADP are indicated by the arrows. Each trace represent the mean ± 1SEM of three recordings (n=3). (A) Control trace without any treatment, (B) O_2 treatment and (C) O_3 treatment with 80 µg/ml O_3. Respiration states are indicated by the numbers 1-4.

Figure 4.16 shows representative traces of respiration in the presence of succinate and rotenone. Succinate is the substrate for complex II, thereby stimulating respiration through complex II. Rotenone is added to irreversibly inhibit complex I and NADH stimulating respiration (Rustin *et al.*, 1994). The state 2 respiration in the presence of succinate increased slightly after O_2 treatment, compared to control respiration, albeit not

significantly. The state 3 respiration (after addition of ADP) increased by approximately 17%. The state 2 respiration after treatment with 80 µg/ml O_3 deceased when compared to the control, but not significantly. The state 3 respiration decreased significantly by approximately 87% after O_3 treatment in a similar way as with the NADH linked substrates.

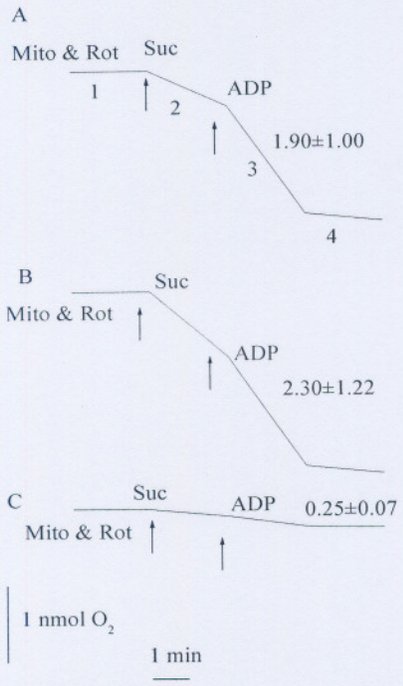

Figure 4.16 Representative traces showing the O_2 consumption of isolated liver mitochondria in the presence of rotenone and succinate. Additions of 0.25 µg/µl mitochondria, 3 mM rotenone, 25 mM succinate and 2 mM ADP are indicated by the arrows. Each trace represent the mean ± SEM of three recordings (n=3). (A) Control trace without any treatment, (B) O_2 treatment and (C) O_3 treatment with 80 µg/ml O_3. Respiration states are indicated by the numbers 1-4.

Figure 4.17 summarises the effects of O_2 and different concentrations of O_3 on the state 3 respirations in isolated liver mitochondria, stimulated via NADH-linked substrates (complex I) or succinate (complex II). The state 3 respiration of the control samples was higher in the presence of succinate than glutamate and malate as substrates. Treatment

with O_2 stimulated respiration slightly, but not significantly, in both cases. Treatment with the different O_3 concentrations clearly and significantly inhibited respiration by approximately 68% for succinate and 40% for glutamate and malate. This inhibition appeared to be intensified at high O_3 concentrations. Inhibition of state 3 respiration decreased by 89% for succinate and 80% for glutamate and malate after treatment with 80 µg/ml O_3. These results correspond with the decreased ATP levels and altered $NADH/NAD^+$ ratio observed with O_3 treatment in white blood cells. The inhibition of respiratory activity by O_3 led to further study of the activity of the different respiratory complexes.

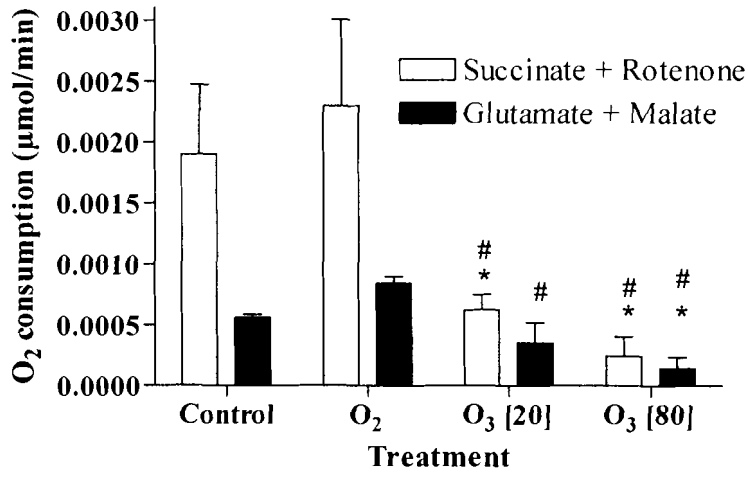

Figure 4.17 Effects of O_2 and 20 and 80 µg/ml O_3 on state 3 respiration. The bars represent the mean ± 1SEM O_2 consumption (µmol/min) measured after the addition of 2 mM ADP (n=3). $p<0.05$ versus the control (*) and O_2 (#) of the different substrates added.

4.6.4 Respiratory chain complex activity

Ozone reacts selectively with the haeme groups of haemoglobin, because it is mistaken as O_2. At the haemoglobin sites, O_3 causes the oxidation of Fe(II) to Fe(III) in the hemin groups and this is followed by a complete breakdown of the porphyrin rings of haemoglobin. This reaction of O_3 with haemoglobin is similar to the reaction of carbon monoxide (CO) with haemoglobin (Cataldo *et al.*, 2005). It is also well known that CO

combines with the haeme containing complex IV (cytocrome c oxidase) of the respiratory chain (Castoldi *et al.*, 2005). It was therefore important to investigate if O_3 also causes the oxidation of complex IV. In addition, the decreased respiration as measured with the oxygen consumption assays made it important to determine if O_3 also affects the other respiratory chain complexes. The same treatment protocol as for the oxygen consumption measurements was used. Table 4.2 shows the effect of O_2 and O_3 treatment on the different respiratory chain complexes.

Table 4.2 The effect of O_2 and O_3 treatment on the different respiratory chain complexes activity.

	Control	O_2	O_3 [20]	O_3 [80]
Complex I	308.1±22.3	210.1±37.8*	375.9±23.2	408.8±20.0
Complex I+III	429.7±19.5	382.5±3.9	413.3±28.4	482.2±34.8
Complex II+III	477.5±6.2	348.1±41.3*	248.5±14.3*	291.1±34.8*
Complex IV	2796.5±166.7	1395.8±100.7*	130.9±12.1*	126.8±6.9*

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

Treatment with O_2 caused significant decreases in complex I, combined complex II+III activity and complex IV activity, by 32, 27 and 50% respectively. It was found that hyperoxia (98% O_2, 2% CO_2) leads to almost 80% inhibition of O_2 consumption in cell culture. This O_2 toxicity is mediated by ROS, such as $O_2^{\cdot-}$, H_2O_2, $OH^{\cdot}$ and singlet oxygen. Under hyperoxia the increased amounts of these radicals overwhelm the cellular defence systems and leads to cell death (Schoonen *et al.*, 1990). This could explain the results found in this study. Schoonen *et al.*, (1990) also found that hyperoxia caused inactivation of complexes I and II of the respiratory chain. Similar to the cells in culture, we found decreased activity of Complex I and combined complex II+II activity, but in contrast additional inhibition of complex IV activity was observed.

Treatment with the two O_3 concentrations had no significant effect on complex I or combined complex I+III activity, but decreased complex II+III activity significantly. Complex IV activity was significantly inhibited by the two different O_3 concentrations by approximately 95%. This corresponds to previous results where low concentrations of O_3 caused 20% inhibition of complex IV activity in fibroblasts (Van der Zee *et al.*, 1987). It is important to note that the inhibition of the combined complex II+III activity was not nearly as significant as the almost complete inhibiiton of complex IV activity.

