

**The effects of ozone exposure on markers of cellular
resilience in cultured human epithelial HeLa cells**

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Dissertation submitted for the degree Magister Scientiae

in

Pharmacology

at the

North-West University (Potchefstroom Campus).

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2008

Potchefstroom

Abstract

Ozone is a natural occurring gas with strong oxidising properties. While its biological effects are associated with toxicity, it has also been used for therapeutic purposes. In biological systems ozone can elicit dose-dependent oxidative stress, which may induce adaptation. The mechanisms for these effects remain illusive. Previous studies in our laboratory indicated that repetitive exposure of cultured human epithelial (HeLa) cells to ozone may induce cytoprotective mechanisms (adaptation), which may include the up-regulation of anti-apoptotic pathways. The aim of the current study was to determine the effects of single and repetitive exposure of HeLa cells to ozone on the expression of genes that encode for anti-apoptotic (Akt, Bcl2, CREB and NFkB) and pro-apoptotic (Bax, caspase 3 and 8) proteins, as well as the corresponding protein expression levels.

Cultured HeLa cells were exposed to control or ozone-saturated glucose-free Krebs-Henseleit solution. Exposures consisted of 4 x 5-minute exposures every four hours, followed by a 16-hour incubation in normal culture medium and then a 25-minute exposure. Cells were then lysed immediately (0 h) or after 8 hours (8 h) incubation in normal culture medium. The relative expression of genes encoding for pro-apoptotic factors and anti-apoptotic factors was then determined with quantitative real-time RT-PCR (RT²-PCR). Protein expression was also investigated for Akt, Akt phospho specific, CREB, CREB phospho specific, pro-caspase 3 and caspase 3 cleaved protein with Western blot after the respective ozone exposure regimens and 8 h after incubation in normal culture medium.

When measuring gene expression at 0 h, genes encoding for Bcl-2, CREB and caspase 3 were down-regulated by ozone administered 4 x 5 minutes every 4 hours, but these returned to pre-treatment values at 8 h. Importantly, cells treated with ozone for 4 x 5 minutes every 4 hours plus a single 25 minute exposure to ozone 16 hours later, showed at 8 h an up-regulation of the genes encoding for the anti-apoptotic factors Akt, and CREB, while corresponding cells at 8 h that treated with ozone either for a single 25-minute exposure or a 4 x 5 minutes plus a single 25 minute exposure showed an up-regulated expression of caspase 3. The Western blot analysis of the proteins showed no significant differences or trends of protein expression for any of the corresponding treatment groups at 8h.

The current gene expression data suggest that up-regulation of Akt and CREB (anti-apoptotic) may be involved in the adaptation of cultured human epithelial HeLa cells after repetitive exposure to ozone. While it up-regulation of caspase 3 (pro-apoptotic) has also been demonstrated, its role and significance in ozone-elicited adaptation is unclear.

Opsomming

Osoon is 'n natuurlik voorkomende gas met sterk oksiderende eienskappe. Hoewel die biologiese effekte hoofsaaklik met toksisiteit verband hou, word dit ook terapeuties gebruik. In biologiese stelsels kan osoon dosisafhanklike oksidatiewe stres veroorsaak wat tot aanpassing kan lei. Die meganisme van hierdie effekte is steeds onopgeklaar. Vorige studies in ons laboratoria het aangetoon dat herhaalde blootstelling van gekweekte menslike epiteelselle aan osoon selbeskermende meganismes (aanpassing) kan induseer wat tot die opregulering van anti-apoptotiese weë kan lei. Die doel van hierdie studie was om die effekte te bepaal van enkele en herhaaldelike blootstelling van 'n menslike epiteelsellyn (HeLa) aan osoon op die uitdrukking van gene wat vir anti-apoptotiese (Akt, Bcl2, CREB en NFkB) en pro-apoptotiese (Bax, caspase 3 en 8) proteïene kodeer asook op die ooreenstemmende vlakke van proteïenuitdrukking.

Gekweekte menslike HeLa-epiteelselle is aan kontrole of osoonversadigde glukosevrye Krebs-Henseleitoplossing blootgestel. Blootstellings was 4 blootstellings van 5 minute elk elke vier uur gevolg deur inkubasie vir 16 uur in normale kultuurmedium en dan 'n blootstelling van 25 minute. Die selle is dan onmiddellik (0 h) of na inkubasie vir 8 h in normale kultuurmedium opgebreek. Die relatiewe uitdrukking van gene wat vir pro-apoptotiese en anti-apoptotiese faktore kodeer, is dan met kwantitatiewe reële-tyd RT-PCR (RT²-PCR) bepaal. Proteïenuitdrukking is ook met die Westelike kladanalise ondersoek vir Akt, Akt-fosfospesifiek, CREB, CREB-fosfospesifiek, pro-caspase 3 en caspase 3 gesplete proteïene na die onderskeie regimes vir blootstelling aan osoon en 8 h na inkubasie in normale kultuurmedium.

Toe die geenuitdrukking onmiddellik na behandeling (0 h) gemeet is, is gevind dat gene wat vir Bcl-2, CREB en caspase 3 kodeer, afgereguleer is deur osoon wat 4 x 5 minute elke 4 uur toegedien is, maar dit het na 8 uur tot waardes soos voor behandeling teruggekeer. Agt uur na behandeling het selle wat herhaaldelik vir 4 x 5 minute elke 4 uur plus 'n enkele blootstelling vir 25 minute 16 uur later behandel is, egter 'n opregulering getoon van gene wat vir die anti-apoptotiese faktore Akt en CREB kodeer, terwyl óf 'n enkele blootstelling van 25 minute óf 4 x 5 minute plus 'n enkele blootstelling van 25 minute van die selle aan osoon die uitdrukking van caspase 3 na 8 uur opgereguleer het. Die Westelike kladanalise van die proteïene van al die behandelingsgroepe het na 8 uur geen beduidende verskille in of tendens van proteïenuitdrukking getoon nie.

Die huidige data van geenuitdrukking suggereer dat opregulering van Akt en CREB (anti-apoptoties) betrokke kan wees by die aanpassing van gekweekte menslike HeLa-epiteelselle na herhaalde blootstelling aan osoon. Die opregulering van caspase 3 (pro-apoptoties) speel ook *via* 'n onbekende meganisme 'n rol.

Acknowledgements

First and foremost, I thank God for giving me the opportunity and perseverance to overcome the obstacles during this study. Without Him nothing is possible.

To my study leader and mentor, Prof. C.B. Brink, my greatest appreciation for your guidance and support during this study, and for sharing your valuable knowledge with me.

To Prof. L. Brand for your support and advice.

To Sharlene Nieuwoudt, for your assistance inside and outside the laboratory.

To my mother and brother for your endless love and encouragement.

To my friends and colleagues (Nico, Bennie, Carl, Leani and Jacques) for your friendship and for making the workplace such an enjoyable place to be.

To the National Research Foundation (NRF) for funding.

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Introduction

Chapter

1

1.1 PROBLEM STATEMENT

The earth's human population is now over 6.65 billion and is expected to grow substantially in the coming years. Moreover, industrialisation of population giants such as China and India is compounding to the current shortage of food and technology. Economic growth, higher educational levels and the world-wide trend of rapid urbanisation, put more pressure on governments to provide more energy, such as electricity, and to elevate industrial production levels, all leading to escalating pollution. In addition, pandemics, such as HIV/AIDS, also put pressure on economies and demand drastic measures for relief.

In all of these abovementioned problems ozone has an important role to play. Ozone is a naturally occurring gas that can be found in the stratosphere, which protects the earth from the UV radiation of the sun (Rozema *et al.*, 2005). Ozone is also found at elevated concentrations in the troposphere, originating from technological and industrial pollution smog (Maynard, 2004) (i.e. not occurring naturally at high levels). When inhaled by humans this may induce oxidative stress, causing a wide range of health problems (Halliwell & Cross, 1994). However, ozone also found application in the medical sciences, such as dentistry (Azarpazhooh & Limeback, 2008) and the topical treatment of skin infections (Valacchi *et al.*, 2005). In alternative medicine, with limited scientific evidence, ozone is also used in the treatment of HIV/AIDS (Bocci, 2006), chronic ulcers (Werkmeister, 1969), retinitis pigmentosa and heart ischemia (Bocci, 1996).

Ozone is a strong oxidising agent that forms reactive oxygen species (ROS) upon contact with biological materials, eventually inducing dose dependent oxidative stress (Cross *et al.*, 1992). When tissue is exposed to ozone it can either damage the tissue (Brink *et al.*, 2008a) or induce an adaptation to the oxidative stressor (STOKINGER, 1956), of which the mechanism is not fully understood. Such adaptation to ozone has been demonstrated in cultured human epithelial (HeLa) cells (Brink *et al.*, 2008a), rodents (Leon *et al.*, 1998), plants (Emberson *et al.*, 2007) and humans (Hackney *et al.*, 1976). In particular, recent studies in HeLa cells indicated that the mitochondrial apoptotic pathway might play an important role in the mechanism of adaptation to ozone (Brink *et al.*, 2008a).

Ozone generates toxic ROS species such as aldehydes, hydrogen peroxide, organic radicals and hydroxyl radicals by means of lipid peroxidation and protein modification (Pryor & Church, 1991). ROS is available systemically and lead to oxidative stress and DNA damage, which signals for mitochondrial mediated apoptosis (Higuchi & Matsukawa, 1997). The mitochondrial pathway of apoptosis is regulated by both pro- and anti-apoptotic genes (Hengartner, 1999). The caspase family include key pro-apoptotic factors and can be further subdivided into two categories, namely the initiator caspases (e.g. caspase 2, 8-10 & 12) and the effector caspases (e.g. caspase 3, 6 and 7) (Earnshaw *et al.*, 1999). In the Bcl-2 family, Bax gene also plays an important role in the destabilisation of the mitochondrial membrane and release of cytochrome C (Armstrong *et al.*, 1996; Borner, 2003), hence promoting apoptosis. Important anti-apoptotic genes in this pathway include the rest of the Bcl-2 family of genes (e.g. Bcl-2 and Bcl-X₁), that stabilizes the mitochondrial membrane and prevent the release of cytochrome C thus preventing apoptosis (McCarthy *et al.*, 1997).

In a recent study, exposing cultured human epithelial cells to various dosing regimens with ozone, caspase 3 and Akt have both been identified to play a role in the adaptation of the cells to ozone (Pretorius, 2005; Brink *et al.*, 2008). In particular, it was found that when cells received repetitive low doses of ozone, followed by a single high dose, adaptation in the cells was accompanied by reversal of initial DNA and membrane damage. An Akt inhibitor was able to block the adaptive process. Akt plays an important role in many biological pathways such as nitric oxide synthesis, protein synthesis and regulation of apoptosis and cellular survival (Downward, 1998; Fulton *et al.*, 1999; Shah *et al.*, 2000).

It is clear from the above that the biomolecular mechanisms involved in cellular adaptation to ozone needs further clarification.

1.2 STUDY OBJECTIVES

1.2.1 Primary Study Aim

The primary objective of the current study was to investigate the role of the pro- and anti-apoptotic pathways in cellular adaptation to ozone.

The investigation was done by exposing cultured, monolayered, human epithelial cells to ozone in aqueous solution, following an existing setup and exposure regimen. In particular the current study investigated the effect of ozone exposure on the gene expression of the pro-apoptotic proteins Bcl2-associated x protein (Bax), caspase 3 and caspase 8 and the anti-apoptotic proteins protein kinase B (Akt), cAMP response element-binding protein (CREB), B-cell

lymphoma 2 (Bcl2), and neurotrophic factor kappa beta (NF κ B). Relative quantification of gene expression was done with quantitative RT²-PCR technology. As a follow-up, relevant protein expression of pro- and anti-apoptotic proteins was also measured with quantitative Western blot technology. No ethics approval was needed for this study, because no genetic manipulation was done and no animals were used.

1.2.2 Specific Objectives

In order to achieve the above mentioned study objectives the following specific objectives were formulated:

- To verify the conditions from a previous study for the exposure of cultured HeLa cells to ozone in aqueous physiological medium (Pretorius, 2005)
- To investigate the gene expression of the pro- and anti-apoptotic proteins in HeLa cells exposed to ozone.
- To investigate the protein expression of the pro- and anti-apoptotic proteins in HeLa cells exposed to ozone.

1.3 PROJECT DESIGN

All studies were preformed in the Laboratory for Applied Molecular Biology at the North-West University (Potchefstroom campus), Potchefstroom, South Africa. In order to address the abovementioned objectives a human epithelial (HeLa) cell line was utilised. The following project layout was followed:

- HeLa cells were exposed to ozone in glucose free Krebs-Henseleit solution with a repeated low dose, single high dose or both, after which cells were immediately used for assays (0 h) or left for 8 hours in normal DMEM and then used for assays (8 h).
- mRNA extractions were performed at 0 h and 8 h, where after real-time reverse transcriptase PCR (RT²-PCR) was preformed to investigate the effects that ozone exposure did to the pro-apoptotic genes (caspase 3, caspase 8 and Bax) and the anti-apoptotic genes (Akt, Bcl2, CREB and NF κ B).
- After the RT²-PCR data was analyzed, these data were used to select appropriate proteins for which to determine further, performing Western Blot analysis, whether expression was modulated by ozone exposure. The selected proteins included Akt, Akt phospho specific, CREB, CREB phospho specific, pro-caspase 3 and cleaved caspase 3 at 8 h.

With this experimental layout it was possible to investigate and achieve the aspects stated in the study objectives.

1.4 DISSERTATION LAYOUT

The dissertation is presented in the University's "Article Format for Dissertations". In short, this implies that there must be at least an introductory chapter with a literature review and problem statement for the study as a whole, thereafter the manuscript(s) as to be submitted to an identified journal(s), followed by a chapter with a summary and conclusions on the study as a whole. Additional experimental data, methods and other relevant information not included in the article(s) should be included in appendixes.

The current dissertation has been divided into the following sections:

- **Chapter 1: Problem statement.** Explains the research question that will be addressed in the study, the aims of this study and the general study and dissertation layouts.
- **Chapter 2: Literature overview.** Summary and interpretation of all the data published on the study theme to date.
- **Chapter 3: Article.** Only relevant data to submit an article is presented in this chapter, in a format as described by the instructions to the author for the identified appropriate international journal.
- **Chapter 4: Summary, conclusion and prospective studies.** This chapter includes a concise summary of all results from Chapter 3 and Appendix B. It then presents a final discussion and conclusion of these results and proposes prospective studies on this topic.
- **Appendix A: Additional materials and methods.** This appendix includes all materials and methods of experiments performed that were not included in the article (Chapter 3).
- **Appendix B: Additional results and discussion.** This appendix includes the results as obtained from the experiments explained in Appendix A, and also provides a discussion thereof.
- **Appendix C: Guide to the author.** This appendix contains the instructions to the author from the selected journal.
- **Appendix D: Congress contribution.** All the conference contributions of the candidate, whether podium or poster, are presented here.

- **Appendix E: Additional article.** A final article (electronic PDF format ahead of hard copy print in April 2008) on data preceding the current study and in which the candidate was a co-author is included here.

Literature overview

Chapter

2

2.1 INTRODUCTION

Ozone is a naturally occurring gas in the stratosphere, chemically defined as a triple oxygen molecule, O_3 . In the troposphere ozone levels are naturally low, while higher levels are produced by mostly industrial pollution, where it also is associated with biological toxicity.

This chapter deals with various topics related to ozone, as of interest for the current study and as reported in literature. The first section will provide an overview of general aspects related to ozone, such as its history in science, its physical-chemical properties, its occurrence and distribution in the atmosphere and the regulation thereof. The second section will discuss biological aspects of ozone, including its biological effects and industrial and medical applications. The third section will focus on the role of ozone in oxidative stress, apoptosis and adaptation, including recent results from our laboratory.

2.2 GENERAL RELEVANT ASPECTS OF OZONE

2.2.1 History in Science

Van Marum (1785), a Dutch chemist, was the first person to smell detect ozone (O_3) gas, describing a characteristic smell around the electrodes used in his experiments. However, the first description of ozone as chemical entity is found in the writings of Schonbein, dating back to 1840, when he denoted to the gas forming at the electrodes of his experimental setup as 'ozone' (from the word ozein, the Greek word for scent). Schonbein was also the first person to research the reaction mechanisms of ozone and organic matter.

The first ozone generator was manufactured in 1857 in Berlin by Von Siemens, who also wrote a book on the applications of ozone in water purification. The French chemist Marius Paul Otto was the first person to start a specialised company for the manufacturing of ozone. This opened the way for large scale use of ozone in water purification and around 1916 there were 49 ozone installations in use throughout Europe.

Key development phases in the scientific discovery and investigation, application and regulation of ozone can be summarised in the following timeline (Pretorius, 2005):

Ozone timeline
• 1840 – O₃ discovered by Schonbein. • 1893 – Application as a disinfectant in drinking water. • 1909 – Application as food preservative for cold storage of meals. • 1939 – Was found to prevent the growth of yeasts and moulds during the storage of fruits. • 1948 – Ozone is discovered to form part of photochemical smog. • 1961 – Ozone first used in autohaemotherapy. • 1991 – EPA restricts exposure levels of ozone to 0.12 ppm for an average of 1 h per day. • 2001 – EPA issues a new ozone exposure level standard of 0.08 ppm for an average of 8 h per day.

2.2.2 Physical-Chemical Properties

2.2.2.1 The Structure of Ozone

To understand chemical and biological reactivity of ozone it is necessary to understand its structure and resonance. The ozone molecule consists of three oxygen atoms that are in an unstable state. These unstable oxygen atoms tend to revert to the more stable state of diatomic oxygen (O₂) by releasing one of the oxygen atoms. The latter release of the oxygen atom renders it a strong oxidising capacity, while it also explains the short half-life ($t_{1/2}$) of ozone in the atmosphere and in aqueous solutions.

2.2.2.2 Physical Properties of Ozone

The physical properties of ozone are summarised in Table 2-1:

Table 2-1: Summary of the physical properties of ozone (Pretorius, 2005)

Property		
Colour	as a gas	Blue
	dissolved in water > 20 ppm	Purple-blue
Molecular weight		48 g/mol
Boiling point		-112°C
Density at room temperature and 1 atmospheric pressure		2.144 g/cm ³
Solubility in water at 0°C		0.64 unit
Half-life in water, pH 7	35°C	7 minutes
	30°C	12 minutes
Electrochemical potential		2.08 V

2.2.3 Atmospheric Occurrence and Regulation

2.2.3.1 Stratospheric Ozone

The stratosphere is the earth's upper atmospheric layer, where higher concentrations of naturally occurring ozone is found (Rozema *et al.*, 2005). Stratospheric ozone also is formed when ultraviolet light (UV) from the sun splits an oxygen molecule (O_2), forming two single oxygen atoms (Cotovio *et al.*, 2001). Each reactive single oxygen atom then binds to an oxygen (O_2) molecule to form ozone (O_3). Since ozone in the stratosphere reflects UV rays from the sun, which may otherwise be harmful to biological life on earth, it is sometimes referred to as "good ozone" (Cotovio *et al.*, 2001).

2.2.3.2 Tropospheric Ozone

The troposphere is the lower layer of the earth's atmosphere, at the surface of land and sea where biological life occurs. While small quantities of ozone are formed naturally, higher levels of tropospheric ozone is produced by industrialisation (i.e. manmade pollutant), as found in photochemical smog (Rozema *et al.*, 2005).

Tropospheric ozone is formed by a photochemical reaction of nitrogen oxides with volatile organic compound and/or carbon monoxide (Maynard, 2004). In urban areas of highly industrialised countries ozone levels are at their highest during the afternoon and early evenings of warm and sunny days, typically in late spring, summer and early fall. Since elevated ozone in

the troposphere is toxic to biological life, it is sometimes referred as “bad ozone” (Rozema *et al.*, 2005).

2.2.4 Ozone Pollution and Toxicity

2.2.4.1 Ozone Pollution

Ozone is one of the components of photochemical smog. Approximately 113 million people in the United States live in areas where ozone levels are above the National Ambient Air Quality standards (N.A.A.Q.) for daily exposure limits (Janic *et al.*, 2003). The standards of ozone exposure is set by the EPA (Environmental Protection Agency) in the US and it states that a person may only be exposed to ozone for 8 h a day at a maximum level of 0.08 ppm.

2.2.4.2 Ozone Toxicity

Ozone is a very reactive oxidising molecule that can be converted rapidly into a variety of reactive oxygen species (ROS). It exhibits its toxicity by reacting with cell proteins and lipids, forming ROS (Shelley *et al.*, 1989; Halliwell & Cross, 1994). While ROS is transported systemically, ozone can cause oxidative stress (thus being potentially toxic) to a wide range of organs and systems, not just in humans but also in plant life and animals. Below is a concise summary of the toxic effect ozone pollution can have on the critical organ systems of the human body.

2.2.4.2.1 Respiratory System

The lungs are essential organs and an major target for ozone toxicity. It is in the lungs that ROS is formed by the interaction of inhaled ozone with the lung lining fluid and from where ROS is transported to the blood and then distributed systemically (Ballinger *et al.*, 2005). The exposure of ozone at higher concentrations or over extended periods has been associated with impaired lung function, typically characterized by pathological changes mainly in lower airways (Janic *et al.*, 2003), such as oedema, inflammation, epithelial cell damage, and surfactant derangement (Shelley *et al.*, 1989; Balis *et al.*, 1991; Putman *et al.*, 1997).

Upon exposure of the lung epithelial cells to ozone injury occurs rapidly (Joad *et al.*, 2000; Postlethwait *et al.*, 2000), suggesting that cell damage in the initial stage is caused by directed toxicity. Although the precise mechanisms whereby ozone initiates acute lung injury remain equivocal, physical contact between inhaled ozone and the epithelia cells may be limited (Uppu *et al.*, 1995; Bush *et al.*, 1996). This is because the lung's surface is overlain by the continuous aqueous epithelial lining fluid (ELF) (Wu *et al.*, 1996) through which inhaled gases must permeate to come in contact with the underlying epithelium. The ELF is a biologically complex mixture (Kubo *et al.*, 1995), generally considered to represent the first line of defence against

inhaled toxicants. The influx of ozone into the ELF is not governed by its aqueous solubility, but largely by its chemical reactions with the ELF (Langford *et al.*, 1995).

Inhaled ozone reacts with ascorbic acid, uric acid, reduced glutathione, proteins and unsaturated lipids (Mudway & Kelly, 1998), and the product that is formed is reactive oxygen species (ROS) (Corradi *et al.*, 2002). The newly formed ROS acts as a secondary messenger of ozone, while also damaging the cells (Connor *et al.*, 2004). The ozone products are also the way the effect of ozone exposure is relayed from the lungs to the rest of the body (Sathishkumar *et al.*, 2005). Ozone and the formed ROS activate neutrophils (Vagaggini *et al.*, 2001) which induce a respiratory burst resulting in overproduction of more ROS and oxidative stress and this damages the cells even more (Fievez *et al.*, 2001). The activation of the neutrophils is responsible for the inflammatory response after ozone exposure (Corradi *et al.*, 2002).

Inhalation of ozone at levels higher than 0.2 ppm may cause the following effects in the respiratory tract (EPA, 1986):

- bronchoconstriction & dyspnoea, mediated by an increase in airway reactivity;
- bronchial inflammation (secondary to oxidative stress and tissue damage);
- decreased tidal volume, resulting in tachypnoea;
- altered permeability of alveolar walls.

2.2.4.2.2 Cardiovascular System

The relation between ozone and myocardial diseases has been reported in several epidemiological studies (Koken *et al.*, 2003; Ruidavets *et al.*, 2005). Reports on the cardiovascular effects of ozone are very contradictory, and some researchers reported increased heart rate, decreased mean arterial blood pressure, arrhythmias and reduced maximal oxygen uptake, while others found no significant changes in these parameters.

Elevated blood cholesterol is associated with increased risk to develop cardiovascular disease, and in particular oxidised forms of cholesterol is more atherogenic. ROS from ozone may oxidise cholesterol to form cholesterol secoaldehyde (CSeco) (Sathishkumar *et al.*, 2005). Cholesterol is one of the most abundant neural lipids in biological membranes and is a component of pulmonary surfactant which is a component of ELF (Sadana *et al.*, 1988). The double bond in cholesterol is susceptible to oxidation by ROS and ozone (Smith, 2004). One of the products of the reaction between ozone and cholesterol is CSeco, which then can get into the blood from the lungs (Sathishkumar *et al.*, 2005). This product has been shown to be toxic to bronchial epithelial cells (Pulfer & Murphy, 2004) and a number of other cell types (Schroepfer, Jr., 2000). In recent studies on H9c2 cardiomyoblasts it was found that CSeco induces a dose dependent apoptosis (Balis *et al.*, 1991). A report by Wentworth *et al.*

demonstrates the formation of CSeco in arterial plaques following treatment with phorbol esters indicating the production of endogenous oxidants under inflammatory conditions (Sathishkumar *et al.*, 2005).

