THE EFFECTS OF OZONE EXPOSURE ON THE VIABILITY AND FUNCTION OF CULTURED HUMAN CELL LINES

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B.Pharm.

Dissertation submitted in partial fulfilment of the requirements for the degree

MAGISTER SCIENTIAE

in

PHARMACOLOGY

at the

NORTH-WEST UNIVERSITY

(POTCHEFSTROOM CAMPUS)

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POTCHEFSTROOM

2005

ABSTRACT

THE EFFECT OF OZONE EXPOSURE ON THE VIABILITY AND FUNCTION OF CULTURED HUMAN CELL LINES

Ozone exposure (O_3) has been shown to have systemic biological effects, including dose-dependent oxidative stress and adaptation. However, the mechanisms of these effects remain elusive.

The aims of the current study were to i) establish appropriate conditions for *in vitro* O_3 acute and repeated exposures, utilising cultured human epithelial (HeLa) cells, and ii) investigate effects of acute and repeated O_3 exposure on cell viability. The involvement of enzymatic pathways in observed cellular adaptation was investigated by including selective enzyme inhibitors and observing changes in DNA integrity with O_3 exposures.

Cultured HeLa cells were exposed to O_3-saturated Krebs-Henseleit solution using various dosing regimes, including acute (0-55 minutes) and repeated exposures (4 × 5 minute O_3 exposure every 4 hours, followed by overnight incubation, 25 minutes O_3 re-exposure and 0, 4, 8 or 12 hours incubation). Thereafter cell viability was determined utilising the trypan blue, MTT and DNA-fragmentation assay. O_3 exposures were also performed in the presence or absence of ME10092 (xanthine oxidase and NFκβ inhibitor), Z-DQMD-FMK (caspase-3 and -6 inhibitor) and (-)-deguelin (Akt inhibitor).

According to the trypan blue test, acute O_3 exposure compromised cell membrane integrity, while the MTT test indicated only a slight reduction in mitochondrial function. A repeated exposure regime consisting of multiple small dose exposures followed by a single high dose exposure was associated with a protective adaptation in cell membrane integrity. This was reversed by inhibition of Akt, caspase-3, xanthine oxidase and NFκβ. Repeated O_3 exposures increased DNA integrity and repair capacity.

In conclusion, the current data suggest that acute *in vitro* O_3 exposure decreases HeLa cell membrane integrity, with no significant effect on mitochondrial function. Importantly, regime-specific multiple exposures to O_3 induces an adaptive response, whereby cell plasticity is upregulated. The latter adaptive effect is associated with the modulation of apoptotic and anti-apoptotic pathways.

OPSOMMING

DIE EFFEK VAN OSOON BLOOTSTELLING OP DIE OORLEWING EN FUNKSIE VAN MENSLIKE SELLYNE

Daar is aanduidings dat osoonblootstelling (O_3) sistemies biologiese effekte veroorsaak, insluitend dosis-afhanklike oksidatiewe stres en adaptasie. Die meganisme(s) van hierdie effekte is egter nog onbekend.

Die doel van die huidige studie was om i) geskikte kondisies vir *in vitro* akute en herhaalde O_3 blootstellings vas te stel deur gebruik te maak van menslike epiteelselle (HeLa), en ii) om die effekte van akuut en herhaalde O_3 blootstellings op sel-lewensvatbaarheid te bepaal. Die ensiemstelselbane betrokke by die waargenome adaptasie is ondersoek deur geselekteerde ensieminhibeerders in te sluit en die verandering in DNA integriteit met O_3 blootstellings waar te neem.

Gekweekde HeLa selle is blootgestel aan O_3-versadigde Krebs-Henseleit oplossing vir verskeie doseringsregimes, insluitend akute (0-55 minute) en herhaalde blootstellings (4 × 5 minute O_3 blootstelling elke 4 ure, gevolg deur oornag inkubasie, 25 minute O_3 herblootstelling en 0, 4, 8 of 12 ure inkubasie). Daarna is die sel-lewensvatbaarheid bepaal deur gebruik te maak van die trypan blou, MTT en DNA-fragmenteringsbepalings. O_3 blootstellings is ook uitgevoer in die teenwoordigheid of afwesigheid van ME10092 (xantienoksidase- en NFκβ-inhibeerder), Z-DQMD-FMK (caspase-3- en –6-inhibeerder) en (-)-deguelin (Akt-inhibeerder).

Volgens die trypan blou toets, het akute O_3 blootstelling die selmembraanintegriteit benadeel, terwyl die MTT slegs 'n minimale vermindering in mitochondriale funksie aangetoon het. Herhaalde blootstellings-regime wat uit meervuldige klein dosis-blootstellings bestaan het, gevolg deur 'n enkele hoë dosis O_3, het 'n beskermende effek in selmembraanintegriteit tot gevolg gehad. Dit is omgekeer deur inhibisie van Akt, caspase-3, xantienoksidase en NFκβ. Herhaalde O_3 blootstellings het DNA-integriteit en -herstelkapasiteit verbeter.

In samevatting, suggereer die huidige data dus dat akute *in vitro* O_3 blootstellings HeLa selmembraanintegriteit verminder met geen betekenisvolle effek op mitochondriale funksie. Belangrik is dat regime-spesifiek meervuldige O_3 blootstellings seladaptasie t.o.v. O_3 induseer, waardeur selplastisiteit opgereguleer word. Die laasgenoemde adaptiewe effek word geassosieer met die modulering van apoptotiese en anti-apoptotiese bane.

ACKNOWLEDGEMENTS

To my heavenly Father, for being with me every step of the way, carrying me and giving me the strength and perseverance to accomplish my goal. Lord, it is in the small detail that we often find You. I stand astonished by Your creation once again.

I would like to express my sincere appreciation to the following people:

To my study leaders, **Prof. C.B. Brink, Prof. D.W. Oliver** & **Prof. D.P. Venter**, for advice, support, assistance, guidance and their invaluable contributions, especially during the compilation of this dissertation.

Mrs. Maureen Steyn & **Sharlene Nieuwoudt**. Thank you for assisting me with many of my experiments in the cell culture laboratorium and for always lending a helping hand.

Mrs. Anriette Pretorius for assistance with the bibliography of this dissertation.

The **Department of Physics**, North-West University (Potchefstroom Campus), for kindly supplying the ozone generator for the period of my study.

The **NRF**, for funding this project and making it possible to complete the project.

To the people who shared every moment of joy, sorrow, anguish, uncertainty and computer illiteracy with me. **Jacolene, Izelle, Anél, Tanya, Elzeri** & **Tania**. You were my colleagues and my friends. Your support throughout these two years is greatly appreciated.

Jaco Lotriet, for advice and assistance during the project.

To my **Mother, Father** & **Michelle**, thank you for supporting me throughout my master study. Your input and continued motivation have helped me to achieve success. I love you very much!

A very special thanks to **Cules**. You are a big reason for this project being such a success. May we always find our strength in God and one another.

"To do successful research, you don't need to know everything.

You just need to know of one thing that isn't known."

~ Arthur Schawlow ~

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CHAPTER 1

INTRODUCTION

1.1. PROBLEM STATEMENT

During the last century, man has made significant achievements such as figuring out how to travel to the moon, make computers and transplanting human organs. But as a new century begins, humans still have far to go in meeting their energy needs without destabilising the atmosphere. Air pollution, including ozone, affects the health of thousands of people each year - some severely enough to require hospitalisation. It may also have detrimental effects on the environment.

The attention of scientists, politicians and the general public was focussed on ozone when the so-called "hole" in the ozone layer (higher atmospheric layer), due to CFCs and other gaseous pollutants of industrialised countries, was discovered. However, more and more research now also focuses on the excessive ozone levels in the troposphere (atmosphere close to earth's surface) due to gaseous pollutants and claims for medical and industrial applications of ozone as well as the associated health risks thereof.

Since the discovery of ozone in 1840, significant research has been done on the possible uses in the industry and medical fields as well as on the toxicological and pharmacologic effects of ozone on the environment and human body. In addition, ozone is increasingly being marketed as disinfectant for both air and water in industries, offices and even houses. In the medical field, ozone is being utilised to treat wounds, cancers, bacterial and viral infections (including HIV). These increasing utilisations and applications of ozone have given rise to the question about the safety of ozone when humans are exposed to the gas. It is of the utmost importance to identify probable therapeutic pharmacological effects and to determine whether acute or chronic exposure has toxic, carcinogenic and/or other long-term side-effects. Current scientific research data are scarce and too many unfounded claims and misinterpreted or low-quality research data are in circulation.

Ozone is naturally present in the troposphere at ultra low concentrations, while industrialisation is associated with raised concentrations of ozone and its reaction products. Numerous studies during the past four decades have shown that inhalation of low-level (<1.0 part per million) ozone may cause lung injury as suggested by studies investigating biochemical, pathological and physiological alterations in experimental animals and humans (DeLucia *et al.*, 1975;

Menzel, 1984; Mustafa, 1990; Devlin *et al.*, 1991; Mustafa, 1994). Many untoward effects of ozone exposure have already been documented including increased sensitivity of the airways, epithelial damage and neutrophil infiltration in the airways. Isolated organ (guinea pig trachea) investigations conducted by the Pharmacology Department of the North-West University (Potchefstroom campus) also suggested many toxicological effects of ozone (Lotriet, 2003). In the long term, ozone can aggravate existing health problems such as asthma, emphysema, pneumonia and bronchitis. Exposure of experimental animals to ozone for periods of up to a few days causes extensive airway epithelial cell damage and major epithelial cell populations and non-ciliated secretory intermediate cells display metaplasia and hyperplasia (Harkema *et al.*, 1987), while chronic exposure of tracheal epithelium does not affect the epithelium as significantly (Nikula *et al.*, 1988).

Investigations have now turned to *in vitro* exposure techniques to allow the investigation of the effects of ozone exposure on cells under controlled conditions. The mechanism(s) underlying the systemic effects of ozone in humans are still unclear. The ability of ozone and its secondary reaction products to cause cellular damage is linked to its powerful oxidative capacity and involves the peroxidation of cell membrane components (Wright *et al.*, 1990). It is hypothesised that ozone itself cannot penetrate deeply into the lung, based on its high reactivity with unsaturated fatty acids (Pryor & Church, 1991). Rather, ozone rapidly reacts with the polyunsaturated fatty acids in the epithelial lining fluid and airway epithelial cells to produce reactive oxygen species (ROS) such as hydrogen peroxide and aldehydes as intermediates (Pryor & Church, 1991; Pryor, 1994). These products are more stable than ozone and diffuse into the underlying tissues reacting with other bio-organic molecules in the body and causing cellular damage. The damage caused may be *via* the release of inflammatory factors and cytokines (Kafoury *et al.*, 1999) or by inducing oxidative stress in the cells. There are suggestions that ozone may elicit possible protective mechanisms following chronic low-dose exposures (Rahman *et al.*, 1991; Tepper *et al.*, 1989; van der Wal *et al.*, 1994; Devlin *et al.*, 1997; Frank *et al.*, 2001).

This study is aimed at investigating the effects of ozone exposure on cultured human epithelial cells and the possible mechanism for alterations in the cell viability.

1.2. STUDY OBJECTIVES

1.2.1. CENTRAL STUDY OBJECTIVE

The central theme of this study involves the subcellular mechanism(s) of the modulatory effects of ozone on cellular plasticity, in particular in cultured human epithelial cells. By understanding

the mechanism and influence of ozone on cellular plasticity, we will further our understanding of the potential therapeutic and toxic effects of ozone exposure.

1.2.2. SPECIFIC STUDY OBJECTIVES

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

1. To establish the appropriate conditions for the *in vitro* exposure of cultured human epithelial (HeLa) cells to ozone.
2. To determine the effects of acute and repeated ozone exposure on the viability of the HeLa cells *in vitro*.
3. To investigate the mechanism of the modulatory effects of repeated ozone exposure on cellular plasticity, including the involvement of enzymes associated with apoptosis and effects on DNA integrity and repair.

1.3. PROJECT DESIGN

All studies were performed 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 acutely or repeatedly, by adding ozonated Krebs-Henseleit solution to each well for the indicated exposure durations. After acute exposure cells were rinsed and cell viability determined using the trypan blue and MTT tests. After repeated exposure cells were rinsed and incubated for 8 hours in normal growth medium with FBS, whereafter the cell viability assays were performed.
- Enzyme inhibitors such as the caspase-3 and -6 inhibitor Z-Asp(OMe)-Glu-Met-Asp(OMe) fluoromethyl ketone (Z-DQMD-FMK), Akt inhibitor (-)-deguelin and the xanthine oxidase and NFκβ inhibitor N-(3,4-dimethoxy-2-chlorobenzylideneamino)-guanidine (ME10092) were introduced after repeated ozone exposures during the 8 hour incubation period, whereafter the cell viability assays were performed.
- HeLa cells were also exposed to these drugs 24 hours prior to acute ozone exposure. After the exposure, cell viability assays were performed to assess HeLa cell plasticity.
- After repeated ozone exposures, HeLa cells were assayed with the single cell electrophoresis (comet) assay to determine DNA integrity and repair.

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

CHAPTER 2

LITERATURE OVERVIEW

2. INTRODUCTION

Ozone occurs naturally in its gaseous phase in higher layers of the earth's atmosphere, where it serves to filter out ultraviolet sunlight. As it is chemically very reactive (associated with a relatively complex chemistry and a strong oxidising potential), it is usually not found in high concentrations in the lower troposphere, although industrialisation is associated with the production of large amounts of ozone and thereby pollution of the troposphere with ozone and its reaction products. In this regard, ozone is considered harmful to humans and other life forms at concentrations in excess of the maximum allowed by consensus in the troposphere.

Besides the challenges of pollution, it is disturbing that, while there is still relatively little known about the effects of ozone on biological systems, several non-scientific (including pseudoscientific) therapeutic claims for the application of generated ozone exist. Based on anecdotal data, ozone is currently actively employed as "alternative medicine" in humans to treat several conditions, including medical conditions and pathology. Scientific clinical data are incomplete, fragmental and sometimes contradicting in terms of the potential beneficial or harmful effects of ozone in humans, depending also on the conditions (dose, duration and route) of administration. Although it is clear that ozone and its reaction products interact with biological systems upon administration (inhalation or other ways), the underlying mechanism(s) of action leading to the observed effects on, for example, cellular plasticity has not yet been investigated and needs further elucidation.

This chapter will review and discuss the relevant scientific research that has already been done on ozone and the possible effects it may have on cellular plasticity. Chapter 2 is therefore divided into two sections. The first section discusses ozone and its effects on biological systems, while the second section provides a theoretical overview of possible mechanisms involved in cellular plasticity (more specifically oxidative stress, apoptosis and necrosis).

2.1. OZONE

2.1.1. BACKGROUND AND CHEMISTRY OF OZONE

Ozone has been the focus of significant research during the past few years. This gas prevents harmful ultraviolet radiation from penetrating deeper into the earth's atmosphere when present in the stratosphere. However, ozone plays a very different role when present in the lower troposphere together with other air pollutants. It is in the troposphere that the gas causes a variety of harmful effects in humans, animals and the ecosystem due to its strong oxidising properties.

2.1.1.1. IMPORTANT EVENTS IN THE HISTORY OF OZONE

Ozone was first discovered in 1840 by the German chemist Christian Friedrich Schönbein. He observed a characteristic smell during his experiments and named this gaseous substance "ozone" after the Greek word *"ozien"*, meaning odour or smelling (Shanklin, 2005). Schönbein presented his findings in a letter entitled "Research on the nature of the odour in certain chemical reactions" to the Academies des Sciences in Paris. After this, many scientists and researchers have investigated the physical properties, chemistry and biological effects of ozone. A summary of the most important dates in the history of ozone is given in Figure 2-1.

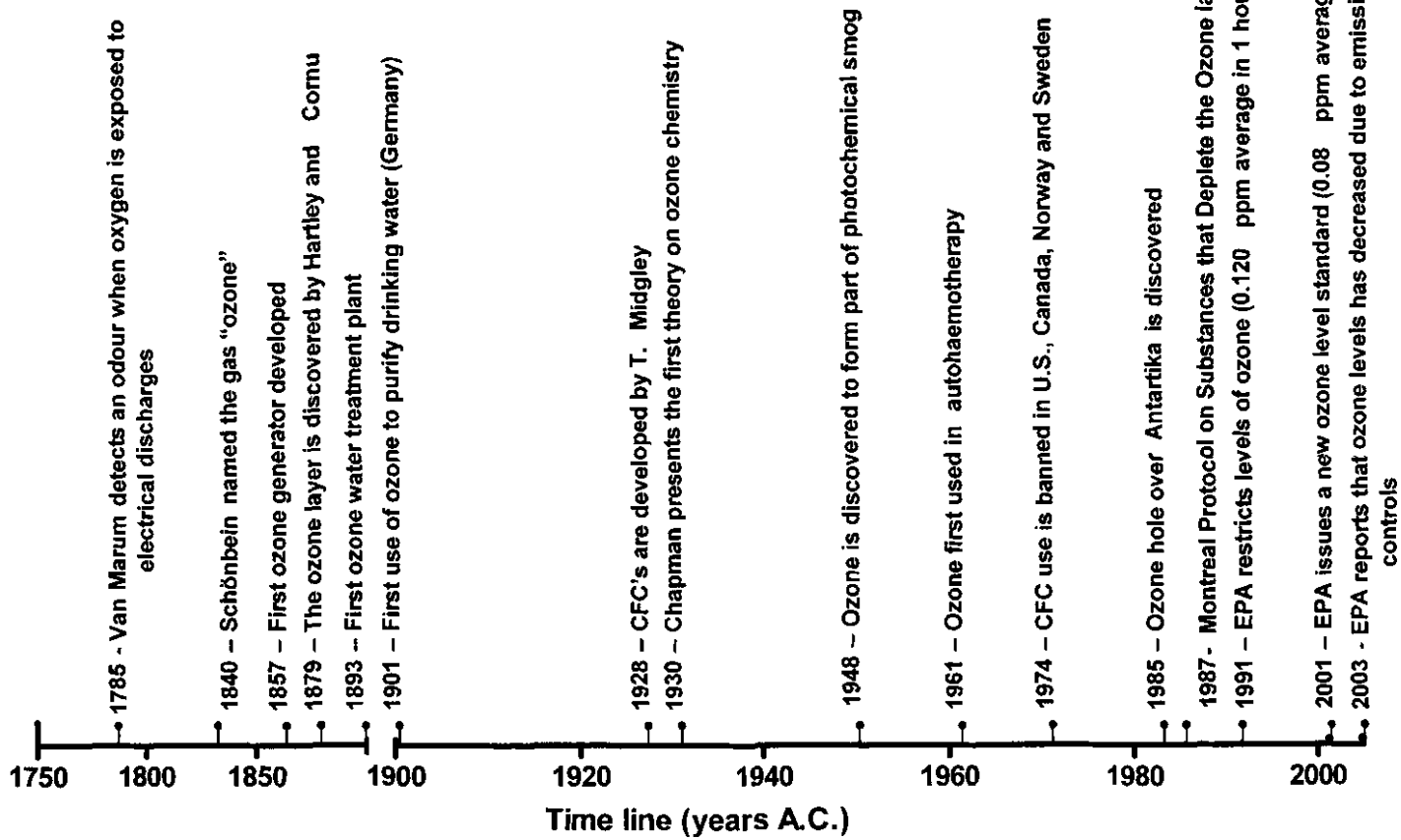


Figure 2-1 Timeline of key historical dates in the history of ozone.

2.1.1.2. THE STRUCTURE OF OZONE

To understand the basis of ozone's reactivity towards other molecules, it is necessary to understand its molecular structure. Ozone, consisting of three oxygen atoms (Figure 2-2), is highly unstable and the molecule tends to revert back to the more stable state of diatomic oxygen (O_2) by releasing one of the oxygen atoms. This is the basis of its oxidising properties, and explains its short half-life (t½) in the atmosphere and in solution, its inability to reach high concentrations systemically in biological systems as intact molecule and its complex chemistry with many reaction products.

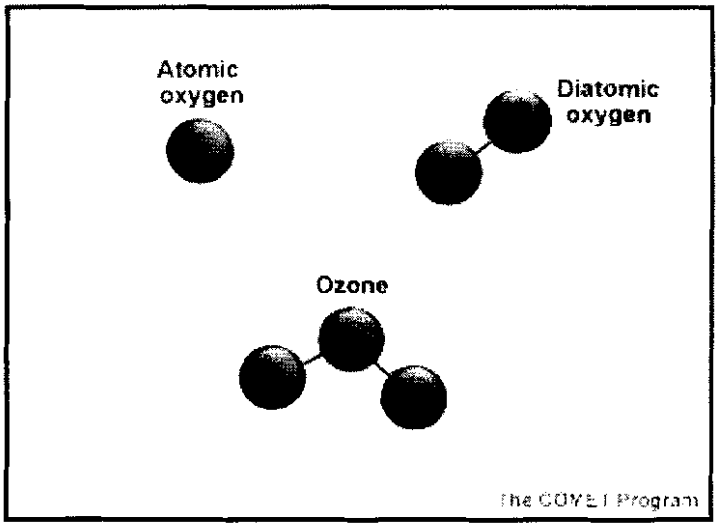


Figure 2-2 Representation of the molecular structure of oxygen (O_2) and ozone (O_3) (UCAR, 2001).

Ozone is not inherently static and has a resonance structure which can be represented as follows:

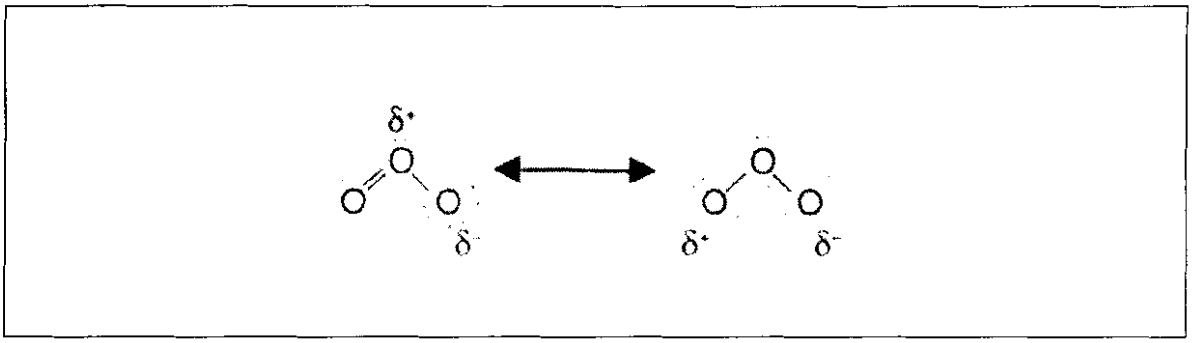


Figure 2-3 Representation of the resonance structure of ozone (Langlais *et al.*, 1991).

The resonance structure of ozone defines the electrophylic nature of its chemical reaction with other molecules.

2.1.1.3. PHYSICAL PROPERTIES

The most important physical properties of ozone are described in Table 2-1.

Table 2-1 Physical properties of ozone (MKS, 2005).

Property	Ozone (O_3)
Colour as a gas	Blue
Colour when dissolved in water	Purple-blue (concentrations > 20 ppm)
Molecular weight	48 g/mol
Boiling point	-112 °C
Density	2.144 g/cm^3
Solubility in water at 0 °C	0.64
Electrochemical potential	2.08 V
Half-life (35 °C) when dissolved in water at pH 7	7 minutes
Half-life (30 °C) when dissolved in water at pH 7	12 minutes

As a gas, ozone only has a half-life of approximately 20 minutes in open areas, while this half-life can increase to hours in enclosed areas with low temperature and humidity. This half-life however, decreases to approximately 7 minutes when ozone is dissolved in water (at 35 °C). In particular, the poor solubility and short half-life of ozone in water poses challenges in establishing appropriate experimental *in vitro* exposure systems for investigating the biological effects of ozone (MKS, 2005).

2.1.1.4. CHEMICAL PROPERTIES

The decomposition of ozone in water has multiple steps and has been described by two different mechanisms, namely the Hoigné-Staehelin-Bader (HSB) and the Gordon-Tomiyasu-Fukutomi (GTF) mechanism. These mechanisms are shown in Figure 2-4.

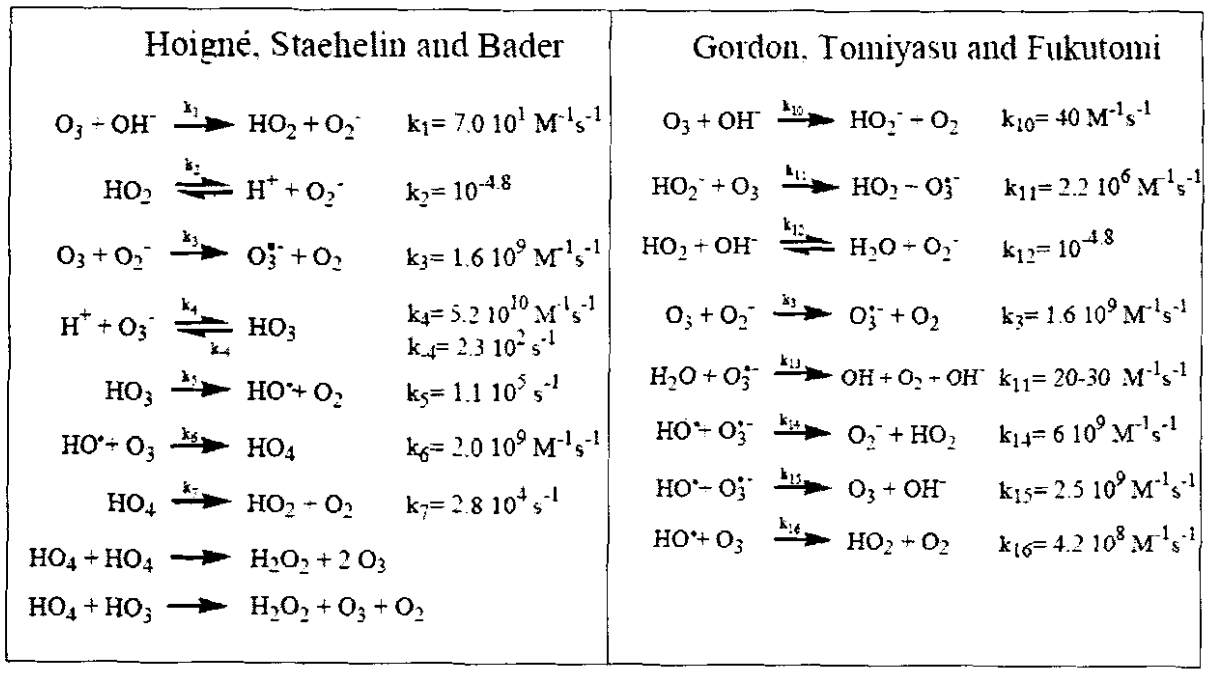


Figure 2-4 The decomposition of ozone in aqueous solution (Langlais *et al.*, 1991).