Control of mitochondrial oxygen consumption and ATP synthesis is shared amongst several steps of oxidative phosphorylation and this can vary according to the type of tissue. This implies that there is no limiting step in the regulation of respiration (Rossingnol *et al.*, 2000). Control of oxidative phosphorylation in the liver is mainly done at the phosphorylation level by ATP synthase and the phosphate carrier. This implies that decreases in complex IV activity have to exceed a critical value (approximately 75% inhibition) before a decrease in mitochondrial respiration can be observed. This is because there is an excess of active respiratory chain complexes that can serve as a reserve to compensate for a deficit. Thus, mobilization of a pool of inactive respiratory chain enzyme complexes takes place. Other possible mechanisms include chemical modification of the kinetic properties of the active complexes and/or modulation of the activity of the complexes by variations in the concentrations of intermediary metabolites (Rossingnol *et al.*, 2003). This implies that, although O_3 inhibits complex IV by 95%, respiration still takes place through the other complexes that was not inhibited, resulting in a less significant inhibition of respiration by O_3 (Figure 4.17).

In conclusion, these results clearly indicates a significant inhibition of mitochondrial respiration and specifically through inhibition of cytochrome c oxidase (complex IV), that may then lead to reduced ATP production, increase of ROS, increased $NADH/NAD^+$ ratio and apoptosis and/or necrosis.

5 Conclusions and recommendations

Ozone as an alternative and complementary form of medicine remains controversial. The pro-lobby for the use of O_3 is of the opinion that, under well controlled conditions, possible toxic effects are negligible and that the medicinal benefit far outweighs the toxic effects. The anti-lobby, on the other hand, feels that the claims of beneficial effects are biased and that it rests on rather unscientific and not well controlled clinical studies. The literature makes it clear that conditions of treatment must be well controlled, especially in the case of autohemotherapy. Too low a concentration will have a placebo effect whereas too high a concentration will be toxic. It has been proposed that the powerful antioxidant defences in blood negates any negative effects of O_3 since ROS that forms are quenched by the antioxidant system. This then generates a therapeutic response (Bocci, 2002). The hypothesis that the antioxidant defence system in blood provides protection against harmful effects of O_3, were investigated.

In view of these opposing opinions, the antioxidant defences in human blood were investigated to determine if sufficient to protection against harmful effects of O_3 exists. To achieve this, blood from apparently healthy subjects were treated with 20 and 80 μg/ml O_3 for 20 minutes. It is important to note that the acute effect of O_3 treatment was investigated and no follow up treatments were done. As positive control, O_2 was used to assess whether changes are due to O_2 or O_3. In order to assess the role of plasma antioxidants, the interventions were done on whole blood as well as blood where the plasma was replaced with a PBS buffer. Subsequently, several selected parameters associated with cell death, oxidative stress and mitochondrial function were investigated in isolated white blood cells.

To give an overview of the main findings of this study, the following paragraphs summarize the results. White blood cell viability as measured by trypan blue exclusion and LDH release was adversely affected by treatment with both O_2 and O_3. These effects were exacerbated in the absence of plasma antioxidants, i.e. in buffered cells. Also, the

effect of treatment with 80 μg/ml O_3 was more pronounced than with O_2 and 20 μg/ml. Furthermore, the results suggest that apoptosis, as measured by caspase 3/7 activity, was increased by O_2 and even more so by both concentrations of O_3. This was similar in whole blood and in buffered cells. The threefold increase in caspase release could probably be attributed to increased levels of lipid ozonation products, which have been proven to cause apoptosis in cultured cells and murine models (Kircichenko *et al.*, 1996; Larini *et al.*, 2004).

Several parameters were investigated to evaluate oxidative stress in the intervention groups. Hydroperoxide production was not very high in plasma from whole blood and the effect of O_2 and O_3 treatment was similar. In the buffered cells, hydroperoxide production increased to almost threefold when compared to whole blood. The effect of O_3 on the glutathione redox status, as measured by the GSH/GSSG ratio, was dose-dependent and also considerably and significantly higher than for O_2, especially after treatment with 80 μg/ml. In buffered cells, the redox status increased to above 65% in both O_2 and O_3 treated groups. The antioxidant capacity of whole blood, as measured by ORAC and FRAP assays, decreased markedly following treatments with both concentrations of O_3. Oxygen treatment had no marked effect. The antioxidant capacity of plasma was significantly lower in the buffered cells than in the whole blood. Treatment with O_2 or O_3 had no additional effect on the antioxidant capacity in the buffered cells. The antioxidant enzymes in white blood cells, catalase, SOD, GR and GPx were not significantly affected by treatment with O_2 and both concentrations of O_3. The effect of treatment was also similar in whole blood and in buffered cells. Thus, the breakdown of H_2O_2 by catalase, conversion of $O_2^{\cdot-}$ to H_2O_2 by SOD and breakdown of H_2O_2 to H_2O by GPx was similarly affected. The only enzyme that was significantly affected by the high O_3 concentration was GR, whose activity was decreased by approximately 20%. Thus, the conversion of GSSG to GSH was somewhat impaired, results that may have contributed to the changes in the GSH/GSSG ratio. Although slight, but still statistically significant modulation of the activities of the antioxidant enzymes were observed, it is concluded that these activities remained relatively constant over the incubation period and O_3 concentrations

used. It may be that more significant changes of these activities can occur during longer treatment regimes, resulting from modulation of gene expression or allosteric regulation.

Parameters that reflect the energy metabolism in the cell were also affected by O_3 treatments. The $NADH/NAD^+$ ratio in plasma of whole blood and buffered cells increased (10 – 35%) significantly with O_3 treatment, but were not statistically significant after treatment by O_2. The ATP levels of white blood cells in whole blood decreased by 15 – 20%, irrespective of the treatment. ATP levels in white blood cells of the buffered cells were slightly lower than the whole blood. These results pointed to an insult of O_3-treatment on energy metabolism and prompted further investigation in isolated mitochondria.

The results of O_3 treatment on O_2 consumption and respiratory chain inhibition are of interest. Treatment with O_3 inhibited state 3 respiration in liver mitochondria by 75 – 87%. Treatment with O_2 inhibited the activity of complex I (32%), complex II+III (27%) and complex IV (50%). Ozone treatment had no effect on complex I and complex I+II. Complex II+III activity decreased significantly by 48 and 39% after treatment with 20 and 80 µg/ml O_3 respectively. The most dramatic effect however, was seen in the inhibition of complex IV activity. Both concentrations of O_3 decreased the activity by approximately 95%. Although complex IV activity was almost completely inhibited, respiration still occurred through the other complexes, which resulted in a less significant inhibition of state 3 respiration. This is because control of mitochondrial O_2 consumption and ATP synthesis is shared amongst several steps of oxidative phosphorylation (Rossingnol *et al.*, 2000).