2.2.4.3 Central Nervous System

If the lung antioxidant defence system is overwhelmed by the ROS, it may cross the lung membranes and interstitial fluids to reach the systemic blood circulation. From here it can reach the rest of the body and, as ROS readily crosses the blood brain barrier, also the central nervous system (Rivas-Arancibia *et al.*, 2003). These free radicals in the systemic circulation may induce an oxidative stress state.

The nerve system is more vulnerable to free radicals than other tissues (Colton & Gilbert, 1993). This vulnerability is due to its high lipid content and oxygen consumption, low catalase and superoxide dismutase activity, and also to the moderate activity of glutathione peroxidase (Rivas-Arancibia *et al.*, 2003). The entire brain is susceptible to oxidative stress, but cretin regions are more than others. In particular, oxidative stress produces an increase in the release and in the oxidation of dopamine. Reactive dopamine metabolites, such as dopamine quinines and ROS, directly alter protein functioning via oxidative modifications that induce a mitochondrial permeability transition pore (LaVoie & Hastings, 1999) and ATP depletion. Nitric oxide metabolites formed in the presence of oxidative stress contribute to the selective vulnerability of dopaminergic neurons, due to dopamine oxidation induced by peroxynitrite and nitrite (LaVoie & Hastings, 1999).

Oxidative stress plays a major role in ageing (Barja & Herrero, 2000) and age-related neurodegenerative diseases such as amyotrophic lateral sclerosis, Parkinson's disease, Alzheimer's disease and Huntington's disease (Beckman & Ames, 1998; Luo & Roth, 2000). Other pieces of evidence have shown that oxidative damage and free radical formation associated with the normal ageing process, may lead to altered hippocampal function and cognitive deficits (Taylor *et al.*, 1995). Also, oxidative damage to the basal ganglia may result in deficits in locomotor function (vila-Costa *et al.*, 2001; Dorado-Martinez *et al.*, 2001).

2.3 BLOOD AND OZONE THERAPY

2.3.1 Blood

Inhaled ozone does not reach the blood in high concentrations, because of the ELF in the lungs. Inhalation is not the only way ozone can enter the human body. Ozone is also used in alternative medicine where ozone is bubbled through blood (autohaemotherapy). Like other

gases ozone must be dissolved in water in order to interact with organic substrates. Upon direct exposure of drawn blood to ozone gas (bubbling), the ozone dissolves in plasma and instantly decomposes in a cascade of ROS, including hydrogen peroxide, superoxide anion, hydroxyl radical and hypochlorous acid (Gutteridge & Halliwell, 1992; Bocci, V, 1998; Bocci *et al.*, 1999a). Some of these species are highly reactive compounds with a short half-life, therefore representing potential toxic compounds that may affect different biochemical targets to form reactive by-products and molecular lesions.

Aerobic organisms have developed several defence mechanisms with antioxidant systems and sacrificial biomolecules (Di *et al.*, 2005), which can be divided into enzymatic and non-enzymatic defences. Non-enzymatic defences include uric acid, ascorbic acid, protein (mainly albumin) and non-protein thiols, vitamin E and bilirubin (Goldstein & Balchum, 1967; Halliwell & Cross, 1994; Los *et al.*, 1995), while enzymatic systems consists of super oxide dismutase, catalase and the glutathione system (glutathione peroxidase, glutathione transferase and glutathione reductase) (Goldstein & Balchum, 1967; Halliwell & Cross, 1994; Los *et al.*, 1995).

While these systems can take care of most of the ozone, it won't eliminate all of the ozone and ROS and this will have an effect on the blood system. The hydrogen peroxide that is formed by ozone has various effects in red blood cells (RBCs). It shifts the haemoglobin-oxygen dissociation curve to the right and facilitates release of oxygen (Freeman & Mudd, 1981; Los *et al.*, 1995; Van, V *et al.*, 1995), and in high concentrations it will haemolyse the RBCs. While in leukocytes and endothelial cells it can stimulate the production of interleukins, interferons, growth factors and nitric oxide (Zangerle *et al.*, 1992). In platelets it favours the release of growth factors (Freeman & Mudd, 1981; Bocci *et al.*, 1998b; Bocci *et al.*, 1999b), while in other cell types, such as macrophages and respiratory epithelial cells, hydrogen peroxide has been shown to stimulate cell activation, cytokine secretion and promote long-term efficiency of antioxidant systems. The latter may follow adaptation to the pro-oxidant action of ozone (Hamilton, Jr. *et al.*, 1996).

2.3.2 Ozone Therapy

By the end of the previous millennium roughly 150 research papers on ozone were published annually, including roughly 15% dealing with the thinning of the earth's ozone layer, and the bulk of the articles addressing the pulmonary toxicity of ozone found in the troposphere (Bocci, 1996). Investigations into other biomedical aspects of ozone are rarely found, probably in part because conventional medicine of the leading western countries disregards a role for ozone in medical therapy. In addition, scientific evidence from expensive and large controlled double-blind studies are rarely found for alternative medicine and non-patentable agents such as ozone.

In an attempt to ensure standardised, optimal and safe administration of ozone, when used in medical therapy, the following measures were suggested to be in place (Bocci, 2006):

- Use a precise ozone generator equipped with a standardised photometer, allowing the measurement of ozone concentration in real time.
- Determine beforehand the optimal dose for achieving the required therapeutic effect, with predictable and limited toxicity.
- Ensure accurate measurements of the ozone dose to be administered by collecting the precise volume of gas with a defined ozone concentration.

Ozone is used mostly administered topically for the treatment of skin infections such as fungal infections. In recent years it has also been used in autohaemotherapy (AHT), where a small amount of blood (usually ± 100 ml) is withdrawn, ozonated in the syringe and then re-injected (Bocci, 1996; Bocci, 2006). Autohaemotherapy is claimed to be useful in the treatment of a variety of systemic diseases, including arteriosclerosis, HIV, autoimmune diseases and back pain (Re *et al.*, 1999; Al-Dalain *et al.*, 2001; Gracer & Bocci, 2005).

2.3.3 Mechanisms of Action of Ozone Therapy

When inhaled, ozone reacts with lipids and other biomolecules to form ROS and lipoperoxide products (LOP), serving as “second messengers” with ROS acting immediately and disappearing and LOP, via the circulation, distribute throughout the tissues and eventually only a few molecules bind to cell receptors. Hydrogen peroxide (H_2O_2) is one of the ROS products of great importance, because it is readily absorbed and is known to trigger a host of pathways depending on the cell type (Gutteridge & Halliwell, 1992).

After exposure to ozone, erythrocytes show an enhanced ability to deliver oxygen to ischemic tissues. The mechanism proposed for this effect, involve the ROS-induced shifting of the oxygen-haemoglobin dissociation curve to the right, by a slight decrease of intracellular pH (Bohr effect) or/and an increase of 2,3-diphosphoglycerate levels. Obviously one autohaemotherapy session may have a minimal effect, whereas a prolonged treatment would have a more optimal effect. During this period LOPs act as repeated stressors on the bone marrow and these frequent stimuli cause the adaptation to the ozone stress during erythropoiesis, with up-regulation of antioxidant enzymes. As a consequence, a patient with chronic limb ischemia undergoing ozone therapy can have clinical improvement due to the formation of successive cohorts of erythrocytes progressively more capable of delivering oxygen to the ischemic tissues (Bocci, 2006). Several sets of criteria to determine the appropriate doses and treatment times with ozone have been reported, but these are mostly unsubstantiated, lacking supportive and comprehensive, high quality clinical data.

When administered via AHT, ozone has been shown to act as a mild enhancer of the immune system by activating neutrophils and stimulating synthesis of some cytokines (Cotovio *et al.*, 2001; Ballinger *et al.*, 2005; Sathishkumar *et al.*, 2005). Once again the crucial messenger is hydrogen peroxide which, after entering into the cytoplasm of blood mononuclear cells (BMC) by oxidising selected cysteines, activates a tyrosine kinase, which then phosphorylates the transcription factor nuclear factor kappa beta ($\text{NF}\kappa\beta$). This complex migrates to the nucleus and switches on a few hundred of genes that are eventually responsible for the synthesis of a myriad of proteins, among which are the acute-phase reactants and numerous interleukins (Bocci *et al.*, 1998a).

During ozonation of blood, particularly if it is anticoagulated with heparin, an ozone-mediated, dose-dependent increase of activation of platelets, with a consequent release of typical growth factors, were observed (Valacchi & Bocci, 1999). These will enhance the healing of chronic ulcers. There is also a release of NO from the endothelial cells that will facilitate vasodilatation (Valacchi & Bocci, 2000; Frehm *et al.*, 2004).

2.4 SUBCELLULAR EFFECTS OF OZONE

2.4.1 Effects on DNA

Ozone can directly damage DNA, or indirectly by means of the ROS and LOP formed by exposure of biological membranes, fluids and tissue to ozone (Pryor, 1992). Ozone can readily oxidise cell lipids and proteins forming reaction products such as hydroxyl radicals, hydrogen peroxide, superoxide anion radicals, carbonyl substances and lipid hydroperoxides. These highly unstable molecules are recognised for their DNA damaging effects (Pryor *et al.*, 1991), including DNA cleavages, such as single-strand breaks, double-strand breaks and nucleotide base oxidative modifications (Halliwell & Aruoma, 1991).

Any agent that causes DNA damage increases the probability of error in the DNA repair process (Steinberg *et al.*, 1990). These changes can lead to mutations and alteration of DNA bases, which may lead to malignant transformations in cells (Victorin, 1992).

Studies have shown that 8-oxoguanine is one of the most prevalent DNA adducts caused by ROS (Loft & Poulsen, 1996; Marnett, 2000). Production of 8-oxoguanine leads to G-A transversion *in vitro* and in animal studies G-A transversion is a common mutation in the p53 genes (Loft & Poulsen, 1996). In recent studies, using more sensitive methods for determining 8-oxoguanine and DNA single strand breaks, it has been shown that breaks and 8-oxoguanine formation can be caused at levels as low as 60-80 p.p.b. for 1 hour (Cheng *et al.*, 2003).

DNA repair is also decreased by ozone exposure. Cellular repair is dependent on the formation of poly (ADP-ribose) polymerase (PARP), which is catalysed by PARP synthetase, as shown in studies investigating this enzyme activity after 0.3 p.p.m. ozone exposure (Bermudez, 2001).

2.4.2 Effects on Proteins

Proteins are very important in the maintenance of homeostasis. It is found throughout the body and is essential to normal functioning of all living organisms. Proteins have a variety of roles in an organism, where they regulate catalysis and synthesis of biologically active substances, the transmission of information via membranes and the formation of connective tissues and cartilage. A particular protein is defined biochemically by a specific sequence of amino acids, which is determined by the gene (expressed in the mRNA sequence). The sequence of amino acids of the protein determines the conformational structure, chemical-physical nature and biochemical properties of the protein. Other factors that are important in the biological function of proteins include the secondary and tertiary structure and macromolecular folding thereof (Styrer L, 1995).

Ozone and ROS damage proteins by oxidising their functional groups (Freeman & Mudd, 1981). Ozone typically oxidise the alcohol and aldehyde functional groups. Ozone reacts mainly with the thiol groups and the aromatic amino acids. The amino acids most affected by ozone are tryptophan, methionine, cysteine, tyrosine and phenylalanine. When ozone reacts with proteins, only the secondary and tertiary structures are modified (Cross *et al.*, 1992; Cataldo & Gentilini, 2005). As can be seen from § 2.3.2 the literature on ozone is not complete and more research is needed on the effects of ozone on proteins.

2.5 BIOMOLECULAR EFFECTS OF OZONE EXPOSURE

2.5.1 Acute Ozone Exposure

The respiratory tract is very susceptible to airborne toxins such as ozone, because this tract comes into direct contact with the environment. However, the amount of ozone that reacts with the deeper respiratory tract tissue is less than the amount inhaled, since ozone first reacts with the protective lung lining fluid (LLF) in the airway. In lung tissue a biphasic response has been observed with short-term exposure to ozone. At first there is an injury phase that is characterised by cellular damage and loss of enzyme activity, followed by a repair phase, associated with increased metabolic activities (adaptation) (EPA, 1986).

In a study performed in our laboratory (Pretorius, 2005), cultured HeLa cells were treated with ozone-saturated physiological solution for 5-minute increments up to 55 minutes. It was found

that, when measuring membrane integrity with the trypan blue assay, significant membrane damage occurred after acute exposure of the cells to ozone with almost no integrity remaining after 55 minutes. The MTT assay, measuring mitochondrial activity, suggested a significant reduction in mitochondrial activity, but adaptation after about 45 minutes. These data suggested that acute exposure to ozone damages cell integrity and mitochondrial function differentially.

2.5.2 Repetitive Ozone Exposure

The effects that occurs after acute ozone exposure, such as cellular damage, inflammation and lung dysfunction, are attenuated with repetitive exposure to ozone (Horvath *et al.*, 1981; van der Wal *et al.*, 1994; Leon *et al.*, 1998). This ability of cells to return to normal function after repetitive ozone exposure is referred to as adaptation, tolerance or attenuation. Ozone adaptation was first documented by Stonkinger in 1956, reporting that after multiple ozone exposures, the expected normal topical effect of ozone was attenuated (Re *et al.*, 1999). The exact mechanism of this physiological adaptation to ozone is still unknown. Several mechanisms have been proposed to explain the adaptation. Of these, enhancement of antioxidant systems and replacement of sensitive cells by ozone-resistant cells are the most plausible (Rahman *et al.*, 1992).

Adaptation to ozone has been investigated in a number of biological systems. In cultured HeLa cells it was found (Pretorius, 2005) that a single high dose of ozone, or a series of four repetitive low doses of ozone induced a severe decrease in membrane integrity, as measured by the trypan blue test. However, after repetitive low dose ozone exposure, followed by a single high dose exposure (i.e. a combination of the repetitive low dose and single high dose ozone), membrane integrity was restored (adaptation). As a matter of fact, membrane integrity was found to be higher than that of control. This adaptation has been observed also in a wide range of rat organs and tissue after repeated exposure (Al-Dalain *et al.*, 2001; Ajamieh *et al.*, 2004), and also in humans. In a small study that compared life-long versus short-term exposure to higher ambient ozone concentrations, as in urban areas (i.e. long-term city residence from birth versus recent city residency), it was found that the life-long residents showed less cellular damage and respiratory effects than the new residents (Hackney *et al.*, 1976). It can be deduced from these results that the life-long residents adapted physiologically to the higher exposure to ozone.

Adaptation may, at least in part, explain why ozone therapy could be of benefit in the treatment of diseases that are associated with changes in physiological redox potential (Bocci, 1996).

2.5.3 Ozone and Oxidative Stress

Oxidative stress is by definition a “imbalance of the steady-state level of oxidative damage-repair processes” in a cell, tissue or organ, caused by an imbalance between the production of ROS and the availability of functional antioxidant molecules (Fernandez-Checa *et al.*, 1998). ROS consists of oxygen free radicals and associated entities that include superoxide free radicals, hydrogen peroxide, singlet oxygen, nitric oxide and peroxynitrite. Several of these species are produced at low levels during normal physiological conditions and are scavenged by endogenous antioxidant systems that include superoxide dismutase, glutathione peroxidase, catalase, and small molecules such as vitamins C and E (Chong *et al.*, 2005).

The production of ROS can lead to cell injury through cell membrane lipid destruction and cleavage of DNA (Vincent & Maiese, 1999). Oxidative stress, such as hydrogen peroxide, results in nucleus condensation and DNA fragmentation (Vincent & Maiese, 1999; Chong *et al.*, 2003), and also impairs mitochondrial function and increases levels of pro-apoptotic gene products (de la Monte *et al.*, 2003). This leads to apoptosis and cell death.

The injury caused by oxidative stress to cells can mediate the initiation of many diseases (Genox Corporation, 1996) such as cancer, Alzheimer’s disease, cardiovascular disease, diabetes and macular degeneration.

2.5.4 Ozone and Apoptosis

Programmed cell death or apoptosis is an evolutionarily conserved process of eliminating unwanted, damaged, aged and misplaced cells during embryonic development and tissue homeostasis (Meier *et al.*, 2000). Apoptosis can be triggered in a diversity of cells by various signals derived from either the extracellular or intracellular milieu. Triggers include activation of tumour necrosis factor (TNF), heat shock, viruses, oxidative stress, hypoxia and nitric oxide (Leist & Nicotera, 1997). When apoptosis is induced inappropriately or is not regulated, it may, similarly as for oxidative stress in general mentioned above, result in the development of cancer, neurodegenerative diseases such as Alzheimer’s disease, Hodgkin’s diseases and transplant rejection (Thompson, 1995).

The distinct morphological changes of a cell undergoing apoptosis are sequentially characterized by shrinkage of the cell, hypercondensation of chromatin, cleavage of chromosomes into nucleosomes, violent bubbling of the plasma membrane and packaging of cellular contents into membrane-enclosed vesicles called apoptotic bodies (Shi, 2001).

There are three phases of apoptosis (Kroemer *et al.*, 1995). In the induction phase apoptosis is initiated via a stimulus promoting cellular death. The signal may be externally delivered through

surface receptors or may originate from within the cell (Raff, 1992). The cell now enters the effector phase of apoptosis and in this phase proteins are induced that relay the initial diverse signals into a few stereotyped pathways. These proteins can be either anti- or pro-apoptotic (Kerr *et al.*, 1972). The final phase of apoptosis is the degradation phase, where chromatin is condensed and DNA degraded, leading to the characteristic morphology and biochemistry of apoptosis (Kerr *et al.*, 1972). Not a lot of literature is available on ozone and its effects on apoptosis (see § 2.3.2).

2.5.4.1 Extrinsic and Intrinsic Pathways of Apoptosis

Programmable cell death can be initiated by activating one of two pathways. The extrinsic apoptotic pathway is activated by binding of a “death ligand” to a “death receptor” on the cell surface. Death receptors, such as the TNF receptor family (e.g. Fas) are stimulated and activate proteins that in turn activate caspases 6, 8 and 9 (Krebs *et al.*, 1999). For example, the cytokine TNF can bind to the death receptor, TNF receptor type 2 (TNF-R2), thereby recruiting two signal molecules, namely TNF-R2-associated death domain protein and Fas-associated protein death domain. These complexes then bind to pro-caspase 8 to activate it, forming caspase 8. This in turn activates the cascade of caspase proteins and result in apoptosis (Reed, 2000).

The intrinsic apoptotic pathway is mediated by the mitochondrial release of cytochrome c (Zimmermann *et al.*, 2001). This pathway is activated if DNA is damaged and is not sensed and repaired by checkpoint genes. Apoptosis can occur immediately, or be delayed after the damage has occurred. In this regard, ROS induce cell death via lipid peroxidation and DNA damage (Higuchi & Matsukawa, 1997), to activate the intrinsic pathway.

2.5.4.2 The Mitochondrial Apoptotic Pathway

Genetic studies have identified four genes that act sequentially to control the onset of apoptosis in *Caenorhabditis elegans*. In contrast to the mammalian pathway, CED-3 is the only known apoptotic caspase in *C. elegans*, and this is both the initiator and the effector caspase (Shi, 2001). CED-4 is an ATP-requiring adaptor protein that forms a casposome (also dubbed an apoptosome) (Hengartner, 1999) with the inactive zymogenic form of CED-3. This brings sufficient CED-3 zymogens to close proximity for autoprocessing and the formation of an active dimeric caspase.

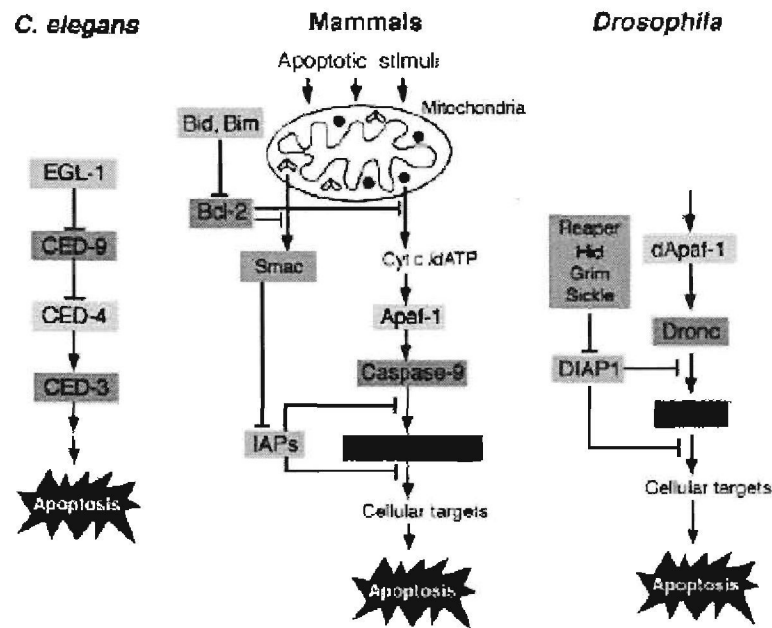


Figure 2-1: A conserved apoptotic pathway in *C. elegans* (left), mammals (middle) and *drosophila* (right) (Shi, 2001).

In healthy cells the formation of the CED-3/CED-4 casposome is prevented by the CED-9 (Chinnaiyan *et al.*, 1997; Spector *et al.*, 1997). This protein sequesters CED-4 to the mitochondrial membrane thereby preventing the adaptor from activating the CED-3 caspase (Wu *et al.*, 1997). In response to a developmentally regulated death stimulus a distant homolog of the Bcl-2 family, called EGL-1, is transcriptionally induced and binds to CED-9 via an amphipathic helical structure called the BH3 domain (Conradt & Horvitz, 1998). Binding of the BH3 domain liberates CED-4 from CED-9, allowing CED-4 translocation to the cytosolic face of the nuclear envelope, where it can activate CED-3 and induce the programmed cell death.

This relatively simple adaptor/caspase principal is conserved in worms and flies. However, because of the complex nature of higher eukaryotes and their need to respond to different endogenous and environmental death stimuli, the regulatory components EGL-1, CED-3, CED-4 and CED-9 are all believed to have evolved into protein families. EGL-1 has evolved into BH3 proteins, CED-9 has evolved into Bcl-2 like survival factors, CED-4 evolved into Apaf-1 and CED-3 evolved into initiator and effector caspase 1-12 (Strasser *et al.*, 2000). This pathway is summarised in Fig 2-1.

For the purpose of this study only the following proteins will be discussed in more detail: caspase family, Bcl-2 family, Akt, BDNF, CREB and NF κ B.

2.5.4.2.1 The Caspase Family of Proteins

The caspase family of proteins are cysteine proteases that cleave vital cellular substrates to aspirate residues (Earnshaw *et al.*, 1999). These enzymes are minimally active in healthy cells and require further activation in response to apoptotic stimuli (Shi, 2002). They are divided into

two categories, namely the initiator caspases (including caspases 2, 8-10 and 12) and the effector caspases (including caspases 3, 6 and 7) (Earnshaw *et al.*, 1999).

An initiator caspase acts at an early point in the apoptotic signalling pathway and has an extended N-terminal pro-domain. This pro-domain interacts with a specific scaffold of adaptor proteins, of which the role is to cluster the respective initiator caspase (Kumar & Colussi, 1999). This proximity enhances the autoproteolysis of the caspase, forming an active dimeric complex. The active initiator caspase then activates an effector caspase by cleavage at specific internal Asp residues, thereby amplifying the apoptotic signal (Earnshaw *et al.*, 1999). Effector caspases are responsible for the dismantling of the cells into apoptotic bodies and subsequent phagocytosis by macrophages (Savill & Fadok, 2000).

2.5.4.2.1.1 Caspase activation and control

When receiving an apoptotic stimulus cytochrome c (Cyt c) is released from the intermembrane space of mitochondria into the cytoplasm, a process regulated by the Bcl-2 family of proteins (Adams & Cory, 1998; Adams & Cory, 2001). Once in the cytosol, Cyt c binds tightly to Apaf-1, changing its conformation from an inhibitory to an active form. The complex of Cyt c and Apaf-1 then binds its critical cofactor dATP or ATP, forming a complex called a apoptosome. The only function of the apoptosome is to recruit and to facilitate activation of pro-caspase 9. Once activated, caspase 9 remains located with the apoptosome as a holoenzyme to maintain its catalytic activity, since the free caspase 9 is marginally active. In this respect the apoptosome serves as an allosteric regulator for the enzymatic activity of caspase 9. The primary target of the holoenzyme is pro-caspase 3, one of the most deleterious effector caspases. This protein cascade activates all the other caspases.

The inhibitor of apoptosis (IAP) family of proteins interacts with and inhibits the enzymatic activity of mature caspases. In normal surviving cells that have not received an apoptotic stimulus, aberrant activation of caspase can be inhibited by IAPs. In cells signalled to undergo apoptosis, however, this inhibitory effect can be suppressed via a process mediated by a mitochondrial protein named second mitochondria derived activator of caspases (Smac). Smac is synthesized in the cytoplasm and is targeted to the intermembrane space of mitochondria. Upon apoptotic stimuli Smac is released from mitochondria into cytosol together with Cyt c. Whereas Cyt c directly activates Apaf-1 and caspase 9, Smac interacts with multiple IAPs and relieves their inhibitory effect on both initiator and effector caspases.