The fundamental principle of both reaction mechanisms in Figure 2-4 is the initial step, where ozone reacts with OH^-. Since the reaction with OH^- is the initial decomposition step, the stability of an ozone solution is thus highly dependent on the pH of the solution. Hoigné and Bader described the reaction of ozone in aqueous solution towards other compounds in two ways, namely by direct reaction or by indirect reaction with radical species (OH^-, O_2^-, $OH^\bullet$ etc.) formed in ozone decomposition (Langlais *et al.*, 1991). Hence, it is these secondary reaction products and not ozone itself that initiate the characteristic series of biological responses at the lung surface.

2.1.1.5. ATMOSPHERIC OZONE

The atmosphere around the earth consists of a number of layers (McIlveen, 1992) of which the stratosphere and the troposphere are the most important in the cycle of ozone formation, occurrence, reactivity and destruction.

2.1.1.5.1. STRATOSPHERE

The stratosphere, in which ozone is most abundant, is found between 25 and 40 km above the earth's surface. The formation and destruction of ozone in the stratosphere is driven by energy absorbed from ultraviolet rays from the sun. Wavelengths of 290 nm and shorter is absorbed by

ozone in this layer. If the ozone in this layer is therefore depleted, shorter wavelengths of UV light (<320 nm) can penetrate to the lower troposphere.

2.1.1.5.2. TROPOSPHERE

This layer extends up to 18 km into the atmosphere from the earth's surface. Here ozone is usually only present in relatively low concentrations in clean troposphere. Ozone concentrations become problematic when the troposphere is filled with other photochemical air pollutants such as nitric oxide species (NO_x), volatile organic compounds (VOCs), odd hydrogen species, peroxy radicals, hydrocarbons and carbon monoxide (CO). It is these compounds that react with ozone and oxygen to produce more ozone, increasing its concentration in the lower troposphere.

2.1.1.6. OZONE IN THE WORKPLACE

Ozone can be generated as a by-product by many processes where ultraviolet light (of the appropriate frequency) is present.

Photocopy machines and laser printers create low quantities of ozone. Zhou *et al.* (2003) examined the levels of ozone produced in offices with little ventilation. Ozone is formed in high quantities during the photocopying process and its levels increase dramatically as the office volume and/or ventilation is decreased. Most modern photocopiers and laser printers are however equipped with activated charcoal filters, which limit the amount of ozone released into the surrounding atmosphere.

Excessive ozone exposure is also a risk when using electric arc welding. UV light from the arc and oxygen in the surrounding air produces significant amounts of ozone, which may easily rise above maximum specified levels at the site of welding (Cole, 2001). Other sources for exposure to ozone are ultraviolet lamps and high voltage electric equipment.

In the United States of America, the Federal Occupational Safety and Health Administration (OSHA) regulate the maximum level (0.1 parts per million (ppm) average over an eight hour workday) of environmental ozone permitted in the workplace and indoors. In addition to the OSHA standards, the Food and Drug Administration (FDA) has established a level of 0.05 ppm as the maximum level allowable in an enclosed space intended to be occupied by people for

extended periods of time (Vistanomics, 2002). South Africa has now implemented similar air quality standards in September last year (SABS, 2004). In the document containing the proposed limits for air pollutants, the target limit for ozone measured over a one hour period was set at 200 $\mu g/m^3$ or 102 parts per billion (ppb), while a standard of 120 $\mu g/m^3$ or 61 ppb was set for the eight hour period (SABS, 2004).

2.1.1.7. POLLUTION AND ENVIRONMENTAL IMPACT

At ambient concentrations of 0 to 1 ppm, ozone can affect various aspects of plant growth (Grunhage & Jager, 1994). Effects on plant species and the surrounding environment include visible leaf injury (changes in foliar pigmentation and development due to impaired physiological processes), growth reductions, reduced net carbon dioxide (CO_2) exchange rate, increased leaf senescence, reduced leaf duration, increased production of ethylene, changes in the allocation of carbohydrates and altered sensitivity to biotic and abiotic stressors (Munster, 1998, EPA, 1996). Long term exposure to ozone may induce effects such as reduction of yields and relative growth rate due to reduced carbohydrate production and decreased allocation and resources needed for plant growth processes may be observed (Munster, 1998; EPA, 1996).

2.1.2. APPLICATIONS OF OZONE

2.1.2.1. COMMERCIAL APPLICATIONS

Ozone generators are marketed commercially for domestic use, or industrial use in public areas such as offices, hotels, restaurants and hospitals for (Finnegan Reztek, 1986):

- the purification of drinking water;
- the production of chemicals including synthetic fibres, jet lubricants and pharmaceuticals;
- the treatment of industrial liquid waste, such as cyanides and phenols;
- deodorisation of sewage gases, rendering plant exhausts and exhausts from other industrial processes;
- deodorising air in inhabited areas;
- food and plant preservation in cold storage (including eggs, vegetables, apples, cheeses, citrus and other fruits, nuts, poultry and meats); and
- sterilisation of containers for aseptic packaging.

The FDA has only approved the use of ozone in the treatment, storage, and processing of meat and poultry (Bureau of National Affairs, 2001).

2.1.2.2. MEDICAL APPLICATIONS

As mentioned in the introduction, ozone has already been utilised for the therapeutic treatment of certain medical conditions. External conditions are usually treated topically with ozone to inactivate or inhibit secondary pathogenic infection or to improve circulation to the affected area. The dosage of ozone is adjusted according to the condition. Some of the external conditions treated include (Sunnen, 2004):

- wounds (including poor healing wounds, decubitus ulcers (*via* ozonated olive oil); burns, infected wounds and frostbite);
- circulatory disorders such as diabetes and arteriosclerosis obliterans;
- lymphatic diseases such as lymphedema;
- fungal skin infections (i.e. *Candida albicans, Tinea pedis*) and nail afflictions;
- eczema and ulcers (with ozonated water); and
- in dental procedures (i.e. reversal of root caries).

In the past few years ozone has also been introduced into the circulation *via* major and minor autohaemotherapy (AHT). In major AHT, 50 to 100 ml blood is withdrawn from a patient, mixed with a predetermined dose of ozone and reinjected *via* intravenous catheter, while in minor AHT only 10 ml blood is withdraw and injected intramuscularly after ozonation. Some conditions that have already been treated with ozone include conditions such as (Sunnen, 2004):

- cancer (major and minor AHT);
- bacterial and viral infections (major AHT)
- acne (minor AHT);
- asthma and other allergic reactions (minor AHT); and
- ulcerative collitis, fistulae, haemorrhoids and proctitis (*via* rectal insufflations).

It is important, however, to note that these therapeutic applications of ozone is applied with little scientific basis of efficacy, side-effects and potential toxicity.

2.1.3. BIOLOGICAL EFFECTS OF OZONE

In the following section, an in depth overview is provided on the effects of ozone on different biological systems within mammals (including humans). Knowledge of these effects is crucial to the understanding of the mechanism by which ozone induces changes to biological molecules, tissue and systems.

2.1.3.1. EFFECTS OF OZONE ON ORGAN SYSTEMS

2.1.3.1.1. BASIC MECHANISM FOR OZONE'S REACTION WITH BIOLOGICAL MOLECULES

Ozone enters the lung on inspiration by a process of reactive absorption (Pryor, 1992). The respiratory tract surface is lined with a thin aqueous layer from the nasal cavity to the alveoli, sometimes referred to as the lung lining fluid (LLF). In aqueous environments such as within this lining, ozone displays a limited solubility (Miller *et al.*, 1993). Its half-life within the LLF has been estimated as 7×10^{-7} s (Pryor, 1992). Thus, when ozone is inhaled, it cannot penetrate deep into the tissue due to its high reactivity and decomposition in aqueous environments.

The composition of the LLF compartment determines the precise nature and extent of the reactions that take place. If there are sufficient antioxidants present, they will react with the inhaled ozone. When the incident level of ozone is low, the majority of ozone will be "neutralised" in the nasal passages and upper airways through reactions with ascorbate and glutathione (Pryor, 1992; Pryor, 1993). At higher ozone levels, or when LLF antioxidant defence concentrations are low or compromised, reactions between ozone and macromolecule targets such as proteins and lipids occur. In general, two main mechanisms are important, namely the oxidation of polyunsaturated fatty acids to acid peroxides and the oxidation of sulfhydryl groups and amino acids of enzymes, proteins and peptides.

Most of the ozone reacts with polyunsaturated fatty acids within the LLF (see § 2.1.3.3.5) (Pryor & Church, 1991). During the peroxidation of these lipids a cascade of secondary, free radical-derived, ozonation products or lipid peroxidation products (LOPs) are formed that mediate the cellular responses to ozone (Pryor, 1991). There is thus only a very small fraction of the total dose of inhaled ozone that passes through the bilayer membrane of the respiratory tract and none that passes through the cells (Pryor, 1992, Pryor, 1993).

The structures of the products formed after the ozonation of the lipids in the LLF can be determined by the Criegee mechanism of ozonation. Examples of these products include ozonides, aldehydes and free radicals such as the hydroxyl radical (•OH), singlet oxygen and hydrogen peroxide (Pryor, 1994). These secondary products are highly reactive and react with other biomolecules such as protein residues of lycine, histidine, tyrosine and tryptophan (Weiner *et al.*, 1999; Bisby *et al.*, 1999) or stimulate pro-inflammatory lipid mediators such as eicosanoids (Eling *et al.*, 1988), platelet activating factor (PAF) (Wright *et al.*, 1994), other reactive oxygen species (Menzel *et al.*, 1991) and cytokines (Noah *et al.*, 1991) that relay the effects of ozone.

2.1.3.1.2. RESPIRATORY SYSTEM

As explained above, the respiratory tract is the first system that comes into contact with the inhaled ozone. Inhalation of ozone in levels higher than 0.2 ppm may cause the following reactions in the respiratory tract (Environmental Protection Agency (EPA), 1986):

- bronchoconstriction mediated by an increase in airway reactivity (*via* cholinergic and vagal stimulation);
- dyspnoea (especially deep inspiration - humans)
- decreased tidal volume leading to tachypnoea; and
- increased pulmonary obstruction (signalling underlying inflammation (Aris *et al.*, 1993) and permeability changes).

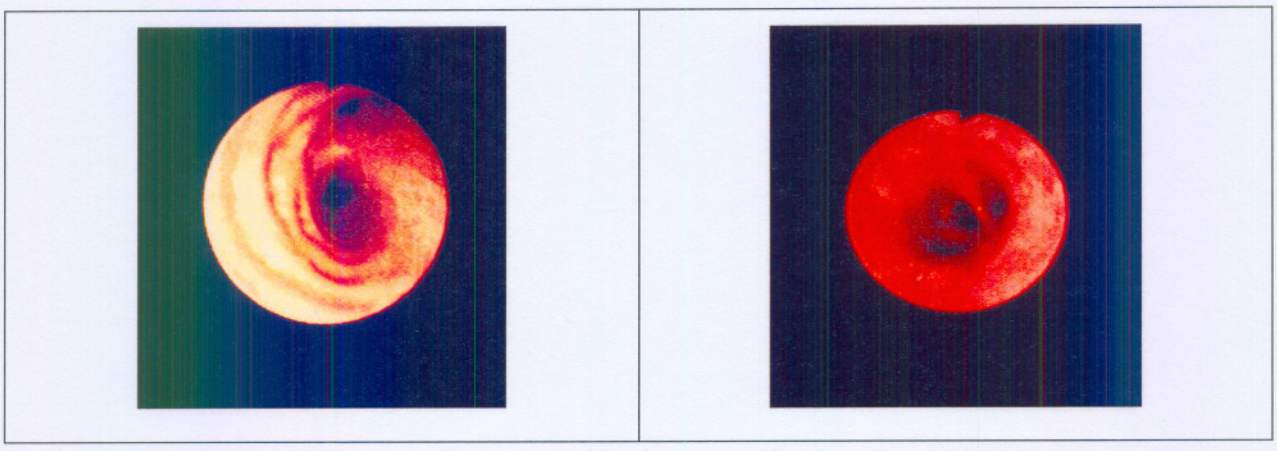


Figure 2-5 Comparison of a normal trachea (left) and an ozone-exposed trachea (right). Note the significant inflammation in the trachea after ozone exposure (EPA, 1999).

These symptoms may lead to decreased exercise tolerance and exasperated pulmonary diseases (such as asthma and emphysema) in humans.

2.1.3.1.3. SYSTEMIC EFFECTS

From the evidence collected so far, it is virtually impossible for any intact ozone molecule to enter the circulation *via* inhalation (Pryor, 1992). Nevertheless, a number of systemic effects after ozone inhalation have been demonstrated. These effects are believed to result from secondary products produced *via* the reaction of ozone with the LLF. Some effects include increased serum levels of hormones (e.g. thyroid-stimulating hormone, thyroid hormones, protein-bound iodine and prolactin) and xenobiotic metabolism of the liver (EPA, 1986) due to increased liver antioxidant enzymes.

2.1.3.1.4. HAEMATOLOGICAL AND SERUM EFFECTS

Whole blood contains various substances (amongst others free fatty acids) and enzymatic systems that are highly reactive with ozone. These substances and enzymes may have some protective effects on erythrocytes and help to reduce the impact of ozone exposure. Cataldo and Gentilini (2005) investigated the effect of ozone on whole blood. Their results show that ozone reacts specifically with haemoglobin, binds to the haeme and damages the prosthetic groups. Further, it was deduced that excessive exposure of blood to ozone causes the expansion and rupture of erythrocytes (haemolysis) (Cataldo & Gentilini, 2005). This haemolysis most likely involves the damage of the erythrocyte membrane since it is comprised of proteins, lipids and carbohydrates which are known to interact with ozone and LOPs. Cholesterol, also an important blood component, contains a double bond rendering it susceptible to reaction with ozone.

Ozone may therefore cause alteration of red blood cell morphology, its ability to bind oxygen as well as the osmotic fragility. These results clearly suggest that blood ozone therapy (i.e. AHT) may have far reaching implications and that the eventual unproven benefits (stimulation of defence systems in the body) may not justify the associated risks of haemolysis and other toxic products produced. In fact, ozone could be viewed as a toxicological agent in this setting (Cataldo & Gentilini, 2005). For ozone to be viewed as a pharmacological agent and a medicine it must comply with the same requirements of effectiveness, safety and quality as other medications.

2.1.3.1.5. CARDIOVASCULAR SYSTEM

Significant research has been done on the effect of ozone on human haemodynamic parameters, but results are very contradictory. Some researchers reported increased heart rate, decreased mean arterial blood pressure (Uchiyama *et al.*, 1986), arrhythmias (including atrioventricular block and premature atrial contractions) (Gong *et al.*, 1998), bradycardia (Arito *et al.*, 1990) and reduced maximal oxygen uptake (which may involve cardiac and/or ventilatory limitations) (Linder *et al.*, 1988), while others found no significant effect on these haemodynamic parameters (Superko *et al.*, 1984; Drechsler-Parks, 1995). Other effects observed by researchers included acute cardiovascular dysfunction (Uchiyama *et al.*, 1986; Uchiyama & Yokoyama, 1989; Arito *et al.*, 1990), microscopic myocardial pathology (Rahman *et al.*, 1992), acute reductions in cardiac output in anaesthetised dogs (Friedman *et al.*, 1983) and abnormal myocardial protein synthesis (Kelly & Birch, 1993).

The effects of ozone inhalation on blood pressure, heart rate, coronary artery tone and myocardial function (increased oxygen consumption and demand) was postulated to be due to adrenergic stimulation and elevation of catecholamines. This was investigated by Gong *et al.* (1998) and even though "baseline" catecholamine levels were elevated, most of their results did not support the hypothesis of adrenergic stimulation and catecholamine involvement as the catecholamine response pattern did not significantly differ between the control and ozone exposed subjects.

2.1.3.1.6. IMMUNE SYSTEM

The effect of ozone on the immune system has been examined only to a limited extent (Peterson *et al.*, 1981; Aranyi *et al.*, 1983; Dziedzic & White, 1986; Orlando *et al.*, 1988; Cohen *et al.*, 1996, 1998). The available data do, however suggest that T lymphocyte-mediated immunity, i.e. cell-mediated immunity (involved in host defence against infectious agents) is more susceptible to ozone than humoral (B lymphocyte-mediated) immunity, with respect to stimulation and activation of T lymphocytes by ozone (Dziedzic & White, 1986).

Pulmonary cells shown to be affected by exposure to ozone are the pulmonary alveolar macrophages (PAMs). These cells represent a primary defence of the lung and provide a link between non-immunologic and immunologic defence mechanisms. As immune cells, their major functions are to ingest and process antigens for presentation to naive T lymphocytes, to non-specifically kill micro-organisms and tumour cells, to kill antibody- and complement-tagged

cells and to secrete cytokines involved in auto-regulation and activation/deactivation of other immune cell types. Ozone exposure can alter PAM membrane fluidity and structure (Dormans *et al.*, 1990), resulting in changes of several membrane-related functional characteristics, such as agglutinability, mobility and F_c-mediated phagocytosis (McAllen *et al.*, 1981; Koren *et al.*, 1987; Prasad *et al.*, 1988; Oosting *et al.*, 1991; Becker *et al.*, 1991).

Cohen *et al.* (2001) examined the effects of ozone exposure on pulmonary cell-mediated immunity and whether local immune cell capacities could interact with immunoregulatory cytokines. Their data suggest that ozone does not alter cell-mediated responses *in situ* by modifying lung lymphocyte stimulation or production of IFNγ, but that it does alter the *in situ* production of IL1α, with levels recovering at after two days.

Therefore ozone may have various effects on the immune system by inducing cell-mediated immunity and the production of cytokines such as IL1α.

2.1.3.1.7. CENTRAL NERVOUS SYSTEM

The nervous system is the most susceptible to the deteriorating effects of the free radicals formed from ozone due to its high lipid content and oxygen consumption and low antioxidant activity (Rivas-Arancibia *et al.*, 2003).

In humans common complaints associated with ozone exposure affecting the central nervous system (CNS) include changes in mental performance, headache (due to constriction of the airways and bronchioles), lethargy, fatigue, nausea and dizziness (Hackney *et al.*, 1975). Animal research reported CNS effects such as impairment of behaviour, decreased locomotor activity and alterations in sleep patterns after acute ozone inhalation at doses higher than 0.5 ppm (Tepper & Wood, 1985; Dorado-Martinez *et al.*, 2001).

One of the most remarkable effects of ozone in the CNS has been observed when rats were exposed to 0.5 ppm for 6 hours and 1.0 ppm ozone for 3 hours. Electroencephalogram (EEG) activity, sleep-wakefulness and heart rate were examined and results suggest decreases in paradoxical sleep and increases in slow-wave sleep (Arito *et al.*, 1992). Arito *et al.* (1992) proposed that the changes in wakefulness and slow-wave sleep may be secondary to the ozone-induced bradycardia and that the bradycardia may result from enhanced

parasympathetic cardiac nerve stimulation. This stimulation may be due to the inactivation of presynaptic muscarinic acetylcholine receptors (mAChRs) by ozone.

In another study conducted by Rivas-Arancibia *et al.* (2000) significant alterations in short-term and long-term memory were observed in young and old rats after a 4 hour acute ozone exposure with doses ranging from 0.7 to 0.8 ppm. They observed a significant increase in lipid peroxidation levels in the striatum (necessary for memory acquisition and memory transference from short-term to long-term memory) and deterioration of long-term memory in the rats. In a follow-up study (Rivas-Arancibia *et al.*, 2003) rats received only a single dose of 1 ppm ozone for the duration of 4 hours. As with their previous results, ozone induced long-term memory deterioration, decreased motor activity (ozone may have an effect on dopamine and its metabolites generating changes in movement) and elevated superoxide dismutase levels in the brain tissue. After termination of exposure, these parameters returned to normal.

Thus, even though ozone as such may not reach the CNS, secondary active products formed through lipid peroxidation (i.e. LOPs) in the LLF diffuse through the blood brain barrier and cause CNS effects.

2.1.3.2. EFFECT OF OZONE ON CELLULAR BIOLOGY

The previous section reviewed the effects of ozone inhalation on whole biological systems, while the current section will discuss the effects of ozone on specific cell types within the body.

2.1.3.2.1. EPITHELIAL AND CILIATED CELLS

The effects of ozone on bronchial epithelial cells are of significant importance as these epithelial cells are the first to come in contact with the inhaled ozone and because these cells form a barrier that hinders antigen interaction with sub-epithelial inflammatory cells.

The most important factor determining the barrier properties of epithelia in the airways is the integrity of the tight junctions (formed by adjacent cell membranes). Under the circumstances of normal epithelial function and intact tight junctions, only a small amount of tracers inhaled finds its way to the underlying cells through *transcellular* pathways. Disruption of this epithelial barrier *via* ozone may result in some intact ozone reaching the underlying cells (through *transcellular* and *paracellular* mechanisms) due to increased barrier permeability (Kehrl *et al.*, 1987).

Disruption of the epithelial barrier following inhalation of ozone results in an increase in airway mucosal permeability (Bhalla & Crocker, 1986; Bhalla & Hoffman, 1997; Yu *et al.*, 1994). Thus, ozone can alter the maintenance of the epithelial barrier (Young & Bhalla 1992; Kleeberger & Hudak, 1992) and particle clearance function (Foster *et al.*, 1987). Barrier disruption is only transient in nature and an intact barrier is restored within a few days. During the state of breach in the lung epithelial barrier, it is, however, likely to be more susceptible to further injury through a simultaneous exposure to co-pollutants and allergens.

After acute inhalation of ozone, epithelial cells (especially type I epithelia) display degenerative changes such as increased membrane permeability, increased extracellular space, an increase in small mucous granule cells, increased cell density (hyperplasia) (Barry *et al.*, 1985, 1988; Moffatt *et al.*, 1987; Chang *et al.*, 1988; Chang *et al.*, 1992; Barr *et al.*, 1988) and replacement by underlying, ozone-resistant type II cells (Pino *et al.*, 1992). Cilia cells become shorter or are completely absent following acute ozone inhalation (Boorman *et al.*, 1980; Wilson *et al.*, 1984). These changes, however, resolve over a period of weeks following a single exposure and return to the pre-exposure state. Ozone may dose-dependently decrease the replicative ability of human bronchial epithelial cell cultures (Gabrielson *et al.*, 1994) and induce apoptosis and necrosis in epithelial and ciliated cell cultures (Cheng *et al.*, 2003; Boorman *et al.*, 1980; Wilson *et al.*, 1984).

2.1.3.2.2. GOBLET CELLS

Goblet cells are also present among the epithelial cells in the airway and are responsible for conditioning inspired air and the secretion of mucous assisting in the removal of inhaled air-borne particles. Upon exposure to ozone, goblet cells exhibit qualitative changes such as a decrease in secretory granules and dilated endoplasmic cisternae. These changes initiate hypertrophy of the lower tracheal submucosal glands leading to the hypersecretion of mucus in the conducting airways (Phipps *et al.*, 1986). This slows mucociliary clearance of inhaled particles and causes pulmonary obstruction.

2.1.3.2.3. MAST CELLS

Mast cells play a central role in inflammatory and immediate allergic reactions. They are able to release potent inflammatory mediators, such as histamine, proteases, chemotactic factors, cytokines, leukotrienes and metabolites of arachidonic acid that act on the vasculature, smooth

muscle, connective tissue, mucous glands and inflammatory cells. Increased submucosal mast cells in healthy subjects occur hours after ozone exposure is discontinued (Blomberg, 1999). The increase of mast cells in the airways and the subsequent release of histamine induce airway hyperresponsiveness and bronchoconstriction, which in turn may lead to increased asthma attacks in persons with this airway disease (Galàn *et al.*, 2003).

2.1.3.2.4. COLLAGEN

Inhaled ozone may induce fibrotic alterations in various animal lung tissues (Barr *et al.*, 1990; Boorman *et al.*, 1980). Studies found an increase in lung collagen, collagen synthesis and prolyl hydroxylase activity associated with fibrogenesis in rodents and primates acutely exposed to ozone (EPA, 1986; Last *et al.*, 1981). Collagen isolated from these ozone-exposed lungs showed abnormalities and collagen deposits in the lungs.

2.1.3.2.5. NEURONS

Many of the neurobehavioral changes have been linked to both structural plasticity (including changes in dendritic spine densities) and neurochemical plasticity. Avila-Costa *et al.* (1999) revealed that the pyramidal neurons of the hippocampus of rats exposed to 1 ppm ozone for 4 hours reduced the number of secondary and tertiary dendritic spines when compared to the control group. Their results were consistent with reports of Lescaudron *et al.* (1989), also observing reduced spine density on the dendrites of CA1 pyramidal cells after chronic ethanol consumption. As with acute ozone inhalation, chronic ethanol consumption also induces neuronal plasticity in adult animal brain. A reduction in spine density in the neurons of striatum and prefrontal cortex were also reported by Avila-Costa *et al.* (2001) after exposure to 1 ppm ozone for 4 hours.