Figure 5.1 is a summary of the main findings and proposals of possible mechanisms whereby O_3 may interact with cells. When O_3 is introduced to biological fluids, it apparently acts in two ways. The first is an O_3-olefin reaction where O_3 reacts with the double bonds in organic substances such as fatty acids to produce H_2O_2, aldehydes and other peroxides. The second mechanism is an O_3-electron donor reaction where a hydroxyl radical (OH^-) is formed. The hypothesis behind O_3-AHT is that the generated

ROS is involved in upregulation of the antioxidant defence mechanisms, which can combat chronic oxidative stress (Bocci, 2002). The damaging effects of ROS on cells are well known. The main ROS that is formed as a result of O_3 is $OH^{\cdot}$, which can oxidise lipids, inactivate proteins and cause strand breaks in DNA. Small, but significant increases in hydroperoxides formation in whole blood were found (5%), when compared to previous results found by Bocci (2002). When the plasma was removed, hydroperoxides formed increased approximately threefold (Figure 5.1, see also Figure 4.4). This clearly demonstrates that the antioxidants in plasma were sufficient to inhibit the formation of hydroperoxides. The result also indicated that lipid peroxidation occurred. The lipid peroxidation may result in changes in membrane fluidity, alterations in ion transport and possibly membrane rupture (Berman and Brodaty, 2004)

The GSH/GSSG ratio decreased markedly because GSH decreased and GSSG increased (Figure 5.1, see also Figure 4.5). This suggested that the small increases in hydroperoxides, could be attributed to GSH acting as a sacrificial antioxidant. Oxidative stress was markedly increased when the plasma was removed, as was expected. The depletion of the GSH when O_3 is introduced can at least in part be responsible for increased cell death, because of the decreased antioxidant capacity (Pastore *et al.*,2003). This is consisted with previous results which indicated that O_3 depletes GSH in red blood cells (Bocci, 2002) and in plasma of baboons (Van Helden, 2005).

O_3-AHT decreased both the radical scavenging capacity and the ferric reducing ability of plasma. It is likely that the ROS depleted the antioxidants present in plasma (Figure 5.1, see also Figure 4.6 and 4.7). This is supported by the finding that hydroperoxide formation was not markedly increased in whole blood, but increased by approximately threefold when the plasma antioxidants were removed. The acute state of oxidative stress did not markedly affect catalase, SOD, GR and GPx activity in white blood cells as discussed before (see Figure 4.8 - 4.11).

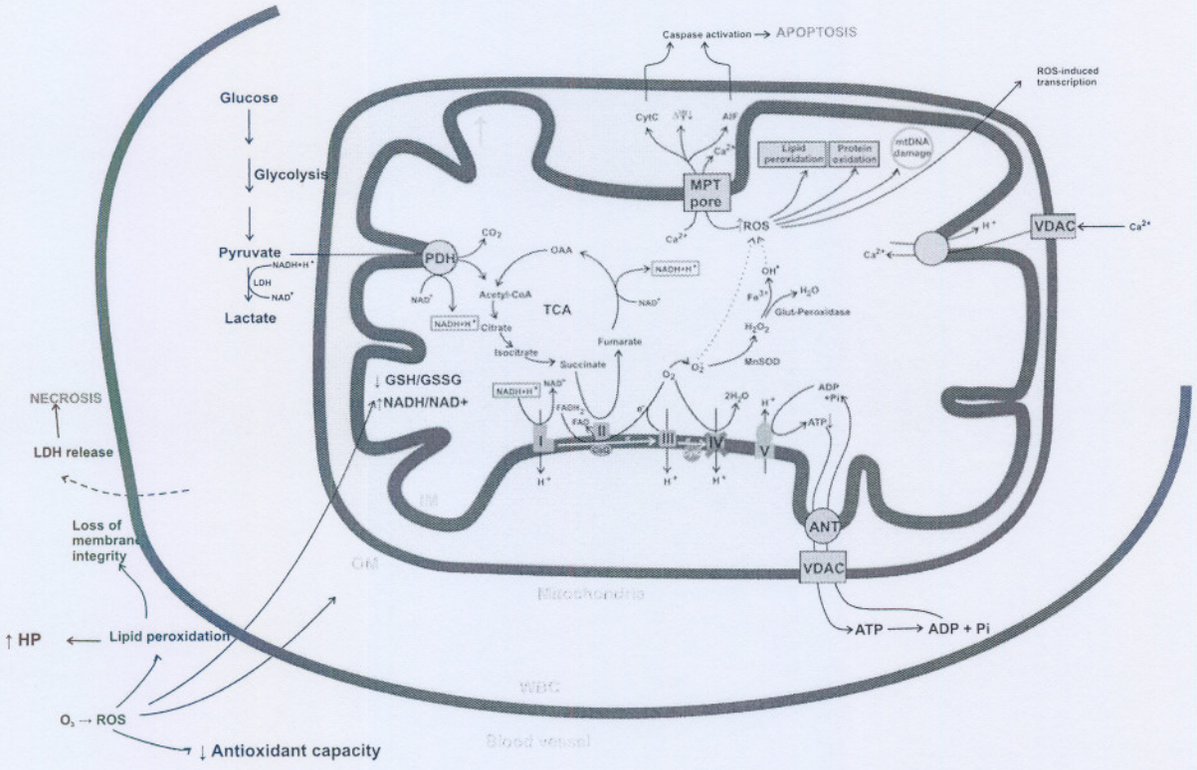

Figure 5.1. Proposed summary of biochemical consequences in plasma and white blood cells during acute treatment of O_3-AHT. This figure shows generally accepted cell biological processes in the cell associated with oxidative stress and energy metabolism. Ozone forms ROS, which can act with the lipids in membranes leading to lipid peroxidation and hydroperoxide formation. This in turn leads to loss of membrane integrity and LDH release, which is indicative of necrosis. The ROS reduce the antioxidant capacity (ORAC and FRAP). Inside the mitochondrion the ROS cause a decreased GSH/GSSG ratio. The most important finding however was the dramatic decrease of complex IV activity (indicated by red x). This decreased respiration causes diminished ATP production and an increased NADH/NAD^+ ratio. This in turn led to increased ROS production ($O_2^{\cdot-}$) by the mitochondria which can lead to apoptosis via the opening of the mitochondrial permeability transition pore (MPT) and subsequent cascade of events, including activation of caspase 3/7. See text for detailed discussion.

O_3–AHT caused small decreases in the white blood cell count in whole blood and in buffered solution by approximately 25%. LDH release from the remaining white blood cells was also significantly increased following treatment with O_3 which can suggest necrosis. On the other hand the lack of change in the morphology of the white cells argues against necrosis (Figure 4.3). It is important to keep in mind that light microscopy is not the most suitable vehicle to asses morphology with. Necrosis of cells is

characterised by cytoplasmic and mitochondrial swelling, rupturing of the plasma membrane and release of cellular content. In the case of treatment with O_2 and O_3, lipid peroxidation probably affected membrane integrity and so increased permeability of the membrane with increased release of LDH (Figure 5.1, see also Figure 4.1 and 4.2). It is likely that 20 minutes exposure to O_2 and O_3 was too short to cause mitochondrial and cytoplasmic swelling and that loss of membrane integrity was the start of necrosis. The caspase activity on the other hand increased threefold, which can indicate that cell death was also caused by apoptosis.