2.5.4.2.2 Bcl-2 Family of Proteins

Bcl-2 is a tumour suppressor gene that was first discovered and identified in B-cell lymphoma. Since then many other proteins have been added to the family. The Bcl-2 family can be divided

into two functional protein groups that can either promote apoptosis, or assist the proteins that inhibit apoptosis (Pretorius, 2005).

The Bcl-2 family are membrane proteins located mainly on the outer mitochondrial membrane, endoplasmic reticulum and the nuclear membrane. The proteins of the Bcl-2 family contain conserved domains, such as BH1 to BH4, and most members contain a COOH-terminal serving as a transmembrane anchor sequence (Lithgow *et al.*, 1994; Reed, 2000). Due to these attributes the Bcl-2 family can remain membrane-bound and regulate the threshold at which mitochondria release apoptogenic factors.

The anti-apoptotic family members Bcl-2 and Bcl-X₁ (Borner, 2003) prevent pro-caspase activation and the initiation of apoptosis by inhibiting the initial release of Cyt c from mitochondria (Armstrong *et al.*, 1996). This is done by stabilising the mitochondrial membrane and stopping depolarisation (McCarthy *et al.*, 1997). It also inhibits the binding of Apaf-1 and caspase 9 to form the apoptosome and stop apoptosis (Borner, 2003).

The pro-apoptotic members of the family Bax, Bad, Bik, Bim and Bid (Borner, 2003) interact with the anti-apoptotic members to form heteromers and inhibit their action (Jurgensmeier *et al.*, 1998). Bax also has the ability to form ion channels in membranes and release Cyt c (Minn *et al.*, 1997; Borner, 2003).

2.5.4.2.3 Protein Kinase B (Akt)

Akt, also known as protein kinase B, is a group of phosphatidylinositol-3-kinase-regulated serine/threonine kinases that form part of the Arabidopsis gene (AGC) subfamily (Franke *et al.*, 1995; Burgering & Coffey, 1995). The proteins of the AGC gene are activated by phosphorylation of two residues. These kinases mediate their cellular effects by phosphorylating key regulatory proteins (Coffey *et al.*, 1998).

The mechanism for the activation of Akt (shown in Fig 2-2) has been elucidated as illustrated in Figure 2-2. The phosphatidylinositol 3-kinase (PI3-k) enzyme produces two lipid products, namely phosphatidylinositol-3,4,5-triphosphate (PIP₃) and phosphatidylinositol-3,4-bisphosphate (PI[3,4]P₂). These lipid products are able to bind to the PH domain of Akt (Burgering & Coffey, 1995; Klippel *et al.*, 1997; Coffey *et al.*, 1998), but the binding of the abovementioned lipids to the PH domain does not activate the enzyme. Rather, it causes conformational changes that unmask the active site, leading to the relocation of Akt from the cytosol to the membrane, thereby bringing the enzyme in close proximity to regulatory kinases that phosphorylate and activate Akt (Klippel *et al.*, 1997).

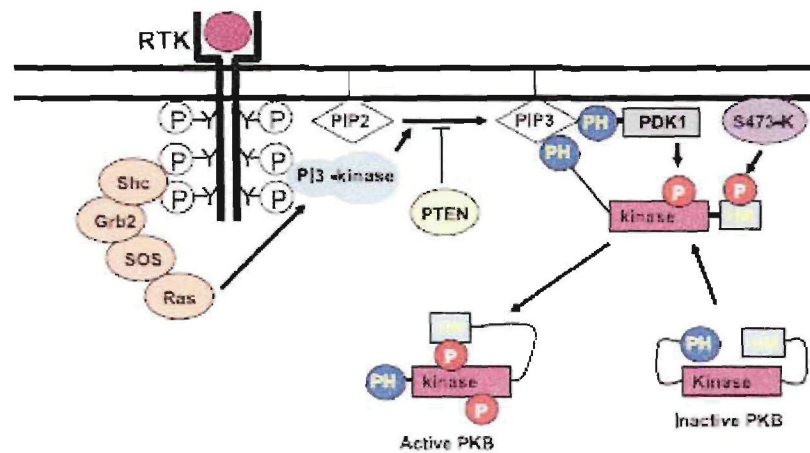


Figure 2-2: Schematic representation of Akt activation (Hanada *et al.*, 2004).

2.5.4.2.3.1 *The role of Protein Kinase B in apoptosis*

Akt plays an important role in many biological pathways (Downward, 1998) such as nitric oxide (NO) synthesis (Fulton *et al.*, 1999), protein synthesis (Shah *et al.*, 2000) and regulation of apoptosis and cellular survival. In Figure 2-3 the role of Akt in the anti-apoptotic pathway is illustrated.

Akt can inhibit the hyperpolarisation of the mitochondria so that Cyt c is not released and apoptosis does not occur (Kennedy *et al.*, 1999). Akt can prevent the disruption of the mitochondrial inner membrane potential by inhibiting conformation changes and redistributing Bax to mitochondrial membranes (Yamaguchi & Wang, 2001). Akt can also break the bond between Bad and anti-apoptotic proteins of the Bcl-2 family, leaving the free anti-apoptotic protein to signal for cell survival (Trencia *et al.*, 2003). The promoter region of Bcl-2 contains a cAMP-response element (CRE) site and the transcription factor CREB (CRE binding protein) has been identified as a positive regulator of Bcl-2 expression. Akt has been shown to activate CREB and increase Bcl-2 anti-apoptotic genes (Du & Montminy, 1998).

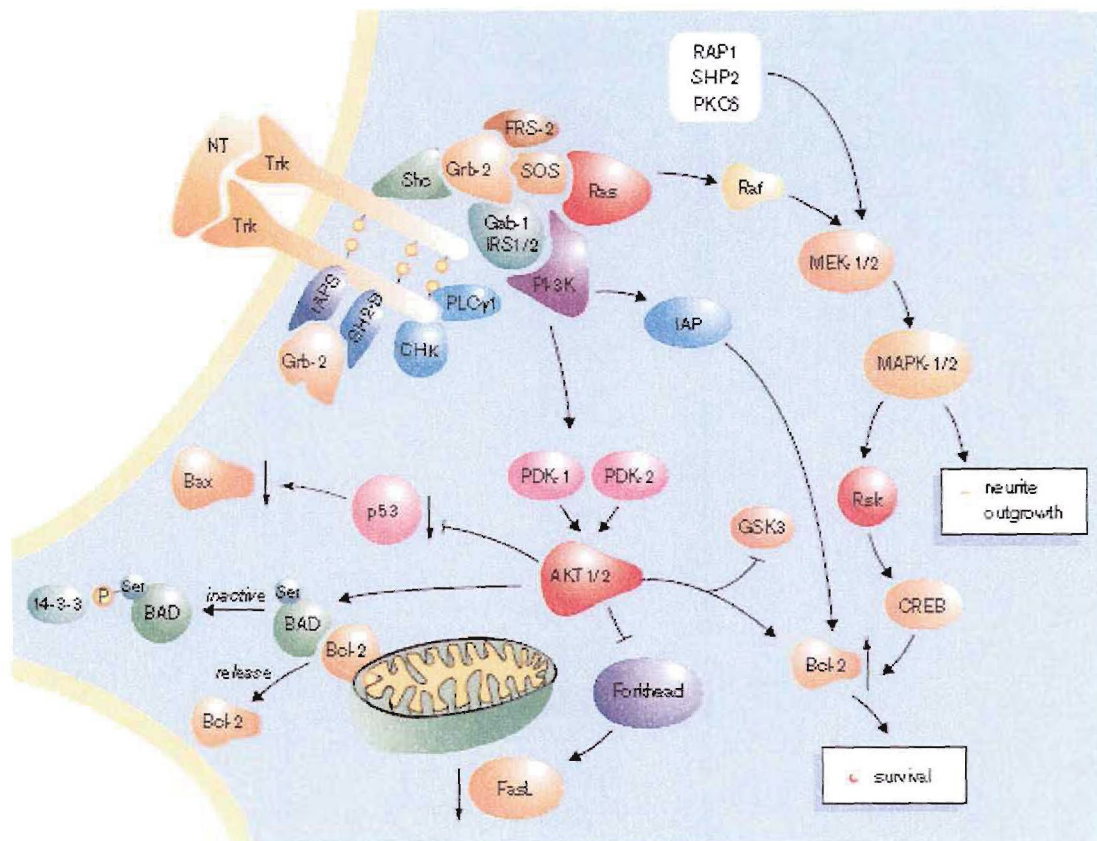


Figure 2-3: The role of Akt in mitochondrial apoptotic pathway (Kaplan & Miller, 2000).

Down stream of the mitochondria Akt can inhibit caspase 9 by phosphorylating the protein, thereby inhibiting the formation of the apoptosome (Cardone *et al.*, 1998). Akt can phosphorylate I kappa which is directly responsible for the regulation of nuclear factor kappa beta ($\text{NF}\kappa\beta$) which is the activation signal for cell survival (Marte & Downward, 1997; Ozes *et al.*, 1999). This may play a role in ozone adaptation and how it signals for cell survival.

2.5.4.2.4 Nuclear Factor Kappa Beta

Nuclear factor kappa beta ($\text{NF}\kappa\beta$) is a dimeric transcription factor consisting of Rel family members (Li & Verma, 2002). $\text{NF}\kappa\beta$ contains a highly conserved Rel-homology domain (RHD) that is responsible for DNA binding, dimerisation, nuclear translocation and interaction with I κ B proteins. The protein family of I κ B binds to $\text{NF}\kappa\beta$ via akryin, repeats and block its nuclear import leaving it in an inactive state (Pomerantz & Baltimore, 2002).

$\text{NF}\kappa\beta$ is activated by the canonical and the non-canonical pathways, depending on whether activation involves I κ B degradation or p100 processing, respectively. The canonical pathway (which is the predominant $\text{NF}\kappa\beta$ signalling pathway, stimulating cells with an agonist, such as tumour necrosis factor α ($\text{TNF } \alpha$) or interleukin-1 β ($\text{IL-1}\beta$)) activates IKK complex that is composed of two catalytic subunits, IKK α and IKK β and regulatory subunit MEMO. These factors induce the phosphorylation of I κ B for polyubiquitination and subsequent degradation by

the proteasome, thus releasing NF κ B (Chen *et al.*, 1995). The non-canonical pathway of NF κ B activation operates mainly in B cells in response to stimulation of a subset of the TNF receptor superfamily. Stimulation of these receptors activates the protein kinase NIK, which in turn activates IKK α . IKK α then phosphorylates p100, leading to the selective degradation of its I κ B-like domain by the proteasome (Xiao *et al.*, 2001; Senftleben *et al.*, 2001).

NF κ B can also be activated by active Akt. Activated Akt up-regulates kinase activity of the IKK complex, leading to NF κ B activation, and this signals for cell survival (Ozes *et al.*, 1999; Romashkova & Makarov, 1999).

2.5.4.2.5 cAMP Response Element Binding Protein (CREB)

cAMP response element binding protein (CREB) is known to be activated by various extracellular stimuli and play important roles in cell proliferation, differentiation, adaptation and survival (Mayr & Montminy, 2001).

CREB requires phosphorylation of the serine residue at 133 to be activated (Gonzalez & Montminy, 1989). Upon phosphorylation at serine 133, the CREB binding protein (CBP) is recruited to CREB. CBP is a transcriptional co-activator with histone acetyl transferring activity that activates gene transcription (Lu *et al.*, 2003). CREB is activated by a wide range of extracellular stimuli through distinct signalling pathways (Johannessen *et al.*, 2004). The different pathways can be seen in Figure 2-4, including the Akt activation pathway.

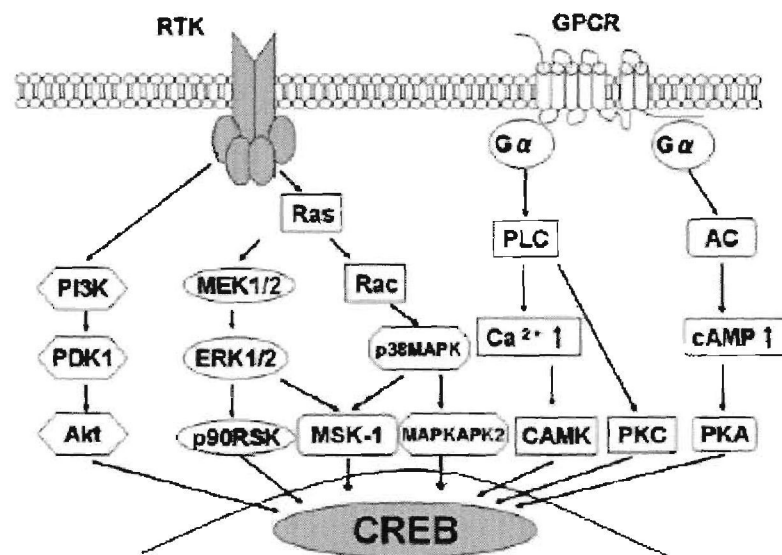


Figure 2-4: Illustrates signalling pathways that activates CREB. Several protein kinase pathways involved in CREB activation are shown (Ichiki, 2006).

2.5.4.2.6 Brain Derived Neurotrophic Factor (BDNF)

Brain-derived neurotrophic factor (BDNF) is a neurotrophic factor, which play a critical role in cell and especially neuronal survival and phenotypic differentiation for cells (Connor & Dragunow, 1998).

Two classes of receptors mediate the effects of neurotrophins, namely the Trk family of tyrosine kinase receptors and the p75 receptors (lacking intrinsic kinase activity). Recent studies have revealed that BDNF can prevent apoptosis. BDNF binds to high affinity receptors or influences apoptotic cell death by binding to low affinity receptors p57. These changes in BDNF levels, as well as changes in distribution and the degree of binding to their receptors in tissue, can trigger a cascade of different effects, which may result in cell survival (Dechant & Barde, 1997; Yoon *et al.*, 1998). BDNF can also be activated by NF κ B, Akt or CREB (Ozes *et al.*, 1999). BDNF was not considered for this study, because a HeLa cell line was used. HeLa cells are epithelial cells and not an appropriate cell line to investigate BDNF.

2.6 RECENT EVIDENCE OF ADAPTION TO OZONE IN HUMAN EPITHELIAL HeLa CELLS

In a recent study conducted in our laboratory (Pretorius, 2005) HeLa cells were exposed to ozone in different exposure regimens, namely repeated low (RL) dose (5 min every 4 h for 16 h), single high (SH) dose (25 min) and a repeated low dose + single high (RL+SH) dose.. After exposure of the cells to ozone, they were incubated for a further 0, 4, 8 and 12 h before analysis of cell viability (following the time course after exposure). Cell membrane integrity (trypan blue assay), mitochondrial activity (MTT test) and DNA integrity (DNA comet assay) were evaluated as markers of cell viability.

When HeLa cells received either the RL or SH exposure regimens and the cells were incubated for another 8 hours, membrane integrity and DNA damage occurred, as can be seen in Figure 2-5. However, when the cells received the RL+SH exposure regimen, adaptation occurred, such that the ozone-induced damage was reversed. In fact, it was found that membrane activity was improved above that of the control. Data measuring mitochondrial activity and DNA integrity supported this finding (Brink *et al.*, 2008a).

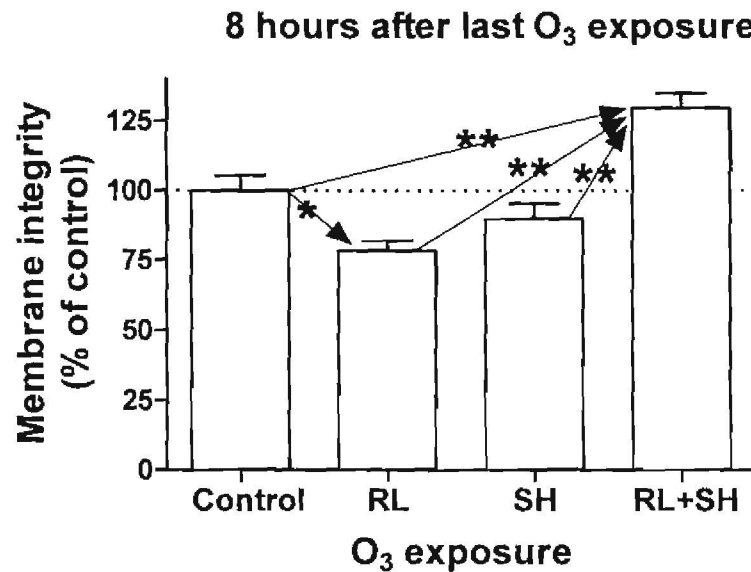


Figure 2-5: Membrane integrity as determined with the trypan blue assay on HeLa cells receiving RL, SH an RL+SH ozone treatment. The data were analyzed statistically by performing a one-way ANOVA and then implementing the Tukey-Kramer post-test, with * indicating $P < 0.05$ and ** indicating $P < 0.01$ (Brink *et al.*, 2008a).

In addition, when anti-apoptotic pathways were blocked (with ME10092 as Akt inhibitor) or pro-apoptotic pathways were blocked (with Z-DQMD-FMK as caspase 3 inhibitor), the adaptation was also inhibited. The data suggested that both pro- and anti-apoptotic pathways play a role in ozone-mediated adaptation.

2.7 SUMMARY AND CONCLUSION

Ozone is a strong oxidising agent that can react with biological systems. More and more people are exposed to this reactive gas whether it is through pollution or alternative medicine. When ozone comes into contact with biological systems it reacts with lipids and proteins to form a seconded messenger ROS. ROS and ozone can place biological systems under dose dependent oxidative stress which can lead to cell damage or ozone adaptation.

The exact mechanism for ozone adaptation is not known. It is known that ROS causes DNA damage and this activates the mitochondrial apoptotic pathway, which is regulated by pro- and anti-apoptotic proteins. From data that were obtained from recent studies it can be hypothesised that the pro- and anti-apoptotic proteins governing the mitochondrial pathway might play an important role in understanding ozone adaptation.

Article for submission	Chapter 3
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Chapter 3 contains a proposed article to Free Radical Biology & Medicine (not yet submitted). The article only contains gene expression data collected after HeLa cells were exposed to ozone in different regimens. Article format was obtained from the guide to authors that can be seen in Appendix C.

The effects of ozone exposure on pro- and anti-apoptotic gene expression in cultured human cell lines

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Support: The project was supported financially by a grant from the South African National Research Foundation.

Running head: Ozone and cellular resilience

Abstract

While the biological effects of the gaseous oxidant ozone (O₃) are associated with toxicity, it has also been used for therapeutic purposes in alternative medicine. In biological systems ozone elicits dose-dependent oxidative stress, which may induce adaptation. We recently demonstrated that repetitive exposure of cultured cells to ozone may induce adaptation, inhibited by the Akt inhibitor (-)-deguelin. In the current study we measured the modulating effects of single and repetitive exposures of human epithelial HeLa cells to ozone on the expression of genes encoding for anti-apoptotic (Akt, Bcl2, CREB, NFκβ and BDNF) and pro-apoptotic (Bax, caspase 3 and 8) proteins. HeLa cells were exposed to control or ozone-saturated glucose-free Krebs-Henseleit (gf-KH) solution for 4 x 5 minutes (spaced 4 hours), followed by 16 hours incubation in culture medium and then a 25-minute exposure to control or ozone-saturated gf-KH. Thereafter the cells were lysed immediately (0 h) or after 8 hours (8 h) and gene expression determined with real-time PCR. When exposed to ozone for 4 x 5 minutes alone, genes for Bcl-2, CREB, BDNF and caspase 3 were down-regulated at 0 h, but these returned to pre-treatment values at 8 h. However, when treated with ozone 4 x 5 minutes + 25 minutes, Akt, and CREB were up-regulated at 8 h. In conclusion and in line with previous results, the current data suggest that anti-apoptotic mechanisms may be involved in the adaptive effects following repetitive exposure to ozone. This adaptation may be preceded by the down-regulation of pro- and anti-apoptotic enzymes.

Key-words

Ozone, adaptation, apoptosis, AKT, CREB, HeLa cells.

Introduction

Ozone (O₃), a strong oxidising gas, is rapidly converted to reactive oxygen species (ROS) upon contact with cell proteins and lipids [1; 2]. While ozone from industrialized air pollution is associated with bio-toxicity, it has also been proposed to have therapeutic applications in alternative medicine [3], dentistry [4] and topical treatment of skin infections [5]. In alternative medicine claims (some unsubstantiated) have been made for the treatment of HIV-AIDS [3], chronic ulcers [6], retinitis pigmentosa and cardiac ischemia [7]. While sufficient evidence for therapeutic efficacy and safety are available for some applications (such as in dentistry), it is limited or absent for most.

Ozone has been associated with cellular damage [8], including dose-dependent oxidative stress to cells exposed to ozone [9]. Yet, biological adaptation to these stressor effects may also occur [10], as has been observed in cultured cells [8], rodents [11], plants [12] and humans [13]. The mechanism of adaptation to ozone has not been fully elucidated, while recent studies in cultured cells indicate that the mitochondrial apoptotic pathway may play an important role in the mechanism of ozone-mediated adaptation [8].

In biological systems ozone generates toxic ROS species, such as aldehydes, hydrogen peroxide, organic radicals and hydroxyl radicals through lipid peroxidation and protein modification [14]. This formation of ROS leads to oxidative stress and DNA damage, which signal for apoptosis via the mitochondrial pathway [15]. This pathway is regulated by pro- and anti-apoptotic genes [16]. The pro-apoptotic caspase family is divided into the initiator caspases (e.g. caspase 2, 8-10 and 12) and the effector caspases (e.g. caspases 3, 6 and 7), being key role players in apoptosis [17]. In particular, caspase 3 has been identified in a recent study as the potential initiator of adaptation [8]. In the Bcl-2 family Bax also plays an important role in the destabilisation of the mitochondrial membrane and the release of cytochrome C [19; 18], promoting apoptosis.

However, other family members such as Bcl-2 and Bcl-X₁ stabilize the mitochondrial membrane and prevent the release of cytochrome C, thereby preventing apoptosis [20].

In a recent study we showed that Akt (protein kinase B) may play an important role in ozone-mediated adaptation [8]. When human epithelial (HeLa) cells were first exposed repetitively to low doses of ozone, DNA and membrane damage was prevented (adaptation) during a consecutive exposure to a high dose of ozone. The addition of an Akt inhibitor reversed this adaptation. In fact, Akt plays an important role in many biological pathways, such as nitric oxide synthesis, protein synthesis and the regulation of apoptosis and cell survival [21; 22; 23]. In particular, Akt is known to activate nuclear factor kappa beta (NFκβ) and cAMP response element binding protein (CREB), which are both anti-apoptotic proteins and strong signals for cell survival [24; 25].

The objective of the current study was to investigate the role of the pro- and anti-apoptotic pathways in ozone-mediated cellular adaptation to oxidative stress. This was done by investigating the gene expression (mRNA) of the pro-apoptotic proteins Bcl2-associated X protein (Bax), caspase 3 and caspase 8, and the anti-apoptotic proteins protein kinase B (Akt), cAMP response element binding protein (CREB), B-cell lymphoma 2 (Bcl2), and neurotrophic factor kappa beta (NFκβ) in human epithelial (HeLa) cells.

Materials and methods

The reagents NaCl, KCl, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, NaHCO_3 and NaH_2PO_4 were from Sigma (Sigma-Aldrich Johannesburg SA). Cell culture media, serum, antimicrobials and trypsin/versine (0.25% 1:250 trypsin + 0.38% EDTA) were from Gibco (Invitrogen, Scotland, UK). FastLane Cell cDNA kit, QuantiTect SYBRGreen and QuantiTect Primer Assays, specific, optimised and validated for each gene to be investigated, were from QISGEN USA. 96-well PCR plates and internal well factors were from Bio-Rad Johannesburg SA. DNase and RNase free tips and tubes were from Whitehead Scientific. An ozone generator (Sterizone P-HP 250 – PCT/ZA00/00031) was manufactured and patented by the Department of Physics, North-West University, Potchefstroom, South Africa. All water used was of double distilled, RNase-free quality.

Cell cultures

A adherent human servical epithelial cell line (HeLa, from American Type Culture Collection (ATCC), cat. no. CCL-2) was employed as before [8]. HeLa cells have been well characterized [26] and oxidative stress has been extensively studied in this cell line [27]. The cells were maintained and grown in monolayers to approximately 90% confluence in Dulbecco's modified Eagle's medium (DMEM), 10% foetal bovine serum (FBS), 100 units/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin and 0.25 $\mu\text{g}/\text{ml}$ fungizone in 150 cm^2 culture flasks at 37° C in 5% CO_2 and humidified atmosphere. Cells were detached from the flask bottom surfaces by means of trypsinization (trypsin/EDTA). For experiments with ozone exposure, cells were seeded in 24-well plates at a density of 2×10^5 cells/well.