Colin-Bareque *et al.* (1999) investigated the cytological alterations of the olfactory bulb after acute ozone exposure (1–1.5 ppm for 4 hours). They found that after rats were exposed to these ozone doses spine density was decreased in both primary and secondary dendrites. There was also evidence of vacuolation of dendrites and spines. This loss in dendritic spines may be associated with transneuronal degeneration and/or circulating free radicals in the blood. These fatty acid derived free radicals provoke membrane alterations and cause secondary neuronal damage (Sinet *et al.*, 1980).

2.1.3.3. SUBCELLULAR EFFECTS OF OZONE

2.1.3.3.1. PHARMACOLOGICAL RECEPTORS

Acute ozone exposure induces reversible airway hyperresponsiveness and bronchoconstriction (Schultheis *et al.*, 1994, Seltzer *et al.*, 1986). One proposed mechanism for the hyperreactivity is the inhibition or down-regulation of M_2 mAChRs. The parasympathetic nerves control the airway smooth muscle tone *via* the release of acetylcholine which interacts with postsynaptic M_3 mAChRs. Inhibitory M_2 mAChRs are situated presynaptically acting as autoreceptors so that activation of M_2 mAChRs decreases neural acetylcholine release. If these receptors are therefore down-regulated or inactivated, the release of acetylcholine is enhanced and this increases vagus-mediated bronchoconstriction. The blockade of M_2 mAChRs appears to be related to the release of major basic protein (MBP), an allosteric antagonist of M_2 mAChRs by eosinophils (Yost *et al.*, 1999).

2.1.3.3.2. PROTEINS

Proteins exert diverse functions throughout the body including the catalysis of the synthesis of biologically active substances, the transmission of information *via* membranes and the formation of connective tissues and cartilage (Styrer, 1995). Proteins are formed from a random sequence of twenty different amino acids. The sequence of these amino acids determines the primary structure and the nature of the protein. Other factors associated with the secondary and tertiary protein structure include the interactions involved in the macromolecular folding such as hydrogen bonds and disulfide cross-links.

Ozone inflicts damage upon proteins by oxidising a range of functional groups either by direct oxidation or *via* free radical mediated reactions (Freeman & Mudd, 1981; Cataldo, 2003). Functional groups that can be oxidised by ozone include sulphydryls, amines, alcohol and aldehydes. The "attack" of ozone on proteins is mainly directed towards the thiol groups and the aromatic amino acids (Cross *et al.*, 1992). The amino acids that are most susceptible to ozone are (Cataldo, 2003):

Tryptophan > Methionine >> Cystine >> Tyrosine >>> Phenylalanine

When ozone reacts with the amino acid structure, only the secondary and tertiary structures of proteins are modified (Cataldo, 2003). This is clearly shown *in vitro* by the change in optical rotation of a protein solution exposed to ozone and the precipitation of some protein from the exposed solution. The amide bond is resistant towards ozone attack and no chain scission takes place in the protein (Cataldo, 2003). Tsong (1974) investigated the effect of ozone on tryptophan and postulated that the reactivity of tryptophan in a protein with ozone depended on a number of factors:

- the position of the tryptophan in the membrane structure;
- the tertiary structure; and
- the chemical interaction within the protein.

Oxidation of tryptophan residues destabilise the proteins and the most significant destabilisation is caused by oxidation of the least exposed tryptophan.

2.1.3.3.3. ENZYMES

Various cytosolic, microsomal and mitochondrial enzymes show a decrease in activity immediately after short-term exposure to ozone (EPA, 1986; Mustafa, 1990). Antioxidant enzyme levels may, however, be increased after ozone inhalation. The effect of ozone on these enzyme systems is discussed in § 2.2.2.3.1 below.

2.1.3.3.4. DNA

As early as 1954 it was shown that bubbling ozone through a solution of DNA causes a rapid change in the UV spectra of the DNA sample, probably resulting from effects on the constituent purines and pyrimidines (Christensen & Giese, 1954). Ozone's ability to react with cellular DNA of the respiratory tract is fivefold, namely (Borek *et al.*, 1989; Last *et al.*, 1987):

- *via* its attack on DNA and other nucleic acids;
- *via* reactions with polyunsaturated fatty acids to form reactive aldehydes and free radicals;
- *via* its interaction with other molecules and the formation of free radicals;
- *via* the stimulation of tissue inflammation and
- an elevated influx of polymorphonuclear leukocytes, with subsequent increased myeloperoxidase activity.

A number of authors (Hamelin *et al.*, 1978; Van der Zee *et al.*, 1987; Rithidech *et al.*, 1990; Lee *et al.*, 1996; Ferng *et al.*, 1997) have clearly demonstrated that ozone causes DNA damage.

Ozone-related DNA damage can be induced both directly (*via* ozone molecules) and indirectly (*via* reactive oxygen species (ROS), inflammation, macrophages and leucocytes). In addition to its direct and potent oxidising capacity, ozone can readily oxidise cell lipids and proteins (Pryor, 1992), forming reaction products such as hydroxyl radicals (•OH), hydrogen peroxide (H_2O_2), superoxide anion radicals (O_2-), singlet oxygen, carbonyl substances and lipid hydroperoxides. These highly unstable molecules are recognised for their DNA damaging effects (Pryor *et al.*, 1991), which may be classified into DNA cleavages such as single-strand breaks, double-stand breaks and nucleotide base oxidative modifications (Halliwell & Aruoma, 1991; Kozumbo *et al.*, 1996). Indirectly DNA damage *via* ozone may be due to inflammation which results from the release of ROS as well as phospholipase A2-induced release of arachidonic acid and other fatty acids from membrane glycerophospholipids (Leikauf *et al.*, 1993; Salgo *et al.*, 1994). These free fatty acids may be oxidised by ozone and converted to harmful secondary reaction products. DNA damage may also be mediated *via* activated polymorphonuclear leukocytes and macrophages (Frenkel *et al.*, 1986; Cerutti *et al.*, 1983).

Any agent that causes DNA damage increases the probability of error in the DNA repair process. These errors can lead to cell mutations and alteration of DNA bases (Steinberg *et al.*, 1990) increases the possibility that DNA damage may lead to biochemical alterations that may induce malignant transformations in cells (Victorin, 1992; Cerutti *et al.*, 1983; Birnboim, 1983). It is in particular the nucleotides thymine and guanine that are the most sensitive to ozone (Shinriki *et al.*, 1984).

The relaxation, linearisation and degradation of supercoiled plasmid DNA, indicating single– and double-strand breaks, were described by Haney *et al.* (1999), Hamelin (1985), Nover *et al.*, (1985) and Sawadaishi *et al.* (1994, 1986). Double-stranded DNA breaks are generally thought to have greater biological consequences than single-stranded DNA breaks, since this may directly lead to chromosomal aberrations and more frequently to the loss of genetic information (Bryant, 1984). Ozone can also induce generation of DNA-interstranded cross-links (Van der Zee *et al.*, 1987). Nucleic acid base damage, DNA strand breaks and DNA stranded cross-linkage may result in reversible or irreversible consequences including cellular repair (Hamelin *et al.*, 1978; Hamelin, 1985), proliferation, differentiation, transformation and cell death (Cochrane, 1991).

DNA repair rate is decreased by ozone exposure (Hanley *et al.*, 1994). Cellular DNA repair is dependent upon the formation of poly (ADP-ribose) polymerase (PARP), which is catalysed by

PARP synthetase (activated in response to DNA damage). Bermudez (2001) and Hussain *et al.* (2001) investigated this enzyme activity after 0.3 ppm ozone exposure. Synthetase activity is stimulated with ozone exposure, resulting in lung cellular DNA repair initiated by the catalyses of PARP (Rasmussen, 1986).

The radical reaction of ozone with DNA is also applicable to the RNA molecule. This was investigated by Cataldo (2005), who found that purine and pyrimidine pendant groups were very susceptible to ozone damage, with guanidine being the most sensitive moiety.

2.1.3.3.5. POLYUNSATTURATED FATTY ACIDS

Polyunsaturated fatty acids are considered to be the most likely initial target of ozone in the lung. These fatty acids are abundant in the LLF and in cellular membranes. When ozone reacts with these lipids in cellular membranes, ozonation products such as lipid hydroperoxides are formed through so-called Criegee ozonation. This mechanism enables us to predict the structure of the secondary lipid peroxidation products formed after ozone exposure.

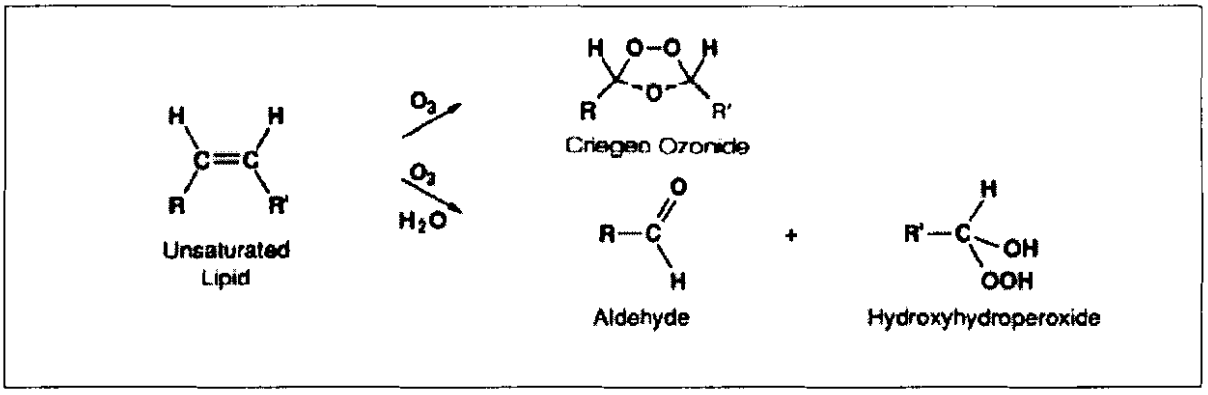


Figure 2-6 Representation of the lipid peroxidation products formed *via* the Criegee mechanism after ozone exposure (Pryor *et al.*, 1991).

In lipophylic environments ozonides are formed through the Criegee mechanism, while carbonyl oxide intermediates (aldehydes and hydroxyl radicals) are formed in an aqueous environment. Three major aldehydes are formed during ozone exposure: malonaldehyde, 4-hydroxy-2-nonenal (HNE) and 4-hydroxy-2-hexenal (Estbauer *et al.*, 1991). Of these metabolites, 4-hydroxy-2-nonenal is the most toxic. In addition to aldehydes, hydrogen peroxide and ozonides, short-lived organic radicals can be produced. All these intermediates act as second messengers of ozone and causes further damage to membranes and underlying cells (Pryor & Church, 1991; Teige *et al.*, 1974; Pryor, 1991).

2.1.3.4. EFFECT OF ACUTE AND REPEATED OZONE EXPOSURE

2.1.3.4.1. ACUTE EXPOSURE

The respiratory tract is particularly susceptible to damage acquired by inhaling toxic agents such as ozone, because it is directly exposed to the external environment. The amount of ozone that reaches the underlying respiratory tract tissue is less than the amount that is inhaled because ozone rapidly reacts with the protective LLF in the airway. A typical short-term exposure of lung tissue to ozone results in a biphasic response. There is an initial injury phase that is characterised by cellular damage and loss of enzyme activity, followed by a repair phase associated with increased metabolic activities, which coincide with a proliferator or metabolically active cells (EPA, 1986).

2.1.3.4.2. REPEATED EXPOSURE

Some untoward effects that occur after acute ozone exposure such as cell damage, inflammation and lung dysfunction seem to attenuate when ozone exposure is repeated for several days (Horvath *et al.*, 1981; van der Wal *et al.*, 1994; Leon *et al.*, 1998). This phenomenon of apparent recovery to normal values is frequently referred to as "adaptation", tolerance or attenuation. To the best of our knowledge, the first study on ozone "adaptation" was done in the late 1970s (Farrell *et al.*, 1979). Several mechanisms have been proposed to explain the observed adaptation. Of these, enhancement of antioxidant systems and replacement of sensitive cells by ozone-resistant cells are the most probable (Rahman *et al.*, 1991).

In toxicological studies of laboratory animals even a single exposure to ozone can produce adaptation to the acute injurious effects of a subsequent ozone challenge. With repeated short-term exposures to ozone, animal studies have consistently shown evidence of attenuation of pulmonary function responses (Tepper *et al.*, 1989; van der Wal *et al.*, 1994). The same observations were made in human studies. Repeated daily exposures to ozone in humans have induced adaptive responses involving forced expiratory volume (FEV), forced vital capacity (FVC), and bronchial reactivity (Hackney *et al.*, 1977; Horvath *et al.*, 1981). What has not been adequately characterised in humans and animals is the nature of the inflammatory response to ozone with repeated short-term exposures.

Christian *et al.* (1998) investigated the effect of single and repeated 0.2 ppm ozone exposures (for 4 hours on four consecutive days) on inflammation within the airways. The results of this study did not show progression of the acute inflammatory response to ozone with repeated short-term exposures. Neutrophils, fibronectin and IL_6 were significantly decreased in bronchoalveolar lavage (BAL) after the four day exposure, but there was no attenuation of IL_8, total protein or lactate dehydrogenase (LDH). This study indicates that although ozone-induced neutrophil recruitment to the respiratory tract is attenuated with repeated short-term exposures, airway epithelial injury may continue to occur. Persistence of such injury may lead to airway remodelling. Other investigations also reported this decline in the inflammatory response after repetitive ozone exposures (Devlin *et al.*, 1997; Frank *et al.*, 2001).

On the other hand, however, some studies suggest the opposite effect of repeated exposures of ozone on inflammation. Jörres *et al.* (2000) exposed healthy human volunteers to 200 ppb ozone for 4 hours on four consecutive days and concluded from their findings of increased total protein, IL_6, IL_8, reduced glutathione and tyrosine that airway inflammation persists after repeated ozone exposure, despite attenuation of some inflammatory markers in BAL. There is thus still more research to be conducted on the effect of repeated ozone exposures on the attenuation of inflammation in the airways.

2.2. OZONE AND CELLULAR PLASTICITY

The body is a dynamic system of which normal functioning depends on the capability to change/adapt at both molecular and cellular levels. Plasticity enables the cells in the body to adapt to environmental changes and challenges such as increased exposure to ozone. In the following section of this chapter the mechanism(s) of cellular plasticity will be discussed, focussing specifically on apoptosis, necrosis and the initiation of oxidative stress responses following ozone exposure.

2.2.1.1. OXIDATIVE STRESS AS TRIGGER FOR CELL DEATH

Oxidative stress is a general term used to describe the steady-state level of oxidative damage in a cell, tissue or organ, caused by an imbalance between the production of ROS and the availability and action of antioxidant molecules (Fernández-Checa *et al.*, 1998). This can affect either a specific cell, organ or the entire organism.

ROS such as singlet oxygen, hydroxyl, superoxide, nitric oxide peroxyl, lipid peroxyl, free radicals and lipid peroxides may be generated by different sources. Endogenous sources include the generation *via* normal metabolic reactions, while exogenous generation may be mediated by cigarette smoke exposure, a poor diet, various types of xenobiotics, environmental pollutants such as ozone, emissions from automobiles and industries, exposure to ionising radiation and bacterial, fungal or viral infections (Genox Corporation, 1996; Kirichenko *et al.*, 1996; Corradi *et al.*, 2002; Kelly, 2003).

Free radicals, as mentioned, are also classified as ROS. All sources of free radicals, both enzymatic and non-enzymatic, have the potential to inflict oxidative damage on a wide range of biological macromolecules including lipids, nucleic acids and proteins. One site that is particularly susceptible to peroxidising reactions is the cellular membranes (Lippmann, 1983). One primary effect of lipid peroxidation is a decrease in membrane fluidity, which alters membrane properties and can significantly disrupt membrane-bound proteins (Tappel, 1975). Free radical activity has also been shown to oxidise and cross-link proteins (amino acid residues are particularly susceptible to oxidative attack), enzymes and connective tissue.

2.2.1.1.1. OXIDATIVE STRESS AND OZONE

Ozone may produce a dose-depending oxidative stress state due to the increased production of free radicals *via* cell membrane lipid peroxidation (with subsequent cleavage of arachidonic acid), protein oxidation, enzymatic inactivation, destruction of DNA and cell apoptosis (Cross *et al.*, 1992).

Pryor and Church (Pryor, 1994; Pryor & Church, 1991) described how inhaled ozone generate toxic ROS species such as aldehydes, hydrogen peroxide, organic radicals and hydoxyl radicals through lipid peroxidation and protein modification (Theile *et al.*, 1997a; Weber *et al.*, 1999). Ozone inhalation also increases cellular and biochemical markers of inflammation in the BALF (Samet *et al.*, 2001; Vagaggini *et al.*, 2001) associated with the influx and activation of neutrophils in the airways (Vagaggini *et al.*, 2001). This inflammatory reaction results in an overproduction of ROS and hydrogen peroxide (Fievez *et al.*, 2001). Therefore, ozone inhalation not only induces oxidative stress (Mustafa, 1990; Postelthwait *et al.*, 1998; Wu *et al.*, 1999; Freed *et al.*, 1999), but also generates non-oxidant bioactive species that contribute to the oxidative stress state (Lippman, 1993; Uhlson *et al.*, 2002).

One of the toxic second messengers produced after ozone inhalation *via* lipid peroxidation is HNE (Kirichenko *et al.*, 1996). HNE induced an oxidative stress state in murine lung cells, leading to the induction of apoptosis (at low concentrations of HNE) and necrosis (at higher concentrations of HNE). In another study, the levels of two oxidative stress markers, 8-isoprostane and hydrogen peroxide, were increased immediately after the ozone exposure (Corradi *et al.*, 2002). These findings correlated well with previous observations of short-term ozone exposure increasing oxidative stress in the respiratory tract (Montuschi *et al.*, 2000). From these studies it is clear that inhaled ozone may produce secondary products that induce an oxidative stress state, leading to a decrease in cellular plasticity due to apoptosis or necrosis.

2.2.1.1.2. OXIDATIVE STRESS AND DISEASES

Oxidative stress can mediate the initiation of many diseases e.g. (Genox Corporation, 1996):

- Autoimmune Disease
- Alzheimer's Disease
- Cancer
- Cardiovascular Disease
- Cataractogenesis
- Diabetes
- Iron Overload
- Ischemic-Reperfusion Injury
- Ischemic-Reperfusion Injury
- Macular Degeneration
- Multiple Sclerosis
- Muscular Dystrophy
- Pancreatitis
- Parkinson's Disease
- Rheumatoid Arthritis
- Segmental Progeria Disorders

Ozone exposure induces the production of ROS (§ 2.1.3.1.1) and other free radicals *via* lipid peroxidation of airway cell membranes. As mentioned in § 2.2.1.1, the increased production of ROS may induce an oxidative stress state in cells. Therefore, long-term or chronic exposure to ozone may possibly contribute to the induction and progression of the above mentioned.

2.2.2. MECHANISMS OF CELLULAR DEATH

Each cellular organelle has the ability to sense stressful and pathogenic alterations and to initiate local or global responses to stress. This can lead ultimately to adaptation or, once a critical threshold of insult or damage has been reached, to cell death.

2.2.2.1. APOPTOSIS

Originally, apoptosis or programmed cell death (PCD) was defined as a distinct mode of cell death on the basis of a series of characteristic ultra structural features according to the following sequence of events: nuclear and cytoplasmic condensation, cell fragmentation and phagocytosis (Kerr *et al.*, 1972). Kerr *et al.* (1972) initially used the term "shrinkage necrosis" to describe this form of cell death. Subsequently they coined the term "apoptosis" (from the Greek apo for "apart" and ptosis for "fallen" to describe the falling of the leaves). One can also describe this type of cell death as a genetically-controlled "suicidal program" (Wyllie *et al.*, 1980).

Apoptosis may occur during normal cellular differentiation and plays an integral part in the development of multicellular organisms. It maintains tissue homeostasis, pathological conditions and ageing. The purpose of this process is the elimination of unwanted cells occurring in three situations, namely (1) development and homeostasis, (2) cellular defence and (3) ageing (Thompson, 1995). Divers cell types can be triggered to undergo apoptosis by various signals derived from either the extracellular of intracellular milieu. Triggers include activation of tumour necrosis factor (TNF), heat shock, viruses, protein synthesis inhibition, oxidative stress, hypoxia, and nitric oxide (Leist & Nicotera, 1997). When apoptosis is induced inappropriately or is dysregulated *via* abiotic or biotic stimuli, it can result in development of cancer, neurodegenerative diseases such as Alzheimer's, Hodgkin's disease and transplant rejection (autoimmunity) (Thompson, 1995).

2.2.2.1.1. CHARACTERISTICS OF APOPTOSIS

There is a series of stereotyped, morphologically well-defined phases that are most clearly evident at microscopic level when apoptosis is initiated. Changes occurring during apoptosis are schematically presented in Figure 2-7 and include plasma membrane blebbing with maintenance of membrane integrity (zeiosis), cell body shrinkage, and formation of membrane-bound apoptotic bodies, which are quickly engulfed *in vivo* by neighbouring healthy cells. The cytoskeleton of an apoptotic cell undergoes profound changes as the nucleus fragments while the cell shrinks and becomes detached from the basal membrane and surrounding cells.

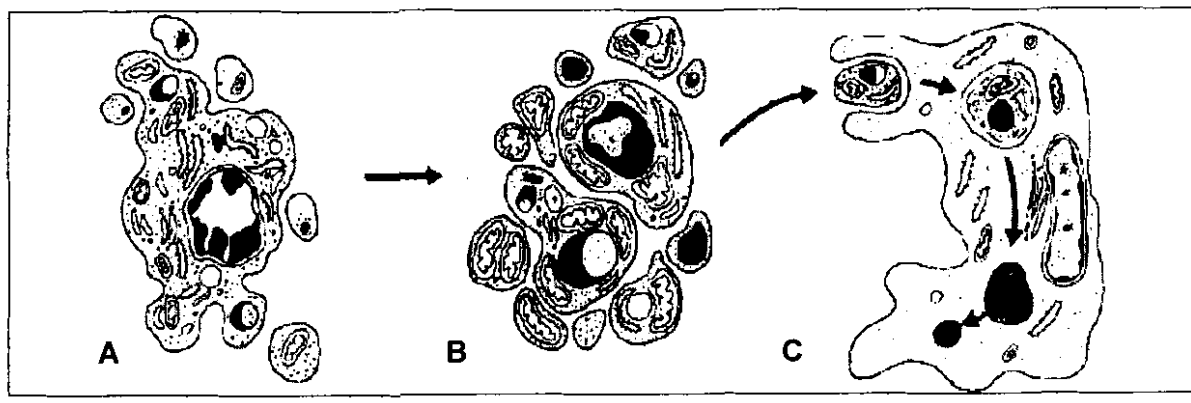


Figure 2-7 Apoptotic cell death: A.) Chromatin is condensed and DNA fragmented, B.) cells form apoptotic bodies C.) which is then engulfed by neighbouring cells (Walker *et al.*, 1988).

Changes in the nucleus represent the first explicit evidence of apoptosis. Chromatin condensation and segregation into sharply delineated masses adjacent to the nuclear envelope are typically observed at the onset of apoptosis. Chromosomal DNA fragmentation during apoptosis may be due to single-strand DNA breaks or activated topoisomerase II (Li *et al.*, 1999). The DNA fragmentation may include or exclude the characteristic internucleosomal DNA cleavage (ladder-like) pattern.

The initial condensation of DNA eventually leads to true nuclear pyknosis (group of dense bodies). In parallel with nuclear changes, cytoplasm condensation also occurs and the cell membrane becomes convoluted with the onset of protuberances of various sizes that may give the cell a star-like appearance. As the cytoplasm density increases, some vacuoles may become evident, but the cell organelles remain unaffected, although they become abnormally closely packed (Lossi & Merighi, 2003).

As the process continues, the cell and its nucleus take on a more irregular shape and nuclear budding occurs to produce discrete fragments. Eventually the cell is fragmented into membrane-bound apoptotic bodies which still display a sharp segregation of condensed chromatin in nuclear fragments. Apoptotic bodies are rapidly cleared out in tissues by macrophages or neighbouring cells and are degraded within heterophagosomes (Lossi & Merighi, 2003).

2.2.2.1.2. PHASES OF APOPTOSIS

The apoptotic process takes place in three apparent phases, namely the induction phase, the effector phase and the degradation phase (Jones & Dangl, 1996; Kroemer *et al.*, 1995).

- During 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 inside the cell because of a drug action, toxin or radiation. Stimuli can include activation of TNF, survival factor withdrawal (Raff, 1992; McCabe *et al.*, 1997), heat shock (Wang *et al.*, 1996a), hypoxia or oxidative stress (Hockenbery *et al.*, 1993; Zamzami *et al.*, 1995). The cellular sensor detects the presence of a death-inducing signal which activates an apoptotic signal-transduction pathway.
- After this initial phase, the *effector phase* follows. During this phase, proteins are induced that relay the initial diverse signals into a few stereotyped pathways. These proteins, such as the Bcl-2 family members, can be either anti- or pro-apoptotic (Kerr *et al.*, 1972). At this point the initiation of cell death passes the point of no return.
- In the *degradation phase* chromatin is condensed and DNA degraded, leading to the characteristic morphology and biochemistry of apoptosis (Kerr *et al.*, 1972).