ROS, cell death and apoptosis can not be mentioned in the same sentence without including the role of the mitochondria. Treatment with O_3 decreased ATP levels (see Figure 4.14), increased the NADH/NAD^+ ratio (see Figure 4.13) and caspase activity (see Figure 4.12). This indicated that mitochondrial function was affected. To clarify this, mitochondrial respiration in rat liver homogenates was investigated. Chronic ROS exposure can result in oxidative damage to mitochondrial and cellular proteins, lipids and nucleic acids. Acute ROS exposure, on the other hand, can inactivate the iron sulphur (Fe-S) centres of the electron transport chain complexes, resulting in the shutdown of mitochondrial energy production (Wallace, 1999). It was found that O_3 reacts with haemoglobin and oxidizes the iron groups, similar to the mechanism whereby carbon monoxide acts (Cataldo *et al.*, 2005). It is also well known that carbon monoxide combines with the haeme containing complex IV (cytocrome c oxidase) of the respiratory chain (Castoldi *et al.*, 2005). The results of this study indicated that O_3 treatment decreased the rate of oxygen consumption and state 3 respiration. It also inhibited complex II+III and especially complex IV activity (Figure 5.1, see also Figure 4.17), probably by interacting with the Fe-S clusters in the respiratory complexes. This could in part explain the results, such as decreased ATP levels and increased NADH/NAD^+ ratio found in the white blood cells. One has to bear in mind that the isolated mitochondria were exposed to O_3 directly and did therefore not have the protective effect of plasma antioxidants. The effects will therefore be more pronounced than in blood. Endogenous ROS production could increase when the electron transport chain is inhibited, as is this case with complex IV inhibition by O_3 (Figure 5.1). This is because electron flow is

disrupted and "leaked" at complex I and III, where they can be donated to O_2 to form $O_2^{\cdot-}$ (Turrens, 1997). Superoxide is detoxified by MnSOD to give H_2O_2 which in turn is converted to H_2O by GPx. The increased ROS production can result in oxidative damage to mitochondrial and cellular proteins, lipids and nucleic acids (Wallace, 1999).

ATP can act as a switch between apoptosis and necrosis. Cells that are more dependent on ATP from oxidative phosphorylation are more likely to undergo necrosis, whereas cells that are more dependent on ATP from glycolysis are more likely to undergo apoptosis when the ATP levels decrease (Li *et al.*, 2003). White blood cells obtain most of their ATP by metabolism and oxidation of glucose (Arslan *et al.*, 1984), but it is not clear whether they depend more on oxidative phosphorylation or glycolysis for ATP. Apoptosis is common in white blood cells, but is dependent on the specific subtype of cells, with T-lymphocytes more likely to undergo apoptosis, than other cells (LaCasse *et al.*, 2005). The inhibited mitochondrial function and decreased ATP levels could however have contributed to the initiation of apoptosis (Figure 5.1). This is achieved by the opening of the mitochondrial permeability transition pore (MPT) located in the inner mitochondrial membrane (IM). A number of cell death-promoting factors are located in the mitochondrial inner membrane space, including cytochrome c (cyt c), apoptosis-inducing factor (AIF) and caspases. The opening of the MPT causes collapse of the membrane potential, selling of the mitochondrial IM and release of the death promoting-factors. Opening of the MPT can also be mediated by excessive calcium (Ca^{2+}) uptake (Wallace, 1999). This, together with the elevated caspase activity argues strongly in favour of apoptosis as the mechanism of cell death in white blood cells after exposure to O_3. Unfortunately it has to be concluded that the results as to the mechanism of cell death are inconclusive. I therefore propose to design a study that will specifically look if O_3 cause necrosis of apoptosis. This will have to include long term exposure with a follow up of several hours after blood is exposed to O_3. Such a study will include an in depth investigation in morphological and biochemical changes brought about by O_3.

In conclusion, O_3-AHT caused damage to white blood cells, but these effects were limited by effective antioxidant defence systems. This indeed proves that the plasma antioxidant defences plays a major role in protecting against the harmful effects of O_3. The hypothesis is therefore accepted. However, the antioxidant system could be overwhelmed and the question remains: Is it safe and effective to use O_3-AHT? From the results of this study it can only be concluded that O_3-AHT can be dangerous if not done properly. More research is needed, especially human clinical trials, before it can be used as a therapy. The inhibition of respiratory complex IV and subsequent apoptosis has to be considered when O_3-AHT is used. The differences in treating with 20 or 80 µg/ml O_3 is also inconclusive and does not clearly indicate that higher concentrations are more dangerous or more advantageous. The results obtained in this study did however contribute to the existing knowledge in the O_3 research field.

Recommendations and future directions

The results of this *in vitro* study gave some insight into the effects of O_3 on white blood cells. A very important finding was that O_3 almost completely inhibited complex IV. As far as could be ascertained, this finding has not been described in the literature. The next step would be to repeat the study, possibly in an *in vivo* study in experimental animals and ultimately in humans. Another approach can be to repeat the *in vitro* study but to use patients with diseases associated with chronic oxidative stress. Patients with cancer, diabetes, cardiovascular diseases or neurodegenerative diseases will be excellent candidates. The effect on individuals that are on vitamin supplementation will further test if an increased antioxidant defence system will protect against this effect of O_3. Comparing the results from patients with increased oxidative stress with those where people have increased antioxidant capacity may give new insights into the mechanisms of oxidative damage to cells. Another possible follow up can be to use cell culture models, e.g. cancer cell lines to determine the effect of O_3 on diseased cells. Although this has been investigated (Sweet *et al.*, 1980, Zänker *et al.*, 1990), different concentrations of O_3 were used. A part that could not be evaluated in this *in vitro* study is the effect of repeated O_3 exposures. The cumulative effect of O_3 could further damage mitochondria and so exacerbate the toxic effects of O_3. This should be investigated in both cell culture

and humans. Various other biochemical analyses can also be done to verify the results of this study.

It will be important to further investigate the apoptotic and possible necrotic effects of O_3. A molecule that causes apoptosis is always considered as an attractive approach to treat certain types of cancers. In order to get a more complete picture of the effects on apoptosis, one must look at different techniques that measure apoptosis. The mitochondrial membrane potential ($\Delta\psi$) is often used as a reliable indicator of cellular viability and apoptosis because the proton gradient across the inner membrane drives ATP production. Other methods will include determination of DNA fragmentation and assessing the mitochondrial permeability transition pore.

It is vitally important to further evaluate the inhibition of complex IV and to determine if it also happens during *in vivo* treatment. If this indeed the case, the future of using O_3 as a form of treatment must be reconsidered.

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Appendix A

Table A1 The results of the dROMs (CARR U) of the subjects

	Whole blood				Buffered cells			
	Control	O_2	$O_{3[20]}$	$O_{3[80]}$	Control	O_2	$O_{3[20]}$	$O_{3[80]}$
P1	494.93	534.03	479.26	500.93	626.72	558.38	562.27	573.69
P2	515.38	533.86	522.33	522.71	559.39	564.63	565.94	561.57
P3	725.69	738.33	692.36	732.00	639.29	758.08	686.46	725.76
P4	269.33	280.00	296.53	344.63	355.43	381.44	393.64	386.43
P5	599.74	632.94	565.62	659.76	682.88	731.61	742.56	760.77

Table A2 The GSH/GSSG ratios of the subjects

	Whole blood				Buffered cells			
	Control	O_2	$O_{3[20]}$	$O_{3[80]}$	Control	O_2	$O_{3[20]}$	$O_{3[80]}$
P1	560.12	581.82	353.93	223.81	326.64	131.30	127.87	123.97
P2	596.60	576.73	204.30	153.92	251.44	170.51	133.54	123.31
P3	401.23	494.89	352.19	128.39	326.44	96.03	94.75	94.81
P4	416.35	491.71	480.10	249.26	295.82	95.54	93.25	94.20
P5	761.80	612.14	463.10	234.38	239.99	285.59	208.88	178.50
P6	502.51	251.10	513.49	215.07	189.91	223.32	202.88	171.03