Ozone generation, setup and choice of medium

The ozone generation, setup and exposure medium were implemented as before [8]. Briefly, the generator was connected to ultra-pure oxygen for ozone generation. The generated ozone was

constantly bubbled through glucose-free Krebs-Henseleit (gf-KH) solution (119 mM NaCl; 4.7 mM KCl, 1.9 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$; 0.54 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$; 24.0 mM NaHCO_3 ; 1.0 mM NaH_2PO_4) at 37° C in a fume hood for at least 30 min (time to reach O_3 saturation) [8]. It should be noted, however, that ozone may form secondary products in an aqueous medium, such as hydrogen peroxide and hydroxyl radicals [28], which may contribute to the oxidative stress observed in cells.

Ozone exposure

Cells were exposed to ozone-saturated or control (no ozone) glucose-free Krebs-Henseleit 4 x 5 min, spaced 4 h apart (designated repetitive low dose, RL), then left over night (16 h), where after the cells were exposed to ozone-saturated or control gf-KH for 25 min (gf-KH replaced every 5 min, designated single high dose, SH). Cells were the either immediately used for assays or left for 8 h in culture medium before the assays. In this study cells were exposed in 24 well plates and received the RL or SH exposures alone, or the combination of the two exposures (RL+SH).

mRNA extraction and cDNA synthesis

mRNA extraction and cDNA synthesis were performed with the FastLane Cell cDNA kit. All reagents were supplied in the kit and the manufacturer's instructions and conditions were followed. Cells were washed with 500 μl of washing buffer, followed by 200 μl of lysating buffer and left for 5 min at room temperature. Thereafter, 4 μl of lysate was transferred to a micro centrifuge tube and 2 μl of gDNA wipeout buffer and 8 μl of PCR quality water was added. The mRNA concentration was not determined, as indicated unnecessary by manufacturers of the validated kit and since mRNA was extracted from the same number of homogenously seeded cells per well. Thereafter the tubes were placed in a heating block at 42 °C for 5 min and then placed directly on ice. 1 μl Quantiscript Reverse Transcriptase (RT), 4 μl

Quantiscript RT Buffer 5x and 1 µl RT Primer Mix were added to the tubes and placed in the heating block at 42 °C for another 30 min to allow synthesis of cDNA. Tubes were then placed at 95 °C for 5 min to stop the reaction. One additional control tube to exclude genomic DNA contamination went through all the steps, but without reverse transcriptase. cDNA was then analysed quantitatively by real-time reverse transcriptase PCR (RT²-PCR).

PCR and primer efficiency

Primer efficiency was calculated to exclude the formation of primer-dimers. Briefly, this was according to principles of standard practice, by taking 4 µl of cDNA from all experimental and control samples. From this cDNA mix five serial dilutions were made for each gene analysed and 1 µl of each dilution added to 12.5 µl of QuantiTect SYBR Green master mix, 2.5 µl of QuantiTect Primer Assay (specific for each gene) and 9 µl of PCR quality water in a 96-well plate. The samples were then analysed by a Bio-Rad iCycler, with RT²-PCR conditions as supplied by QIAGEN. Efficiency per gene was calculated from the data with the following equation:

$$E\% = (10^{(-1/s)} - 1) \times 100$$

where E% is the efficiency (in percentage) and it is the slope of the linear regression of the Ct values (of the respective dilutions) against the log of the dilution factor.

RT²-PCR

After cDNA synthesis, 1 µl of each sample was placed in a 96-well PCR plate with 12.5 µl of QuantiTect SYBR Green master mix, 2.5 µl of QuantiTect Primer Assay (specific for each gene) and 9 µl of PCR quality water and analysed by a Bio-Rad iCycler, with RT²-PCR conditions as supplied by QIAGEN. In addition, a reverse transcriptase blank and PCR blanks (five across the plate) were included on each 96-well plate. Relative RT²-PCR quantification was used to

determine the ratio of gene expression relative to the reference (housekeeping) gene ubiquitin C (UBC). The relative gene expression was calculated from the Ct results with the following formula [29].

$$Ratio = \frac{(E_{sample})^{Ct(control-sample)}}{(E_{REF})^{Ct(control-sample)}}$$

where E_{sample} is the efficiency of the gene that is being investigated and E_{Ref} is the efficiencies of the reference gene or housekeeping gene UBC.

Statistical analyses

Data from assays were obtained as triplicate observations from at least five separate and independent experiments. Graphs were constructed utilizing the computer software Graph Pad Prism® (Version 5.01 for Windows®, GraphPad Software, San Diego, CA, U.S.A., www.graphpad.com). Graph Pad Prism® was also utilized for all statistical analyses of data. For multiple comparisons the one-way ANOVA comparison was performed followed by either the Dunnett's post-test (for comparing groups to the control) or the Tukey-Kramer post-test (for comparing all groups to one another). For all reported statistical probability values, $p < 0.05$ was regarded as statistically significant.

Results

Primer efficiency

As can be seen from Fig.1 the efficiency of primers were within the norm for efficient primers [29], being close to 100% (varying between 97.7% and 112.6%) and suggesting the absence of double folding.

Anti-apoptotic gene expression

From Fig. 2A, C, E and G it can be seen that at time point 0 h the gene expression was significantly reduced only with the repetitive low dose (RL) ozone for Bcl2 relative to control (36.21 ± 7.28 versus 99.99 ± 14.4 , $p < 0.05$, $n = 12$) and CREB relative to control (49.56 ± 10.46 versus 99.99 ± 12.88 , $p < 0.05$, $n = 12$). The gene for Akt also showed a tendency for reduced expression with RL, but did not reach statistical significance, while gene expression was not altered for the gene for NF κ B.

However, it can be seen in Fig. 2B, D, F and G that after 8 h the gene expression was significantly up-regulated only with repetitive low dose plus single high dose (RL+SH) ozone for Akt versus control (257 ± 47.2 versus 100 ± 20.4 , $p < 0.05$, $n = 12$) and CREB versus control (161.3 ± 19.73 versus 100.00 ± 12.88 , $p < 0.05$, $n = 15$), but not for Bcl2 or NF κ B. No significant changes in gene expression at time point 8 h were observed with either repetitive low dose (RL) or single high dose (SH) ozone alone.

Pro-apoptotic gene expression

In Figure 3A, C and E it can be seen that at time point 0 h the gene expression was significantly reduced only with the repetitive low dose (RL) ozone for caspase 3 (Casp3) relative to control (21.92 ± 6.59 versus 99.99 ± 12.50 , $p < 0.05$, $n = 12$), and a trend for decreased expression for Bax, but not for caspase 8.

However, it can be seen in Fig. 3B, D and F that after 8 h the gene expression was significantly up-regulated for caspase 3 (Casp3) versus control with the single high dose (SH) ozone (440.2 ± 104.2 versus 100 ± 18.73 , $p < 0.05$, $n = 12$) and with the repetitive high dose plus single high dose (RL+SH) ozone (394.5 ± 62.95 versus 100 ± 18.73 , $p < 0.05$, $n = 12$). No statistically significant changes in gene expression were observed for Bax or caspase 8 (Casp8).

Discussion and conclusion

From the data in Fig. 1 sufficient efficiency within the norms of the primers, as provided by QIAGEN, was demonstrated.

The genes encoding for the anti-apoptotic enzymes Akt and CREB, and the pro-apoptotic enzyme caspase 3, were down-regulated at time point 0 h (16 hours after the last low dose ozone) only when the repetitive low dose (RL) ozone treatment regimen was given (Figs. 2 & 3). The significance of these down-regulations is not known. One could speculate that the down-regulation of the anti-apoptotic enzymes is in line with previous findings of initial cellular damage by ozone, while these down-regulations were reversed 8 hours later (time point 8 h).

Importantly, it was shown that the anti-apoptotic enzymes Akt and CREB were up-regulated with repetitive low doses, followed by a single high dose ozone (RL+SH), at time point 8 h (Fig. 2). This suggests potential cytoprotective properties (adaptation) by the RL+SH treatment regimen and supports our previous findings (using the same ozone treatment regimen with HeLa cells) of up-regulated membrane integrity and reversal of DNA damage [8]. In particular, we also reported [8] that these adaptive effects are reversed in the presence of an Akt inhibitor, and therefore suggested Akt, which is strongly linked with anti-apoptotic signalling [30], to play a role in these cytoprotective effects. Another study also showed Akt up-regulation in alveolar macrophages upon ozone exposure [31].

The gene encoding for CREB, an anti-apoptotic factor known to be activated by Akt [32], was also up-regulated by the RL+SH treatment, concurrent with Akt gene up-regulation (Fig. 2). CREB was identified as another possible role player in ozone adaptation and cell survival. While Akt is known to activate NF κ B [24; 25], it was not up-regulated in the current study.

Caspase 3 was up-regulated when receiving the SH treatment. This indicates cell damage and apoptosis [17]. This is in line with a study [8] that showed that cells receiving only the SH

treatment showed DNA and membrane damage. DNA damage is a strong signal for the activation of the mitochondrial apoptotic pathway [15]. Caspase 3 was also up-regulated when receiving the RL+SH treatment. In the recent study [8] it was hypothesized that caspase 3 does not indicate apoptosis but is an upstream signal for cell survival. This was identified when (-)-deguelin (caspase 3 blocker) was added to cells that received the RL+SH treatment and no reversal of damage in contrast to the cells that did not receive the blocker. This study strengthened the hypothesis that caspase 3 might play a role in signalling for ozone adaptation and cell survival.

In conclusion, this study has supported the hypothesis that the pro- and anti-apoptotic genes play a role in ozone-mediated adaptation to oxidative stress. In particular, we showed here that after repetitive low dose exposure to ozone, followed by a single high dose, result in up-regulation of the genes encoding for Akt, CREB and caspase 3 over a period of 8 hours. While the up-regulation of the anti-apoptotic genes may explain adaptation, it is not clear what the role or implication is of the up-regulated pro-apoptotic gene caspase 3. If one compares the current data with that of previously published work, reporting improved membrane and DNA integrity under the same experimental conditions, it can be deduced that the anti-apoptotic effects supersedes any pro-apoptotic effects. The current findings warrant further research into the potential interaction between the pro- and anti-apoptotic pathways in adaptation. Furthermore, the *in vivo* relevance of the current findings could be investigated in animals exposed to ozone, while the relevance of the current findings on eventual protein expression and activity also needs to be unravelled.

Acknowledgments

The project was supported financially by a grant from the South African National Research Foundation. The authors wish to express their gratitude towards Mrs. Maureen Steyn and Mrs. Sharleen Nieuwoudt for general laboratory assistance and the handling of logistic matters related to the project.

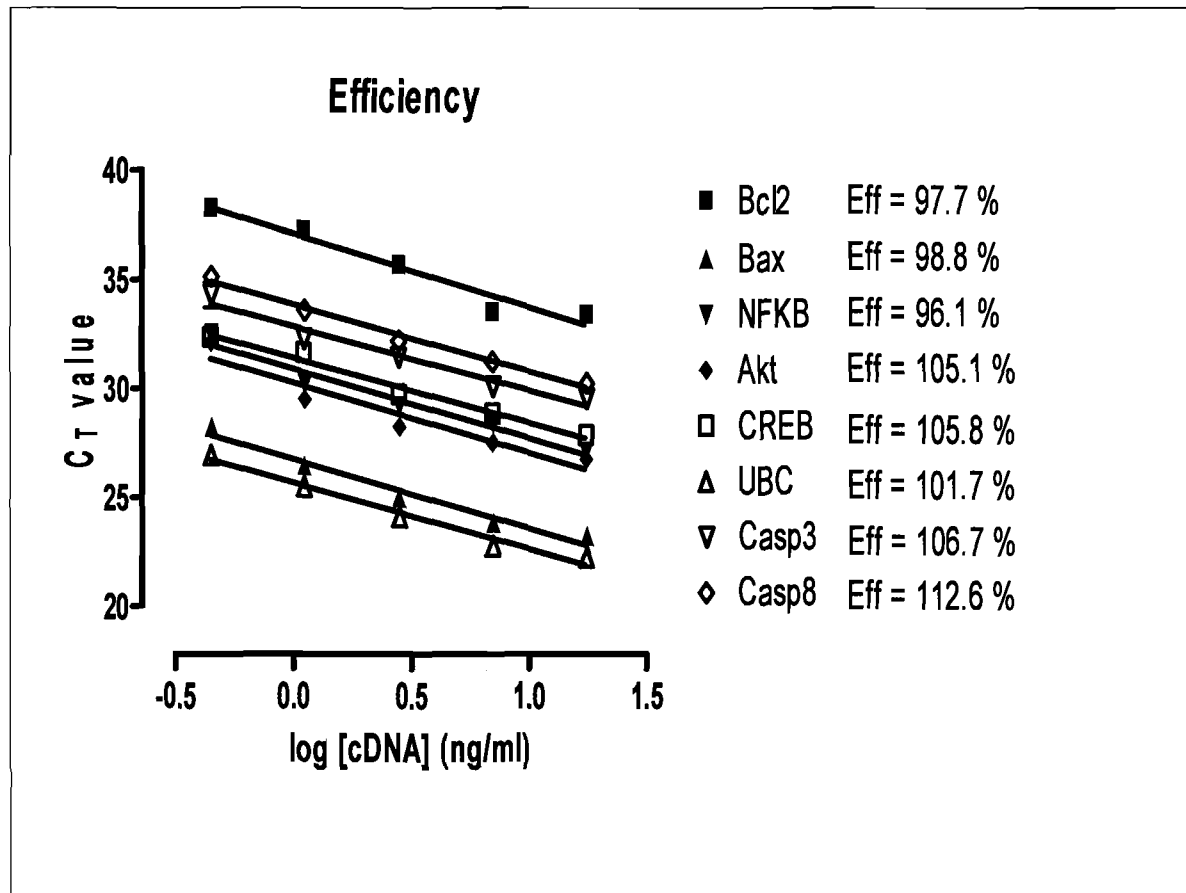
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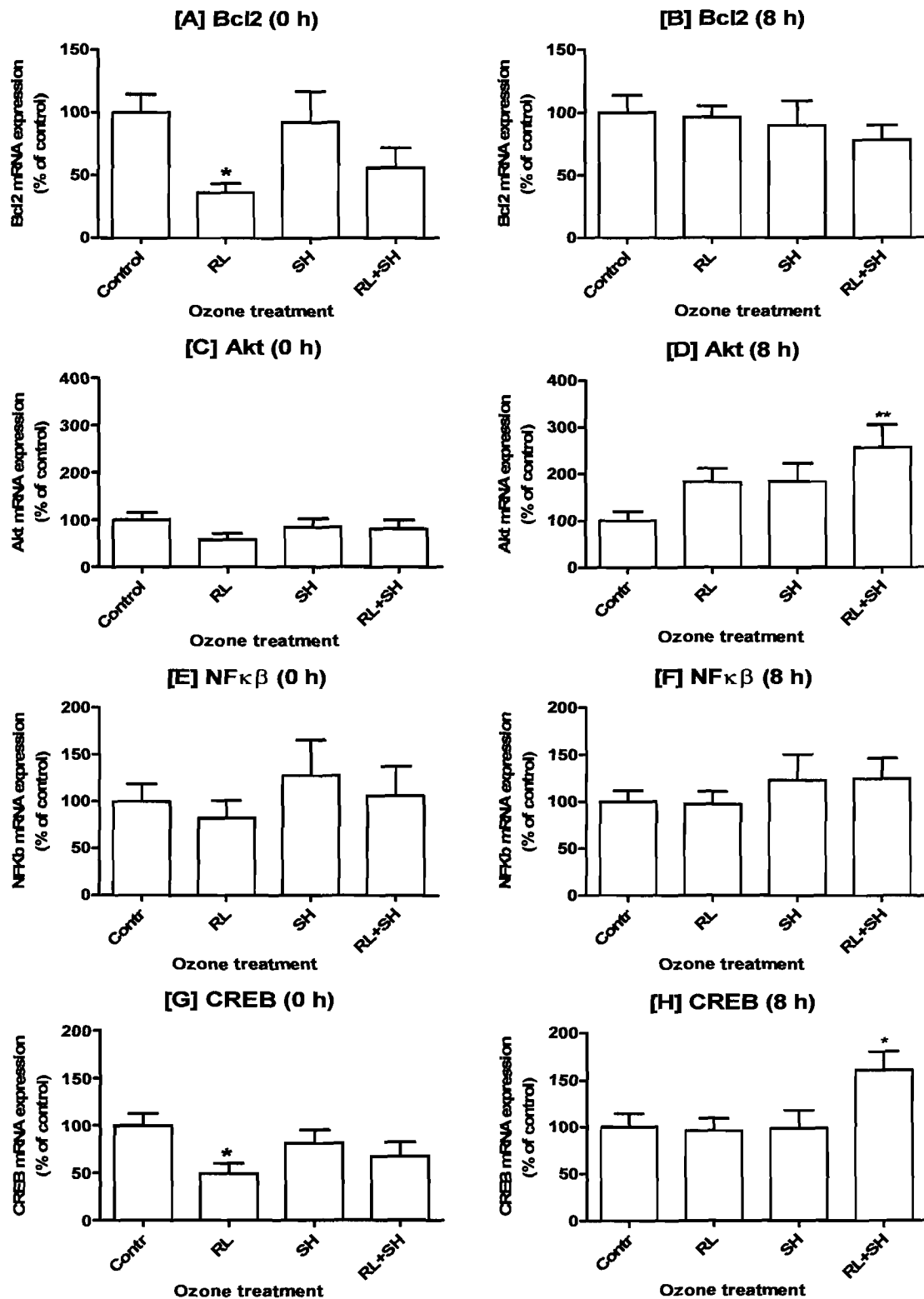
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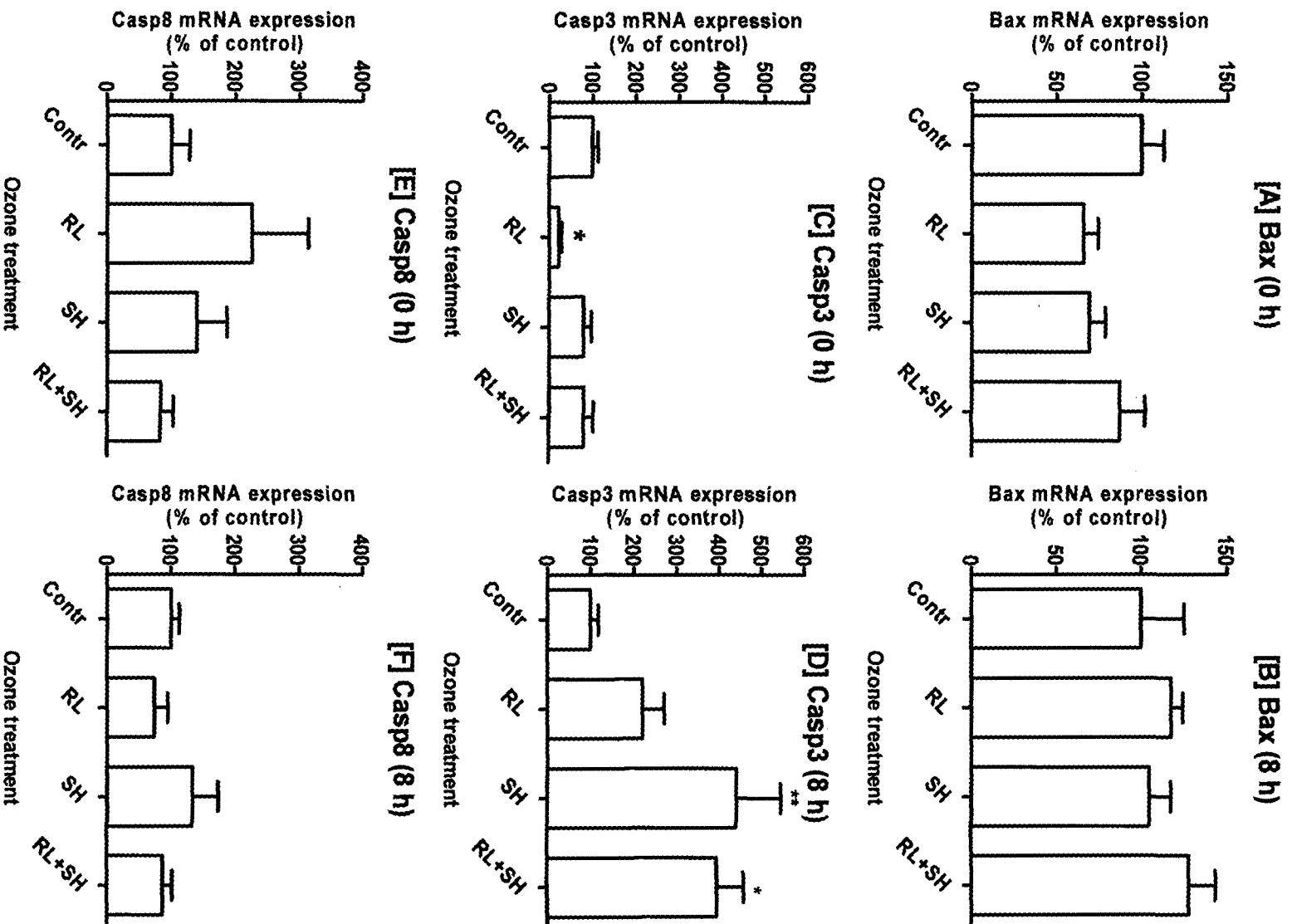
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Anti-apoptotic genes



Pro-apoptotic genes



Legends

Figure 1 shows the primer efficiencies for Akt, Bcl-2, caspase 3 and 8, CREB and NF κ B. The graph is the log of the dilution factor (x axis) against the Ct value of each primer at the dilution. Efficiency is expressed in percentage %.

Figure 2 shows the gene expression of the anti-apoptotic genes Bcl-2, Akt, NF κ B and CREB after receiving the different ozone treatments (RL, SH and RL+SH) and control at 0 and 8 h, as determined by RT²-PCR. A one-way ANOVA followed by the Dunnett's multiple comparison test was performed for statistical analysis. Data are averages of four independent experiments with triplicate observations expressed as the mean $\pm$ SEM, with * $p < 0.05$; ** $p < 0.01$.

Figure 3 shows the gene expression of the pro-apoptotic genes Bax, Casp3 and Casp8 after receiving the different ozone treatments (RL, SH and RL+SH) and control at 0 and 8 h, as determined by RT²-PCR. A one-way ANOVA followed by the Dunnett's multiple comparison test was performed for statistical analysis. Data are averages of four independent experiments with triplicate observations expressed as the mean $\pm$ SEM, with * $p < 0.05$; ** $p < 0.01$.

Summary & Conclusion

Chapter

4

This section gives a summary and final conclusion of the whole study, including results from Chapter 3 (article) plus additional results from Appendix B (not included in the article). It therefore endeavours to unify the outcomes of the whole study and draws overall conclusions from all data, points out relevance and specific scientific advances made by the study and finally points out remaining scientific questions and proposes appropriate prospective studies.

4.1 Summary

In the first pilot section of this study project (see Appendix B, § 1.1) it was aimed to replicate and verify data from a previous study (Brink *et al.*, 2008a) regarding the half-life of ozone in physiological solution (glucose-free Krebs Henseleit (gf-KH)) at 37 °C, as well as the pH of gf-KH after saturation with ozone. Thereafter, in the primary study, human epithelial HeLa cells were exposed to different treatment regimens with ozone-saturated gf-KH, as also implemented before (Brink *et al.*, 2008a), denoted RL, SH and RL+SH treatments as defined in Chapter 3, and then to investigate the effects of these treatments on the pro- and anti-apoptotic gene and protein expression.

Pilot study

It was shown from additional data of the current study (see Appendix B § 1.1) that glucose-free Krebs Henseleit solution at 37°C becomes saturated with ozone after 30 minutes of bubbling at a concentration of **26.7 mg/l**. Taking into account the estimated half-life of **1.64 min**, this implies that the concentration of ozone would vary between roughly 27 mg/l to >2.2 mg/l within each exposure cycle of 5 min, as employed before (Pretorius, 2005) and in the current study. Previous data, however, suggested a saturation concentration of 3.38 mg/l and a half-life of 5.6 min (Pretorius, 2005) and it cannot be explained why the two sets of data yield different results. The data collected in the current study, however, correlates with the literature in that ozone has been reported to be unstable in solution and to have a short half-life in aqueous solution (Chapter 2, § 2.2.2) (Lotriet *et al.*, 2007).

As found before (Pretorius, 2005), the current study also indicated that the **pH of gf-KH was increased during a period of 90 minutes** when bubbled with ozone. The largest increase in

pH is seen between 0 and 30 min (from 7.4 to 8.3), and then a small increase (from 8.3 to 8.6) until 90 min. gf-KH was therefore bubbled with ozone for at least 30 min before use, and up to a maximum of 90 minutes.