2.2.2.1.3. GENERAL MECHANISMS AND PATHWAYS

The process of apoptosis is induced *via* two major signalling pathways. The *extrinsic apoptotic pathway* is initiated by death receptors and requires the binding of a ligand to a death receptor on the cell surface. This extrinsic pathway is schematically presented in Figure 2-8. Signals that originate from the TNF receptor family, such as Fas, are largely Bcl-2 insensitive and these signals activate the upstream caspase-6, -8 and -9 (Krebs *et al.*, 1999). For example, the cytokine TNF binds to the death receptor, TNF receptor type 2 (TNF-R2), which recruits two signal transducing molecules, namely TNF-R1-associated death domain protein and Fas-associated protein death domain. This complex then binds to procaspase-8 to activate caspase-8, in turn initiating the protease cascade leading to apoptosis (Reed, 2000).

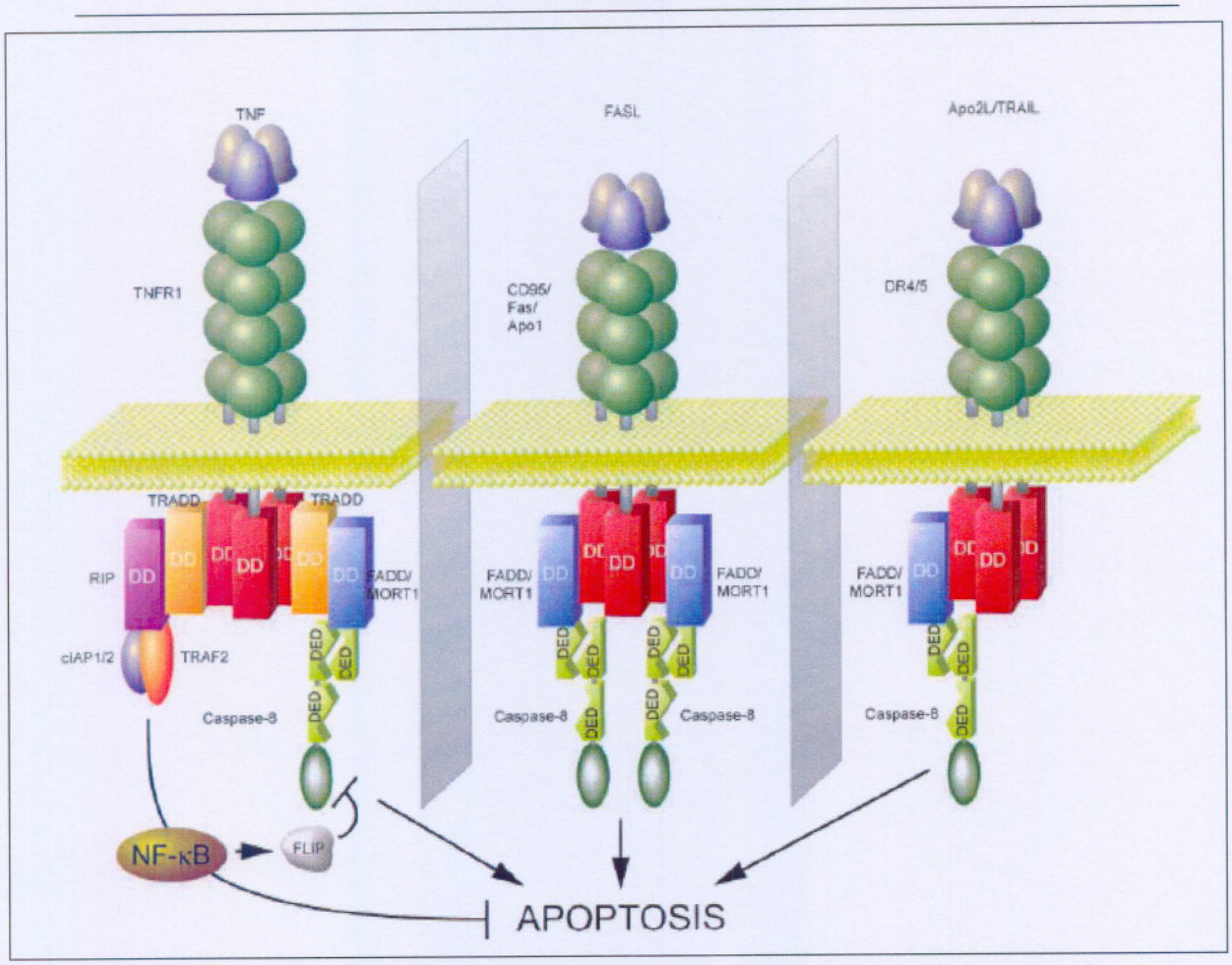


Figure 2-8 The extrinsic apoptotic pathway (Danial & Korsmeyer, 2004).

The *intrinsic apoptotic pathway* (shown in Figure 2-9) is mediated by the mitochondrial release of cytochrome c (Zimmerman *et al.*, 2002). This pathway is activated if damaged DNA is not sensed and repaired by checkpoint genes. Initiation of apoptosis may occur immediately or be delayed after the DNA damage has occurred. The response may or may not be dependent on the presence of the nuclear transcription factor p53 (an important tumour suppressor gene that is involved with the induction of apoptosis and cell cycle regulation in cells that have sustained DNA damage) (Shuler *et al.*, 2000). Bcl-2 (§ 2.2.2.1.3.2) potently inhibits the pathway activated by signals that originate from other stimuli, such as UVB irradiation, growth factor deprivation and p53 up-regulation. Most agents that produce ROS (including ozone) induce cell death *via* lipid peroxidation and DNA damage (Higuchi & Matsukawa, 1997). DNA strand breakage by ROS triggers secondary effects including the upregulation of p53 and activation of the nuclear enzyme poly(ADP-ribose)polymerase.

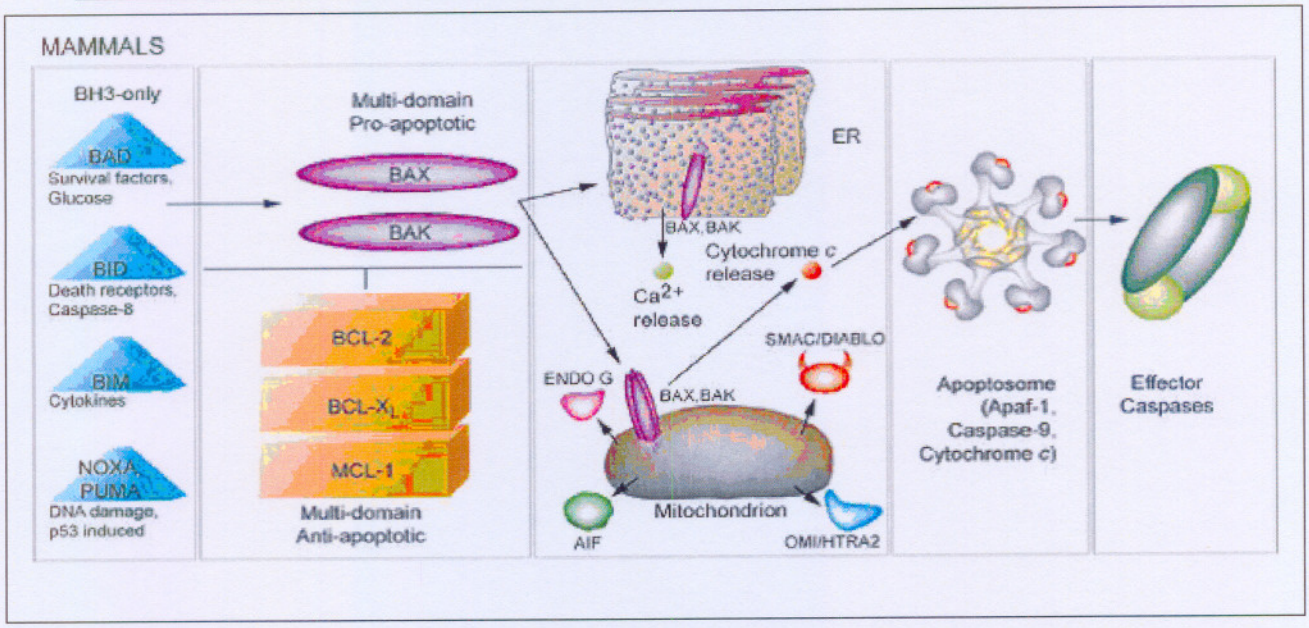


Figure 2-9 The intrinsic apoptotic pathway (Danial & Korsmeyer, 2004).

Both the extrinsic and intrinsic pathways eventually meet, leading to the activation of the caspases (§ 2.2.2.1.4) (Thornberry & Lazebnik, 1998).

2.2.2.1.3.1. THE MITOCHONDRIAL APOPTOTIC PATHWAY

Researchers have investigated the role of the mitochondria during the initiation of apoptosis (Green & Reed, 1998). Three general mechanisms are known by which mitochondria induce apoptosis in the cell (Green & Reed, 1998):

- *via* the disruption of the electron transport, oxidative phosphorylation and ATP production;
- *via* the release of proteins that initiate the activation of caspases; and
- *via* alteration of cellular reduction-oxidation potential.

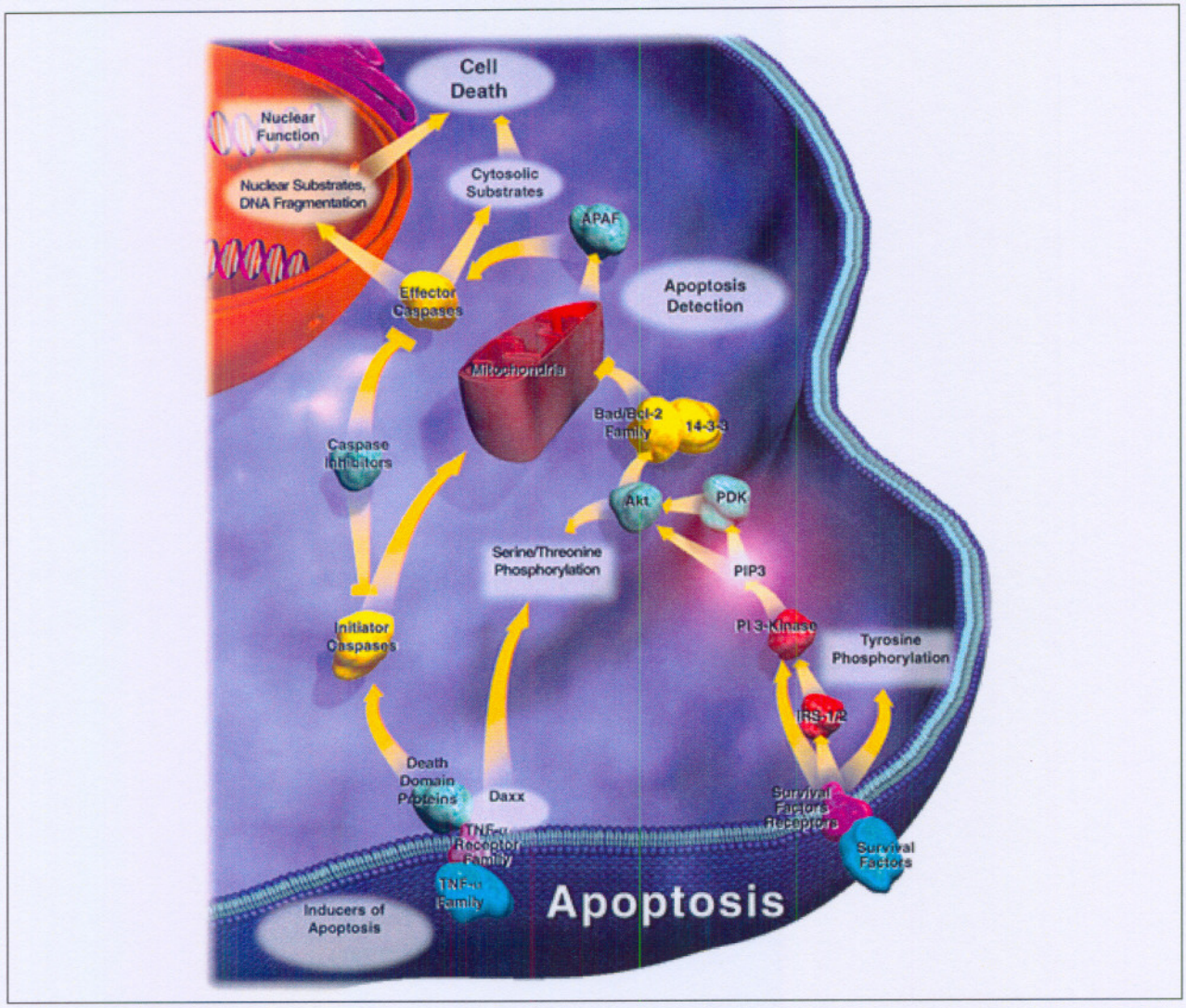


Figure 2-10 The apoptotic pathway (Biomol, 2005).

Apoptosis, programmed cell death, is triggered by a variety of stimuli, including mitochondrial response to stress and activation of cell surface receptors such as Fas. Caspases convey the apoptotic signal in a proteolytic cascade, cleaving and activating other caspases which in turn degrade other cellular targets that lead to cell death. The caspases at the upper end of the cascade include caspase-8 and caspase-9. The mitochondrial stress pathway begins with the release of cytochrome c from mitochondria, which then interacts with Apaf-1, causing self-cleavage and activation of caspase-9. Caspase-3, -6 and-7 are downstream caspases that are activated by the upstream proteases and act themselves to cleave cellular targets

The protein named cytochrome c is released from the mitochondria into the cytoplasm as soon as an apoptotic stimulus reaches the cell. The mitochondria also contain other apoptogenic factors (e.g. Omi/Htra2 and SMAC/Diablo) in the intermembrane space that may be released to commit a cell to apoptosis (Zimmerman *et al.*, 2002). Cytochrome c normally resides in the space between the outer and inner membrane of the mitochondria and is a vital component for

the generation of ATP *via* the respiratory chain. Various cellular stressors, including cytotoxic drugs, growth factor withdrawal and DNA damage can trigger the release of cytochrome c. It is released from the mitochondria by either physical disruption of the outer mitochondrial membrane as a result of inner membrane hyperpolarisation and matrix swelling, through regulation *via* the Bcl-2 family of proteins (§ 2.2.2.1.3.2) or *via* outer membrane rupture occurring as a secondary consequence of mitochondrial permeability transition. When cytochrome c is released into the cytoplasm of the cell, it associates with a protein named apoptosis protease activating factor (APAF1) (also known as death-inducing signalling complex (DISC)) and ATP, forming a complex referred to as an apoptosome. This complex is necessary to activate pro-caspases which in turn leads to the destruction of cellular proteins and ultimately apoptosis (Earnshaw *et al.*, 1999) (see Figure 2-10).

Although many proteins and enzymes are involved in the apoptotic pathway, only two enzyme families have been selected for the current study namely, the caspases and the anti-apoptotic enzyme Akt. These two enzyme families will be discussed in greater detail in the following section.

2.2.2.1.3.2. BCL-2 FAMILY OF ENZYMES

Bcl-2 is a tumour suppressor gene that was first discovered and named in association with B-cell lymphoma. Since then multiple related proteins have been found to be involved with either pro-apoptotic events or inhibition of apoptosis.

The Bcl-2 family members were first discovered and named in association with B-cell lymphoma and since their discovery multiple related proteins have been found to be involved with either pro-apoptotic events or inhibition of apoptosis. The Bcl-2 family are integral membrane proteins located mainly on the outer mitochondrial membrane, endoplasmic reticulum and the nuclear membrane. They contain conserved domains (Bcl-2 homologies) designated BH1 to BH4 and most members contain a COOH-terminal transmembrane anchor sequence which allows for their association with cellular membranes (Krajewski *et al.*, 1993, Lithgow *et al.*, 1994; Yang *et al.*, 1995; Reed, 1997). The Bcl-2 proteins are critical regulators of apoptosis, monitoring and integrating death and survival signals while setting the threshold at which mitochondrial release of apoptogenic factors occurs.

There are both pro-apoptotic and anti-apoptotic family members. The *anti-apoptotic* family members (Bcl-2 and Bcl-X_L) prevent pro-caspase activation (Kluck *et al.*, 1997; Yang *et al.*, 1997) and the initiation of apoptosis (Armstrong *et al.*, 1996) by inhibiting the initial release of cytochrome c from mitochondria. This is achieved by preventing early depolarisation of the mitochondrial membrane. However, once apoptosis is initiated the process cannot be delayed by these proteins (McCarthy *et al.*, 1997).

The *pro-apoptotic* members (Bax, Bad, Bik, Bim and Bid) interact with the anti-apoptotic members to form heteromers and inhibit their survival activity (Jurgensmeier *et al.*, 1998). Some members such as Bax also has the ability to form ion channels in membranes and release cytochrome c *in vitro* (Muchmore *et al.*, 1996; Minn *et al.*, 1997; Schendel *et al.*, 1997; Schlesinger *et al.*, 1997) leading to the hypothesis that anti-apoptotic family members might function as transmembrane channels (Vander Heiden *et al.*, 1997) in cells.

2.2.2.1.4. CASPASES

Our current knowledge on the gene regulation of apoptotic cells is mainly derived from studies on the nematode worm *Caenorhabditis elegans* (Yuan *et al.*, 1993). In *C. elegans* 131 out of 1090 somatic cells undergo developmental apoptosis. Four genes named ced3, ced4, ced9 and egl1 form the "death machinery" responsible for the execution programmed cell death (reviewed by Hergartner, 1997). With the discovery of the four genes the focus shifted to the mammalian caspases as initiators of apoptosis (Yuan *et al.*, 1993; Thornberry *et al.*, 1992).

ICE was the first caspase identified through its ability to convert the precursor of interleukin-1β to its mature form, a potent mediator of inflammation (Cerretti *et al.*, 1992). Although caspase-1 has no obvious role in cell death, it became the first identified member of a large family of proteases whose members have distinct roles in apoptosis. To date, 15 members of the caspase family have been identified.

In general, caspases are proteins that cleave their substrates at aspartate sites. The term caspase (**c**ysteinyl **asp**artate specific protein**ases**) denotes two key characteristics of these proteases (Alnemri *et al.*, 1996):

- cysteine forms the nucleophilic group for substrate cleavage; and
- the peptide bond c-terminal to aspartic acid residues is cleaved.

All caspases contain three domains: (1) a NH_2 terminal (which varies in length and sequence for each caspase, (2) a large subunit and (3) a small subunit. The three-dimensional structure of caspase-3 protein is shown in Figure 2-11.

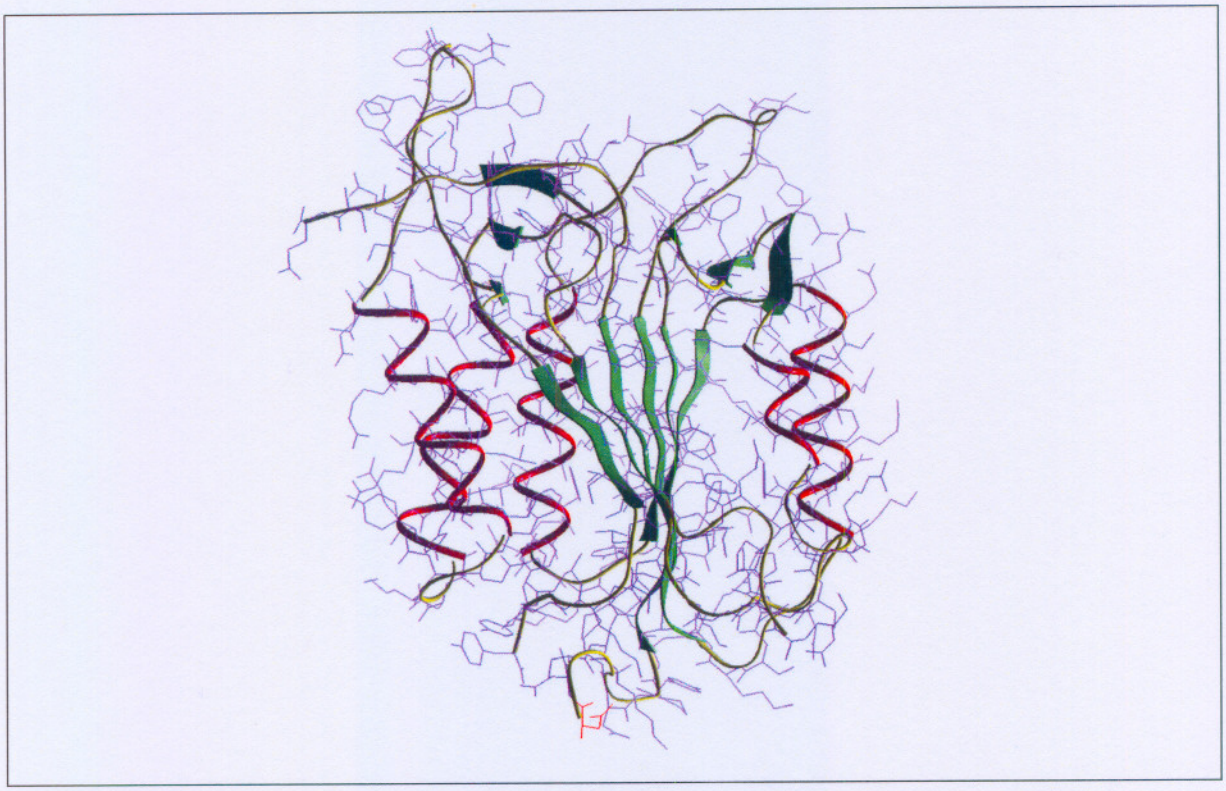


Figure 2-11 Caspase-3 three-dimensional structure (Jèkely, 2002).

2.2.2.1.4.1. CLASSIFICATION OF CASPASE GROUPS

Caspases are classified according to their function and structure. Activator caspases (upstream caspases, including caspases 1, 2, 4, 5, 9, 11 and 13) respond to apoptotic stimuli. They usually have long pro-domains and are responsible for the activation of the downstream caspases. The effector caspases (downstream caspases, including caspases 3, 6, 7 and 14) cleave cellular proteins leading to DNA and cell destruction. They have shorter pro-domains and are activated by the upstream caspases. The caspases may also be divided into subfamilies on the basis of the peptide-sequence preferences of their substrates (Lossi *et al.*, 2003):

- the ICE-protease subfamily (caspases 1, 4, 5, 13, 14, 11 and 12);
- the ced-3 subfamily (caspases 3, 6, 7, 8, 9 and 10); and
- the caspase-2 subfamily.

2.2.2.1.4.2. ACTIVATION OF CASPASES

Caspases usually exist as inactive zymogens (pro-caspases), remaining latent until a pro-apoptotic stimulus initiates their activation *via* one of two mechanisms (Cryns & Yuan, 1998):

- a Bcl-2-regulated pathway specific to membranes; and
- a cytoplasmic activation pathway (directly activating the pro-caspases *via* cytochrome c release without the direct regulation of Bcl-2).

Caspases are activated when pro-apoptotic signals activate co-factors such as cytochrome c and APAF1. Two or more pro-caspases are brought in close proximity during this activation, allowing intermolecular proteolytic processing after the aspartic acid site. This is followed by the association of one large and one small subunit, forming a heterodimer. Two heterodimers then associate to form a tetramer containing two catalytic sites that appear to function independently (Walker *et al.*, 1994; Wilson *et al.*, 1994; Rotonda *et al.*, 1996).

Caspase-3 (also known as CPP32, YAMA or Apopain) may be activated along with caspase-6 and caspase-8 *via* the Fas/TNF-mediated death pathway or participate in mitochondria-associated cell death together with APAF1 and cytochrome c (Zimmermann *et al.*, 2001). These two pathways do not seem to be completely independent since Bid mediates cytochrome c release from the mitochondria in response to activation of cell surface death receptors (Li *et al.*, 1998; Luo *et al.*, 1998).

2.2.2.1.4.3. CONSEQUENCES OF CASPASE ACTIVATION

Caspases are very specific endopeptidases. One major role of the caspase family is to inactivate proteins that protect living cells from apoptosis when continued growth is unwanted. They may cut off contact with surrounding cells, reorganise the cytoskeleton, shut down DNA replication and repair, interrupt splicing, destroy DNA, disrupt the nuclear structure, induce the cell to display signals that mark it for phagocytosis and disintegrate the cell into apoptotic bodies (Chang & Yang, 2000).

Within the nucleus of the cell, caspases initiate the degradation of DNA after the induction of apoptotic cells by cleaving and inactivating ICAD and PARP. In non-apoptotic cells, caspase-activated deoxyribonuclease (CAD), a DNase responsible for DNA fragmentation, is complexed with its inhibitor ICAD and is functionally inactive. In apoptotic cells, ICAD is cleaved and

inactivated by caspase-3 and caspase-7, releasing CAD to function as a nuclease to degrade nuclear DNA (Enari *et al.*, 1998; Liu *et al.*, 1997a). PARP is a nuclear enzyme that senses DNA nicks and catalyses the ADP-ribosylation of histones and other nuclear proteins in order to facilitate DNA repair. It may be cleaved by caspase-3 during apoptosis, separating the N-terminal DNA-binding domain from the catalytic domain and inactivating the enzymatic activity. DNA fragmentation by CAD during apoptosis may also cause significant activation of PARP.

Caspases directly initiate the disassembly of cell structures, such as the nuclear lamina (a rigid structure underlying the nuclear membrane involved in chromatin organization) by cleaving them at a single site. The lamina collapses and chromatin condensates (Takahashi *et al.*, 1996; Orth *et al.*, 1996). Acinus, a caspase-3-acivated factor, induces this chromatin condensation within the cell nucleus.

Caspases can also reorganize cell structures indirectly by cleaving proteins involved in cytoskeleton regulation. Gelsolin, cleaved by caspase-3, effects membrane blebbing (Kothakota *et al.*, 1997), while cleavage of targets such as α-fodrin and focal adhesion kinase (FAK) may lead to cell body shrinkage and allow apoptotic cells to detach from neighbouring cells and basement membrane (Wen *et al.*, 1997). Cell cycle regulators such as Cdc27 (component of the ubiquitin ligase complex mediating the degradation of mitotic cyclins) and Wee 1 (a kinase that provides inhibitory phosphorylation on cyclin-dependent kinase (Cdk)) may also be cleaved and inactivated by caspases (Zhou *et al.*, 1998).