Table A3 The NADH/NAD ratios of the subjects

	Whole blood				Buffered cells			
	Control	O_2	$O_{3[20]}$	$O_{3[80]}$	Control	O_2	$O_{3[20]}$	$O_{3[80]}$
P1	1.88	1.83	1.86	3.43	5.75	4.93	3.51	2.86
P2	1.69	1.76	1.84	1.83	1.84	2.07	2.65	2.85
P3	1.76	2.47	3.44	3.45	2.20	3.95	4.66	4.53
P4	0.68	0.63	0.78	1.07	1.21	1.26	1.80	2.84
P5	0.96	1.39	1.82	1.69	2.54	3.23	2.50	3.17
P6	0.60	0.55	0.74	1.85	2.49	3.02	1.42	1.62

Table A4 The ORAC values (μM TE) of the subjects

	Whole blood				Buffered cells			
	Control	O_2	$O_{3[20]}$	$O_{3[80]}$	Control	O_2	$O_{3[20]}$	$O_{3[80]}$
P1	1497.20	1454.62	1460.39	1468.97	1212.19	1345.23	1441.67	1400.43
P2	1318.26	1366.36	1401.53	1169.44	1256.18	1352.21	1283.28	1172.03
P3	1302.64	1439.57	1358.68	664.14	463.54	724.80	658.80	922.00
P4	1916.00	1914.00	1789.00	1680.18	822.31	604.79	614.45	558.83
P5	1709.48	1369.91	550.70	1403.01	945.64	694.00	828.86	700.79
P6	1851.89	1339.67	1407.26	1501.43	275.24	452.04	535.70	682.07

Table A5 The FRAP values (μM) of the subjects

	Whole blood				Buffered cells			
	Control	O_2	$O_{3[20]}$	$O_{3[80]}$	Control	O_2	$O_{3[20]}$	$O_{3[80]}$
P1	293.38	290.31	188.00	154.15	159.54	174.54	126.08	119.15
P2	270.31	271.08	166.08	153.00	147.61	125.69	123.38	116.46
P3	208.00	213.00	192.61	174.54	163.77	166.46	157.23	155.31
P4	325.69	248.38	146.08	137.23	131.08	93.38	65.31	181.85
P5	255.68	239.63	138.05	127.53	65.16	61.74	58.58	71.74
P6	213.84	265.16	156.21	138.05	137.53	76.47	128.05	71.47

Table A6 The Catalase activity (U/μg) of the subjects

	Whole blood				Buffered cells			
	Control	O_2	$O_{3[20]}$	$O_{3[80]}$	Control	O_2	$O_{3[20]}$	$O_{3[80]}$
P1	14.40	14.85	14.28	13.77	14.97	14.53	14.34	13.77
P2	14.02	11.23	12.25	13.77	14.15	14.02	12.50	12.82
P3	14.28	13.96	13.45	13.00	15.16	13.26	12.69	12.37
P4	19.86	16.05	15.86	14.21	18.46	15.29	14.34	13.96
P5	17.26	15.16	15.04	14.34	15.10	14.85	14.40	13.45
P6	19.67	19.73	17.89	16.69	19.48	18.72	17.58	14.97

Table A7 The SOD (ng) activity of the subjects

	Whole blood				Buffered cells			
	Control	O_2	$O_{3[20]}$	$O_{3[80]}$	Control	O_2	$O_{3[20]}$	$O_{3[80]}$
P1	441.44	419.76	394.49	392.81	444.44	372.98	356.86	366.67
P2	560.95	527.60	401.63	417.26	544.12	482.18	447.69	473.59
P3	452.53	417.39	446.82	422.90	468.64	380.48	388.24	398.59
P4	376.00	305.05	326.21	338.33	471.51	452.82	365.06	396.15
P5	498.28	450.00	356.59	391.05	458.75	321.03	315.62	309.17
P6	570.94	491.21	493.39	435.75	559.61	453.20	460.89	461.51

Table A8 The GR activity (U/μg protein) of the subjects

	Whole blood				Buffered cells			
	Control	O_2	$O_{3[20]}$	$O_{3[80]}$	Control	O_2	$O_{3[20]}$	$O_{3[80]}$
P1	0.05	0.05	0.05	0.04	0.05	0.05	0.05	0.04
P2	0.03	0.03	0.03	0.03	0.03	0.04	0.03	0.03
P3	0.04	0.03	0.03	0.03	0.03	0.031	0.031	0.03
P4	0.05	0.04	0.04	0.04	0.05	0.04	0.03	0.04
P5	0.03	0.03	0.03	0.03	0.03	0.036	0.03	0.03
P6	0.04	0.03	0.04	0.04	0.04	0.04	0.04	0.04

TableA9 The GPx activity (U/μg) of the subjects

	Whole blood				Buffered cells			
	Control	O_2	$O_{3[20]}$	$O_{3[80]}$	Control	O_2	$O_{3[20]}$	$O_{3[80]}$
P1	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02
P2	0.03	0.04	0.03	0.03	0.03	0.03	0.03	0.03
P3	0.05	0.06	0.05	0.05	0.06	0.05	0.05	0.05
P4	0.07	0.06	0.06	0.06	0.07	0.06	0.06	0.06
P5	0.04	0.05	0.05	0.04	0.03	0.04	0.04	0.04
P6	0.07	0.07	0.07	0.06	0.06	0.04	0.05	0.04

Table A10 The cell viability (%) of the subjects

	Whole blood				Buffered cells			
	Control	O_2	$O_{3[20]}$	$O_{3[80]}$	Control	O_2	$O_{3[20]}$	$O_{3[80]}$
P1	94.94	90.54	68.29	79.13	90.41	89.61	57.59	64.41
P2	91.38	92.7	79.14	69.36	90.48	80.28	72.86	66.34
P3	91.15	82.13	77.57	70.18	91.14	89.9	72.07	70.45
P4	96.68	89.53	75.46	75.95	93.51	89.8	72.96	74.87
P5	94.04	95.18	90.41	86.41	92.58	83.43	74.9	72.28
P6	91.01	97.05	80.21	84.79	90.2	84.44	74.81	63.92

Table A11 The LDH release (%) of the subjects

	Whole blood				Buffered cells			
	Control	O_2	$O_{3[20]}$	$O_{3[80]}$	Control	O_2	$O_{3[20]}$	$O_{3[80]}$
P1	100.00	120.45	122.35	136.59	117.15	185.14	191.86	200.25
P2	100.00	103.38	108.82	164.75	104.60	172.79	206.26	222.91
P3	100.00	160.48	226.57	219.56	119.72	179.99	204.63	206.85
P4	100.00	233.85	158.06	232.61	132.30	257.96	251.48	216.65
P5	100.00	119.44	110.22	160.64	115.74	237.91	286.55	331.66
P6	100.00	154.27	175.68	157.03	116.21	302.85	359.24	313.54

Table A12 The Caspase 3/7 (AU/min/μg) activity of the subjects

	Whole blood				Buffered cells			
	Control	O_2	$O_{3[20]}$	$O_{3[80]}$	Control	O_2	$O_{3[20]}$	$O_{3[80]}$
P1	1.19	1.48	2.67	3.67	1.05	1.53	3.05	4.14
P2	1.23	1.50	2.381	3.34	1.60	1.51	2.42	3.90
P3	1.08	1.20	3.07	3.48	1.61	1.64	2.94	4.48
P4	0.88	1.93	1.89	2.87	0.86	0.75	2.74	3.01
P5	0.75	1.29	2.17	3.19	1.76	1.48	2.71	2.69
P6	1.12	1.18	1.94	2.73	0.94	0.98	1.56	2.78