Main study

The data collected from RT²-PCR regarding gene expression before and after HeLa cells were exposed to ozone (see Chapter 3) revealed ozone-induced changes in gene expression of specific pro- and anti-apoptotic genes. The results of the relative gene expression are summarised in Table 4-1.

Table 4-1 Gene expression results of the study (Chapter 4), for all genes and treatments are summarised in the table, where ▲ = significant gene up-regulation, ▼ = significant gene down-regulation and – = no significant change.

Gene	RL		SH		RL+SH	
	0 h	8 h	0 h	8 h	0 h	8 h
Akt	–	–	–	–	–	▲
Bcl-2	▼	–	–	–	–	–
Bax	–	–	–	–	–	–
CREB	▼	–	–	–	–	▲
NF-κβ	–	–	–	–	–	–
Caspase 3	▼	–	–	▲	–	▲
Caspase 8	–	–	–	–	–	–

The lack of modulation of protein expression was not expected, especially in the light of marked changes in corresponding gene expression. The significance of this finding is discussed below.

4.2 Conclusion

The current study supports the findings that gf-KH is an appropriate physiological solution for the administration of ozone in solution to cultured HeLa cells. The different treatment regimens and the corresponding measurements of appropriate biomolecular markers yielded valuable cues for potential mechanisms of ozone-induced adaptation. This method of ozone administration is practical, affordable and renders useful results.

On the downside, however, there were small inconsistencies in the estimated half-life of ozone in gf-KH as determined in the current study and the study of Pretorius (Pretorius, 2005). In addition, the current and previous studies have shown that the bubbling of gh-KH with ozone causes a marked rise in pH after 30 min (from 7.4 to 8.4), suggesting that an alternative physiological solution may be sought. For this study the gf-KH was sufficient to obtain good results. The article published by Brink *et al* (Brink *et al.*, 2008b) showed that the pH differences did not significantly influence their results.

The down-regulation of the pro- and anti-apoptotic genes CREB, Bcl-2 and caspase 3, and the tendency of all the other genes to be down-regulated, after 0 h when receiving the RL dose alone, is not fully understood. It has been found in a other study that the RL treatment alone induces membrane and DNA damage of HeLa cells (Brink *et al.*, 2008a). The down-regulation of the above mentioned anti-apoptotic genes may explain initial reduced protective mechanisms and may explain concurrent membrane and DNA damage. However, one should be careful to assume a linear (in-time) relationship between gene expression and eventual translation into protein expression and biological effect.

Importantly, it was shown that the anti-apoptotic enzymes Akt was up-regulated with repetitive low doses, followed by a single high dose of ozone (RL+SH) at 8 h. This suggests potential cytoprotective properties (adaptation) by the RL+SH treatment regimen and supports our previous findings (using the same ozone treatment regimen with HeLa cells) of up-regulated membrane integrity and reversal of DNA damage (Brink *et al.*, 2008a). In particular, we also reported that these adaptive effects are reversed in the presence of the Akt inhibitor Akt, and therefore suggested Akt, which is strongly linked with anti-apoptotic signalling, to play a role in these cytoprotective effects (Kennedy *et al.*, 1999). Another study also showed Akt up-regulation in alveolar macrophages upon ozone exposure (Laskin *et al.*, 2002).

The current study also found that CREB gene expression was up-regulated by the repeated low plus single high regimen (RL+SH). It is known that Akt is an strong activator of CREB, a strong anti-apoptotic signalling agent (Johannessen *et al.*, 2004). It is also known that Akt is a strong activator of NF κ B (Marte & Downward, 1997; Ozes *et al.*, 1999), but this study found no up-regulation or activation at all time points or treatment regimens.

Caspase 3 was up-regulated by the SH treatment, indicating cellular damage and apoptosis (Earnshaw *et al.*, 1999). This is in line with a study (Brink *et al.*, 2008a) that showed that cells receiving only the SH treatment showed DNA and membrane damage. DNA damage is a strong signal for the activation of the mitochondrial apoptotic pathway (Higuchi & Matsukawa, 1997). Caspase 3 was also up-regulated when receiving the RL+SH treatment. In the a recent study (Brink *et al.*, 2008a) it was postulated that caspase 3 gene up-regulation may not necessarily constitute apoptosis, but may be an upstream signal to stimulate processes that, in response, activate cell survival (anti-apoptotic) mechanisms. This was identified when (-)-deguelin (caspase 3 blocker) prevented the adaptation in HeLa cells seen after the RL+SH treatment. The current study is therefore congruent with the previous suggestion that caspase 3 up-regulation may play a role in promoting adaptation and cell survival.

No significant changes were found in the protein expression, when correlated with the gene expression data. These results were disappointing, but may be explained. Firstly, gene

expression is not always translated directly into protein expression. Also, we observed the most prominent changes in gene expression only after 8 hours (same time point as measuring protein expression here), so that translation to protein expression may have occurred later. It could be that significant changes will be observed a few hours later. Lastly, while we were able to detect protein expression, the current approach is not designed to measure protein expression in real time and also may not be sufficiently refined and sensitive to measure protein expression under these conditions.

In conclusion, the current study supports the hypothesis that the pro- and anti-apoptotic genes play a role in ozone-mediated adaptation to oxidative stress. In particular, we showed here that after repetitive low dose exposure to ozone, followed by a single high dose, resulted in up-regulation of the genes encoding for Akt, CREB and caspase 3 over a period of 8 hours. While the up-regulation of the anti-apoptotic genes may explain adaptation, it is not clear what the role or implication is of the up-regulated pro-apoptotic gene caspase 3. If one compares the current data with that of previously published work (Brink *et al.*, 2008a) reporting improved membrane and DNA integrity under the same experimental conditions, it can be deduced that the anti-apoptotic effects supersedes any pro-apoptotic effects. Furthermore, limited data on the subcellular, biomolecular effects of ozone on cell systems and its association to adaptation are available, so that the current study is one of the first to investigate this phenomenon at this biomolecular level.

4.3 Prospective studies & recommendations

The current study provides supporting evidence for previous suggestions that pro- and anti-apoptotic pathways are involved in the ozone-mediated adaptation seen in HeLa cells. Valuable gene expression data were obtained from the current study, so that prospective studies will have to investigate the correlation between gene expression and protein expression and the functional significance of the current results. The possible role of additional genes and proteins in adaptation should also be investigated to clarify the pathways involved.

Another important question that needs clarification involve the role of caspase 3 in the adaptation to oxidative stress, and in particular whether it is necessary for activation of the anti-apoptotic pathway. For example, it remains to be confirmed whether the inhibition of caspase 3 will prevent the up-regulation of Akt and CREB with the RL + SH treatment regimens.

It is also important to investigate the *in vivo* relevance of the current results by comparing the *in vitro* to the *in vivo* effects of ozone in different tissue. The effects of ozone on various systemic organ systems and in the central nervous system are also important to be investigated.

Further clarification of the role of different members of the pro- and anti-apoptotic families in ozone-induced adaptation is necessary. It may be important to distinguish the role of the intrinsic and extrinsic pathways of apoptosis in this regard.

Finally, it is generally recommended that more pre-clinical investigation be undertaken to gain an understanding of the biological effects, mechanisms and toxicity of ozone before any extended clinical experimentation is undertaken. Indeed, any therapeutic claim and application should be based on sound scientific evidence, ensuring responsible, accountable and professional practices.

Additional materials and methods

Appendix

A

1.1 MATERIALS

1.1.1 Chemicals

The following chemicals were purchased from the specified companies:

- Bovine serum albumin (BSA), protease inhibitor cocktail, phosphatase inhibitor cocktail 2, Tris base, glycine, Tris HCl, glycerol, mercaptoethanol, Bradford solution and methanol was from Sigma Aldrich (Johannesburg, RSA).
- The 30% acrylamide/bisacrylamide solution 29:1, Immun-Star™ WesternC™ Chemiluminescent kit, ammonium persulphate, bromophenol blue, 10% Tween 20 and N,N,N',N'-tetramethylethylenediamine (TEMED) was from Bio-Rad (Cape Town, South Africa).
- PAGERuler™ prestained protein ladder plus was from Fermentas (Lithuania).
- Indigo trisulphonate potassium salt redox indicator was from Riedel-de Haen (Johannesburg, South Africa).
- Research antibodies anti-AKT phospho specific, anti-CREB phospho specific, anti-AKT, anti-CREB1 and peroxidase conjugate affinity purified anti-rabbit IgG were from Rockland Immunochemicals (USA).
- Research antibodies anti-caspase-3, anti-caspase-8, and anti-mouse IgG HRP-linked antibody were from BioVision (USA).
- Nonidet P-40 was from Roche (Johannesburg, South Africa).
- ECL Advance™ blocking reagent was obtained from GE Health Care (UK).

1.1.2 Instruments

The following instruments were employed in the experiments as indicated:

- Bench top centrifuge 3K15 Sigma Aldrich (Johannesburg, RSA).
- 96-well plate reader, with a 560 nm filter (Labsystems Multiscan RC).
- SmartSpec™ Plus Spectrophotometer (Bio-Rad USA).
- Power Pac 200 (Bio-Rad, USA).
- Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad, USA).
- Mini-Protean 3 Cell (Bio-Rad, USA).
- ChemiDoc™ EQ (Bio-Rad, USA)

1.1.3 Methods

1.1.3.1 Ozone Exposure System

To expose the HeLa cells to ozone, ozone was continuously bubbled through a sample solution of glucose-free Krebs-Henseleit and 1 ml of this solution was added to each cell containing well and replaced every 5 minutes (compensating for the short half-life of ozone in solution 1). The system setup for the exposure of this solution to ozone was done as shown in figure A-1. To prevent the reaction of ozone with the non-biological components of the system, all the apparatus that were exposed to ozone were made of inert materials, for example glass, Teflon® and silicon.

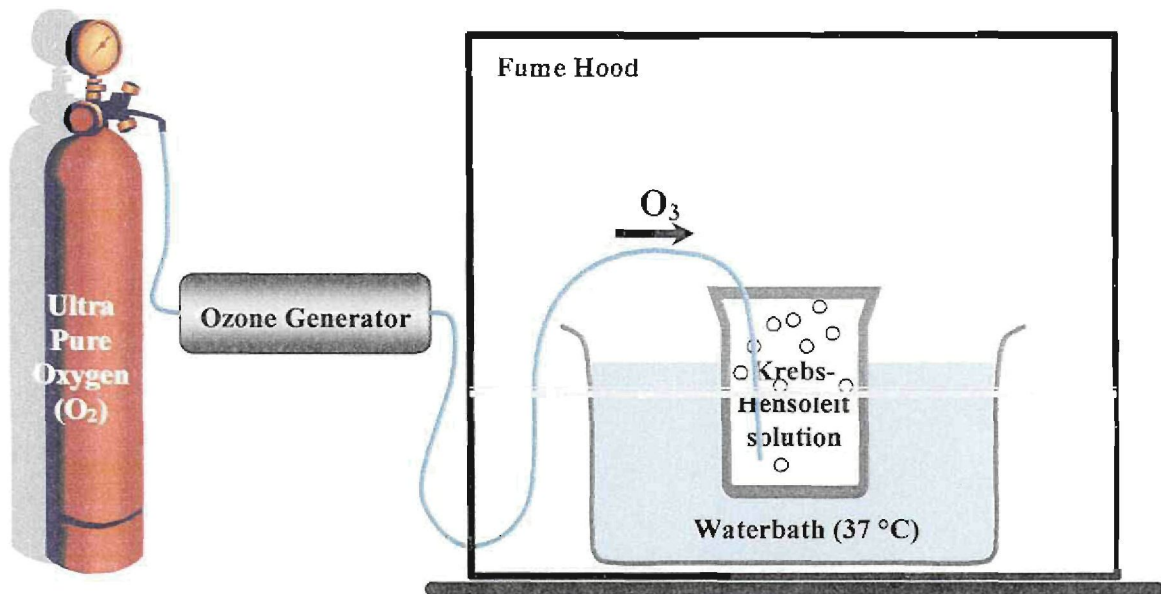


Figure A-2: Ozone exposure system set-up in cell culture laboratory.

1.1.3.2 Indigo Colorimetric Method for Determining Ozone Concentration

1.1.3.2.1 Introduction

The aim of the indigo colorimetric assay was to determine the concentration of ozone in the glucose-free Krebs Henseleit (gf-KH) solution that cells are exposed to. The detection of ozone in liquid phase can be done using indigo-reagent in the potassium indigotrisulphonate colorimetric method (Hougue *et al*, 1980). This method is regarded as an international standard and is a quantitative and selective assay for the determination of ozone in aqueous solutions. It is based on the decolourisation of indigo by ozone in an acidic solution, with the absorbance measured at a wavelength of 600 nm. The decrease in absorbance is linear with an increase in the concentration of ozone, expressed as ppm or mg/l.

1.1.3.2.2 Assay

This protocol describes the measurement of ozone concentrations in gf-KH solution:

- Indigo stock solution was prepared by adding 5 ml distilled water and 0.01 ml concentrated phosphoric acid to a 10 ml volumetric flask. To this 7.7 mg potassium indigo trisulphonate was added and the solution was stirred while adding distilled water to 10 ml.
- From the stock solution of the indigo reagent was prepared by adding 1 ml of stock solution to a 50 ml volumetric flask. To this 0.5 mg sodium dihydrogen phosphate and 350 μ l concentrated phosphoric acid was added. The volumetric flask was filled to 50 ml with distilled water.

- The gf-KH solution at 37°C was bubbled with ozonated super pure oxygen for 30 min (time to reach saturation) before experiment starts and the ozone exposure continues throughout the experiment.
- For the negative control 1 ml indigo reagent was added to a 10 ml volumetric flask and the flask was filled to 10 ml with unexposed gf-KH solution.
- From the gf-KH solution 5 ml samples were taken every minute for 10 minutes after discontinuation of the ozone exposure.
- To each of these samples 1 ml indigo reagent was added and the absorbance immediately determined spectrophotometrically at 600 nm.
- The ozone concentration was then determined using the following equation:

$$[O_3](mg/l) = 100 \times \frac{Abs_{blank} - Abs_{sample}}{f \times b \times V}$$

Where;

Abs_{blank} = absorbance of the blank solution

Abs_{sample} = absorbance of sample solution

$F = 0.42$ (sensitivity factor)

b = path length of cell in cm

v = volume of the sample in ml

1.1.3.3 Protein Expression

1.1.3.3.1 Introduction

From the data measuring gene expression before and after exposure of HeLa cells with ozone (see Chapter 3, Discussion and Conclusion), it was shown that repetitive low dose exposure + single high dose exposure to ozone (RL + SH) up-regulate the gene expression of Akt, CREB and caspase 3, following 8 hours of incubation. Therefore, we investigated whether this up-regulation of genes was translated into protein expression. Thus, relative quantification of protein expression was measured with Western blots for the expression of Akt, Akt phospho specific, CREB, CREB phospho specific, pro-caspase 3 and cleaved caspase 3. It was decided

to include the phospho specific antibodies for Akt and CREB, because ROS can activate the proteins and elicit an effect without a change in protein expression.

1.1.3.3.2 Sample Preparation

- After the different ozone exposure regimens (RL, SH and RL+SH, as described in Chapter 3, Materials and methods) the cells were incubated for 8 hours in normal DMEM.
- The medium was then aspirated and the cells rinsed with 2 ml of trypsin-versin, where after the cells in each well were incubated with 2 ml fresh trypsin for 10 min at 37°C.
- The loosened cells were then transferred to microcentrifuge tubes and centrifuged at 2000xg for 6 min to pellet the cells.
- The supernatant was discarded, 1 ml of ice-cold PBS added, the pellet resuspended (vortex) and centrifuged again at 2000xg for 6 min to pellet the cells.
- The supernatant was discarded and 0.3 ml of cell lysis buffer (5 ml of 1M Tris base solution, 3 ml of 5 M NaCl solution and 1 ml of Nonidet P-40) was added to form a stock solution) was added to the cell pellet vortexed and left on ice for 5 min.
- Samples were then sonicated for 7 seconds at a medium setting and then centrifuged at 2000xg for 10 min.
- Supernatant was split in aliquots, snap frozen and kept at -80 °C until used within 6 months.

1.1.3.3.3 Protein Determination

For measurement of protein concentration of samples the standard Bradford assay was used. In particular it was necessary to ensure that equal quantities of total protein were added to each well on the SDS page gel.

- The Bradford reagent was gently shaken in the bottle; 5 ml withdrawn and allowed to reach room temperature in a dark environment.
- Protein standards were prepared by dissolving 2 mg bovine serum albumin (BSA) in 1 ml double-distilled water (to produce a 2 mg/ml solution) and then making a series of 100 µl dilutions as indicated in the table below.

Table A-1 Preparation of protein standards

Protein concentration	Dilution procedure	
	Volume of 2 mg/ml BSA	Volume of lysis buffer
0 mg/ml	0 μ l	100 μ l
0.1 mg/ml	5 μ l	95 μ l
0.4 mg/ml	20 μ l	80 μ l
0.7 mg/ml	35 μ l	65 μ l
1.0 mg/ml	50 μ l	50 μ l
1.4 mg/ml	70 μ l	30 μ l

- 2 $\times$ 5 μ l of each protein standard dilution, as well as 2 $\times$ 5 μ l of the sample suspension were added to separate wells of a 96-well plate, i.e. all in duplicate.
- 250 μ l of Bradford reagent was added to each well, mixed by a 30 second vibration and incubated for 15 minutes at room temperature.
- The absorbance in each well was determined in a 96-well plate reader using a 560 nm filter.
- The protein concentration of the sample suspension was calculated using the mathematical equation obtained when net absorbance was plotted against the protein concentration of the standards.

1.1.3.3.4 SDS-PAGE Electrophoresis and Immunoblotting

- 10% polyacrylamide resolving gel (i.e. 30% acrylamide/bisacrylamide, ddH₂O, 1.5 M Tris HCl and 10% SDS. Polymerisation of the gel was initiated by adding 10% ammonium persulphate and TEMED) was prepared and poured into a gel cassette up to 75% capacity. The gel was immediately overlaid with ddH₂O and left for 40 min to set.
- After removal of the overlaying ddH₂O, the remaining 25% capacity of the cassette was filled with 5% polyacrylamide stacking gel (i.e. 30% acrylamide/bisacrylamide, ddH₂O, 0.05 M Tris

HCl and 10% SDS. Polymerisation of the gel was initiated by adding 10% ammonium persulphate and TEMED). A 10-well comb was inserted and the gel left to set for 40 min.

- The gel cassette assembly was removed from the casting stand, placed into the electrode assembly, clamped in the frame and submerged in the mini-tank, filled with running buffer (0.1% SDS, 0.25 M Tris base, 1.92 M glycine and pH was adjusted to pH 8.3).
- Just before use, the protein samples were thawed and mixed 1:1 with loading buffer (62.5 mM Tris HCl, 2% SDS, 25% glycerol, 0.01% bromophenol blue and mercaptoethanol 50 μ l for every 950 μ l of buffer) and placed on a heating block at 95 °C for 5 min. Samples were then loaded into the wells according to Table A-2. Each gel also received a page ruler with molecular weight markers.
- After 105 min of electrophoresis at 100 V the gels were removed and equalised in transfer buffer (39 mM glycine, 48 mM Tris HCl, 0.0375% SDS and 20% methanol) for 15 min.
- The PVDF membrane was briefly soaked in methanol and then placed in transfer buffer for 15 min. Extra thick blotting paper was cut to size and then also placed in transfer buffer for 15 min.
- In the semi-dry transfer unit a gel sandwich was prepared as follows: Two blotting papers, PVDF membrane, SDS-PAGE gel and another two blotting papers. The proteins were then transferred from the SDS-PAGE gel to the PVDF membrane at 20 V for 25 min.
- Membranes were removed and placed in 2% ECL advance blocking reagent in washing buffer (10 mM Tris HCl pH 7.5, 100 mM NaCl and 0.2% Tween-20) for 2 h at room temperature on a rocking boat at low speed.
- The membranes were then washed with washing buffer for 30 min on a rocking boat at high speed, replacing the buffer every 10 min.
- Primary antibody was diluted in washing buffer and 2% ECL advance blocking reagent specific for each antibody Table A-2. The membranes were left overnight in primary antibody at 4 °C on a rocking boat at low speed.

Table A-2: The concentrations of the different proteins that were loaded into the wells and primary and secondary antibody dilutions for the specific proteins.

Protein	Protein concentration mg/ml	Primary antibody	Secondary antibody
Akt	4	1:8000	1:15 000
Phospho Akt	8	1:8000	1:10 000
CREB	4	1:8000	1:10 000
Phospho CREB	8	1:8000	1:10 000
Pro-caspase 3	8	1:200	1:500
Cleaved caspase 3	4	1:1000	1:2000
Pro-caspase 8	8	1:200	1:500
Cleaved caspase 8	4	1:1000	1:2000

- The next morning the membranes were washed in washing buffer for 30 min on a rocking boat at a high speed, changing the buffer every 5 min.
- Secondary antibody was diluted with 2% ECL advance blocking reagent in washing buffer (Table A-2). The membranes were incubated in secondary antibody solution for 1 h on a rocking boat at low speed.
- Thereafter, the membranes were removed and washed with washing buffer for 30 min on a rocking boat at high speed, changing the buffer every 5 min.
- ECL was then used for detection and for this the Immun-Star™ WesternC™ Chemiluminescent kit was used. From the kit 2.5 ml of reagent A was mixed with 2.5 ml of reagent B, the mixture was then added on to the membranes. The membranes were then left for 5 min on a rocking boat at a low speed.
- Membranes were dried and placed in the Chasidic for analysis. The Chasidic was set to take 20 pictures between 2000 and 3500 seconds. The best picture was chosen for band intensity analysis. Data were expressed as % of control.

Additional Results	Appendix B
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In this section all the results of additional experiments, not presented in the article (see Appendix A) are given. The first section presents results of the measurements of ozone concentration in glucose-free Krebs Henseleit (gf-KH) solution, while the second section depicts results from the Western blots, measuring protein expression.

1.1 Establishing Conditions for *in vitro* Ozone Exposure

The conditions for the exposure of cultured, monolayer human epithelial (HeLa) cells, as employed in the current study, has been used before (Brink *et al.*, 2008a). While other complex experimental systems for the exposure of cells to gaseous ozone have been developed, this represents a simple setup that is easy to deploy. Briefly, the cells are exposed to ozone-saturated physiological solution according to a specified exposure and incubation regimen with defined durations, followed by an appropriate biomolecular assay.

The unique challenges associated with this approach of *in vitro* exposure of cultured cells to ozone in solution, include the choice of appropriate treatment physiological solution to administer the ozone:

- **The physiological solution of choice must be relatively inert to reaction with ozone.** Ozone is a strong oxidant that may react with chemicals in the solution (e.g. organic molecules) to alter the composition thereof and even produce biologically active (toxic or otherwise) by-products.
- **The physiological solution should, in the presence of ozone, retain a pH that is compatible with physiologically active cultured cells.** Ozone may affect the pH of the physiological solution to create non-physiological conditions.
- **The half-life of ozone in the physiological solution at 37°C should be sufficiently long to allow the cells to be exposed significantly to the ozone in solution.** Ozone has a relatively short half-life in aqueous medium.

The data presented in this section are from experiments investigating whether these conditions for physiological solution as carrier of ozone are met.

1.1.1 Decomposition of Ozone in Glucose-free Krebs-Henseleit Solution

We have previously shown that glucose in Krebs-Henseleit solution reacts with ozone to form an unidentified toxic by-product (Pretorius, 2005). It was here important to determine the saturation concentration of ozone in glucose-free Krebs-Henseleit solution at physiological temperature (37 °C), when ozone was bubbled through the medium. It was also important to determine the half-life of dissolved ozone in this physiological solution at 37 °C.

Figure B-1 depicts the decay of ozone in gf-KH at 37 °C, following saturation of gf-KH with ozone.

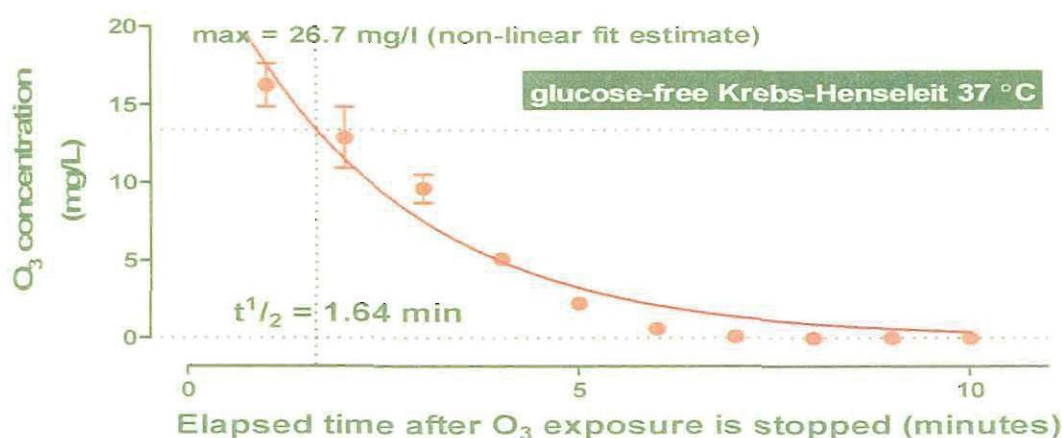


Figure B- 1: Ozone decay in gf-KH solution at 37 °C over a period of 10 min, measuring ozone concentration (mg/l). Data points are averages $\pm$ standard error of the mean of triplicate observation from three independent and comparable experiments and data are presented as the concentration of ozone in solution at the indicated time-point after saturation and cessation of ozone bubbling. The half-life of ozone was estimated from a one phase exponential decay non-linear fit of the data points.