Two other important proteins affected by caspases are Bcl-2 (§ 2.2.2.1.3.2) and cytochrome c. Cleavage of Bcl-2 by caspases not only inactivates these proteins, but also produces a fragment that promotes apoptosis (Xue *et al.*, 1996: Cheng *et al.*, 1997; Adams & Cory, 1998). While cytochrome c release and activation of caspases can occur before any detectable changes in the mitochondrial morphology are noted, activation of caspases can also induce cytochrome c release. It is thus possible that cytochrome c release occurs in two steps. At first only some cytochrome c molecules are released due to outer membrane rupture in a small number of mitochondria leading to caspase activation. Active caspases then cause changes in most mitochondria and consequently almost all cytochrome c molecules are released.

Other caspase substrates include (Chang & Yang, 2000):

- *Cell death proteins* (Bcl-x_L, Bid, CrmA, IAP, p28, Bap31, p35 and procaspases);
- *Cell cycle regulation* (Cyclin A, MDM2, p27, PITSLRE kinases and retinoblastoma protein);
- *Cytoskeleton* (Catenin, fodrin, Gas2, plakoglobin, keratin-18 and -19 and lamins);
- *Cytokine precursors* (Pro-IL1β, pro-IL16 and pro-IL18);
- *DNA metabolism* (DNA-dependent protein kinase, topoisomerase1, MCM3 DNA replication factor, NuMA and DNA replication complex c);
- *Neurodegenerative disease proteins* (APP, ataxin3, androgen receptor, atrophin1, huntingtin and presenilins);
- *RNA metabolism* (Eukaryotic initiation factor 2α, 70-kDa U1-snRNP and heteronuclear ribonuclear proteins C1 and C2);
- *Signal transduction* (Adenomatous polyposis coli protein, calmodulin-dependent kinase IV, c-Raf, D4-GDP dissociation inhibitor, fyn tyrosine kinase, MEKK1, MST/Ksr, PAK-2/hPAK65, protein kinase C delta, protein kinase C theta, TCR-ζ chain, protein phosphatase 2A, Ras, GTPase activating protein and protein kinase C-related kinase 2);
- *Transcription factors* (Heat shock factor, GATA1, IκBα, NFκB, NRF2, Sp1, STAT1 and sterol-regulatory element-binding proteins); and
- *Others* (Calpastatin, Hsp90, Nedd4, transglutaminase, rabaptin-5, phospholipase A2).

2.2.2.1.4.4. INHIBITION OF CASPASES

Among the most important regulators of the caspases are the inhibitors of apoptosis proteins (IAPs) (Deveraux & Reed, 1999). They are not active-site-specific inhibitors and their inhibition of apoptosis does not require cleavage by caspases. Evidence suggests that IAPs inhibit apoptosis through inhibition of effector caspases and that the baculoviral IAP repeat (BIR) domain may be responsible for direct and specific inhibition. For example, the aspartic acid (Asp^{148}) residue in the XIAP (X-linked IAP) domain of the BIR2 is critical for the inhibition of caspase-3 (Deveraux *et al.*, 1998).

Another protein involved in regulating caspases is Smac (Chai *et al.*, 2000). This protein, normally situated in the intermembrane space of mitochondria, is released into the cytosol during apoptosis together with cytochrome c and AIF. The activated Smac binds to several

IAPs and removes the ability of IAPs to block caspase-mediated apoptosis (Du *et al.*, 2000; Verhagen, 2000). Smac may also interact with the BIR2 domains of XIAP (Chai *et al.*, 2001). How Smac opposes the inhibition of caspase-3 by the XIAP BIR2 domain is less clear. XIAP might be hindered sterically by the BIR2 domain from simultaneously binding to caspase-3 and Smac (Chai *et al.*, 2001).

Phosphorylation and nitrosylation may also modulate caspase activity. Akt (see § 2.2.2.1.5 below) phosphorylates and inhibits caspase-9 activity *in vitro* and activation *in vivo* (Cardone *et al.*, 1998). This phosphorylation may affect assembly of the caspase tetramer or alternatively regulate caspase activity allosterically. Another way to modify caspase-3 posttranslationally is by S-nitrosylation of Cys 163 which is a functionally essential amino acid conserved among the caspase protein family. Nitric oxide (NO) and related molecules have been found to inhibit apoptosis by S-nitrosylation (Mannik *et al.*, 1999).

2.2.2.1.5. AKT

Protein kinase B (PKB), also known as Akt or RAC kinase, is a group of phosphatidylinositol-3-OH-kinase-regulated serine/threonine kinases that form part of the Arabidopsis gene (AGC) subfamily (Franke *et al.*, 1995; Burgering & Coffer, 1995). Members of the AGC subfamily of protein kinases are activated and stabilised by phosphorylation of two residues, one that resides in the T-loop of the kinase domain and the other that is located in the C-terminal to the kinase domain in a region known as the hydrophobic motif. These kinases mediate their cellular effects by phosphorylating key regulatory proteins (Coffer *et al.*, 1998).

Akt was discovered in 1977 through experimental work of Staal and co-workers who transformed the murine retrovirus (Akt-8) and found that DNA fragments of the virus contained two human homologues, namely Akt1 and Akt2 (Staal *et al.*, 1997). In 1991, Akt was identified independently by three different groups. Two of these groups identified the kinase as a result of its homology to both protein kinase C and protein kinase A, giving rise to the alternative name, protein kinase B (Coffer *et al.*, 1991). The inactive form of the protein is presented three-dimensionally in Figure 2-12.

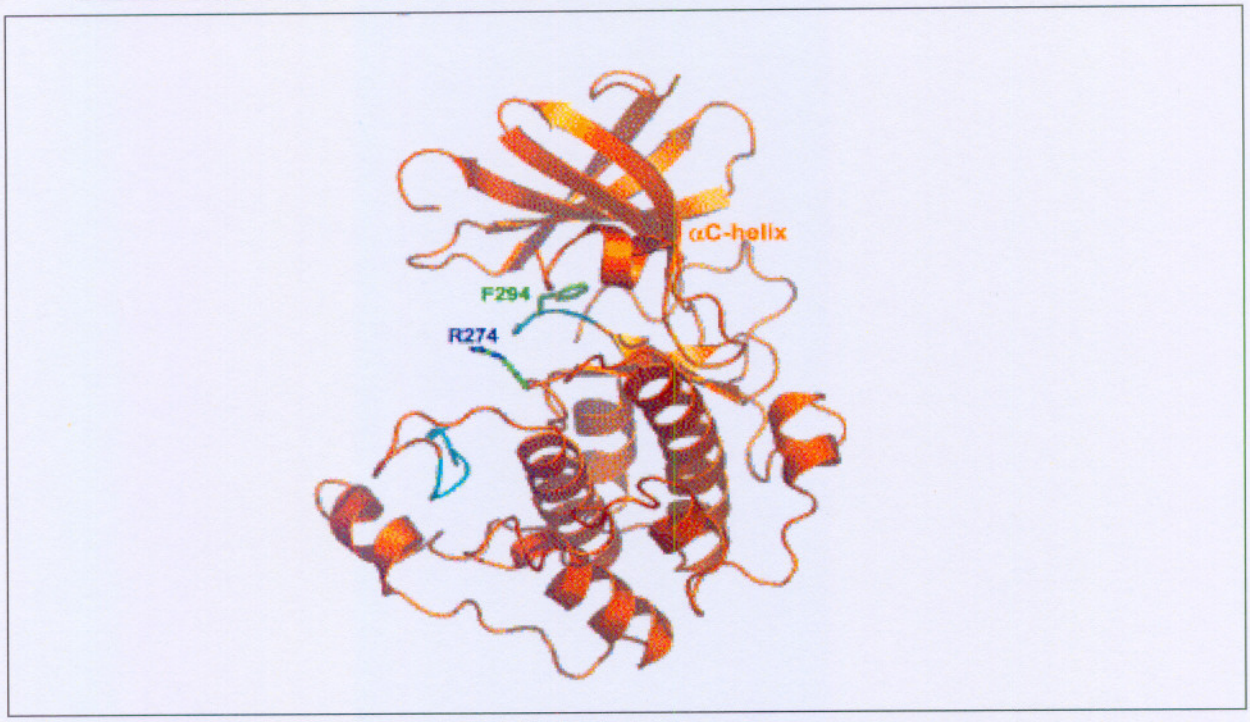


Figure 2-12 Structure of inactive catalytic domain of Akt (Hanada *et al.*, 2004).

2.2.2.1.5.1. AKT FAMILY OF ISOFORMS

In addition to the first characterised member of the family (PKBα/Akt1), two other very closely related enzymes have now been identified, namely (1) PKBβ/Akt2 (Jones *et al.*, 1991) and (2) PBKγ/Akt3 (Konishi, 1995).

The three known mammalian Akt family members are expressed differentially at both the mRNA and protein levels (Bellacosa *et al.*, 1993; Altomare *et al.*, 1995; Brodbeck *et al.*, 1999). However, it is presently unclear whether Akt1, Akt2 and Akt3 are functionally redundant or whether each one performs specific functional role(s) (also see Table 2-2).

Table 2-2 Isoforms of Akt: expression and function.

Isoform	Expression	Stimuli	Functions
Akt1	In every tissue in the body.	Cytokines, chemokines, growth factors, survival factors, integrin ligation (Datta *et al.*, 1999; Koh *et al.*, 1998); heat shock, oxidative stress (Yuan *et al.*, 2002a); insulin (Barthel *et al.*, 1998; Cho *et al.*, 2001a).	Protection against cell death induced by: • hyperglycaemia (Ido *et al.*, 2002), • hypoxia (Chong *et al.*, 2001; Scott *et al.*, 2002), • free radical exposure (Yamaguchi *et al.*, 2001; Chong *et al.*, 2003), • ionising radiation (Edwards *et al.*, 2002), • oxidative stress (Ikeyama *et al.*, 2002). Phosphorylation of Bad Postmitochondrial release of cytochrome c (Cardone *et al.*, 1998; Zhou *et al.*, 2000) Normal cell growth (Cho *et al.*, 2001a)
Akt2	Highest expression in the skeletal muscles, but also present in β-islet cells (pancreas) and brown fat.	Growth factors released *via* Ras and PI3-K signalling pathways, insulin (Barthel *et al.*, 1998; Cho *et al.*, 2001a)	Activated in cancers such as human ovarian cancer (Yuan *et al.*, 2002b).
Akt3	Restricted to the heart, placenta and brain (Morimoto *et al.*, 2000).	Heat shock, oxidative stress (Yuan *et al.*, 2002a) Insulin, (Barthel *et al.*, 1998; Cho *et al.*, 2001a)	Involved (however not principal function) in regulating insulin signalling (Masure *et al.*, 1999) Estradiol-independent tumour growth (Faridi *et al.*, 2003)

All isoforms contain the same domains (as can be seen in Figure 2-13). In addition to a serine/threonine kinase domain, there is also a pleckstrin homology (PH) domain at the amino-terminal end (amino acids 1-106). The integrity of the pleckstrin homology domain has been found to be essential for activation of Akt in intact cells in response to various growth factors (Franke *et al.*, 1995; Andjelkovic *et al.*, 1996) and expression of activated PI3-K (Klippel *et al.*, 1996).

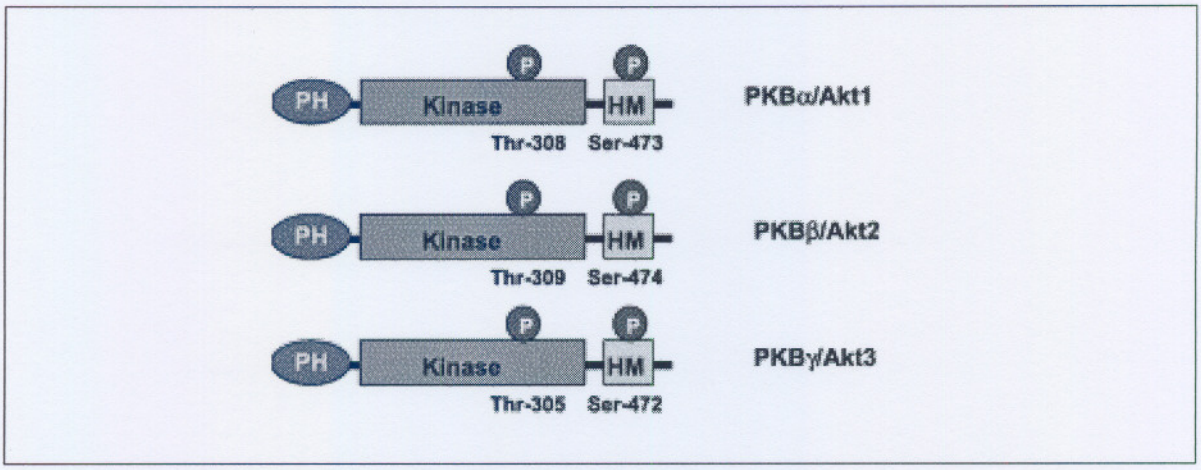


Figure 2-13 Domain structure of Akt isoforms (Hanada *et al.*, 2004).

All the Akt isoforms have a common structure which includes a pleckstrin homology (PH), a hydrophobic motif (HM) located at the carboxyl-terminal and an adjacent kinase domain. Phosphorylation sites in the activation loop in the kinase domain and the hydrophobic motif are indicated.

The most important function of the PH domain is to mediate translocation of the kinase from the cytosol to the plasma membrane following activation of PI3-K. The kinase domain stretches from amino acid 148 to 411, with the carboxy-terminal tail region (amino acids 412-480) accounting for the remainder of the protein. The carboxyl terminus includes a hydrophobic and praline-rich domain.

2.2.2.1.5.2. ACTIVATION OF AKT

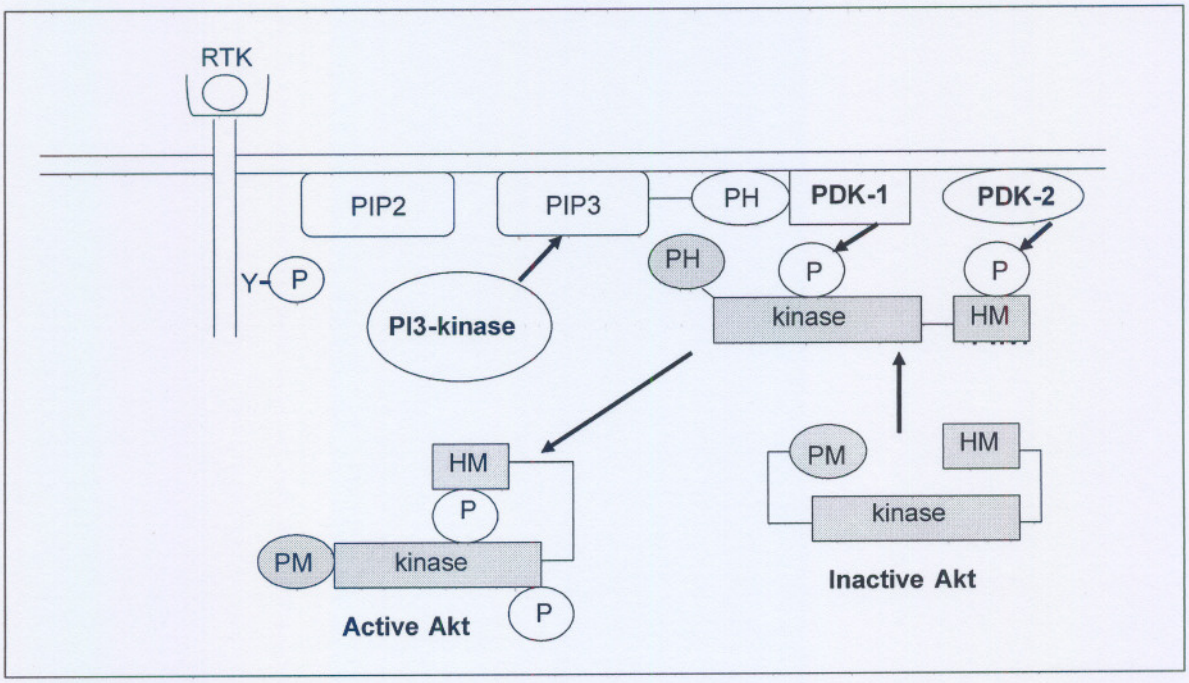


Figure 2-14 Schematic representation of Akt activation (Hanada *et al.*, 2004).

Oligomerization and activation of receptor tyrosine kinase (RTK) by its ligand activates phosphatidylinositol 3-kinase (PI3-kinase) to produce phosphatidylinositol 3,4,5 trisphosphate (PIP_3). PIP_3 organizes Akt, PDK1 and, probably, Ser473-kinase on the plasma membrane, where Akt becomes phosphorylated and activated. Activated Akt then translocates from the cytosol to the nucleus.

The mechanism for the activation of Akt has recently been elucidated (Figure 2-14). The PI3-K produces two lipid products, namely phosphatidylinositol-3,4,5-triphosphate (PIP_3) and phosphatidylinositol-3,4-bisphosphate ($PI[3,4]P_2$). These lipid products are able to bind to the PH domain of Akt with relatively high affinity and specificity (Burgering & Coffer, 1995; Franke *et al.*, 1995, 1997; Klippel *et al.*, 1997), but the binding of PIP_3 to the PH domain does not activate the enzyme (Klippel *et al.*, 1997). It causes conformational changes that unmask the active site leading to the relocation of Akt from the cytosol to the membrane, bringing the enzyme in proximity to regulatory kinases that phosphorylate and activate Akt.

Phosphorylation of Akt occurs at two independent sites, namely threonine in the kinase domain (Thr 308 (Akt1)/Thr 309 (Akt2)/Thr305 (Akt3)) and serine in the carboxy-terminal (Ser 473 (Akt1)/Ser 474 (Akt2)/Ser 472 (Akt3)) (Alessi *et al.*, 1996a Alessi *et al.*, 1997a; Datta *et al.*, 1999). Two different kinases are involved in phosphorylating these sites.

Once the PH domain of Akt engages PIP_3 on the membrane it becomes a substrate for 3-phosphoinositide-dependent protein kinase-1 (PDK1) phosphorylation. PDK1 is located in both the cytoplasm and at the inner surface of the plasma membrane (Currie *et al.*, 1999) and its activity is strongly stimulated *in vitro* by PIP_3 and $PI[3,4]P_2$ (Alessi *et al.*, 1997b; Stokoe *et al.*, 1997). Unlike Akt, PDK1 is a constitutively active enzyme and its kinase activity within cells does not appear to be enhanced by the presence of survival factors that activate Akt (Alessi *et al.*, 1997b). PDK1 can, however, only phosphorylate Akt at the threonine residue (Alessi *et al.*, 1997b; Stokoe *et al.*, 1997).

This suggests that the serine site is phosphorylated by a kinase other than PDK1 or Akt. This unidentified kinase, referred to as PDK2, also appears to be under the control of PI3-K (Alessi *et al.*, 1997b). Other possible mechanisms for serine residue phosphorylation include autophosphorylation of the hydrophobic site (Toker & Newton, 2000) and integrin-linked kinases (Delcommenne *et al.*, 1998). Treatment with phosphatase reverses the phosphorylation of Akt (Burgering & Coffer, 1995).

In addition to PI3-K activation, other cellular stressors such as heat shock and hyperosmolarity are also able to stimulate the activity of Akt (Khwaja *et al.*, 1997).

2.2.2.1.5.3. CONSEQUENCES OF AKT ACTIVATION

There is considerable interest in the substrates of Akt due to its diverse role in biological systems. Akt will be able to phosphorylate a substrate with the following preferred sequence: RxRyz(S/T)(hy), where x is any amino acid, y and z are small residues other than glycine, S and T are the serine and threonine residues while hy is a bulky hydrophobic group (Alessi *et al.*, 1996b).

Akt plays an important role in many biological pathways (Downward, 1998). Some important functions of this enzyme include glucose transport (Kohn *et al.*, 1996), indirect regulation of glucose transport *via* endothelial nitric oxide synthase (Fulton *et al.*, 1999), regulation of insulin secretion (Rondinone *et al.*, 1999), protein synthesis (Shah *et al.*, 2000; Gingras *et al.*, 2001; Ueki *et al.*, 1998; Hajduch *et al.*, 1998), lipogenesis and antilipolysis (Wang *et al.*, 1998) and glycogen synthesis (Cross *et al.*, 1995; Welsh *et al.*, 1996).

Research done during the past few years have, however, focused on another critical role of Akt, i.e. in the regulation of apoptosis and cellular survival. The exact mechanism by which Akt protects cells from apoptosis still remains unclear, but studies have suggested that by inhibiting PI3-K, the ability of survival factors such as Akt to protect cells from apoptosis is also inhibited (Yao & Cooper, 1995, 1996). These anti-apoptotic pathways are depicted in Figure 2-15. There is evidence suggesting that Akt can inhibit the hyperpolarisation of the mitochondria, which precedes cytochrome c release and caspase activation (Kennedy *et al.*, 1999). Akt may prevent the disruption of the mitochondrial inner membrane potential by inhibiting conformational changes and redistribution of Bax to mitochondrial membranes (Yamaguchi & Wang, 2001). Another Bcl-2 family member affected by Akt is Bad. Bad directly interacts with anti-apoptotic members (Bcl-X_L) and through this inhibits cell survival (Yang *et al.*, 1995; Ottilie *et al.*, 1997; Zha *et al.*, 1997). Akt phosphorylates Bad at the Ser136, causing its dissociation from Bcl-X_L, leaving it free to associate with cytoplasmic 14-3-3 proteins (a family of anti-apoptotic adaptor proteins inhibiting caspase-3) instead (Del Peso *et al.*, 1997; Datta *et al.*, 1997b; Zha *et al.*, 1996; Trencia *et al.*, 2003). The association of Bad with 14-3-3 proteins may protect Bad from dephosphorylation (Muslin *et al.*, 1996; Yaffe *et al.*, 1997) and the resulting phosphorylation promotes cellular survival (Zha *et al.*, 1996; Yang *et al.*, 1995). Some evidence suggests that Akt can translocate to the nucleus (Andjelkovic *et al.*, 1997) and possibly regulate Bcl-2 expression. The promotor 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 (Du & Montminy, 1998; Pugazhenthi *et al.*, 2000). Akt has been shown to activate CREB, increasing anti-apoptotic gene transcription such as Bcl-2 (Du & Montminy, 1998).

Downstream of the mitochondrial activity, Akt may phosphorylate caspase-9 on serine 196, inhibiting the activation of pro-caspase-9 *via* cytochrome c (Cardone *et al.*, 1998), attenuating caspase-9 activity. Akt may also inhibit caspase-9 and caspase-3 *via* phosphorylation of x-linked inhibitor of apoptosis protein (XIAP), a direct inhibitor of caspase-3 and caspase-9. This modulates the Bax/cytochrome c pathway (Deveraux *et al.*, 1998) and results in the inhibition of caspase-3 activity and apoptosis (Dan *et al.*, 2003). Akt does, however, not act as a general caspase inhibitor.

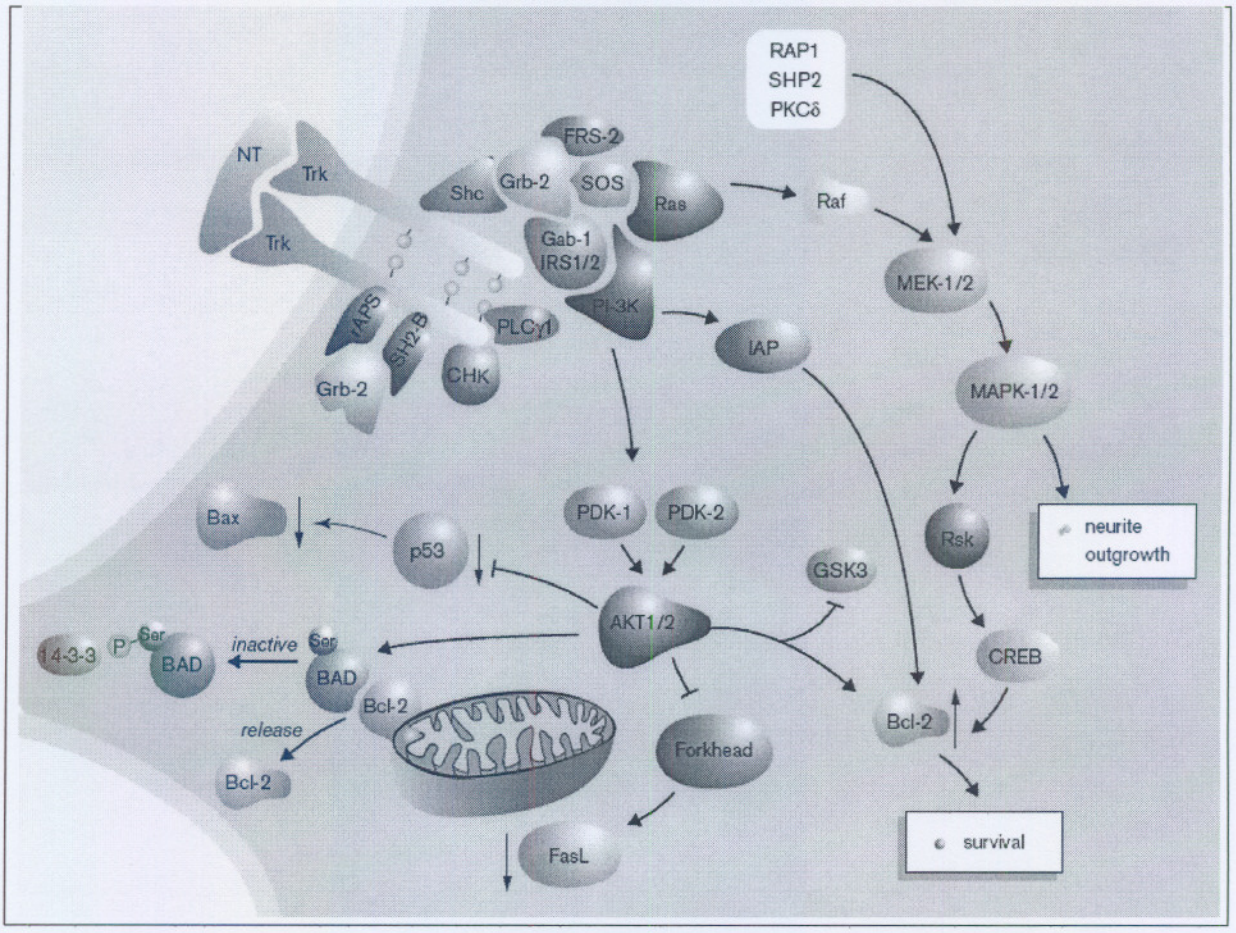


Figure 2-15 Consequences of Akt activation and substrate phosphorylation (Kaplan & Miller, 2000).