Table A13 the ATP levels (%) of the subjects

	Whole blood				Buffered cells			
	Control	O_2	$O_{3[20]}$	$O_{3[80]}$	Control	O_2	$O_{3[20]}$	$O_{3[80]}$
P1	100.00	82.93	76.08	73.64	83.18	73.11	64.11	69.27
P2	100.00	94.95	87.85	86.13	98.63	89.35	86.83	84.74
P3	100.00	88.08	95.18	90.99	97.12	82.27	86.66	80.57
P4	100.00	92.09	93.97	88.69	94.50	87.26	90.07	92.11
P5	100.00	97.64	92.07	88.08	98.46	87.04	86.73	89.15
P6	100.00	87.13	83.05	73.71	95.99	77.55	74.07	69.12

Appendix B

Table B1 Reference intervals for oxidative stress levels

CARR U	mg/100 ml H_2O_2	Oxidative stress level
250-300	16-20	Normal range
300 -320	20-24	Borderline range
320 -340	24-27	Low level of oxidative stress
340 -400	27-32	Middle level of oxidative stress
400 -500	32-40	High level of oxidative stress
>500	>40	Very high level of oxidative stress

Table B2 Conversion values between O_3 concentrations expressed as µg/ml or ppm

0.1 ppm = 0.2 mg/m^3 = 0.2 µg/l = 0.0002 µg/ml

Therefore

1 ppm = 0.002 µg/ml

1000 ppm = 2 µg/ml

10 000 ppm = 20 µg/ml = 0.42 mM

40 000 ppm = 80 µg/ml = 1.66 mM

Ppm = parts per million

Calculation of the O_3 dose for therapeutic uses:

O_3 dose (µg) = Gas volume (ml) × O_3 concentration (µg/ml)

Table B3 Calculation of the O_3 concentrations used in this study

Abs (254 nm)	O_3 concentration	
	c % v/v	µg/ml
0	0	0
0.2019	0.934	20
0.4039	1.868	40
0.8078	3.736	80

Conversion of c % to µg/ml: (%/4.67) x 100 = µg/ml and absorbance to c %: Absorbance x 4.625 = c %.

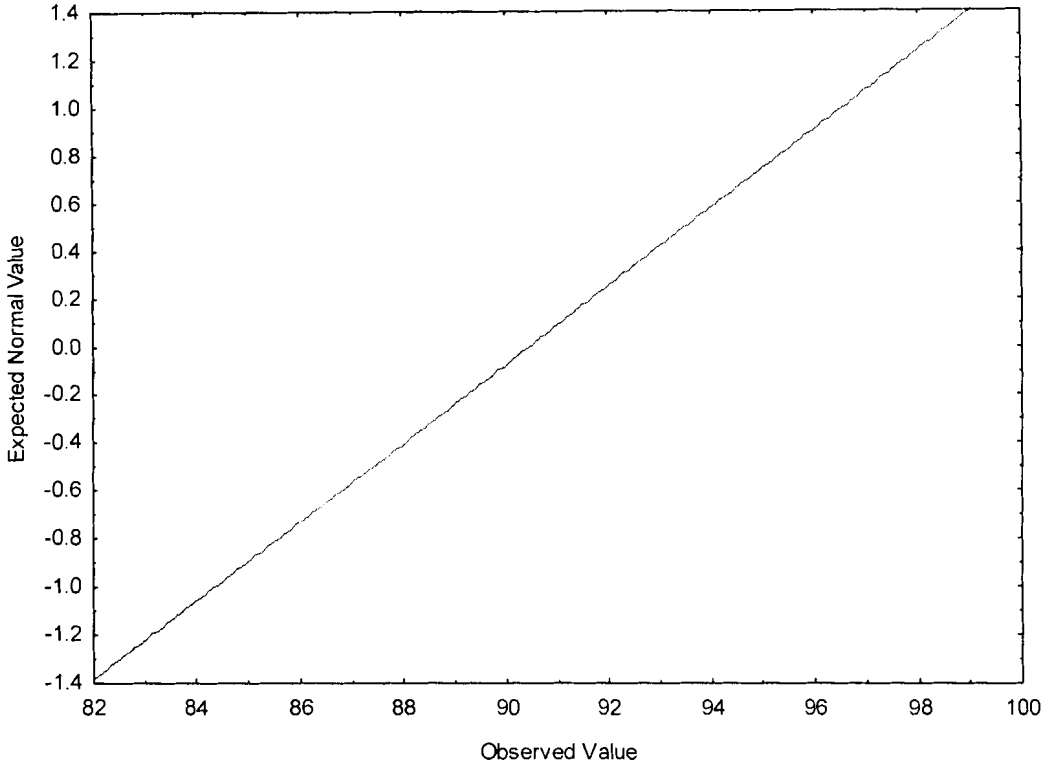

Figure B1 Representation of results calculated with normal probability plots. The standard normal probability plot is constructed with calculated z values (standardized values of the normal distribution) on the y axis and the observed values on the x axis. If the residuals are not normally distributed they will deviate from the line. Outliers can also be determined in this plot.

Appendix C

Conferences at which the research was presented during the study as well as a copy of the article published.

C1. Presentation at National conference

South African Society of Biochemistry and Molecular biology, Pietermaritzburg, July 2006.
L.H. du Plessis, F.H. van der Westhuizen, H.F.Kotze. The effect of different ozone concentrations on white blood cell energy homeostasis. (Poster presentation).

C2. Presentation as International conference

13th Biennial Congress of the International Society for Free Radical Research - SFRR (August 15-19, 2006, Davos, Switzerland).
L.H. du Plessis, F.H. van der Westhuizen, H.F.Kotze. The effect of different ozone concentrations on white blood cell energy homeostasis. (Poster presentation).

C3. Abstract published in Free Radical Research, Official Journal of the Society for Free Radical Research-European Region. 2006. Volume 40, Supplement 1. ISSN 1071-5762.

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There is skepticism about the toxicity and effectiveness of ozone as a therapeutic agent. In order to shed some light on this we examined the effects of ozone autohemotherapy on the energy homeostasis of leukocytes. Venous blood of six healthy human donors was

collected in heparin. In one aliquot a precise volume of blood was mixed with an equal volume of oxygen/ozone gas mixture containing 80, 40 and 20 μg/ml ozone. In another aliquot the plasma was washed out and replaced with a buffered phosphate. The buffered blood cells were treated with ozone, similar to those in plasma. Control samples was either not treated or treated with an equal volume of oxygen. Various biochemical analyses were preformed on isolated white blood cells and plasma samples. Higher doses of ozone caused an increase in oxidative stress and death of white blood cells. Antioxidant enzyme levels were elevated and the serum antioxidant capacity decreased. These effects were more profound when the plasma was removed. i.e. in the absence of plasma antioxidants. Therefore, high concentrations of ozone were damaging to the cells, but this effect was dampened by antioxidants present in plasma.

C4. Article published in Proceedings of the 13th Biennial Congress of the International Society for Free Radical Research -SFRR (August 15-19, 2006, Davos, Switzerland). Indexed by Current Contents of ISI, Philadelphia, USA.