From Figure B- 1 it can be seen that the saturation concentration of ozone after 30 minutes of bubbling in gf-KH is 26.7 mg/l. It has been determined before that 30 minutes is sufficient to obtain saturation (Pretorius, 2005). From the exponential decay non-linear fit it was calculated that the half-life of ozone is 1.64 min (95% confidence interval 1.64 to 2.008). Such a short half life was expected, since it is known that ozone is relatively unstable in aqueous solution at 37

°C (Lotriet *et al.*, 2007). Previously, cells were exposed to ozone in the same experimental setup by replacing the ozone-saturated solution every 5 minutes (Pretorius, 2005; Brink *et al.*, 2008a) and it was decided to follow the same procedure. This implies that the ozone concentration administered to cells would vary from roughly 27 mg/l at 0 minutes to > 2.2 mg/l at 5 minutes (at the time of replacement).

1.1.2 Change in pH after Ozone Exposure

To investigate whether ozone affects the physiological pH (± 7.4) of gf-KH solution, the pH of the solution was determined at various time points (0, 30, 60 and 90 min) during constant ozone exposure. Figure B-2 depicts the change in pH as a function of time of bubbling with ozone.

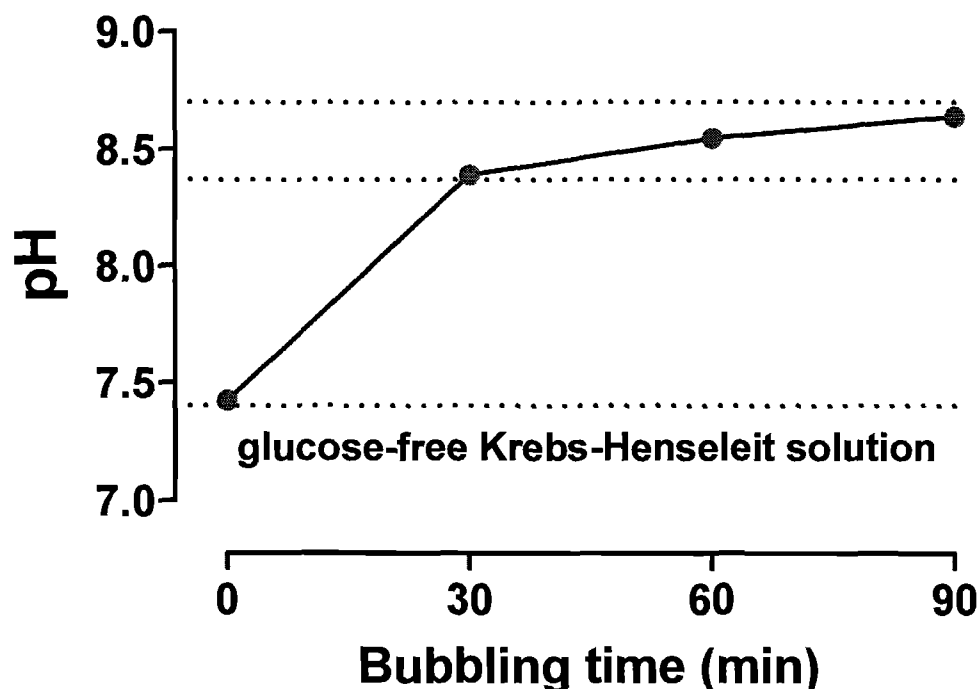


Figure B-2: The increase of pH during gf-KH ozone exposure. Data are averages $\pm$ standard error of the mean of triplicate observations for three independent and comparable experiments. The data were analysed statistically by performing a one-way ANOVA and then implementing the Tukey-Kramer post-test.

As can be seen from Figure B-2 during the 90 min exposure period the pH increased significantly from 7.4 ± 0.012 to 8.6 ± 0.006 . The most significant increase occurred in the first 30 min from 7.4 ± 0.012 to 8.3 ± 0.03 , while the pH was relatively stable for the last 60 min. It has been determined before (Brink *et al.*, 2008a) that a similar change in pH is seen when the solution is bubbled with oxygen, suggesting that this is due to the replacement of CO_2 . The buffer of Krebs-Henseleit solution is dependent of the presence of CO_2 . For this reason the

ozone-saturated gf-KH was extracted from its source for exposure of cells from the medium that has been bubbled with ozone for 30 to a maximum of 90 minutes.

1.2 Protein Expression of Pro- and Anti-apoptotic Proteins after Ozone Treatment

Based on the results from data obtained from RT²-PCR, looking at gene expression before and after the various ozone exposure regimens, we selected target proteins to evaluate further for relative expression before and after ozone exposure with Western blot. In particular, we selected to measure the relative expression of Akt, CREB and caspase 3. To distinguish active and inactive forms of the proteins, we also included measurement of the expression of CREB phospho specific, Akt phospho specific, pro-caspase 3 and the cleaved active form of caspase 3.

Figure B-3 shows the relative expression of these proteins before and after the various ozone treatment regimens and an 8 hour incubation period. We chose to measure protein expression only after 8 hours after the last exposure to ozone because the genes were up-regulated after 8 h and in a previous study the effects of ozone adaptation was only majored after 8 h after ozone treatments stopped (Brink *et al.*, 2008a). This was a good indication for us at which time to look for changes in protein expression.

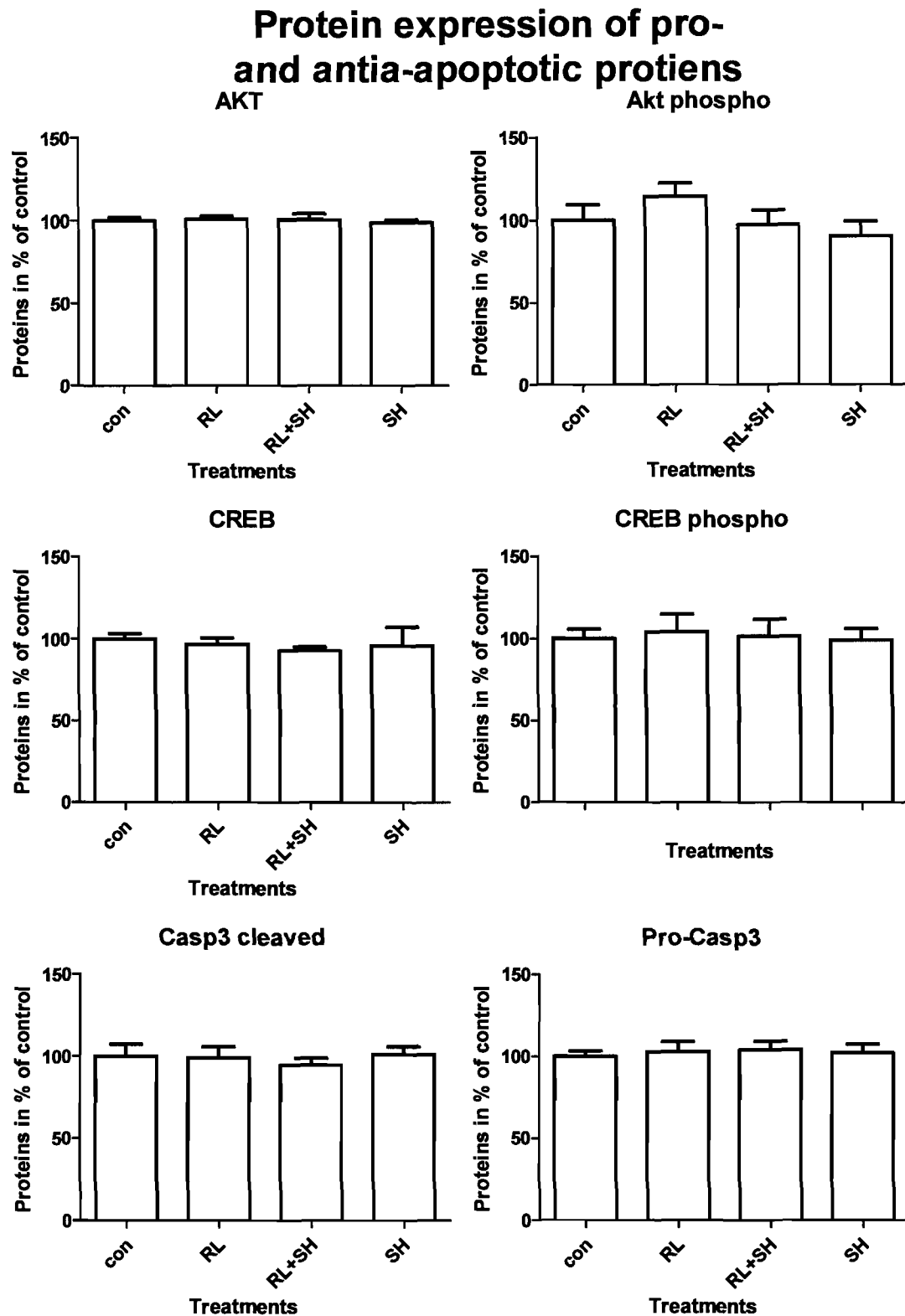


Figure B-3: This figure illustrates the protein expression of pro- and anti-apoptotic proteins after receiving ozone exposure (RL, SH and RL+SH). Cells were left for 8 h in normal DMEM before the experiment was done. Data are expressed in % of control. A one-way ANOVA followed by the Dunnett's multiple comparison test was performed for statistical analysis. Data are averages of four independent experiments with triplicate observations expressed as the mean $\pm$ SEM.

As can be seen from Figure B-3 there were no significant differences or trends noticed from the different proteins for all of the treatment groups (RT, SH and RT+SH). These results were disappointing, but may be explained. Firstly, gene expression is not always translated directly into protein expression. Also, we observed the most prominent changes in gene expression only after 8 hours (the same time as measuring protein expression here), so that translation to protein expression may have occurred later. It could be that significant changes will be observed a few hours later.

Lastly, while we were able to detect protein expression, the current approach is not designed to measure protein expression in real time and also may not be sufficiently refined and sensitive to measure protein expression under these conditions.



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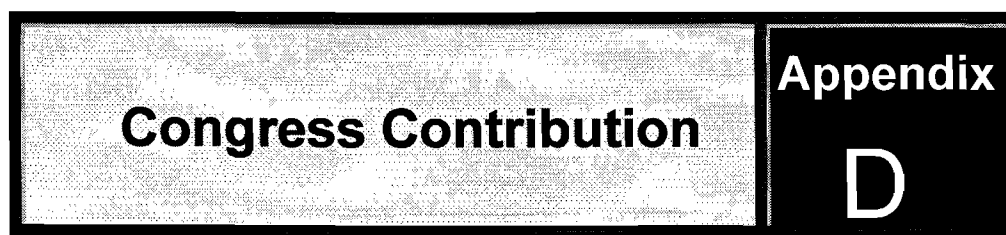
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Congress Contribution

Appendix D

At the SA Pharmacology & Toxicology Congress held from 2 to 5 October 2007 at Buffelspoort Holiday Resort, RSA the following contributions were made: abstract, poster and oral presentation. Only the gene expression data were presented at the congress. In this appendix the abstract and the poster are included.

The effects of ozone exposure on markers of cellular resilience in cultured human epithelial HeLa cells

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Purpose:

Ozone is a natural occurring gas with strong oxidising properties. While its biological effects are associated with toxicity, it has also been used for therapeutic purposes. In biological systems ozone can elicit dose-dependent oxidative stress, which may induce adaptation. The mechanisms for these effects remain illusive. Previous studies in our laboratory indicated that repetitive exposure of cultured human epithelial cells to ozone may induce cytoprotective mechanisms (adaptation), which may include the up-regulation of anti-apoptotic pathways. The aim of the current study was to determine the effects of single and repetitive exposure of humane cell lines to ozone on the expression of genes that encode for anti apoptotic (AKT, Bcl2, CREB, NFKB and BDNF) and pro apoptotic (Bax, Caspase 3 and 8) proteins, as well as the corresponding protein expression levels.

Methods:

Cultured human epithelial HeLa cells were exposed to control or ozone-saturated glucose-free Krebs-Henseleit solution. Exposures consisted of 4 x 5-minute exposures every four hours, followed by a 16 hours incubation in normal culture medium and then a 25-minute exposure. Cells were then lysed immediately (0 h) or after 4h or 8h incubation in normal culture medium. The relative expression of genes encoding for pro-apoptotic factors Bax, caspase-3 and caspase 8, and anti-apoptotic factors BDNF, NF- κ B, Akt, CREB and Bcl-2 was then determined with quantitative real-time RT-PCR. In follow-up studies, currently being conducted, the corresponding expression of these proteins will be quantified with Western blot analyses.

Results:

When measuring gene expression immediately after treatment (0h), genes encoding for Bcl-2, CREB, BDNF and caspase-3 were down-regulated by ozone administered 4 x 5 minutes every 4 hours, but these returned to pre-treatment values after 8 hours. However, 8 hours after the treatments, cells treated with ozone for 4 x 5 minutes every 4 hours plus an acute 25-minute exposure 16 hours later, showed an up-regulation of the genes encoding for the anti-apoptotic factor Akt, and a trend towards up-regulation of CREB, while a single 25-minute exposure of the cells to ozone up-regulated the expression of caspase-3 after 8 hours. It needs to be established how these results relate to the up- or down-regulation of pro-apoptotic and anti-apoptotic proteins, as measured by Western blot analyses. The current data therefore suggest an anti-apoptotic mechanism for adaptive effects seen in cultured human epithelial HeLa cells after repetitive exposure to ozone.

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Additional Article	Appendix E
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In this appendix an additional article is included in which I was involved in and co-authored the article. The data which I was responsible for was additional work on the cell viability assays. The article was submitted to Redox Report and was excepted for publication in the 2nd issue of 2008. A proof is also included in this appendix.

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Studies on cellular resilience and adaptation following acute and repetitive exposure to ozone in cultured human epithelial (HeLa) cells

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Ozone is used to treat several medical conditions, while the underlying mechanisms of action are sometimes poorly understood. In the current study, we exposed cultured human epithelial (HeLa) cells acutely and repeatedly to ozone and investigated the effects thereof on cell viability. The involvement of anti-apoptotic pathways in observed adaptive responses to ozone were investigated by employing the Akt inhibitor (–)-deguelin. Cells were exposed to an ozone-saturated physiological solution using various dosing regimens, including acute exposure and various repetitive exposures. Cell viability was determined with Trypan Blue or MTT tests, or by a DNA-fragmentation (comet) assay. Acute ozone exposure compromised cell membrane integrity severely, while adaptation to reverse an initial reduction in mitochondrial activity was observed. Repetitive, short-duration exposures followed by a single long-duration exposure to ozone furnished a protective adaptation that was reversed by Akt inhibition. Extracellular and intracellular damage (and adaptation) occurs differentially. While acute ozone may decrease cell viability, multiple pre-exposures up-regulates cellular plasticity via induction of anti-apoptotic pathways in a treatment regimen-specific manner.

Keywords: Ozone, adaptation, membrane integrity, mitochondrial activity, DNA integrity, human epithelial cells

INTRODUCTION

While relatively high concentrations and doses of ozone have been associated with biotoxicity, it has also found many useful applications. A comprehensive review of the latest available pertinent information on atmospheric science, air quality, exposure, dosimetry, health effects, and environmental effects of ozone and other related photochemical oxidants are presented in the final *Ozone Criteria Document 2006* of the United States Environmental Protection Agency.¹ In the medical field, ozone is utilized in dentistry,² for the treatment of diabetic wounds,³ cancers⁴ and microbial infections, with admin-

istration ranging from topical application to systemic autohemotherapy.⁵ While ozone is widely utilised in many complimentary and alternative healthcare practices, there is a need for a better understanding of the biological and therapeutic effects and safety of ozone. In particular, the mechanisms of action, including mechanisms of proposed biological adaptive responses to ozone exposure in a therapeutic context, are still poorly understood.

The biological toxicity of ozone is ascribed to its strong oxidizing properties and, in particular, its rapid conversion into a number of reactive oxidant species that react with cellular proteins and lipids.⁶ Ozone has been shown to produce dose-dependent oxidative stress, due to an increase in the production of free radicals via cell membrane lipid peroxidation (with subsequent cleavage of arachidonic acid), protein oxidation, enzyme inactivation, destruction of DNA and apoptosis.⁷ Upon inhalation, ozone reacts with antioxidants and polyunsaturated fatty acids in lung-lining fluids, while the latter reaction forms lipid peroxidation products that can be absorbed

Received 23 August 2007

Revised 25 October 2007

Accepted 2 November 2007

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systemically.^{8,9} Systemic effects include increased serum levels of thyroid-stimulating hormone, thyroid hormones, protein-bound iodine and prolactin and xenobiotic metabolism of the liver¹ due to increased liver antioxidant enzymes. There are contradicting reports on cardiovascular hemodynamic effects of ozone, including its influence on heart rate and rhythm and mean arterial blood pressure.¹⁰⁻¹² The immune system may also be susceptible in that ozone may stimulate and activate T lymphocytes¹³ and modulate cytokine (e.g. interleukin [IL]-1 α) production.¹⁴

The effect of ozone on the respiratory tract has been previously studied, both *in vivo* and *in vitro*. A number of studies have demonstrated that acute ozone exposure is associated with increased airway hyper-responsiveness and impaired lung function. The latter is characterized by pathological changes mainly in lower airways, including edema, epithelial cell damage, surfactant derangement, and accumulation of inflammatory mediators in the pulmonary epithelial lining fluid.¹⁵⁻¹⁷ Inflammation of the respiratory tract after ozone exposure has been observed in nearly all species studied.¹⁸ In general, the *in vivo* studies have shown that homeostasis of certain cellular elements and/or their products (cytokines, growth factors) is disrupted, whilst *in vivo* human studies have also shown reduction of forced expiratory volume FEV₁.¹⁹ On the other hand, numerous studies have shown that repetitive exposures to lower doses of ozone can induce protection or adaptation to these acute injurious effects of ozone.²⁰⁻²² Importantly, repeated daily exposures to ozone in humans have been shown to induce adaptive responses involving forced expiratory volume (FEV), forced vital capacity (FVC), and bronchial reactivity.²⁰

In vitro methods, employing relevant pulmonary cells (including cells isolated from broncho-alveolar lavage [BAL]²³ or cultured epithelial cells²⁴) provide a useful tool to study the complexity of ozone-induced lung inflammation. Alveolar macrophages isolated from ozone-exposed rodents exhibited cell damage,⁶ decreased phagocytosis, and production of chemotactic factors.²⁵ It has also been shown that the exposure of rats to ozone inhalation increases polymorphonuclear leukocytes and fibronectin in the broncho-alveolar lavage.²⁶ The clinical importance of understanding the mechanisms underlying ozone-induced lung injury necessitates further analysis of ozone effects on various pulmonary cell types.

In the present study, we employed an *in vitro* technique to screen for the subcellular effects of ozone, by exposing a cultured human epithelial cell line to ozone in an aqueous medium. We investigated the effects of different ozone exposure regimens (single acute versus repetitive exposures) on markers of cell viability (i.e. membrane integrity, mitochondrial activity and DNA

integrity), as well as the role of anti-apoptotic pathways in this regard, in an *in vitro* system.

MATERIALS AND METHODS

Materials

Cell culture media, serum, antimicrobials and Trypan Blue stain (0.4%) were obtained from Bio-Whittaker (Walkersville, MD, USA). Trypsin/versine (0.25% 1:250 trypsin + 0.38% EDTA) was obtained from Gibco (Invitrogen, UK). CaCl₂·2H₂O, HCl (33%), H₂O₂, KCl, MgCl₂·6H₂O, NaHCO₃, NaH₂PO₄, NaCl, NaOH, ethylenediaminetetraacetic acid di-sodium salt (EDTA), dimethyl sulfoxide (DMSO), low melting point agarose (LMPA), high melting point agarose (HMPA) were from Merck (Darmstadt, Germany). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), *iso*-propanol (99+%), Z-Asp(OMe)-Gln-Met-Asp(OMe) fluoromethyl ketone, (-)-deguelin, Triton X-100, Trizma base and Trizma-HCl were from Sigma Aldrich (St Louis, MO, USA). Indigo trisulfonate potassium salt redox indicator was from Riedel-de Haën (Seelze, Germany). Ethidium bromide was obtained from MP Biomedicals (Eschwege, Germany). Formic acid and orthophosphoric acid (H₃PO₄; 85%) were from SAARChem (Krugersdorp, Gauteng, South Africa). Ultra high purity oxygen (Afrox; 99.995%) used for ozone production was from African Oxygen Limited (Johannesburg, South Africa). The ozone generator (Sterizone P-HP 250 – PCT/ZA00/00031) was manufactured and patented by the Department of Physics, North-West University, Potchefstroom, South Africa. All water used was of double distilled quality.

Cell culture

A standard, cultured, adherent, human cervical epithelial cell line (HeLa, from American Type Culture Collection [ATCC], cat. no. CCL-2) was employed. The current study intended to screen the general (not lung-specific) markers of oxidative stress and adaptation following exposure to ozone. The HeLa cell line was selected since it is well characterized,²⁷ oxidative stress has been extensively studied in this cell line,²⁸ it has appropriate culturing characteristics and was readily available. HeLa cells were maintained and grown to approximately 90% confluency in Dulbecco's modified Eagle's medium (DMEM), 10% fetal bovine serum (FBS), 100 units/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml fungizone in 150-cm² culture flasks at 37°C in 5% CO₂ and humidified atmosphere. Cells were detached from the flask bottom surfaces by trypsinization (Trypsin/EDTA).

For experiments with ozone exposure, cells were seeded in 24-well plates at a density of 3×10^5 cells/well or in 25-cm² culture flasks at a density of 2×10^6 cells/flask.

Ozone generation, set-up and choice of medium

The ozone generator was connected to ultra-pure oxygen for ozone generation. The generated ozone (or pure oxygen for the oxygen control) was constantly bubbled through glucose-free Krebs–Henseleit solution (119 mM NaCl, 4.7 mM KCl, 1.9 mM CaCl₂·2H₂O, 0.54 mM MgCl₂·6H₂O, 24.0 mM NaHCO₃, 1.0 mM NaH₂PO₄) at 37°C in a fume hood for at least 30 min (time needed for saturation, see below) by means of a connected glass straw that was submerged in a beaker with solution. The choice of glucose-free Krebs–Henseleit solution as the ozone exposure system for HeLa cells is primarily based on the need for a chemically simple and lipid- and protein-free physiological incubation medium, to minimize ozone reaction by-products. In addition, this has been used before to expose an isolated guinea pig tracheal preparation to ozone.²⁹ It should be noted, however, that ozone may form secondary products in an aqueous medium (e.g. hydrogen peroxide and hydroxyl radicals³⁰), which may contribute to the oxidative stress observed in cells. The glucose-free Krebs–Henseleit solution does not contain any nitrogen atoms, so that the formation of toxic nitrates is eliminated. As reported before,²⁹ we found that, when glucose was included in the Krebs–Henseleit solution, significant cell damage was evident already after 5 min of exposure to ozone (compared to 0 min), as measured by the Trypan Blue ($42.3 \pm 13.0\%$; $P < 0.05$; $n = 25$) and MTT ($39.0 \pm 1.7\%$; $P < 0.01$; $n = 9$) assays, respectively, with relatively little further cell damage thereafter (data not shown). Notably, this complete toxic effect was absent when glucose was excluded (data shown below), suggesting that glucose may form a putative cytotoxic product during ozone exposure. Glucose-free Krebs–Henseleit was, furthermore, considered an appropriate physiological solution for short-term incubation of the cells. A 55-min incubation period of HeLa cells in glucose-free Krebs–Henseleit solution, as compared to corresponding incubation in normal culture medium with serum, resulted in a moderate reduction in cell membrane integrity and mitochondrial activity, as measured by Trypan Blue ($59.2 \pm 4.8\%$ reduction relative to control; $P < 0.01$; $n = 9$) and MTT ($33.7 \pm 11.4\%$ reduction relative to control; $P < 0.01$; $n = 9$) assays, respectively (data not shown). The residual cell membrane integrity and mitochondrial activity as measured after 55-min incubation in glucose-free Krebs–Henseleit physiological solution was, therefore, sufficient for further experiments investigating the modulation of cell viability by specific interventions, and

being below 100%, any (adaptive) up-regulation of integrity are more likely to be detected.