Another substrate for phosphorylation by Akt is p53 (Zhou *et al.*, 2001). This protein is activated in response to DNA damage, subsequently operating as transcription factor. By promoting the degradation of p53, Akt impairs the cellular stress response, increasing the survival of tumour cells.

Apart from the role Akt plays in the inhibition of apoptosis *via* the mitochondrial pathway, it is also involved in the regulation of several transcription factors, one being nuclear factor kappa beta (NFκβ). Once Akt is activated, it interacts with and phosphorylates Iκβ kinase (IKKa) which is directly responsible for the regulation of NFκβ activity. NFκβ translocate to the nucleus (Ghosh *et al.*, 1998) where it up regulates transcription and increases cellular survival (Ozes *et al.*, 1999; Marte & Downward, 1997; Van Antwerp *et al.*, 1998).

2.2.2.2. NECROSIS

Necrosis (from the Greek expression for "deadness" or "dying") is an uncontrolled, passive form of cell death resulting from the loss of homeostasis. It manifests the death of a cell or group of cells usually as a result of injury, disease or pathological state. When the threshold limits of insults are too severe and the damage is either too extensive to repair or an apoptotic response cannot be initiated, the cells die *via* necrosis.

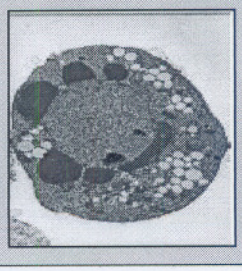
A variety of stimuli such as physicochemical injury (radicals, radiation, temperature, toxic trauma, etc.), osmotic imbalance, bacterial toxins (Warney *et al.*, 2000), abrupt anoxia, energy deprivation, immune defence (Shimizu *et al.*, 2000) and blocked apoptotic execution pathways can induce necrosis. Pathological conditions characterized by inadequate secretions of cytokines, nitric oxide and ROS are also accompanied by extensive initiation of necrosis.

Improvement of methods for differentiating between apoptosis and necrosis has revealed that some biochemical and morphological characteristics of both pathways can be found in the same cell. Members of the TNF receptor family (TNF, Fas and TRAIL) may not only initiate apoptosis, but also necrotic cell death (Holler *et al.*, 2000), while some lipid peroxidation products and ROS species may also induce necrosis (Kruman & Mattson, 1999; Hampton & Orrenius, 1997). Akt and MAP kinase (ERK), which protect cells from stress-induced apoptosis, can also protect against necrotic death (Mochizuki *et al.*, 2002).

2.2.2.2.1. DIFFERENCE BETWEEN APOPTOSIS AND NECROSIS

Necrosis differs from apoptosis in a number of morphological characteristics. In Table 2-3 a summary is given of these different characteristics.

Table 2-3 Necrosis vs. Apoptosis (Adapted from Reuhl, 2005).

Criteria	Necrosis 	Apoptosis 
Patterns of death	Groups of neighbouring cells	Single cells
Stimuli	Cytotoxic agents, severe hypoxia, massive insult conditions with ATP loss	Normal development, TNF, heat shock, viruses, oxidative stress, hypoxia, nitric oxide
Energy requirement	Energy independent, ATP depletion	Requires ATP
Histology	Cell swelling, organelle disruption	Shrinkage of the cell, chromatin aggregation, DNA fragmentation
DNA pattern	Random sized fragments, diffuse and random DNA degradation	Clumps and fragmented, DNA appearing in the cytosol
Mitochondria	Swelling, disordered structure	Increased membrane permeability, but structure is preserved
Organelle shape	Swelling	Contracted (loss of water but remain intact)
Plasma membrane	Early lyses (membrane smooth)	Preserved continuity, blebbed (releasing small cytoplasm-filled vesicles)
Phagocytosis by:	Immigrant phagocytes	Neighbouring cells
Tissue reaction	Inflammation (due to leakage of content)	No inflammation

2.2.2.2.2. CHARACTERISTICS OF NECROSIS

Necrotic cells undergo morphological changes characteristic of necrosis (see Figure 2-16) such as vacuolation of the cytoplasm, breakdown of the plasma membrane and induction of inflammation around the dying cell. This is attributed to the release of cellular contents and proinflammatory molecules. Usually chromatin condenses in multiple small clumps with irregular outlines and is poorly demarcated from the surrounding nucleoplasm. Occasionally densities in the matrix, abnormally swollen mitochondria and local membrane disruption become

evident. At later stages, cell organelles and membranes disintegrate. The chromatin disappears at the end of the process, leaving only "ghost-like" cell residues. Necrotic debris is removed by cells of the mononuclear phagocytic system (Lossi & Merighi, 2003).

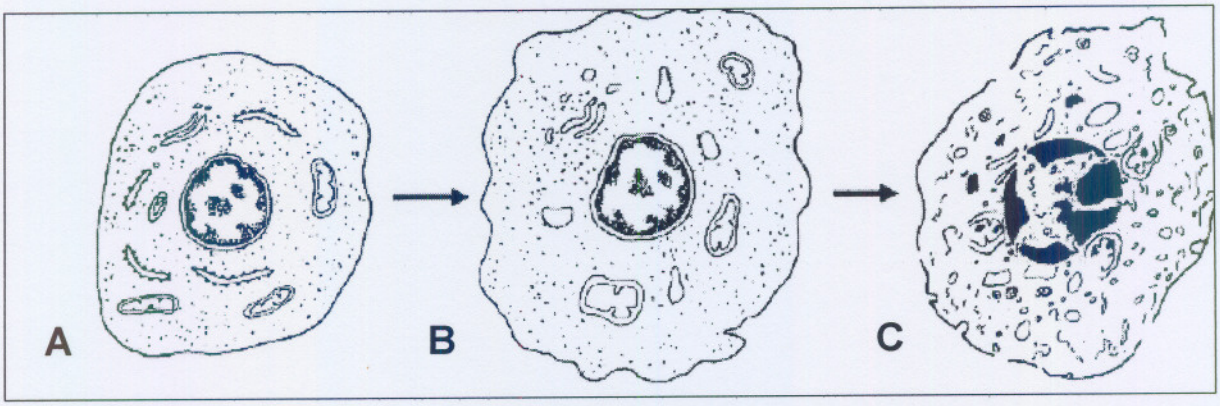


Figure 2-16 The process of necrotic cell death: A.) The normal cell B.) undergo changes such as vacuolation of the cytoplasma and breakdown of the plasma membrane until C.) chromatin disappears and cellular membranes disintegrate (Walker *et al.*, 1988).

2.2.2.2.3. GENERAL MECHANISMS AND PATHWAYS

The extent of the damage and energy supply determines whether necrosis will be induced or not. ATP is considered to be the switch between apoptosis and necrosis (Leist *et al.*, 1997b). Necrosis is independent of ATP, or more accurately, energy depletion leads to necrosis. DNA breaks activate the enzyme PARP (§ 2.1.3.3.4) which in turn attaches oligomers of ADP-ribose to itself and some other nuclear proteins. Excessive activation of PARP by induced DNA breaks may cause cell death due to ATP depletion (Eliasson *et al.*, 1997). This ATP depletion results from use of ATP for the synthesis of the PARP substrate NAD^+. Thus, massive DNA breaks cause activation of PARP, depleting its substrate NAD^+ and, subsequently, ATP, which may lead to necrosis.

Necrosis may be divided into two main stages (Clarke, 1990). At first cellular mechanisms maintaining cellular homeostasis is irreversibly altered, whereafter the cell degenerates and subsequent inflammation follow.

Proskuryakov *et al.* (2003) suggests the following scenario for necrotic death induction. Several receptors are implicated in triggering necrosis including TNF receptors and other receptors of this family (Fas, TRAIL), purinogenic receptors, excitoreceptors (NMDA) (Holler *et al.*, 2000; Boone *et al.*, 2000) and DNA damage which may be induced directly or indirectly (Hampton &

Orrenius, 1997; Palomba *et al.*, 1999; 48). Among the second messengers participating in receptor-mediated necrosis are Ca^{2+} (Yoshioka *et al.*, 2000) and ceramide (Mengubas *et al.*, 1999). Receptor stimulation, oxidative stress and DNA damage activate stress kinases such as JNK and p38, which are components of both apoptotic and necrotic programs. At present it is not clear why the activation of these kinases lead to apoptosis in some cases and necrosis in others, but mitochondria may play the key role in determining the cellular death pathway (Denecker *et al.*, 2001; Kroemer & Reed, 2000).

As with the apoptotic pathway, proteases (such as caspases, calpains, cathepsins and serine proteases) are activated during necrosis (Kohli et al., 1999; Lankiewicz *et al.*, 2000). Research indicates that the inhibition of caspases during stress may, however, trigger necrosis in some cases, rather than to suppress it. For this reason caspase activity is sometimes necessary, paradoxically, for protection of cells from stress, possibly due to the caspase-mediated elimination of ROS-generating mitochondria (Oppenheim *et al.*, 2001).

2.2.2.3. CELLULAR PROTECTION

Cell proliferation and survival during oxidative stress are determined by the ability to adapt to the stress. Adaptation usually presides in the form of upregulation of antioxidant systems within the cells. As most of the inhaled ozone reacts within the LLF of the airways, the antioxidant composition of this fluid is critically important in determining an individual's sensitivity to ozone and other gaseous pollutants. Antioxidants act as ozone scavengers and either react directly with ozone or counteract the oxidative stress caused by the secondary ozonation products in the lung.

2.2.2.3.1. ANTIOXIDANT ENZYMES

It seems that oxidative stress induces an apoptotic response when cells can maintain their reducing capacity against ROS (through natural antioxidant systems), whereas necrosis is triggered when this reducing homeostasis is disturbed (for example by excessive production of ROS).

Antioxidant enzymes present in cells include enzymes such as catalase, superoxide dismutases (SOD), glutathione reductase (GSH), selenium-dependent glutathione peroxidase, selenium independent glutathione peroxidase, glutathione S-transferases, thioredoxin and thioredoxin reductase (EPA, 1986). Their function in protecting the lung against ozone toxicity is still not

clear. Ozone induces a higher mRNA concentration for antioxidants such as SOD, catalase and gluthatione peroxidase, resulting in greater enzyme activity (Rahman *et al.*, 1991). The activity of SOD and catalase therefore increases days after ozone exposure due to increased gene expression and not infiltration of enzyme-rich cells (Zhou *et al.*, 2003; Rahman *et al.*, 1991; Barber *et al.*, 1999). The levels of these antioxidant enzymes may determine whether cell death will be induced in cells exposed to ozone and free radicals generated by ozone.

2.2.2.3.2. WATERSOLUBLE ANTIOXIDANTS

Studies have shown that the LLF contain many antioxidants including low molecular weight antioxidants such as ascorbate (Vitamin C) (Thiele *et al.* 1997a; Thiele, *et al.*, 1997b), α-tocopherol (Thiele *et al.*, 1997a; Thiele *et al.*, 1997b; Valacchi *et al.*, 2000), urate (Housley *et al.*, 1995) and Vitamin E. Both water soluble and lipophylic antioxidants may be depleted by ROS generated through ozone exposure (Kennedy *et al.*, 1992; Lee *et al.*, 2003).

The human body is therefore equipped to counteract the damaging effects induced by ozone and oxidative stress *via* endogenous antioxidant systems. This protection mechanism is however only effective when the levels of oxidative stress are relatively low. When oxidative stress levels increase, the cellular damage cannot be efficiently reduced by these antioxidants.

2.2.2.3.3. ACTIVATION OF AKT

In addition to inducing cell death, secondary oxidants produced by excessive ozone such as hydrogen peroxide, singlet oxygen and peroxynitrate stimulate cell survival signalling pathways. Hydrogen peroxide and peroxynitrate stimulate membrane tyrosine kinase growth factor receptors leading to the activation of Akt (§ 2.2.2.1.5). Cell death induced by singlet oxygen is enhanced by the inhibition of PI3-K, suggesting that this oxidant may also activate Akt, but unlike the response to other oxidants, this activation does not involve growth factor receptors. Activation of neither FAK nor Ras (responsible in mediating responses to ROS) is involved in the singlet oxygen-induced activation of the PI3-K–Akt survival (Zhuang & Kochevar, 2004). Many questions remain unanswered and much research still needs to be done on the mechanism of cellular survival induced by Akt after ozone exposure.

2.3. SUMMARY

The link between ozone and apoptosis therefore lies in the induction of oxidative stress in cells. The induction of oxidative stress is initiated by the overproduction of free radicals such as ROS during lipid oxidation of polyunsaturated fatty acids present in the LLF and lipids in cellular membranes by the inhaled ozone. Once oxidative stress is induced, apoptotic and anti-apoptotic enzymes are activated which may ultimately cause apoptotic cell death in cells exposed to ozone or secondary reaction products of ozone.

2.4. CONCLUSION

Ozone is a highly reactive molecule that may react with a multitude of biological and non-biological molecules. Of particular interest are the effects of ozone on biological molecules in the human body. While it is currently employed in some alternative health care settings, administered systemically (AHT) to treat various diseases, our knowledge of its clinical effectiveness and toxicity is based on anecdotal data. Data from sound biochemical and pre-clinical pharmacological studies are also limited. It is well recognised that ozone produces free radicals and other ROS, which may cause the induction of a state of oxidative stress in cells. Oxidative stress in turn initiates a cascade of protease activations, which ultimately leads to cellular death. Even though ozone may have a negative impact on cell survival *via* this mechanism, it has also been shown that repeated ozone exposures could induce "adaptive" responses in cells (for example upregulation of antioxidant systems). Therefore, ozone may have both protective and damaging effects on cell tissues. This was the focus of our current research project.

EXPERIMENTAL PROCEDURES

CHAPTER 3

3. INTRODUCTION

The primary purpose of this study was to investigate the effect of ozone exposure on cellular plasticity, including the effects of ozone on the pro-apoptotic caspase-3 and the anti-apoptotic protein Akt. For this purpose a human adherent epithelial cell line (HeLa) was utilised. The cells were exposed to ozone-saturated physiological solution acutely or repetitively, whereafter cell viability was evaluated with the trypan blue cell stain or MTT reagent and DNA integrity and repair was evaluated. In addition, the role of selected enzymes involved in cellular plasticity was investigated by addition of the enzyme inhibitors Z-Asp(OMe)-Gln-Met-Asp(OMe) fluoromethyl ketone (Z-DQMD-FMK) (a caspase-3 and -6 inhibitor), (-)-deguelin (an activated Akt inhibitor) and the xanthine oxidase and NFκβ inhibitor, N-(3,4-dimethoxy-2-chlorobenzylideneamino)-guanidine (ME10092).

In this chapter the materials, experimental design, biological model (i.e. cell line used) and various assays for these experiments implemented are explained and discussed.

3.1. EXPERIMENTAL LAYOUT

The study can be divided into four phases (Figure 3-1), namely:

- *Phase 1*: Acute ozone exposure of HeLa cells and determination of cell viability.
- *Phase 2*: Repeated ozone exposure of HeLa cells and determination of cell viability.
- *Phase 3*: Investigation of the possible intervention with the enzyme inhibitors (-)-deguelin, Z-DQMD-FMK and ME10092 in the initiation of apoptosis in the HeLa cells.
- *Phase 4*: Determining the DNA integrity after repeated ozone exposures and the repair rate of HeLa cells after exposure.

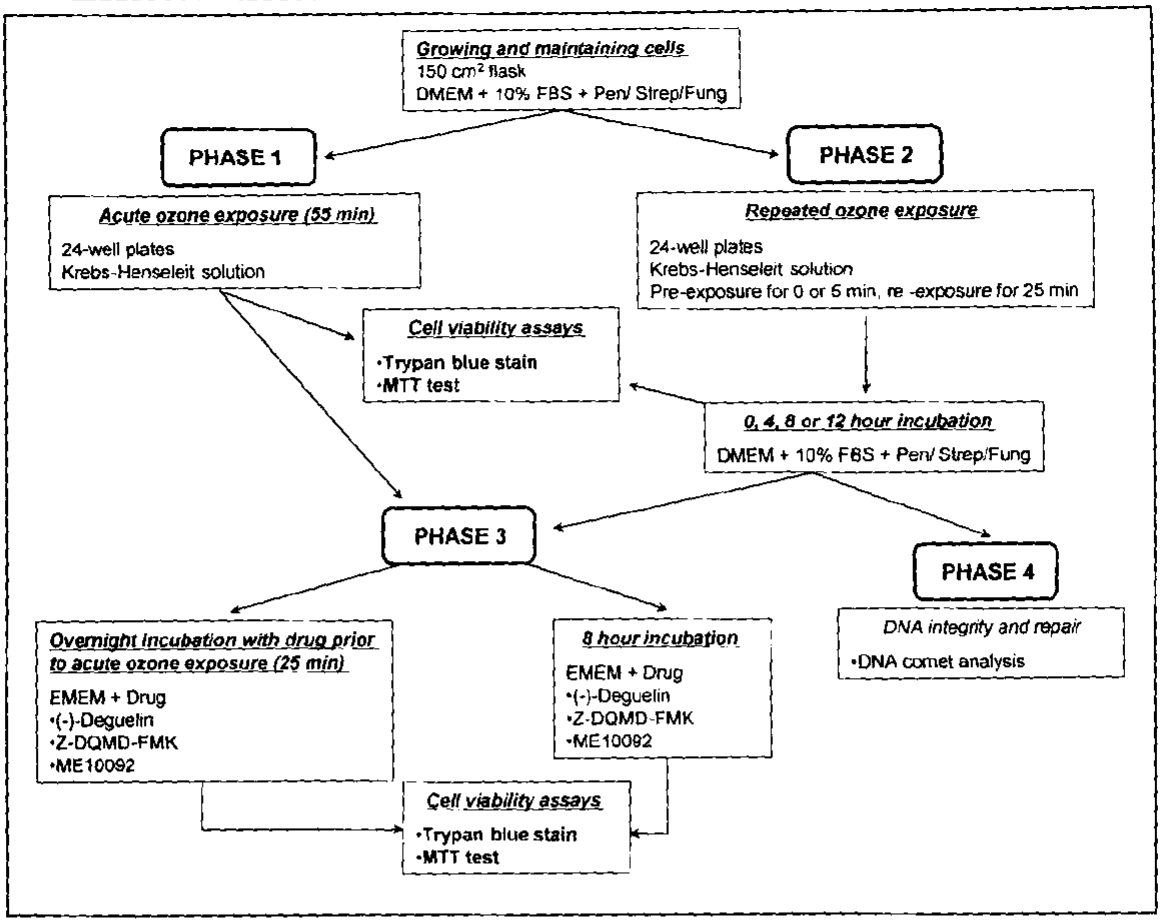


Figure 3-1 Experimental design of the current project.

3.2. CELL LINE EMPLOYED

Inhalation experiments with humans and animal models repeatedly confirm the toxicity of ozone after acute exposure to relatively high doses (Aris et al., 1993; EPA, 1996; Arito *et al.*, 1990; Rivas-Arancibia *et al.*, 2003; Rivas-Arancibia *et al.*, 2000) and some protective effects after repetitive exposures to lower doses (Rahman *et al.*, 1991; Tepper *et al.*, 1989; van der Wal *et al.*, 1994; Devlin *et al.*, 1997; Frank *et al.*, 2001). In recent years, toxicologists used cell cultures to study the biological effects of ozone in more detail. Cell cultures are grown *in vitro* under artificial conditions, and for this reason they sometimes respond slightly differently to drugs than *in vivo* cells. However, the *in vitro* investigations mimic *in vivo* biological systems surprisingly well, allow for strict control of environmental factors and are relatively simple biological systems which improve control of biological variables. Thereby cell cultures enable us to study biochemical mechanisms in more detail.

For this study a standard, well-characterised adherent human epithelial cell line (HeLa cell line from American Type Culture Collection (ATCC – catalogue no CRL-2266)) was used. This cell

line was isolated from the 31-year-old Henrietta Lacksin in 1951 and named HeLa from the first two letters of her name. HeLa cells have been used throughout biomedical research fields to study the biochemical pathways of normal and diseased tissue in human cells.

The HeLa cell line has been selected for this study because epithelial cells lining the small, peripheral airways function as important targets for the action of inspired ozone. Loss of epithelial barrier integrity in these regions is a common element in ozone-induced inflammation. The HeLa cell line can therefore be considered a suitable *in vitro* biological model to study the cytotoxic effects of ozone in the airways, although the biochemical changes measured may apply to other cell types as well.

HeLa cells were maintained in DMEM, 10% foetal bovine serum (FBS), 100 units penicillin/ml, 100 µg streptomycin/ml and 0.25 µg fungizone/ml in culture flasks at 37 °C in 5% CO_2 and humidified atmosphere. Under these conditions the cells attached as a monolayer on culture flask surfaces and had a duplication time of approximately 24 hours. The medium was renewed every three to four days. At approximately 80% confluency cells were detached from the flask bottom by means of trypsination (10 minutes incubation with trypsin/EDTA) and seeded in new culture flasks at a density of no less than $^1/_6{}^{th}$ of confluent. One 95% confluent 150 cm^2 culture flask delivered sufficient cells for seeding 3 x 10^6 cells/well into two 24-well plates for cell treatment and assays (described in § 3.4.3 and 3.4.4).

3.3. MATERIALS

3.3.1. COMPOSITION OF KREBS-HENSELEIT SOLUTION

A modified Krebs-Henseleit solution was chosen as cell medium for ozone exposure to avoid the possible reaction of ozone with amino acids and glucose present in the normal DMEM growth medium (Cataldo, 2003; Tsong, 1974). During the initial experiments, glucose was included in the Krebs-Henseleit solution. However, since the MTT test suggested about 30% decrease in mitochondrial function (compared to control) after as little as 5 minutes exposure to ozone-saturated Krebs-Henseleit medium, sustained for 55 minutes and since this effect was absent in the absence of glucose, subsequent exposures to ozone was performed in glucose-free Krebs-Henseleit medium (see data and discussion in § 4.1.2). The glucose-free Krebs-Henseleit solution was prepared as described by Patil and Jacobowitz (1968), but with omission of 11.1 mM glucose:

Table 3-1 Composition of glucose-free Krebs-Henseleit solution.

Chemical salt	Concentration
NaCl	119 mM
KCl	4.7 mM
$CaCl_2.(2H_2O)$	1.9 mM
$MgCl_2(6H_2O)$	0.54 mM
$NaHCO_3$	24.0 mM
NaH_2PO_4	1.0 mM

The salts in Table 3-1 were dissolved in demineralised and double distilled water (ddH_2O). Ingredients were added after the previous salt was dissolved, while KCl and $MgCl_2$ were added last to prevent the formation of precipitate. The solution was then heated to 37 °C and kept at this temperature throughout the experiment.

When indicated, ozone or ultra pure oxygen was bubbled through the solution in a fume hood for at least 30 minutes to ensure a saturated solution of the gas (see § 3.3.4).

3.3.2. CHEMICALS

3.3.2.1. CHEMICALS USED FOR CELL CULTURES

- *From Bio-Whittaker (Walkersville, MD, U.S.A.):*
  Dulbecco's Modified Eagles Medium (DMEM), Minimum Essential Media with Earle's Base (EMEM), foetal bovine serum albumin, foetal bovine serum (FBS), penicillin, streptomycin, fungizone and Human Serum Type AB (Mycoplasma treatment).

- *From Scientific Group (Midrand, South Africa) (Gibco):*
  Trypsin/versine (0.25% 1:250 + 0.38% EDTA).

- *From Afrox (Johannesburg, South Africa):*
  Liquid N_2.

3.3.2.2. CHEMICALS USED FOR ASSAYS

- *From Bio-Whittaker (Walkersville, MD, U.S.A.):*
  Trypan blue stain (0.4%).

- *From Sigma Aldrich (Missouri, U.S.A.):*
  Thiazolyl blue tetrazolium bromide (MTT) ($C_{18}H_{16}N_5SBr$) reagent, Isopropranol (99+%) (C_3H_8O), Z-Asp(OMe)-Gln-Met-Asp(OMe) fluoromethyl ketone, (-)-deguelin, Triton X100, and Tris buffer.

- *From Saarchem-Holpro Analytic (Krugersdorp, Gauteng, South Africa):*
  Formic acid.

- *From Riedel-de Haën:*
  Indigo trisulfonate potassium salt redox indicator ($C_{16}H_7K_3N_2O_{11}S_3$).

- *From Saarchem, Unilab (Krugersdorp, Gauteng, South Africa):*
  Phosphoric acid (85%).

- *From Merck (Johannesburg, South Africa/Darmstadt, West Germany):*
  NaCl, KCl, $CaCl_2.2H_2O$, $MgCl_2.6H_2O$, NaOH, ethylenediaminetetra acetic acid di-sodium salt, dimethyl sulfoxide, HCl, NaCl, hydrogen peroxide, low melting point agarose, high melting point agarose, $NaHCO_3$ and NaH_2PO_4.