The effect of different ozone concentrations on white blood cell energy homeostasis

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Summary

Our aim was to evaluate the toxicity and effectiveness of ozone as a therapeutic agent. Two exposure models were used to assess the effect of ozonation on white blood cells in the presence and absence of plasma antioxidants. In the first case whole blood was used and in the second case plasma was washed of and the cells resuspended in phosphate buffered saline. Each model was then mixed with an equal volume of oxygen/ozone gas mixture containing 80 or 20 μg/ml ozone. Control samples were either not treated or treated with an equal volume of oxygen. Higher doses of ozone caused an increase in oxidative stress and death of white blood cells. These effects were more pronounced when the plasma was removed, which is probably due to the antioxidant effect of plasma. Therefore, high concentrations of ozone were damaging to the cells, but this effect was buffered by the presence of plasma.

Introduction

Ozone therapy is an alternative medical approach that has been proposed as an adjunct to conventional treatment in a variety of conditions [1]. The therapeutic mechanism through which ozone elicit its effect is not well understood. Strong evidence suggests that it upregulates the immune system through increased white blood cell and cytokine production. Another mechanism seems to be increased production of reactive oxygen species (ROS) which upregulates antioxidant defense systems. During ozone-autohemotherapy a precise volume of blood is collected from a patient, mixed with the same volume of a oxygen/ozone gas mixture, having a predetermined concentration and then returned to the patient [1]. Although ozone therapy seems to have positive therapeutic effects, there is some concern regarding its toxicity and effectiveness. It is well known that ozone is toxic when inhaled, in spite of the fact that the surfactant contains sufficient numbers of antioxidant systems to minimize toxcicity [2]. Since the antioxidant systmes in plasma is almost equivalent to that in surfactant the question to be answered is if ozone can also be toxic to the blood. The aim therefore was to determine if the plasma provides protection against ROS generated due to ozone.

Materials and Methods

The study was approved by the ethics committee of the North-West University, Potchefstroom (project number 05M07). Ozone was generated from ultrapure oxygen (>99.9 %) by an ozone generator (School of Physics, North-West University, Potchefstroom, USA patent 09/914,199). The ozone concentration was determined by a UV/Vis spectrophotometer at 254 nm. Blood (100 ml in EDTA) was collected from six apparently healthy human donors, who gave informed consent. The blood was divided into two samples. In the first sample, venous blood was used directly for the treatment and in the second sample the blood plasma was removed by differential centrifugation and the cells resuspended in phosphate buffered saline before the treatment. These two samples of blood were then divided into four treatment groups that consisted of i) a control sample that received no treatment, ii) a sample treated with an equal volume of oxygen, and samples treated with an ozone/oxygen gas mixture that contained either iii) 20 or iv) 80 μg/ml ozone. The ozone treatment was done according to standard procedures [1]. The samples were gently and continuously mixed with the gas for 20 minutes and prepared for biochemical analysis.

Hydroperoxides in serum were measured with a commercially available kit (the d-ROMs test; DIACRON International, Grosseto, Italy) and results expressed as Carratelli units (CARR U) where 1 CARR U is equal to 0.08 mg/100 ml H_2O_2 [3]. The ratio of reduced and oxidized glutathione was measured as described [4] but with slight modifications. The total antioxidant capacity in plasma was measured by using the ferric reducing antioxidant potential (FRAP) assay [5]. Antioxidant enzymes were determined using the methods described by Ellerby and Bredesen [6]. White blood cell viability was determined with the trypan blue exclusion assay. The positive control (PC) was treated with 6 % acetic acid. All statistical analyses were done with Graphpad Prism® (version 4) and Statistica (version 7; Statsoft). Results were expressed as the mean ± SEM.

Differences in mean values were considered significant when $p < 0.05$. Treated samples were compared to the untreated or oxygenated samples.

Results and discussion

When ozone dissolves in plasma it generates reactive oxygen species (ROS), which oxidizes with various substances including polyunsaturated fatty acids and antioxidants [1]. These two interactions should observed by a decreased antioxidant capacity as measured by FRAP and increase in the amount of hydroperoxides. As shown in Figure 1A and B, a clear and significant decrease (10.8 and13.8 % respectively) of FRAP could be observed with ozone treatments of whole blood but not a significant increase of hydroperoxides. However, with buffered cells, which do not contain plasma, FRAP values were only slightly but not significantly decreased. Hydroperoxide levels in these cells were slightly, albeit significantly increased compared to untreated cells with both ozone and oxygen treatments. Furthermore ozone treatment of whole blood, which contains much more (~ 1000 times) more glutathione than white blood cells, significantly decreased the glutathione redox state levels to levels approximately 150 % lower (at 80 µg/ml ozone) compared to untreated of oxygen treated samples (Figure 1C). Cell viability in both treatment groups decreased significantly after treatment with 20 and 80 µg/ml ozone respectively, albeit somewhat more in buffered cells (24 and 26.3 %) compared to whole blood (15.8 and 16.7 %) (Figure 1D). The concentration of the antioxidant enzymes catalase, glutathione reductase and glutathione peroxidase all decreased slightly but significantly after treatment with ozone whereas treatment with oxygen had no significant effect. Superoxide dismutase increased significantly with ozone treatment (7.3 and 14.6 % respectively). In the buffered cell group these effects were generally more pronounced than in the whole blood group (Table 1). The results clearly indicate that ozone treatment with 20 and 80 µg/ml ozone causes increased oxidative stress and decreased the antioxidant defenses. This could explain why white blood cell viability also decreased. In addition our results indicate that plasma antioxidant defenses are important when ozone is used as an adjunct form of therapy and perhaps other types of treatments when ROS formation is increased. The plasma antioxidant components have a definite protective effect on damage caused by ozone.

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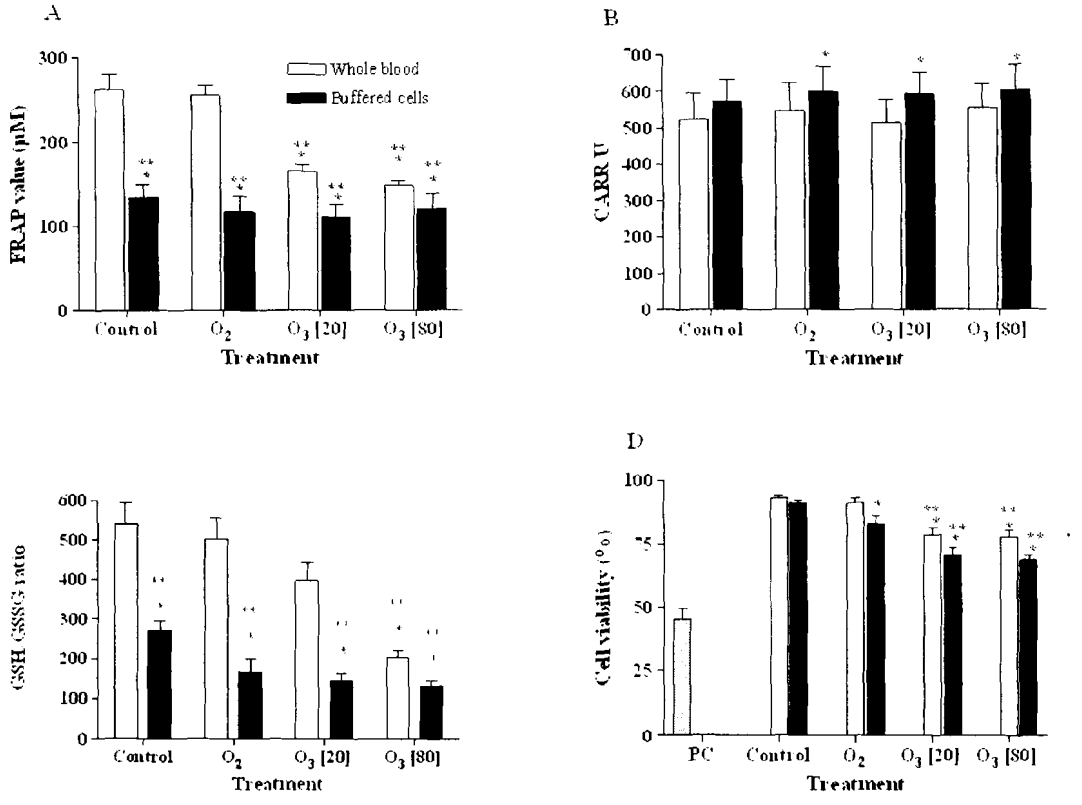