Indigo colorimetric method for determining ozone concentration

The concentration-dependent decolorization reaction of the indigo reagent by ozone, measured at a wavelength of 600 nm and with absorbance inversely linear with ozone concentration, is utilized as an international standard method for measuring ozone concentration in aqueous solutions.³¹ Indigo stock solution (0.077% w/v potassium indigo trisulfonate; 0.085% v/v H₃PO₄) was used to prepare fresh indigo reagent (0.0015% m/v potassium indigo trisulfonate; 0.6% v/v H₃PO₄; 1% m/v NaH₂PO₄) before measuring the ozone concentration. After appropriate exposure (bubbling) of glucose-free Krebs–Henseleit solution at 37°C with ozone, the ozone concentration in solution was determined by adding 1 ml of indigo reagent to 5 ml of sample and the absorbance was immediately measured spectrophotometrically at 600 nm. The ozone concentration was calculated by employing Equation 1:³¹

$$[\text{O}_3] \text{ (mg/ml)} = 100 \times \frac{\text{Abs}_{(\text{blank})} - \text{Abs}_{(\text{sample})}}{f \times b \times V} \quad \text{Eq. 1}$$

where Abs_(blank) is the absorbance of the blank solution, Abs_(sample) is the absorbance of the sample solution, $f = 0.42$ (sensitivity factor, determined by the change in absorbance per mole of added O₃ per liter), b is the path length of the cell (cm) and V is the volume of the sample (ml).

Ozone exposure procedure

All the apparatus exposed to ozone gas was made of ozone-inert materials, for example glass, Teflon® and silicon. When constantly bubbled with ozone, glucose-free Krebs–Henseleit solution at 37°C becomes saturated (3.38 ± 0.58 mg/l) within 30 min, with a half-life of 5.6 min (95% CI 4.5–7.5 min; $n = 16$; data not shown).

For ozone exposures, HeLa cells were seeded in 24-well plates at a density of 3×10^5 cells/well (verified by visual cell counting in a hemacytometer under a microscope). Growth medium was aspirated and HeLa cells in each well were exposed to 1 ml untreated, or ozone-saturated, or oxygen-saturated glucose-free Krebs–Henseleit solution at 37°C for different time intervals as indicated in the results, while the medium was replaced every 5 min in all instances to compensate for the short half-life (5.6 min), ensuring the maintenance of an ozone concentration ranging between roughly 1.7–3.4 mg/ml

(~0.00025% w/v), see above) of ozone under the experimental conditions employed. The total number of solution replacement steps was always kept similar between all treatment groups for the total duration of the longest ozone treatment (*i.e.* 55 min or less, as indicated). After ozone exposures, cells were rinsed once with untreated (ozone-free) glucose-free Krebs–Henseleit solution and the cell viability assay immediately performed (see below) or normal growth medium added and the cells maintained under normal conditions as described above. When DNA integrity was evaluated, cells were seeded at a density of 2×10^6 cells per 25-cm² culture flask and ozone exposures performed in these flasks using 10 ml solution, similar to exposures in 24-well plates, as described above.

Ozone treatment regimens

Following the ozone exposure procedures described above, HeLa cells were exposed to ozone according to acute or repetitive ozone exposure regimens. In the acute treatment regimens, cells were exposed to glucose-free Krebs–Henseleit solution for a total of 55 min, of which the solution was ozone-saturated or oxygen-saturated for the last 0, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50 or 55 min (as indicated). The repetitive treatment regimens included exposure of the cells to normal, ozone-saturated or oxygen-saturated glucose-free Krebs–Henseleit solution for 4×5 min (5-min exposures spaced 4 h apart in normal growth medium – designated pre-exposure P), followed by an overnight (16 h) incubation in growth medium, then a single 25-min exposure of the cells to normal, ozone-saturated or oxygen-saturated glucose-free Krebs–Henseleit solution (designated re-exposure R), followed by a 0-, 4-, 8- or 12-h incubation period in normal cell culture medium.

Where appropriate, oxygen-saturated controls mentioned above were included to verify that observed effects were due only to generated ozone and not to remnant oxygen that was not converted to ozone by the ozone generator. Since bubbling of the physiological solution with ozone also increases the pH (see experimental results), which was mimicked by bubbling with oxygen, the oxygen controls also corrected for any effects of a change in the pH of the physiological solution on the observed effects with ozone treatments. For all ozone exposures longer than 5 min and the corresponding controls, all medium was replaced every 5 min (due to the short half-life of 5.6 min for ozone in the physiological solution at 37°C, as described above). For example, for acute ozone exposure for a total duration of 55 min, cells that were destined for 0-min ozone exposure received ozone-free solution with every replacement, the cells that were destined for 5-min ozone

exposure received ozone-saturated solution only for the last 5 min, *etc.* After the respective treatment regimen, a cell viability test (Trypan Blue or MTT) or a DNA integrity assay, as described below, was performed on the treated cells.

Trypan Blue

The aim of the standard Trypan Blue assay was to investigate the effect of acute and repeated ozone exposure regimens on cell membrane integrity. It should be noted that blue staining of cells with this assay does not constitute cell lysis or cell death, but merely indicates that the membrane integrity is decreased to a degree where the stain is able to permeate through the membrane. With the concept ‘membrane integrity’ in the current context and as used in this manuscript, one should, therefore, understand ‘permeability to the stain’, so that ‘decreased membrane integrity’ implies ‘increased permeability to the stain’ and ‘increased membrane integrity’ implies ‘decreased permeability to the stain’ relative to control. This assay was performed as follows. After the indicated ozone or control treatments, cells were rinsed twice with EMEM, whereafter the cells were detached from well bottoms by trypsinization and the resulting cell suspension diluted in EMEM. The cell suspension was transferred to microcentrifuge tubes and centrifuged at 5000 rpm (~2300 g) for 10 min at 25°C in a bench-top centrifuge. Each pellet was resuspended in phosphate-buffered saline (PBS) and centrifuged again. The pellet was resuspended in 1 ml PBS with 50 µl of 0.4% w/v Trypan Blue stain immediately prior to visual counting of stained and unstained cells with a hemacytometer under an inverted microscope (optimization showed this to be sufficient time for staining). The percentage viable cells were calculated as follows:

$$\text{Viable cells (\%)} = 100 \times \frac{N_{(\text{white})}}{N_{(\text{white})} + N_{(\text{blue})}} \quad \text{Eq. 2}$$

where $N_{(\text{white})}$ is the number of white (unstained, intact) cells and $N_{(\text{blue})}$ is the number of blue (stained, damaged) cells.

MTT

The aim of the standard MTT cell viability assay³² was to investigate the effect of acute and repeated ozone exposure regimens on mitochondrial activity. This assay was performed as follows. After the indicated ozone or control treatments, cells were rinsed twice with EMEM, whereafter 200 µl of 0.5% MTT in PBS was added to each well. The cells were then incubated for 2 h at 37°C

in 5% CO₂, whereafter the MTT reagent was aspirated. *Iso*-propanol (250 µl) was added to each well and left at room temperature for 5 min to dissolve the purple formazan crystals synthesized in viable cells. From each well, 100 µl *iso*-propanol with dissolved formazan was transferred to a 96-well plate and the absorbance determined at 560 nm with a multiwell scanning spectrophotometer (Labsystems Multiscan RC). While untreated cells served as negative control (optimal cell viability), cells treated with 10 mM formic acid for 30 min at 37°C served as positive control (100% cell death). The percentage of viable cells in each sample was calculated as follows:

Viable cells (%) =

$$100 \times \frac{\text{Abs}_{(\text{sample})} - \text{Abs}_{(\text{positive control})}}{\text{Abs}_{(\text{negative control})} - \text{Abs}_{(\text{positive control})}} \quad \text{Eq. 3}$$

where Abs_(sample) is the absorbance as obtained with the treated cells, Abs_(negative control) is the absorbance as obtained with untreated cells (optimal cell viability) and Abs_(positive control) is the absorbance as obtained with the positive control (100% cell death).

DNA integrity assay

The aim of the DNA integrity (comet) assay was to determine whether the ozone exposure regimens induce DNA damage (single- and double-stranded breaks) in HeLa cells. The assay was performed as described in the literature.^{8,33} In preparation of the assay, microscope slides were covered with a 1.3 mm layer of 1% v/v high melting point agarose in 3.72% v/v EDTA (HMPA), by pipetting 350 µl of pre-heated (80°C) HMPA on the slide, distributing it evenly across the slide with a heated steel scraper on a steel template and then allowing it to cool and set at room temperature (~15 min). Thereafter, 0.5% v/v low melting point agarose in 3.72% EDTA (LMPA) was prepared and kept at 37°C, until loaded with cells as described below.

Ozone-treated and control cells from a 25-cm² culture flask were detached by trypsinization and about 5 × 10⁶ cells transferred to microcentrifuge tubes. The cells were centrifuged at 5500 *g* for 5 min and resuspended in 1 ml PBS. Of this suspension, 20 µl (~100,000 cells) was transferred to 150 µl LMPA at 37°C and immediately transferred onto the centre of a HMPA pre-coated slide. Similar to the HMPA, the cell-containing LMPA was distributed evenly over half of the slide in a 0.8 mm layer, to yield a monolayer of cells. The slide was placed on moist paper towels to cool down to room temperature and set.

Immediately thereafter, the slides with cells were lysed overnight (8 h) in a lysis buffer (2.5 M NaCl, 10

mM Trizma base, 0.1 mM EDTA, 1% v/v Triton X-100, 10% v/v DMSO, 0.2 M NaOH, pH adjusted to 10) at 4°C, while protected from light. Thereafter, the slides were rinsed in H₂O, incubated in the electrophoresis buffer (0.3 M NaOH, 1 mM EDTA, pH > 13) at 4°C for 30 min and then electrophoresed (37 V and 400 mA) at 400°C for 20 min. The slides were rinsed in H₂O and incubated in 0.4 M Trizma-HCl buffer for 15 min at 4°C, rinsed with H₂O, incubated in 10 µM ethidium bromide solution for 15 min at 4°C and rinsed again in H₂O. Within 6 h, the image (×40–×60 enlargements) of the resulting DNA comets of 50 cells per treatment condition was captured and the resulting comets analyzed using the CASP® 1.0.0 computer software program. Analyses compared the amount of DNA in the head (intact DNA) with the amount of DNA in the tail (DNA fragments). DNA integrity was expressed as percentage DNA in tail and calculated as follows:

$$\text{DNA in tail (\%)} = 100 \times \frac{F_{(\text{tail})}}{F_{(\text{tail})} + F_{(\text{head})}} \quad \text{Eq. 4}$$

where F_(tail) = fluorescence in comet tail, F_(head) = fluorescence in comet head.

When DNA repair capacity was to be analyzed, an additional step was introduced just before the cells were suspended in LMPA, where 10 µM H₂O₂ was added to the cell suspension and incubated at 37°C for 40 min. Thereafter, samples were centrifuged for 5 min at 5500 *g*, the supernatant removed and the pellet resuspended in 1 ml PBS, centrifuged for another 5 min at 5500 *g* and resuspended in normal DMEM growth medium. From the resulting cell suspension, 20 µl was immediately mixed with 150 µl LMPA in a microcentrifuge tube and loaded onto the slide (as described above), while the remainder of the cell suspension was incubated for 40 min (allowing DNA repair), whereafter another 20 µl of cell suspension was mixed with LMPA and loaded onto a slide. Thereafter, the assay was continued exactly as described above.

Treatment with enzyme inhibitor

When indicated (with the repetitive exposure regimen), 200 nM (–)-deguelin, an inhibitor of anti-apoptotic pathways, was added to serum-free culture medium (EMEM) for 8 h following the last 25-min ozone exposure (see above). Deguelin has been shown to inhibit phosphatidylinositol-3-kinase (PI3K)/Akt in the nanomolar range (high nanomolar range),³⁴ as well as the survival factor nuclear factor-κB (NF-κB),³⁵ ornithine decarboxylase and mitochondrial function.³⁶ Important for the current study was its general inhibition of anti-apoptotic pathways and, specifically, its significant inhibition of the PI3K/Akt pathway at the concentration employed.

Data analysis

Data from assays were obtained as triplicate observations from at least three separate and independent experiments and expressed as a percentage of control, unless otherwise specified. Graphs were constructed utilizing the computer software Graph Pad Prism® (v.4.01 for Windows; GraphPad Software, San Diego, CA, USA <www.graphpad.com>). Graph Pad Prism® was also utilized for all statistical analyses of data. Each experiment performed on a particular day comprised of a full series of control plus all treatment groups, including three observations per treatment condition. Data obtained per experiment (*i.e.* per day) were converted to percentage of the control and as a result pseudo-replicates from each experiment could be considered as true replicates when pooling data from different experiments. Therefore, $n = 9$ where triplicate measurements from three independent and comparable experiments were included.

For comparison of two values, the Student's *t*-test (one- or two-tailed, as indicated) was implemented. For multiple comparisons the one-way ANOVA comparison was performed followed by either the Dunnett's post-test (for comparing groups to the control) or the Tukey-Kramer post-test (for comparing all groups to one another). For all reported statistical probability values, $P < 0.05$ was regarded as statistically significant.

RESULTS

Effect of ozone bubbling on the pH of the physiological solution

Figure 1 depicts the change in pH of glucose-free Krebs-Henseleit solution at 37°C when constantly bubbled with ozone or oxygen for 0, 30, 60 or 90 min. During the first 30 min of ozone exposure, the pH of the Krebs-Henseleit solution increased significantly from 7.42 ± 0.012 to 8.39 ± 0.030 ($P < 0.0001$; $n = 3$), while the small increases thereafter, rising to 8.64 ± 0.006 during the following 60 min, is statistically insignificant. The changes in pH of the oxygen-treated Krebs-Henseleit solution (control) were indistinguishable from the pH changes of ozone-treated Krebs-Henseleit solution, with the pH increasing within the first 30 min of oxygen exposure from 7.41 ± 0.009 to 8.37 ± 0.038 ($P < 0.0001$; $n = 3$) and then increasing relatively little thereafter, rising to 8.71 ± 0.029 during the following 60 min. These observations can be explained by the displacement of dissolved CO_2 (necessary for buffer action) from the Krebs-Henseleit solution by bubbling with ozone or oxygen, thereby increasing the pH.

Samples for ozone exposure of cells in subsequent experiments were drawn from ozone-saturated source

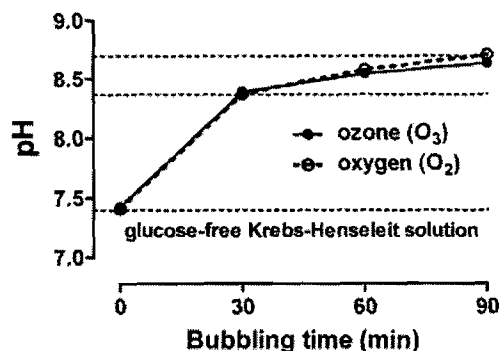


Fig. 1. The effect of bubbling of glucose-free Krebs-Henseleit solution with ozone or oxygen for 0, 30, 60 and 90 min on the pH. Data points are averages $\pm$ SEM of triplicate measurements from three independent and comparable experiments. The data were analyzed statistically by performing a one-way ANOVA and then implementing the Tukey-Kramer post-test (comparing all values), showing that pH values at all four time points differed statistically significantly ($P < 0.05$) from one another, for both the ozone and oxygen bubbled solutions, respectively. However, at no time point did the pH value of the ozone bubbled solution differ statistically significantly from that of the oxygen-bubbled solution.

solutions exposed to ozone for at least 30 min, but not longer than 90 min, so that the pH ranged from approximately 8.4 to 8.7 during cell exposure periods. Also, to verify that any changes in measured parameters of cell viability after ozone exposure is indeed due to ozone, and not to an exposure of the cells to increased pH or to remnant oxygen that were not converted to ozone by the generator, an oxygen control was introduced in subsequent experiments where appropriate.

Effect of acute ozone exposure on cell viability

Figure 2 depicts the time-dependent effects of acute ozone exposure on the membrane integrity and mitochondrial activity of cultured human epithelial HeLa cells, as resulting from exposure of these cells for 0–55 min to ozone-saturated or oxygen-saturated glucose-free Krebs-Henseleit solution. In Figure 2A, it can be seen from the results of the Trypan Blue test that ozone exposure decreases cell membrane integrity rapidly (within 5 min) and significantly ($54.4 \pm 11.0\%$; $P < 0.001$; $n = 9$), deteriorating gradually thereafter to virtually no measurable membrane integrity remaining at 55 min. The parallel oxygen control, however, does not show any deterioration of membrane integrity. Importantly, however, it should be noted that cell staining in the Trypan Blue test indicates an increase in membrane permeability to the dye (as with ozone exposure above). It does not necessarily constitute cell lysis or death and the decrease in membrane integrity may in some cases be preventable, as

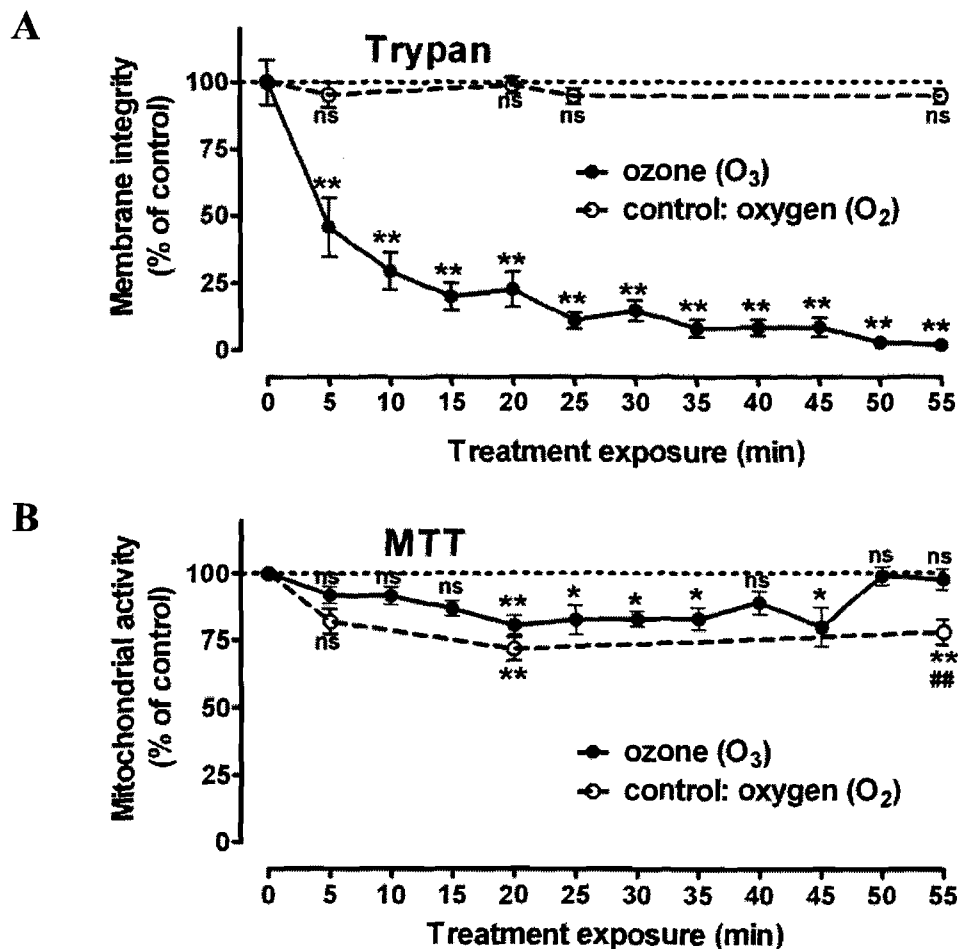


Fig. 2. The effect of acute ozone exposure, or oxygen control, of HeLa cells for 0–55 min on (A) cell membrane integrity, as measured by the Trypan Blue assay or (B) mitochondrial activity, as measured by the MTT cell viability assay. Data points are averages $\pm$ SEM of triplicate measurements from three independent and comparable experiments and are expressed as percentage of the control. The data were analyzed statistically by performing a one-way ANOVA and then implementing the Dunnett's post-test (comparing values to the control), with * $P < 0.05$ and ** $P < 0.01$.

was noted in the current study (see repeated ozone exposures below).

Contrary to the results on membrane integrity, it can be seen from the MTT test in Figure 2B that mitochondrial activity decreases statistically significantly by about 20% only between 20–45 min of ozone exposure, where-after mitochondrial activity returns to pre-treatment values. The oxygen control, however, shows a rapid decline in mitochondrial activity, reaching a 22% decline ($78 \pm 4.6\%$; $P < 0.05$; $n = 9$) after 55 min. At 55 min, there is a statistically significant difference between the ozonated and oxygenated cells ($98 \pm 4.0\%$ versus $78 \pm 4.6\%$; $P = 0.006$; $n = 9$).

In addition to the results described above, a separate set of control experiments were performed, where only the pH of the glucose-free Krebs–Henseleit solution was adjusted to 8.9 with NaOH, without any bubbling with

ozone or oxygen. The results from both the Trypan Blue test and MTT test were indistinguishable from the oxygen control in Figure 2 (data not shown). Again, as with the oxygen control, these results confirm that changes in the pH of the physiological solution does not account for the changes in cell viability elicited by the ozone treatments in Figure 2.