- *From Afrox (Johannesburg, South Africa):*
  Ozone is produced from 99.995% pure (UHP) oxygen.

- *From MP Biomedicals (Eschwege, Germany):*
  Ethidium bromide.

- *From Separations (Eschwege, Germany):*
  Hydrogen peroxide.

3.3.2.3. CONSUMABLES

- *From Corning (New York, U.S.A):*
  Culture flasks (150 cm^2), 24-well plates, 96-well plates, 50 ml sterile conical tubes and serological pipettes.

3.3.3. INSTRUMENTS

The following instruments were employed in the experiments as indicated.

- Eppendorf quantitative micropipettes (10-100 µM, 100-1000 µM and multi-pipette) were used for analytical measurements.
- Ozone generator (Sterizone P-HP 250 – PCT/ZA00/00031) manufactured and patented by the Department of Physics, North-West University (Potchefstroom campus).
- For the trypan blue viability assay a haemocytometer (0.1 mm depth, 0.0025 cm^2), Nikon TMS inverted microscope (model number: 31771) and bench top centrifuge 3K15 (Sigma Aldrich, Johannesburg, South Africa) were used.
- A 96-well plate reader with 560 nm filter (Labsystems Multiscan RC), spectrophotometers (Spectronic 20 from Bausch & Lomb and SmartSpec™ Plus Spectrophotometer from BioRad) were utilised for spectroscopic analyses of the MTT formazan product.
- For the SmartsSpec™ Plus Spectrophotometer, standard quartz QS cuvettes (borrowed from Cenqam) with a path length of 10.00 mm were used.
- Power Pac 200 (from BioRad) was used for electrophoresis during the DNA comet assay.
- Olympus IX70 inverted system microscope was used for the visualisation of the DNA comets.
- The analySIS® program software was used to capture the images of the comets visualised on the microscope.
- The CASP® program software was used for quantification of the comets captured during visualisation.

3.3.4. 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 – see § 2.1.1.3 and data discussion in § 4.1.1). The system setup for the exposure of this solution to ozone was done as shown in Figure 3-2. 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.

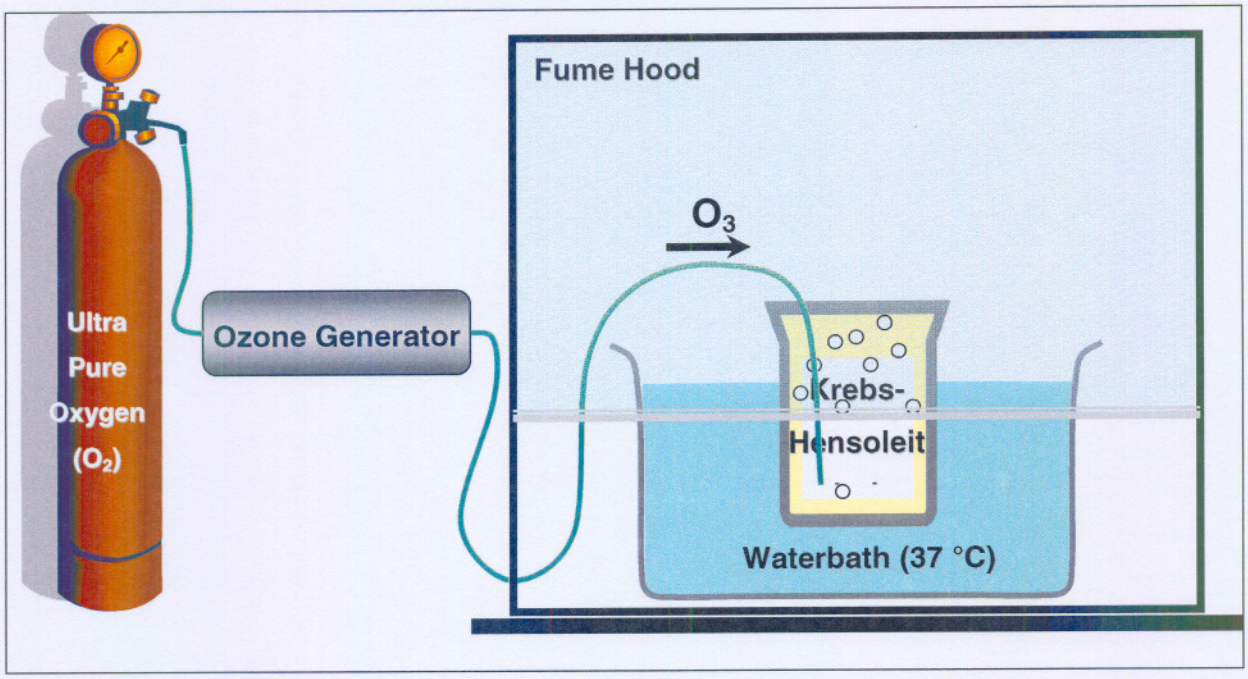


Figure 3-2 Ozone exposure system set-up in cell culture laboratory.

3.4. EXPERIMENTS

3.4.1. SEEDING OF CELLS IN 24-WELL PLATES

The following protocol was followed to seed HeLa cells grown in 150 cm^2 flasks into 24-well plates:

- For experiments, cells were grown and cultured in 150 cm^2 culture flasks with normal culture medium (DMEM + 10% FBS) until 95% confluency (see § 3.2).
- Cells were detached from the flask bottom with trypsin/versine and seeded with normal culture medium in 24-well plates at a density of 3 x 10^6 cells per well (following cell counting (see § 3.4.4.1).
- For each experimental condition three separate wells were seeded, allowing triplicate observations per condition per single experiment.
- Cells were then incubated overnight (for 15 hours) at 37 °C in 5% CO_2 to allow cells to attach to the well bottoms.
- After incubation, cells were exposed to different ozone regimes (see § 3.4.2.1 and § 3.4.2.2).
- At all times, whatever the procedure, all wells underwent the same rinsing and exposure steps to ensure comparable results at the time of analysis.

3.4.2. EXPOSING SEEDED CELLS TO OZONE

3.4.2.1. ACUTE OZONE EXPOSURE

The following protocol describes the steps followed during acute ozone exposure of HeLa cells:

- The glucose-free Krebs-Henseleit (gf-KH) solutions (§ 3.3.1) in the reservoir were kept at 37 °C and utilised untreated (base-line control) or saturated with either oxygen (O_2 control) or ozone (ozone – test groups) by continuous gaseous bubbling for at least 30 minutes before use.
- The growth medium in each well was aspirated.
- Of the gf-KH solution 1 ml was added to each well and cells were exposed for a total of 55 minutes.
- The solution in all wells was replaced every 5 minutes (see § 4.1.1.). Depending on the duration of ozone or oxygen exposure specified, the untreated solution was replaced with the appropriate treated (i.e. oxygen- or ozone-saturated) solution at the appropriate time interval during the 55 minute exposure time.
- After the full exposure time, the gf-KH solution was aspirated and wells were rinsed once with phosphate-buffered saline (PBS).
- The indicated cell viability assay was then performed (as described in § 3.4.4.2).

3.4.2.2. REPEATED OZONE EXPOSURES

For the repeated ozone exposure treatment of HeLa cells, the following protocol was utilised:

- The treatment of gf-KH solution and procedures of cell exposure was in principle as described in § 3.4.2.1.
- Cells were, however, exposed to base-line control (i.e. no oxygen or ozone), oxygen- or ozone-saturated gf-KH solution for a duration of 5 minutes, repeated every 4 hours, for a total of 4 exposures.
- After the last exposure the wells were incubated overnight at 37 °C in 5% CO_2 in DMEM + FBS.
- The following day, the wells were exposed to the indicated treatment (i.e. base-line control (i.e. no oxygen or ozone), oxygen or ozone) for a total duration of 25 minutes, replacing the solution every 5 minutes, as described in § 3.4.2.1.
- After the full exposure time, the gf-KH solution was aspirated and wells were washed with PBS.
- The well-plates were then incubated at 37 °C in 5% CO_2 in DMEM + 10% FBS for 0, 4, 8 or 12 hours, as indicated.

- After this incubation period the cell viability assays were performed (as described in § 3.4.4.2).

3.4.3. ENZYME INHIBITOR INTERVENTION

As indicated in the experimental design (Figure 3-1), the drug treatment involved incubation with (-)-deguelin, ME10092 and Z-DQMD-FMK. The aim of this drug treatment was to determine the mechanism behind ozone-induced effects on HeLa cell viability.

3.4.3.1. (-)-DEGUELIN

Deguelin (Figure 3-3) is a rotenoid isolated from several plant species including *Mundulea sericea*. Deguelin treatment induces both cell-cycle arrest in the G2/M phase and apoptosis. These findings indicate that the pro-apoptotic activity of deguelin results from its ability to inhibit PI3-K/Akt-mediated signalling pathways (Chun *et al.*, 2003). It also inhibits the expression of cyclooxygenase-2 (Lee *et al.*, 2004). This drug was utilised in the study to determine whether ozone inhibits the activation of Akt when HeLa cells are exposed to ozone.

In previous cell culture studies (Chun *et al.*, 2003; *Lee et al.*, 2004) it was observed that deguelin inhibits cell proliferation by inhibiting the PI3-K mediated activation of Akt (with IC_{50} values ranging from 0.01 µM to 10 µM). Therefore 0.2 µM deguelin (i.e. 20x the lower range IC_{50} value) was used for this study in cell drug treatment where indicated.

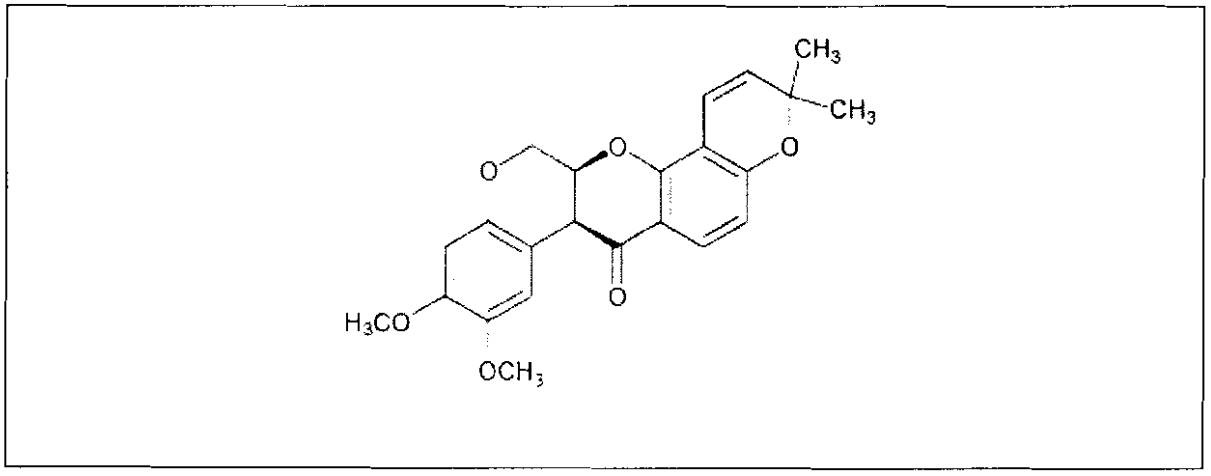


Figure 3-3 The chemical structure of (-)-Deguelin.

3.4.3.2. Z-DQMD-FMK

In order to investigate how cell death is induced by ozone, the caspase-3 and -6 inhibitor, Z-DQMD-FMK was chosen to competitively and irreversibly inhibit caspase activation in the cells. It is well known that, once caspases are activated (especially caspase-9 and -3), important repair proteins are inactivated, ultimately leading to cell death (Chang & Yang, 2000; Enari *et al.*, 1998; Liu *et al.*, 1997a; Xue *et al.*, 1996; Cheng *et al.*, 1997; Adams & Cory, 1998).

No previous cell culture studies were done using this drug as treatment. The product information sheet, however, recommended a final concentration of 5 to 100 µM. For the current study a concentration of 5 µM was chosen for use in HeLa cells where indicated.

3.4.3.3. ME10092

N-(3,4-dimethoxy-2-chlorobenzylideneamino)-guanidine (ME10092) has been utilised experimentally in cardioprotection during ischemic reperfusion injury (reviewed by Oliver *et al.*, 2004), but its pharmacologic mechanism is still relatively unclear.

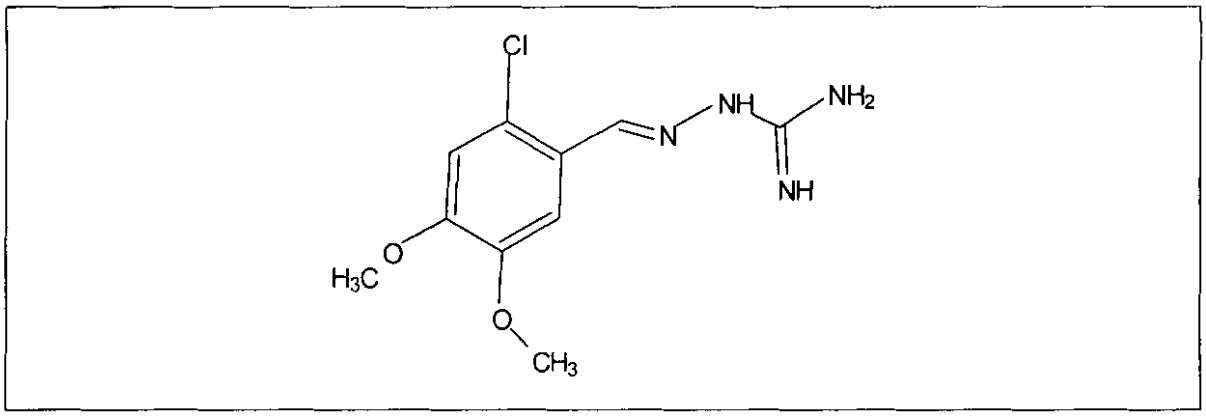


Figure 3-4 Structure of the guanidine ME10092 (Oliver *et al.*, 2004).

Research now focuses on its cellular protection mechanism which may be multiple. It is a weak inhibitor of xanthine oxidase and nitric oxide synthase as well as the Na^+/H^+ exchange inhibitors. It also inhibits free radical formation by NAD(P)H oxidase to some extent, but not very potently (Yildiz *et al.*, 1998; Dambrova *et al.*, 2004). ME10092 can attenuate NO production by inhibiting both constitutive and inducible NOS, significantly reducing TNF-α release *in vitro* and may attenuate poly (ADP-ribose) polymerase activation (Marzocco *et al.*, 2004), while potently inhibiting NFκβ activity (Liepsinsh *et al.*, 2005). Although inhibition of NFκβ translocation to the nucleus by ME10092 increases the number of viable and non-apoptotic cells, concentrations

higher than 1 µM completely reduces the stimulated NFκβ activation, decreasing viable cells and increasing apoptotic cells (Liepsinsh *et al.*, 2005).

After testing the direct effect of various concentrations between the limits 10 mM and 0.1 µM for direct effects on cell viability, it was decided to use 1 µM as concentration in treating HeLa cells (Data not shown). Results from these preliminary experiments showed that high concentrations (10 mM and 10 µM) completely reduce the mitochondrial function of HeLa cells, while the best cell viability was achieved by treating the HeLa cells with 1 µM ME10092.

A summary of the important substrates of these enzyme inhibitors is shown in Figure 3-5 below.

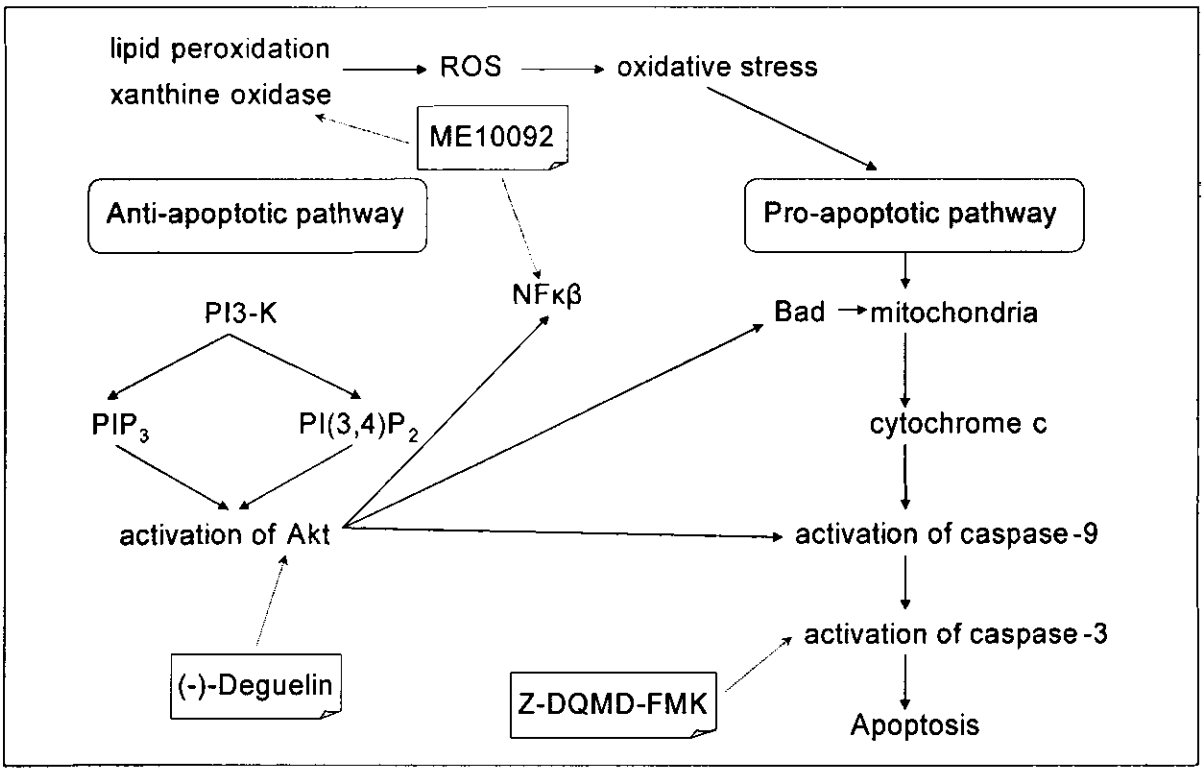


Figure 3-5 Diagram representing (-)-deguelin, Z-DQMD-FMK and ME10092 and the enzymes they inhibit.

3.4.3.4. PROTOCOL FOR ENZYME INHIBITION

After the treatment procedures described in § 3.4.2.1 and § 3.4.2.2 the cells were rinsed twice with 500 µl EMEM medium, whereafter 300 µl of each drug solution was added to the wells. The cells were incubated for 8 hours at 37 °C in 5% CO_2 in serum-free minimum essential medium (Earle's base) (EMEM) after which the cell viability assays were performed (as described in § 3.4.4.2).

3.4.4. ASSAYS

3.4.4.1. CELL COUNTING AND SEEDING ASSAY

The following protocol was used for counting HeLa cells before seeding them into 24-well plates:

- After overnight incubation at 37 °C in 5% CO_2, DMEM growth medium was aspirated from the culture flasks.
- Trypsin/versine (2 ml) was added to each flask and flasks were incubated for 10 minutes in 37 °C and 5% CO_2.
- After the cells were detached from the bottom of the flasks, the cell suspension was pipetted up and down in order to get a homogenous cell suspension and to inhibit the formation of cell clusters.
- Of the cell suspension 20 µl was diluted with 180 µl of DMEM and 20 µl of this diluted suspension was transferred to the haemocytometer for counting.
- The average of the cells counted was then used to determine the dilution factor of the cell suspension necessary for the experimental suspension.
- Of the experimental cell suspension 1 ml was then added to each well in a 24-well plate.
- Seeded cells were incubated overnight at 37 °C in 5% CO_2 to allow cells to attach to the bottom of the wells.

3.4.4.2. INDIGO COLORIMETRIC METHOD FOR DETERMINING OZONE CONCENTRATION

3.4.4.2.1. INTRODUCTION

The aim of the indigo colorimetric assay was to determine the concentration of ozone in the glucose-free Krebs-Henseleit solution that cells are exposed to.

The detection of ozone in liquid phase can be done using the potassium-indigotrisulfonate colorimetric method. The method is quantitative, selective and simple.

The reaction of the indigo-reagent ($C_{16}H_7N_7O_{11}S_3K_3$) is utilised as an international standard method for the determination of ozone in aqueous solutions. The principle of the reaction is based on the decolourisation of indigo by ozone in an acidic solution. This difference in colour can be measured at a wavelength of 600 nm. The decrease in absorbance is linear with

increasing concentration. The ozone concentration is expressed as ppm or mg/l. The detection upper limit for this spectrophotometric method is 2 µg/l ozone (Franson, 1995).

3.4.4.2.2. ASSAY

This protocol describes the method utilised to determine the concentration of ozone 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 trisulfonate 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 stock solution to a 50 ml volumetric flask. To this, 0.5 g sodium dihydrogen phosphate (NaH_2PO_4) and 0.35 ml concentrated phosphoric acid was added. The volumetric flask was filled to 50 ml with distilled water.
- The gf-KH solution was prepared as described in § 3.3.1 and continuously exposed to ozone for the duration of the experiment.
- For the blank, 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 (gaseous bubbling stopped).
- To 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 (Franson, 1995):

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

where:

$Abs_{(blank)}$ = absorbance of the blank solution

$Abs_{(sample)}$ = absorbance of the sample solution

f = 0.42 (sensitivity factor, determined by the change in absorbance per mole of added O_3 per litre)

b = path length of cell (cm)

V = volume of the sample (ml)

3.4.4.3. CELL VIABILITY ASSAYS

3.4.4.3.1. TRYPAN BLUE VIABILITY STAIN

The aim of the trypan blue viability assay was to investigate the effect of acute and repeated ozone exposure on cell viability and cell membrane integrity.

3.4.4.3.1.1 INTRODUCTION

The trypan blue stain assay can rapidly assess cell membrane integrity as a measure of cell viability (oncosis), but cannot detect apoptosis as such.

The trypan blue assay essentially functions on the chromopore, which is negatively charged and the stain does not cross the cell membrane (i.e. it is not found intracellularly), unless the membrane is damaged (Freshney, 1987). Viable cells usually have intact membranes, so that trypan blue does not enter, while the membranes of non-viable (damaged) cells are permeable to trypan blue. Damaged cells (where membrane integrity is damaged) are therefore stained by the dye. Blue stained (damaged) cells can be distinguished visually under the microscope form intact (viable) unstained (white) cells, implementing cell counting for quantification.

3.4.4.3.1.2 ASSAY

The following steps describe the trypan blue assay utilised to determine HeLa cell viability:

- After HeLa cells were exposed to ozone (§ 3.4.2.1 and § 3.4.2.2), the medium was aspirated and cells were rinsed twice with EMEM.
- To detach cells from the well bottoms, 200 µl trypsin/versene was added and well-plates were incubated for 10 minutes at 37 °C in 5% CO_2.
- Serum-free EMEM (800 µl) was added to each well.
- The cell suspension was pipetted up and down to ensure that all cells were detached from the wells.
- Each well's cell suspension was transferred to microcentrifuge tubes and centrifuged for 10 minutes at 5 000 rpm (approximately 2300 x g) at 25 °C in the bench top centrifuge.
- The supernatant (EMEM) was aspirated and the pellet resuspended in 1 ml PBS to remove trysin/versene that may interfere with the trypan blue assay. It was centrifuged again as described above and supernatant aspirated.
- To each microcentrifuge tube 50 µl of trypan blue stain (0.4% w/v) was added just before counting.
- Thereafter the tube was vortexed to resuspend cells in the trypan blue stain.

- Of this suspension 20 µl was taken and diluted with 180 µl of trypan blue. The tube was again vortexed.
- Of this diluted cell suspension, 20 µl was transferred to the haemocytometer.
- Stained cells and unstained cells were counted separately and results were used to calculate the number of viable cells.

3.4.4.3.2. MTT ASSAY

The aim of the MTT assay was to investigate the effect of acute and repeated ozone exposure on cell viability and the effect of ozone on the function of cellular mitochondria.

3.4.4.3.2.1 INTRODUCTION

Mitochondrial damage will inevitably result in loss of the ability of the cell to maintain and provide energy for metabolic cell function and growth. Metabolic activity assays such as the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, are based on the premise of metabolic cell function loss. As the permeability assay mentioned above, this colorimetric assay may underestimate mitochondrial damage and detect cell death only at the later stages of apoptosis when the metabolic activity of the cells are reduced.

The MTT assay was first described by Mosmann in 1983 (Mosmann, 1983). The assay is based on the ability of mitochondria from viable cells to cleave the tetrazolium rings of the pale yellow, water-soluble MTT and produce dark purple, water-insoluble formazan crystals.

MTT is converted to its coloured by-product *via* a group of non specific mitochondrial dehydrogenase enzymes (Figure 3-6), including NADH dehydrogenase, malate dehydrogenase and succinic dehydrogenase (Liu *et al.*, 1997). Dehydrogenases utilised NADH or NADPH as coenzymes to convert the MTT salt to formazan crystals. These enzymes belong to the respiratory chain present in the mitochondria and are only active in metabolically intact cells.

Most cellular reduction of MTT is dependent on the reduced pyridine nucleotides NADH and NADPH, and not succinate as previously believed (Berridge *et al.*, 1993, 1994; Berridge & Tan, 1993). Since most cellular reductions occur in the cytoplasm and involve NADH and NADPH, the MTT assay can no longer be considered strictly a mitochondrial assay.