Figure 1: The effect of oxygen and ozone treatments on oxidant/antioxidant status of plasma and the viability of white blood cells. A represents the reducing ability of plasma measured, B the hydroperoxide content expressed as CARR U, C the glutathione redox status of plasma and D the viability of white blood cells (expressed as a percentage of the control). Data are given as a mean ± SEM. * $p<0.05$ versus control or ** $p<0.05$ versus oxygenated sample of the whole blood group.

Whole blood				
	SOD (ng)#	CAT (U/µg protein)	GR (U/µg protein)	GPx (U/µg protein)
Control	483 ± 31	16.6 ± 1.1	0.041 ± 0.003	0.048 ± 0.01
O_2	435 ± 31	15.2± 1.1	0.038 ± 0.002	0.049 ± 0.01
$O_{3\,[20]}$	403 ± 25*	14.8 ± 0.8*	0.038 ± 0.002	0.047 ± 0.01
$O_{3\,[80]}$	400 ± 14*	14.3 ± 0.5*	0.035 ± 0.003*	0.043 ± 0.01
Buffered cells				
	SOD (ng)#	CAT (U/µg protein)	GR (U/µg protein)	GPx (U/µg protein)
Control	491 ± 20	16.2 ± 0.9	0.041 ± 0.004	0.046 ± 0.01
O_2	410 ± 25*	15.1 ± 0.8*	0.040 ± 0.004	0.042 ± 0.01
$O_{3\,[20]}$	389 ± 23*	14.3 ± 0.7*	0.037 ± 0.003	0.042 ± 0.01
$O_{3\,[80]}$	401 ± 25*	13.6 ± 0.4*	0.033 ± 0.002*	0.040 ± 0.01*

Table 1: Levels of antioxidant enzymes in the experimental groups (n=6). Values given as mean ± SEM. * $p<0.05$ versus the control of the whole blood group. # Amount of protein needed to cause 50 % inhibition of 6-hydroxydopamine auto-oxidation. SOD = superoxide dismutase; CAT = catalase; GR = glutathione reductase; GPx = glutathione peroxidase.

Appendix D

Ethical approval for the project under the title *"In vitro effects of treatment of blood with different O_3 concentrations on cell integrity, plasma antioxidant status and plasma organic and amino acids"* with the reference number 05M07, was obtained. A copy of the informed consent form is attached for experimentation with the subjects.

Informed consent form:

CONFIDENTIAL

Informed consent form
PART 1

1. **School/Institute:**

   .....

2. **Title of project/trial:**

   .....

3. **Full names, surname and qualifications of project leader:**

   .....

4. **Rank/position of project leader:**

   (Professor, Lecturer, research scientist etc.)

   .....

5. **Full names, surname and qualifications of supervisor of the project:**

   (Complete only if not the same person named in 4.)

   .....

   .....

6. **Name and address of supervising medical officer *(if applicable)*:**

   .....

7. **Aim of this project**

   .....

8. **Explanation of the nature of all procedures, including identification of new procedures:**

   .....

9. Desciption of the nature of discomfort or hazards of probable permanent consequences for the subjects which may be associated with the project:

(Including possible side-effects of and interactions between drugs or radio-active isotopes which may be used.)

.....

10. Precautions taken to protect the subjects:

.....

.....

11. Description of the benefits which may be expected from this project:

.....

.....

.....

.....

12. Alternative procedures which may be beneficial to the subjects:

(Complete only if applicable.)

.....

.....

.....

Signature: ..... Date: .....
Project leader

PART 2

To the subject signing the consent as in part 3 of this document:

You are invited to participate in a research project as described in paragraph 2 of Part 1 of this document. It is important that you read/listen to and understand the following general principles, which apply to all participants in our research project:

1. Participation in this project is voluntary.

2. It is possible that you personally will not derive any benefit from participation in this project, although the knowledge obtained from the results may be beneficial to other people.

3. You will be free to withdraw from the project at any stage without having to explain the reasons for your withdrawal. However, we would like to request that you would rather not withdraw without a thorough consideration of your decision, since it may have an effect on the statistical reliability of the results of the project.

4. The nature of the project, possible risk factors, factors which may cause discomfort, the expected benefits to the subjects and the known and the most probable permanent consequences which may follow from your participation in this project, are discussed in Part 1 of this document.

5. We encourage you to ask questions at any stage about the project and procedures to the project leader or the personnel, who will readily give more information. They will discuss all procedures with you.

6. If you are a minor, we need the written approval of your parent or guardian before you may participate.

7. We require that you indemnify the University from any liability due to detrimental effects of treatment by University staff or students or other subjects to yourself or anybody else. We also require indemnity from liability of the University regarding any treatment to yourself or another person due to participation in this project, as explained in Part 1. Lastly it is required to abandon any claim against the University regarding treatment of yourself or another person due to participation in this project as described in Part 1.

8. If you are married, it is required that your spouse abandon any claims that he/she could have against the University regarding treatment or death of yourself due to the project explained in Part 1.

PART 3

Consent

Title of the project:

.....

.....

I, the undersigned .....(full names)
read/listened to the information on the project in PART 1 and PART 2 of this document and I declare that I understand the information. I had the opportunity to discuss aspects of the project with the project leader and I declare that I participate in the project as a volunteer. I hereby give my consent to be a subject in this project

.

I indemnify the University, also any employee or student of the University, of any liability against myself, which may arise during the course of the project.

I will not submit any claims against the University regarding personal detrimental effects due to the project, due to negligence by the University, its employees or students, or any other subjects.

(Signature of the subject)

Signed at .....on .....

Witnesses

1. .....

2. .....

Signed at ..... on .....

For non-therapeutic experimenting with subjects under the age of 21 years the written approval of a parent or guardian is required.

I, ... (full names)
parent or guardian of the subject named above, hereby give my permission that he/she may participate in this project and I also indemnify the University and any employee or student of the University, against any liability which may arise during the course of the project.

Signature: ..... Date: .....

Relationship: .....

For experimenting with married persons the following indemnity from the spouse is required.

I, ... (full names), the spouse of the subject in this application, hereby undertake not to submit any claims against the University regarding treatment in case of death or injuries of this person due to the project as described in this application, due to negligence of the University, its students or another subject, or in any other way.

Signature: ..... Date: .....

Relationship: .....