Effect of repeated ozone exposure on membrane integrity

Figure 3A illustrates that only the 4×25 -min pre-exposure P, or a single 25-min re-exposure R to ozone reduces HeLa cell membrane integrity significantly ($34.6 \pm 5.6\%$, $P < 0.001$ and $42.5 \pm 3.1\%$, $P < 0.05$, respectively) when measured immediately (*i.e.* 0 h)

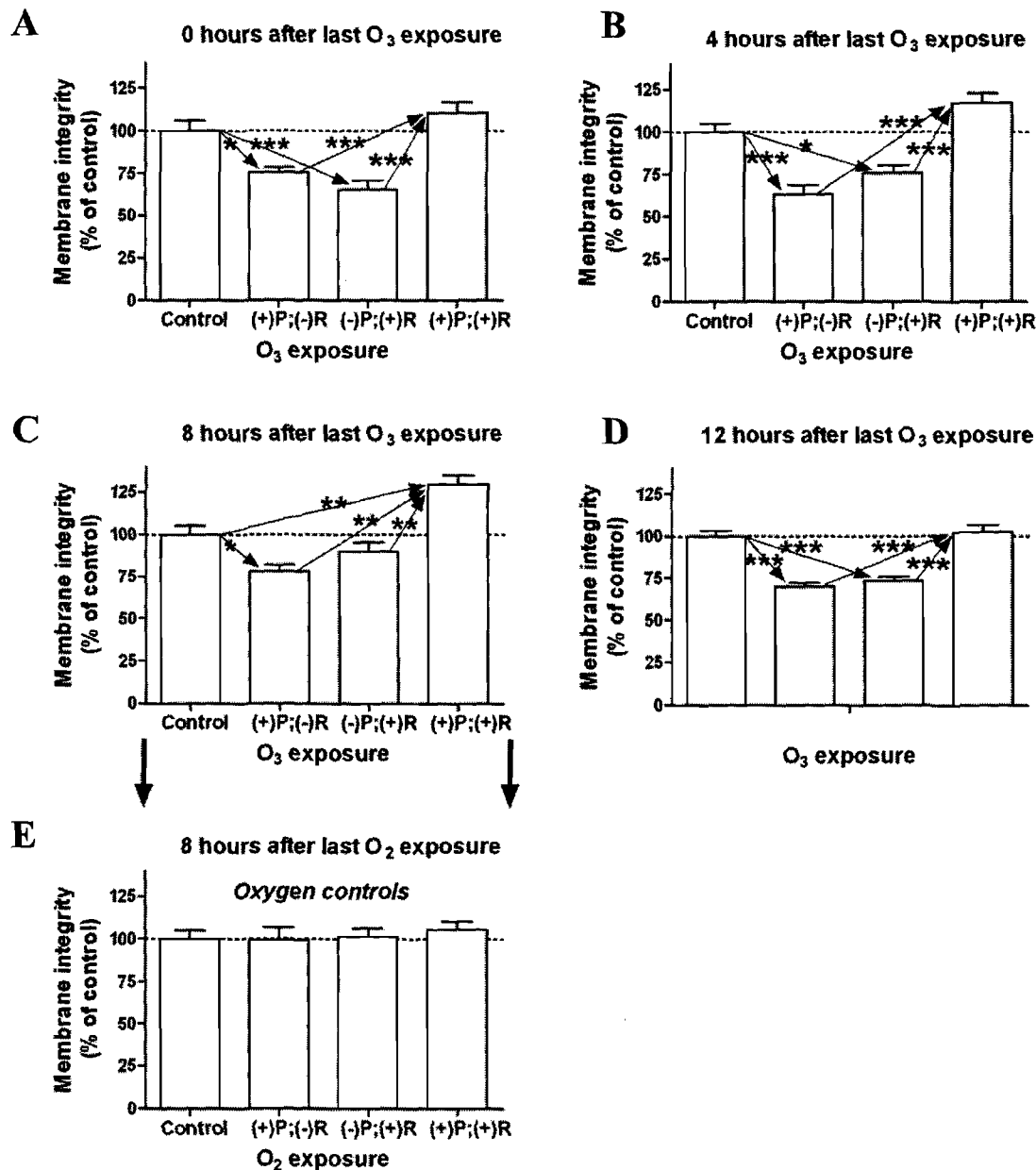


Fig. 3. The effect of different (including repeated) ozone exposure regimens on cell membrane integrity (A) 0 h after the final 're-exposure' step, (B) 4 h after the final 're-exposure' step, (C) 8 h after the final 're-exposure' step and (D) 12 h after the final 're-exposure' step, as measured by the Trypan Blue cell viability assay, as well as (E) an oxygen control for the respective exposure regimens 8 h after the final 're-exposure' step. Below the bars, the 4 × 5-min ozone (or oxygen control) exposures were designated pre-exposure P while the 25-min ozone (or oxygen control) exposure on day 2 was designated re-exposure R, with (+) and (-) before the symbols indicating whether or not ozone (or oxygen) was present during that exposure period. Data are averages ± SEM of triplicate measurements from three independent and comparable experiments and are expressed as percentage of the control. The data were analyzed statistically by performing a one-way ANOVA and then implementing the Tukey-Kramer post-test (comparing all values), with * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

following the 25-min ozone re-exposure R. However, a combination of pre-exposure P and re-exposure R to ozone (i.e. the bar with the (+)P;(+)R label) restores membrane integrity to the control value. The reduction in membrane integrity after the 25-min re-exposure R alone was expected, as this corresponds with the acute

ozone exposure, similar to the results in Figure 2. However, the decrease in membrane integrity with the repeated exposure regimen ($35 \pm 5.6\%$ in Fig. 3A) was not as pronounced as with the acute treatments ($89 \pm 3.0\%$ in Fig. 2A), which may be due to a degree of undefined adaptation induced during the 4×5 -min

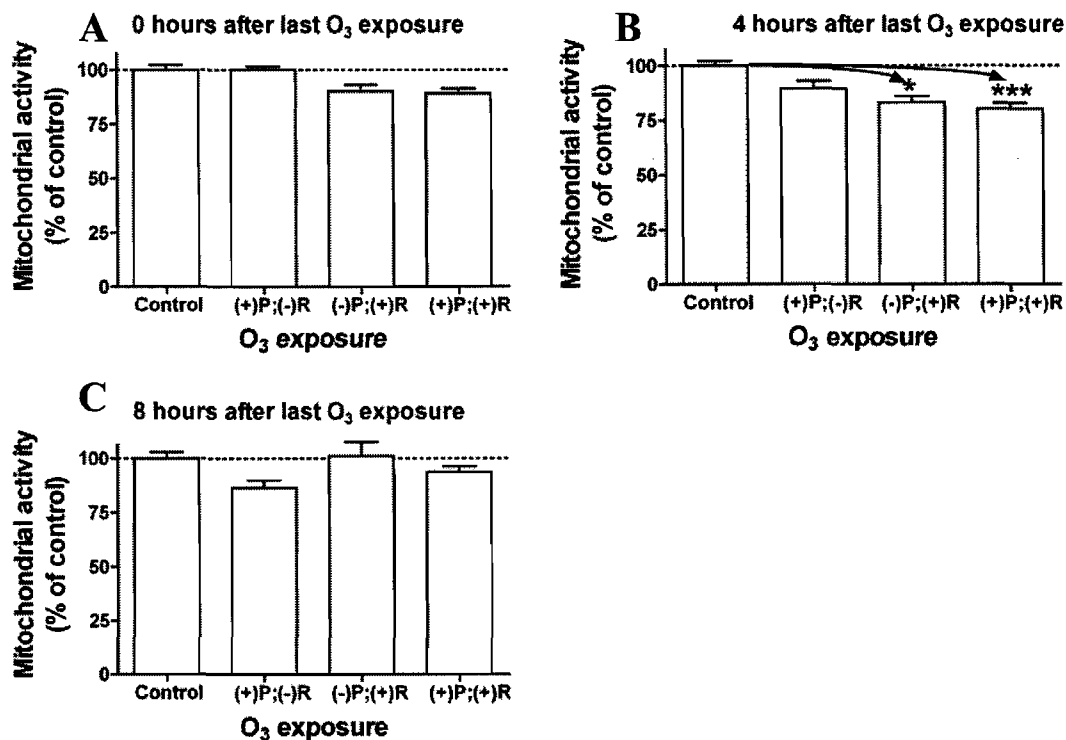


Fig. 4. The effect of different (including repeated) ozone exposure regimens on cell mitochondrial activity (A) 0 h after the final 're-exposure' step, (B) 4 h after the final 're-exposure' step and (C) 8 h after the final 're-exposure' step, as measured by the MTT cell viability assay. In the figure, the 4 × 5-min ozone exposures were designated pre-exposure P while the 25-min ozone exposure on day 2 was designated re-exposure R, with (+) and (-) before the symbols indicating whether or not ozone (or oxygen) was present during that exposure period. Data are averages ± SEM of triplicate measurements from three independent and comparable experiments and are expressed as percentage of the control. The data were analyzed statistically by performing a one-way ANOVA and then implementing the Tukey-Kramer post-test (comparing all values), with * $P < 0.05$ and *** $P < 0.001$.

pre-treatments with glucose-free Krebs-Henseleit solution in the repeated exposure regimen. One should, therefore, be cautious not to compare the two sets of data from Figures 2 and 3 directly. As can be seen in Figure 3B, the same trends as in Figure 3A are also present after a 4-h recovery period following the 25-min re-exposure R.

The most noteworthy observation regarding the effect of ozone on membrane integrity is depicted in Figure 3C. Here, when observing membrane integrity after both pre-exposure P and re-exposure R to ozone (*i.e.* the bar with the (+)P;(+)R label), followed by 8-h recovery, cell membrane integrity is significantly increased from control ($29.5 \pm 5.3\%$; $P < 0.01$). Therefore, this adaptive ('pre-conditioned') increase in cell membrane integrity is observed only after an 8-h incubation period. The observed adaptive response is, therefore, time-dependent, as well as dependent on the treatment regimen with ozone (*i.e.* repeated exposures of short duration to ozone [4 × 5 min], followed by a single exposure of longer duration [1 × 25 min], spaced 4 h apart as specified).

In Figure 3D, it can be seen that after 12 h the trend is again resembling that observed after 0 and 4 h (Fig. 3A,B)

and it is important to note that the increased cell membrane integrity observed for both pre-exposure P and re-exposure R to ozone (*i.e.* the bar with the (+)P;(+)R label) after 8-h incubation (Fig. 3C), now returns to pre-treatment values. The latter suggests a reversal of the cellular adaptation between 8–12 h, while the reduction in membrane integrity following only either pre-exposure P (*i.e.* the bar with the (+)P;(-)R label) or re-exposure R (*i.e.* the bar with the (-)P;(+)R label) to ozone was sustained.

As illustrated in Figure 3E, oxygen control does not elicit any significant changes in membrane integrity, confirming that the observed effects depicted in Figure 3C are indeed the result of ozone as such, and not due to any remnant oxygen that was not converted to ozone by the ozone generator or to higher pH of ozone-saturated physiological medium.

Effect of repeated ozone exposure on mitochondrial activity

It is evident from Figure 4 that mitochondrial activity is affected differently by ozone exposure than membrane

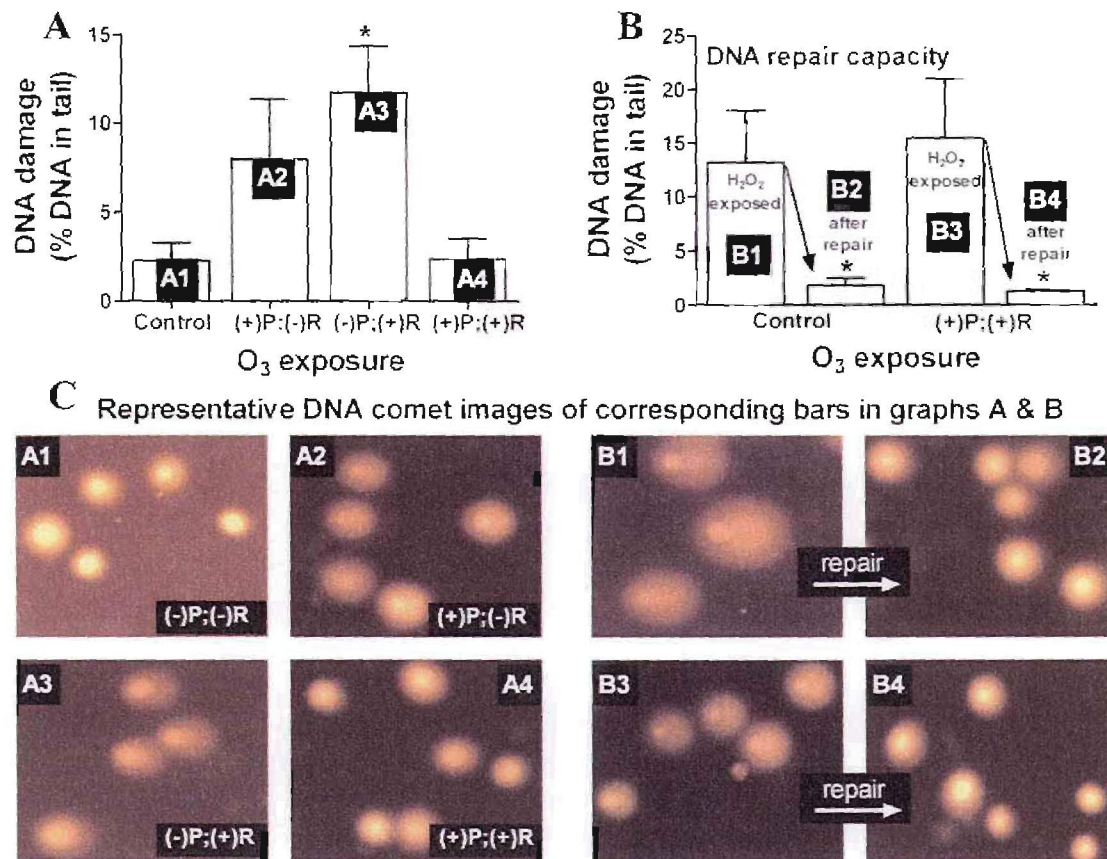


Fig. 5. The effect of different repetitive ozone exposure regimens on (A) DNA integrity and (B) DNA repair capacity in HeLa cells at 0 min and 40 min after a single H₂O₂ challenge, and (C) the corresponding representative DNA comet images of the data in (A) and (B). Data in bar graphs are the averages $\pm$ SEM of triplicate measurements from three independent and comparable experiments and expressed as the percentage of DNA in the tail. The data in (A) were analyzed statistically by performing a one-way ANOVA and then implementing the Dunnett's post-test (comparing values to the corresponding control), while the data in (B) were analyzed by performing one-tailed Student's *t*-test, with **P* < 0.05.

integrity (Fig. 3). The reduction in mitochondrial activity was statistically significant only after re-exposure R ($16.8 \pm 2.8\%$; *P* < 0.05; *n* = 9) or both pre-exposure P and re-exposure R to ozone ($19.8 \pm 2.7\%$; *P* < 0.001; *n* = 9) and only after 4 h following the 25-min re-exposure (Fig. 4B).

Effect of repeated ozone exposure on DNA integrity

Figure 5A depicts the DNA damage after the indicated repeated ozone exposure regimens. It is evident that a 25-min re-exposure (*i.e.* the bar with the (-)P;(+)R label), followed by 8-h incubation in culture medium, resulted in a statistically significant 5-fold increase in DNA damage (2.3 ± 1.0 to 11.8 ± 2.6 ; *P* < 0.05; *n* = 9). A trend towards DNA damage can also be seen after the 4 $\times$ 5-min pre-exposure (*i.e.* the bar with the (+)P;(-)R label), although this did not reach statistical significance. However, the DNA status after the combination of pre- and re-exposures (*i.e.* the bar

with the (+)P;(+)R label) clearly resembles the control without any ozone exposure (*i.e.* the bar with the (-)P;(-)R label), supporting the data obtained in Figure 3C. The representative images of DNA comets, corresponding to Bars A1–A4, can be seen in Figure 5C.

Figure 5B depicts the DNA repair capacity in HeLa cells following H₂O₂ treatment, with control cells (no ozone), corresponding to the (-)P;(-)R ozone treatment regimen, as well as following the repeated full (+)P;(+)R ozone treatment regimen. When comparing bars A1 and B1 of Figure 5, it can be seen that the H₂O₂ treatment alone induced significant damage to DNA (almost a 6-fold increase in DNA fragmentation). In this figure, Bars B2 and B4 demonstrate full repair of DNA, suggesting complete DNA repair capacity under these conditions and, again, supporting the data in Bars A1 and A4, as well as the data in Figure 3C. The representative images of DNA comets, corresponding to Bars B1–B4, can be seen in Figure 5C.

Role of anti-apoptotic and proliferative pathways in the cytoprotective effect of repeated ozone exposure

From Figure 6A it can be seen that (–)-deguelin attenuates the positive effect of repeated ozone exposure on membrane integrity. Also, (–)-deguelin alone, without ozone (*i.e.* white bar), behaves as expected, decreasing membrane integrity (via inhibition of an anti-apoptotic pathway). Figure 6B displays the calculated difference in membrane integrity before and after ozone with the co-treatment of (–)-deguelin (27.9 ± 5.0 versus -77.9 ± 1.9 ; $P < 0.001$; $n = 9$).

In Figure 6C, it can be seen that, although ozone alone does not reduce mitochondrial activity in the control cells (without enzyme inhibitor), ozone treatment in the presence of (–)-deguelin causes a significant decrease in mitochondrial activity. This observation is also supported by the statistically significant decrease in mitochondrial activity seen with deguelin treatment, as compared to control, in Figure 6D.

DISCUSSION

The current study demonstrates that acute exposure of cultured human epithelial HeLa cells to an ozone-satu-

rated physiological solution indeed rapidly and time-dependently induces deterioration of membrane integrity (Fig. 2A, Trypan Blue test). This observation correlates with previous related studies of other investigators.³⁷ This is accompanied by a somewhat delayed and markedly less pronounced decline in mitochondrial activity (Fig. 2B, MTT test), which is reversed over a period of minutes. The fact that the oxygen control shows a sustained decrease in mitochondrial activity, first suggests that the 55 min in glucose-free Krebs–Henseleit solution together with the high concentration of oxygen induce cytotoxic conditions. Second, it contrasts with the ozone-induced adaptation, suggesting that the latter protects the cells against the cytotoxic stressor. Likewise, differences between oxygen- and hydrogen peroxide-induced cellular damage have been described before,³⁸ so that the difference between oxygen and ozone, as seen here, can also be expected. These results from the current study also indicate that, while the cell membrane (the first-line defense against ozone exposure) is damaged most prominently, there may be an adaptive mechanism at the intracellular (mitochondrial) level to counteract the oxidative damage by ozone. Similarly, an adaptation of mitochondrial function in lung epithelial cells, following 4 days of exposure of rats to ozone, has been described by Bassett and co-workers,³⁹

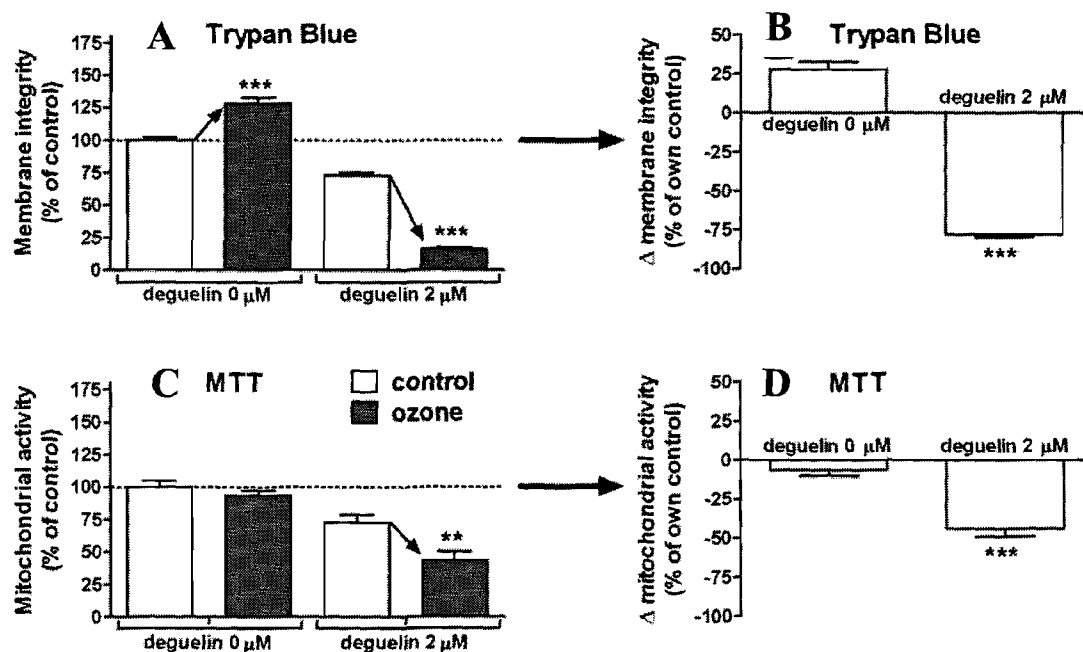


Fig. 6. The effect of the co-treatment of HeLa cells with the Akt inhibitor (–)-deguelin on (A) cell membrane integrity before and after repeated ozone exposure, as measured by the Trypan Blue cell viability assay, as well as (B) the calculated percentage of increase in cell viability (*i.e.* after repeated ozone exposure in comparison to the treatment without ozone) from the corresponding data in (A). Also, the effects of the enzyme inhibitors on (C) mitochondrial activity after repeated ozone exposure as measured by the MTT cell viability assay, as well as (D) the calculated percentage of increase in cell viability (*i.e.* after repeated ozone exposure in comparison to the treatment without ozone) from the corresponding data in (C). Data are the averages $\pm$ SEM of triplicate measurements from three independent and comparable experiments and data in (A) and (C) are expressed as percentage of the control. The data in (A) and (C) were analyzed statistically by performing the two-tailed Student's *t*-test, with $**P < 0.01$ and $***P < 0.001$, while the data in (B) and (D) were analyzed by performing a one-way ANOVA and then implementing the Tukey–Kramer post-test (comparing all columns).

while associated adaptive mitochondrial defense mechanisms have been described.⁴⁰ Adaptation to ozone exposure has also been well documented for lung function and inflammatory markers.^{20,41} It should be noted, however, that mitochondrial adaptation may be different in normal and cancer cells and that cancer cells can usually adapt to conditions under which the survival of untransformed cells would be poor or impossible. In particular, it has been shown that cancerous HeLa cells (as employed in the current study) can source energy entirely from glutaminolysis under conditions of glucose-deprivation,^{27,42} while it is known that cells may also obtain energy from lipolysis and ketogenesis for survival. Nevertheless, the relatively short periods of glucose deprivation in the current study may have influenced the relatively rapid metabolism of the cancerous HeLa cells, so that the current findings may underestimate the effect of ozone in normal cells. Also important to note is that, under the experimental conditions employed in the current study, extracellular (membrane) and intracellular (mitochondrial) effects of acute ozone exposure are not directly correlated.

The present study further found that repeated exposure of cultured human epithelial HeLa cells to an ozone-saturated physiological solution (Fig. 3) influences cell viability in a treatment regimen-dependent and a time-dependent manner. First, the treatment regimen-specific effects show that only when cells are repeatedly exposed for short durations to ozone (*i.e.* 4 × 5-min exposures), followed by a single exposure of longer duration to ozone (*i.e.* 25 min exposure), adaptation and increased cell membrane integrity develops thereafter over a period of hours. Second, the time-dependency for the development of increased cell membrane integrity (*i.e.* adaptation) is evident from data showing that this effect occurs not at 4 h but at 8 h following the last ozone exposure and a reversal of the adaptation after 12 h. The reason for the observed reversal of the adaptation after 12 h has not been investigated. While adaptation can be viewed as a dynamic (not permanent) process, the reversal may also, at least in part, be explained by the normal cell culture growth (multiplication) with gradual addition of new untreated cells. The adaptation in membrane integrity after 8 h is further supported by data regarding DNA integrity and DNA repair capacity (Fig. 5), where repeated ozone exposure is followed within hours by the development of increased DNA integrity and repair capacity. However, changes in mitochondrial activity (Fig. 4) do not parallel the effect on cell membrane integrity for corresponding treatments (Fig. 3). In this regard, the initial decrease of mitochondrial activity after 4 h (Fig. 4B) is restored after 8 h (Fig. 4C).

Another study also suggested that new proteins may be synthesized after pre-conditioning against oxidative stress.⁴³ It was postulated that the proteins synthesized

include heat shock proteins and antioxidant enzymes, although other proteins may also be synthesized. It has also been shown that levels of the caspase-3, superoxide dismutase, glutathione peroxidase and glutathione reductase are increased after 4-hydroxynonenal (HNE) treatment.⁴⁴ HNE, a secondary aldehyde formed during ozone exposure, also promotes the inactivation of the anti-apoptotic enzyme Akt⁴⁵ and induces apoptosis.⁴⁶ Other oxidative substances (such as H₂O₂) also induce apoptosis; however, when cells are pre-conditioned with H₂O₂, they can be protected against apoptosis.⁴⁷ The data from the current study (Fig. 3), therefore, prompted further investigation into the role of apoptotic and anti-apoptotic pathways.

In addition, Hamilton and co-workers⁴⁶ have reported that ozone induces single-stranded DNA breaks and produces cytosolic DNA fragments. With repeated ozone exposures, however, the DNA repair enzyme poly-(ADP-ribose) synthetase and the free radical scavenging enzyme superoxide dismutase are induced,⁴⁸ resulting in cellular DNA repair. Therefore, the data regarding DNA damage and repair in Figure 5 suggest an adaptation mechanism induced by repeated ozone exposure, in particular the exposure of HeLa cells to the (+)P;(+)R ozone treatment regimen, which also support the data in Figure 3C.

Finally, the study shows that the mechanism by which repeated ozone exposure induces increased membrane integrity involves the activation of anti-apoptotic pathways, which may include increased activity of Akt (Fig. 6). The activation of Akt as a protective response mechanism during the exposure of lung epithelial cells to ozone has been demonstrated before.⁴⁹ Anti-apoptotic pathways may, therefore, play a significant role in the adaptation induced by repeated ozone exposure, eventually leading to the improved membrane integrity seen in the current study with the (+)P;(+)R ozone treatment regimen. In fact, it may be reasonable to speculate that, under the experimental conditions employed, the activation of anti-apoptotic pathways by ozone may mask a simultaneous underlying detrimental effect of ozone on the mitochondrial activity in these cells.

The current study did not investigate in further detail the mechanism whereby anti-apoptotic pathways are activated, which is currently being investigated in continued studies. Furthermore, the *in vivo* relevance of these results (from valuable screening tests in an *in vitro* model) needs to be determined in animal studies.

CONCLUSIONS

This study suggests that ozone may induce differential extracellular and intracellular damage and adaptation in a treatment regimen dependent manner (*i.e.* dependent

on both exposure duration and dosing schedule). Adaptation may develop over a period of less than an hour (mitochondrial activity) or several hours (membrane integrity). Adaptation processes also include the induction of anti-apoptotic and DNA reparative mechanisms. While the current data suggest that the effect of ozone on cellular plasticity may be dependent on whether ozone is administered acutely or repetitively, it may be speculated that for the therapeutic use of ozone the specific treatment regimen (dose and dosing schedule) may be critical in determining the outcome. As such, one should be aware of potential inter-individual variation in response due to the suggested delicate balance between the potential beneficial and detrimental biological effects of ozone.

The findings of this study warrant further investigation into the biological effects and mechanisms of ozone on cellular plasticity. While the administration of ozone has been shown to elicit systemic (including central) effects, it may hold therapeutic potential in various degenerative disease states. It should also be verified whether the adaptive and other effects and mechanisms described in the current study are tissue and cell type specific and to what extent they apply to the *in vivo* situation using different administration routes.

ACKNOWLEDGEMENTS

The project was supported financially by a grant from the South African National Research Foundation. The authors wish to express their gratitude to Mr Jaco Lotriet for advice on practical issues related to the generation of ozone and measurement of ozone concentration, and Mrs Maureen Steyn and Mrs Sharleen Nieuwoudt for general laboratory assistance and the handling of logistic matters related to the project.

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