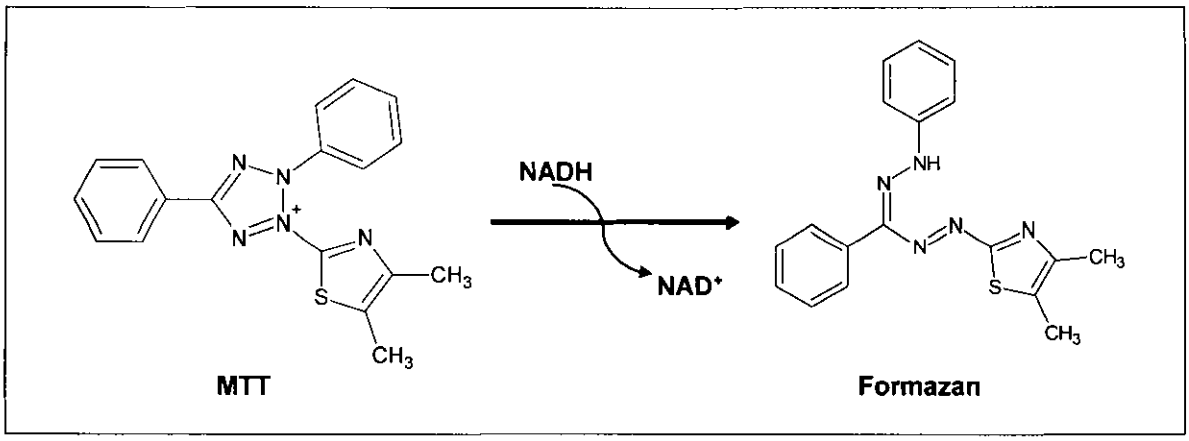


Figure 3-6 Conversion of MTT to formazan product in mitochondria (Berridge *et al.*, 1996).

The formazan crystals are largely impermeable to cell membranes. This results in its accumulation within healthy cells. To measure the amount of formazan product formed, the formazan crystals must be solubilised by the addition of an organic solvent to produce a homogeneous solution suitable for measurement. Organic solvents were tested by Mosmann, but isopropanol was found to be the most suitable solvent (Mosmann, 1983). The colour can then be quantified using a simple colorimetric assay. The results can be read on a multiwell scanning spectrophotometer (ELISA reader).

MTT is best suited for use with adherent cell lines (Scudiero *et al.*, 1988). The number of surviving cells is directly proportional to the level of the formazan product formed and thus the absorbance is directly proportional to the number of viable cells.

3.4.4.3.2.2 ASSAY

The following protocol describes the MTT cell viability test utilised after exposing HeLa cells to ozone:

- MTT[1] was dissolved in PBS at 5 mg/ml and filtered to sterilise and remove a small amount of insoluble residue present in some batches of MTT.
- After HeLa cells were exposed to ozone, the medium was aspirated and cells were rinsed twice with EMEM.
- To terminate cell growth, 200 µl MTT reagent was added to each well.
- The well plates were then incubated for two hours at 37 °C in 5% CO_2 to allow the cells to metabolise the MTT reagent.
- After two hours of incubation, the MTT reagent was aspirated.

[1] The MTT reagent is light-sensitive and must be prepared in the laminar flow chamber in the dark.

- The purple formazan crystals were then dissolved by adding 250 µl isopropanol to the wells and leaving the plates at room temperature (25 °C) for 5 minutes.
- From each well's solution 100 µl was transferred to a 96-well plate.
- This plate was placed in the multiwell scanning spectrophotometer and absorbance was determined at 560 nm.
- These results were used to determine the percentage viable cells after treatment.

3.4.4.4. SINGLE CELL GEL ASSAY (COMET ASSAY)

3.4.4.4.1. INTRODUCTION

The aim of the comet assay was to determine whether ozone exposure can induce DNA damage (single- and double-stranded breaks) in HeLa cells. Ozone, in addition to its direct oxidising capacity, can cause lipid peroxidation and the secondary reaction products arising from this peroxidation include H_2O_2, carbonyl substances and lipid hydroperoxides. All of these products are recognised to be DNA damaging agents through their highly unstable molecular structures and electrophilic reactivities with DNA strands (Pryor *et al.*, 1991).

This assay was first introduced by Ostling and Johanson in 1984 (Ostling & Johanson, 1984) as a microelectrophoretic technique for the direct visualisation of DNA damage in individual cells. The resulting DNA damage was subsequently named for their appearance as "comets". The procedure is based on the principle that strand breaking agents reduce the size of the large duplex DNA molecules and that the electric current migrates the charged DNA from the nucleus causing DNA fragments to migrate freely into the tail of the comet. Ostling and Johanson employed a pH of less than 10. A few years later, procedures were developed involving treatment at a higher pH (pH > 13). The latter procedure was originally described by Singh *et al.* (1988) and is now the standard comet assay used (see Figure 3-7).

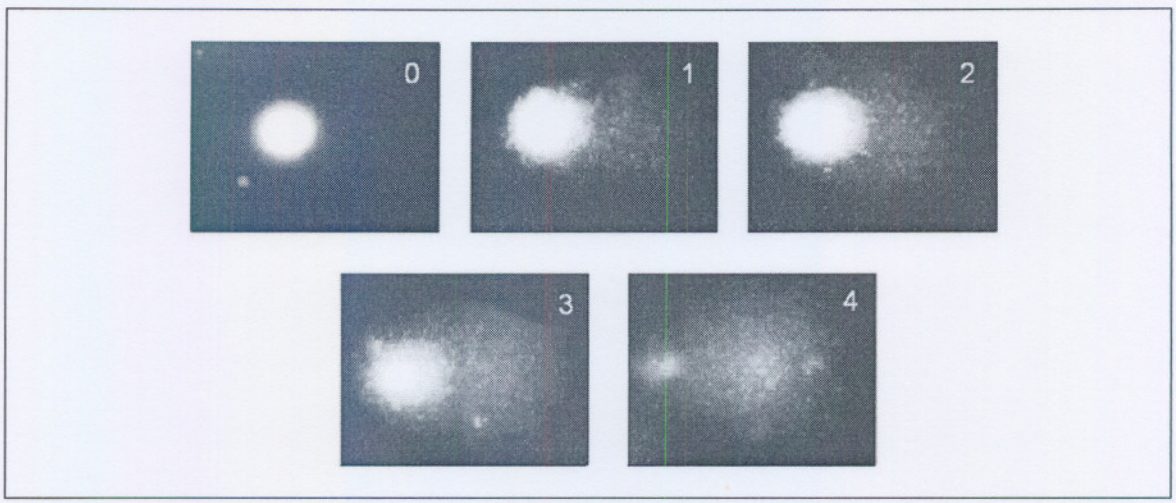


Figure 3-7 Images of comets (from lymphocytes) representing classes 0 to 4 used for visual scoring (Collins *et al.*, 2004).

The most commonly used parameters are the tail length, relative fluorescence intensity of head and tail and the tail moment. The comets are usually divided into 5 classes, from 0 (no tail) to 4 (almost all DNA in tail). This visual scoring is performed as described by Collins *et al.* (1993).

3.4.4.4.2. ASSAY

Preparation of microscope slides

- The following reagents were prepared freshly with each experiment:
  - EDTA – (37.2 g dissolved in 1 litre ddH_2O).
  - High melting point agarose (1% HMPA) – (0.5 g dissolved in 50 ml EDTA).
  - Low melting point agarose (0.5% LMPA)[2] – (0.25 g dissolved in 50 ml EDTA).
- HMPA was heated in the microwave oven until the agarose was dissolved and the gel reached a temperature just below boiling point.
- Of the HMPA 350 µl was pipetted onto the microscope slide and distributed evenly across the rough side of the slide by using the steel template (1.3 mm) and a warmed steel scraper.
- HMPA was allowed to air dry (± 15 min). Slides were left in moist box until use.
- Thereafter LMPA was heated in the microwave oven until the agarose was dissolved and the gel reached a temperature just below boiling point. The gel was cooled down and kept in a water bath at 37 °C, until the loading with cells as described below.

Detaching and preparing cells for assay

[2] Both LMPA and HMPA must be prepared fresh once a week.

- Control and ozone-treated cells in a 25 cm^3 culture flask were detached from the flasks with trypsine/versene[3].
- From each culture flask ¼ of the cells (± 5 million cells) were transferred to microcentrifuge tubes.
- The cells were centrifuged at 5500 x g for 5 min and then resuspended in 1 ml PBS.

Loading of monolayer cells in LMPA onto slides

- Of the cell suspension, 20 µl was mixed with 150 µl LMPA at 37 °C in a microcentrifuge tube.
- From this solution 150 µl was transfered onto the centre of a pre-coated slide (see prepared slides above). The solution was spread evenly across the surface by using the steel template and warmed steel scraper (rendering a 0.8 mm thick LMPA layer containing a spread mono-layer of cells).
- The slide with cell-loaded gel was placed on moist paper towels to cool down and set.

DNA repair capacity slides

When the DNA repair capacity were to be investigated, the same method for preparation and loading of cells were followed as described above, but the cells were treated with 10 µM hydrogen peroxide (H_2O_2) as follows:

- H_2O_2 (8.8 M) was mixed with PBS to give a hydrogen peroxide solution of 3 520 µM.
- The hydrogen peroxide solution (10 µl) was added to the remaining cell solution and incubated for 40 min at 37 °C.
- Thereafter it was centrifuged for 5 min at 5500 x g.
- The supernatant was removed and the pellet resuspended in PBS, whereafter it was centrifuged for another 5 min at 5500 x g.
- The cells were subsequently resuspended in normal DMEM growth medium
- From this cell suspension 20 µl was mixed with 150 µl LMPA in a microcentrifuge tube and loaded onto one of the windows of a pre-coated slide with the use of the steel template (1.6 mm) and warmed steel scraper.
- The remainder of the cell suspension was incubated for 40 minutes, whereafter another 20 µl of cell suspension was mixed with LMPA and loaded onto a slide window.
- Slides were placed on moist paper towel to air dry.

Cell lysis

- The following reagents were prepared freshly every week:
    - NaOH – (40.0 g dissolved in 1 litre ddH_2O).

[3] Trypsine/versene (0.25%) – 1:250 [with EDTA (0.38%)]

- Lysis buffer:
  - NaCl (2.5 M).
  - Trizma base (10 mM).
  - EDTA (0.1 M).

- The mixture was stirred while adding 8 g NaOH and allowing NaOH to dissolve (30 min). The pH was adjusted to 10 if necessary and volume was adjusted to 890 ml with ddH_2O.
- Subsequently the following chemicals were added:
  - Triton X100 – 10 ml (1%)
  - DMSO – 100 M (10%)
- Cells on the microscopic slides were lysed overnight in the lysis buffer at 4 °C, while protecting it from light.

Electrophoresis

- For the electrophoresis buffer the following reagents, freshly prepared, were mixed:
  - NaOH (0.3 M) (pH > 13).
  - EDTA (1 mM).
- The pH of the buffer was measured to ensure a pH > 13.
- The slides were rinsed in ddH_2O and then incubated for 30 minutes at 4 °C in the electrophoresis buffer.
- After this period, the slides were electrophoresed for 20 min at 37 V and 400 mA at 4 °C.
- The slides were then rinsed in ddH_2O.

Neutralisation and staining

- The following reagents were prepared freshly every week:
  - Tris buffer: Tris HCl (0.4 M) (pH = 7.5).
  - Ethidium bromide (light sensitive).
- Slides were incubated in the Tris buffer for 15 min at 4 °C, rinsed with ddH_2O and then incubated in ethidium bromide[4] solution for another 15 min at 4 °C. The slides were rinsed in ddH_2O.
- The image of 50 cells per condition was captured on the same day after performing the electrophoresis stage. Cells were enlarged x40 to x60.
- Comets from the images were analysed using the CASP® program.

[4] After colouring with ethidium bromide slides can be stored for 3 – 4 hours at 4 °C in a container with damp jumbo roll paper underneath.

3.5. STATISTICAL DATA ANALYSIS

Data from assays were obtained as triplicate observations from at least three separate and independent experiments and expressed as percentage of control, unless otherwise specified. Graphs were constructed utilising the computer software Graph Pad Prism® (Version 4.01 for Windows®, GraphPad Software, San Diego, CA, U.S.A., www.graphpad.com).

For estimating the half-life of ozone a one phase exponential decay non-linear fit was performed through the data points. The curve fitting is based on the equation:

$$y = \text{plateau} + \text{span.exp}(-k*x)$$

where: span = distance from the starting concentration to bottom concentration
exp = e to the –k.*x power
k = rate constant
x = x-values
plateau = to where the concentration decays

Graph Pad Prism® was also utilised for all statistical analyses of data. All measurements in each experiment were considered replicates as inter-day variations were overcome by converting each control group to 100%. For comparison of two values, the nonparametric 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 groups to each other). For all reported statistical probability values, $p < 0.05$ was regarded as statistically significant.

CHAPTER 4

RESULTS AND DISCUSSION

In this chapter all the results from the various experiments, as described in Chapter 3, are presented. The results in this chapter have been divided into two parts. The first part represent results from control experiments conducted to establish the conditions for *in vitro* ozone exposure to cultured cells, while the second part focuses on the study objective experiments. A comprehensive discussion of each set of results will follow, while a summary, final conclusions and recommendations are presented in Chapter 5.

4.1. ESTABLISHING CONDITIONS FOR ACUTE IN VITRO OZONE EXPOSURES

The unique challenges associated with studies investigating the effect of ozone on *in vitro* cultured cells include the choice of an appropriate ozone administration vehicle, as well as the choice of appropriate treatment procedures and conditions (ozone concentrations and the exposure durations). It is important to note that such *in vitro* experiments investigate biological mechanisms, screening multiple possibilities. Its relevance to the *in vivo* situation should eventually be verified in smaller, focussed follow-up studies, as suggested by the results from the *in vitro* studies. There is, however, a remarkable correlation between findings from *in vitro* and *in vivo* studies.

4.1.1. DECOMPOSITION OF OZONE IN KREBS-HENSELEIT SOLUTION

Firstly, it was important to determine the saturated concentration of ozone in aqueous solution, such as glucose-free Krebs-Henseleit (gf-KH) solution (see § 4.1.2 below for choice of gf-KH as physiological solution), at physiological temperature (i.e. 37 °C). Secondly, being a highly reactive gaseous molecule at this temperature, it was important to determine the half-life of ozone in this solution. Therefore, gf-KH was exposed to ozone for a period of 30 minutes (to ensure ozone saturation of the solution – Lotriet, 2003), whereafter exposure was stopped and the ozone concentration in solution, as well as its time-dependent decrease measured using the indigo colorimetric assay described in § 3.4.4.3.

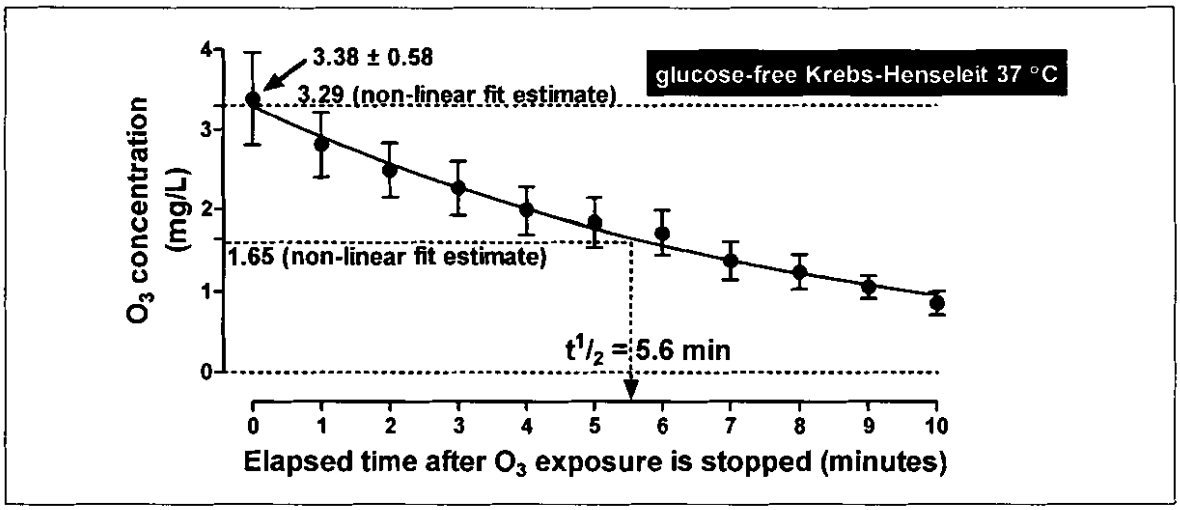


Figure 4-1 Ozone concentration (mg/l) in gf-KH solution at 37 °C over a period of 10 minutes. Data points are averages ± standard error of the mean of triplicate observations from four independent and comparable experiments (n = 16) and data are presented as the concentration of ozone in solution at the indicated time-points. The half-life (t½) of ozone was estimated from a one phase exponential decay non-linear fit of the data points.

In Figure 4-1 it is observed that the saturated concentration of ozone in gf-KH solution at 37 °C is 3.38 ± 0.58 mg/l and that this concentration rapidly decreases with a half-life (t½) of 5.6 minutes (95% confidence interval 4.5 to 7.5 minutes). Such a short t½ was expected, since the highly reactive ozone molecule is known to be relatively unstable in aqueous solution, with also a relatively poor solubility (Langlais *et al.*, 1991) (see § 2.1.1.3). The degradation of ozone in gf-KH at 37 °C follows a typical hyperbolic pattern, approaching zero concentration and from which the t½ can be estimated. Therefore, to ensure a continuous exposure of HeLa cells to a high (i.e. close to saturated) concentration of ozone, the ozone-saturated gf-KH (as well as control ozone-free or oxygen-saturated gf-KH) was replaced every 5 minutes in all subsequent experiments with HeLa cells.

4.1.2. CHANGE IN pH AFTER OZONE EXPOSURE

To investigate whether ozone affects the pH of the gf-KH solution, the pH of the solution was determined at various time points (0, 30, 60 and 90 minutes) during ozone exposure. The results are shown in Figure 4-2.

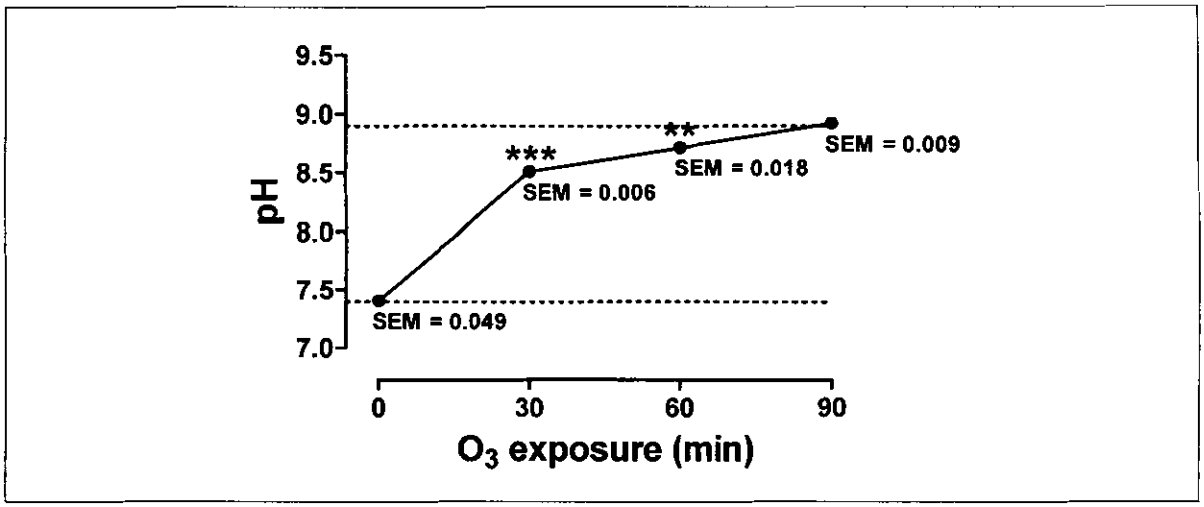


Figure 4-2 The increase of pH during gf-KH ozone exposure. Data are averages ± standard error of the mean of triplicate observations from three independent and comparable experiments (n = 3). The data were analysed statistically by performing a one-way ANOVA and then implementing the Tukey-Kramer post-test (comparing values to each other), with ** indicating $p < 0.01$ and * indicating $p < 0.001$.**

During the 90 minute ozone exposure period the pH increased significantly (from 7.4 ± 0.05% to 8.9 ± 0.01). The most significant increase, however, occurred during the first 30 minutes of ozone exposure (7.4 ± 0.05% to 8.5 ± 0.006%, $p < 0.001$), while the pH remained relatively constant during the last 60 minutes of exposure ($p < 0.01$). It is in this period that the cells are exposed to the gf-KH and therefore pH may not play a significant role in the results obtained.

4.1.3. PHYSIOLOGICAL SOLUTION EXPOSED TO OZONE

Cultured cells need culture medium to grow and can survive for short periods (few minutes or hours) in simple physiological medium. Since culture medium contains many amino acids and other growth factors that may interact with ozone (Cross *et al.*, 1992; Cataldo, 2003), simple ozone-saturated, physiological medium provides an ideal vehicle for ozone administration to cells for short intervals. For this reason physiological gf-KH solution (containing only physiological salts and buffer) was chosen, while results show that ozone saturation upon continuous bubbling of the solution with pure ozone gas has no effect on its pH.

It was important to establish whether the incubation of a standard cultured cell line, human epithelial (HeLa) cells, with gf-KH or Krebs-Henseleit (KH) solution instead of normal culture medium (DMEM + FBS) for a period of 55 minutes has any significant effect on cell viability, as measured by standard cell viability assays. The results are depicted in Figure 4-3.

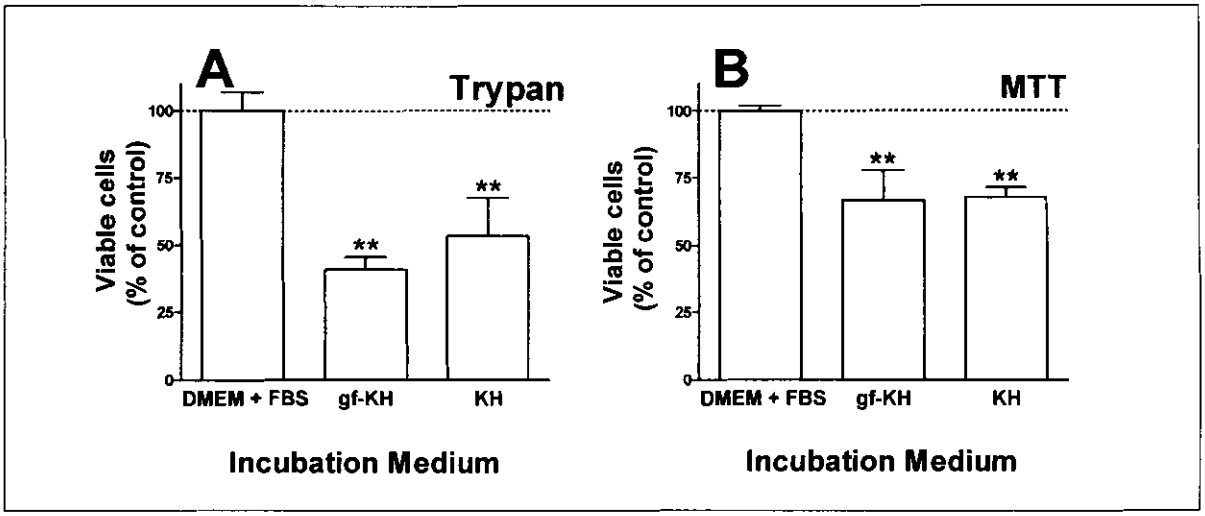


Figure 4-3 The effect of a 55 minute incubation period in DMEM + FBS medium, unexposed gf-KH and KH solution on HeLa cell viability as measured using the A.) trypan blue and B.) MTT cell viability assays. Data are averages ± standard error of the mean of triplicate observations from three independent and comparable experiments (n = 9) and are expressed as percentage of control. The data were analysed statistically by performing a one-way ANOVA and then implementing the Dunnett's post-test (comparing values to the control), with ** indicating $p < 0.01$.

When comparing the percentage viable HeLa cells after incubation with DMEM + FBS, gf-KH and KH solution, it was observed that both gf-KH and KH solutions significantly ($p < 0.05$) decreased cell viability. These observations were made in both the trypan blue (a decrease of 59.2 ± 4.8% and 46.2 ± 13.5% respectively- Figure 4-3 A) and MTT (a decrease of 33.7 ± 11.4% and 32.5 ± 3.7% respectively - Figure 4-3 B) cell viability assays.

These results suggest that the nutrients in the DMEM + FBS growth medium are essential for cell viability (i.e. both mitochondrial activity[1] and membrane integrity, as suggested by the two respective assays), although incubation with gf-KH and KH solution for 55 minutes renders sufficient cells viable for further experimentation. Since the presence of amino acids, glucose and other growth factors in culture medium (which may react with ozone to form unwanted reaction products with possible biological activity) (Cross *et al.*, 1992; Cataldo, 2003) renders it unsuitable for ozone exposure, gf-KH solution was chosen as vehicle for ozone exposure of the HeLa cells for up to 55 minutes.

[1] During the MTT assay, absorbance was linear when compared to the amount of cells (0.5, 1, 3 and 5 million) cells seeded (data not shown).

4.1.4. EFFECT OF GLUCOSE ON CELL VIABILITY AFTER ACUTE OZONE EXPOSURE

As mentioned in the previous section (§ 4.1.2), the gf-KH solution was chosen as the most suitable vehicle for *in vitro* ozone administration to HeLa cells. It was, however, still necessary to investigate the effect of a 0 to 55 minute acute exposure to ozone-saturated KH solution on HeLa cells viability. Figure 4-4 A and B depict the effect of a 0 to 55 minute acute ozone exposure period on the viability of HeLa cells utilising KH solution.

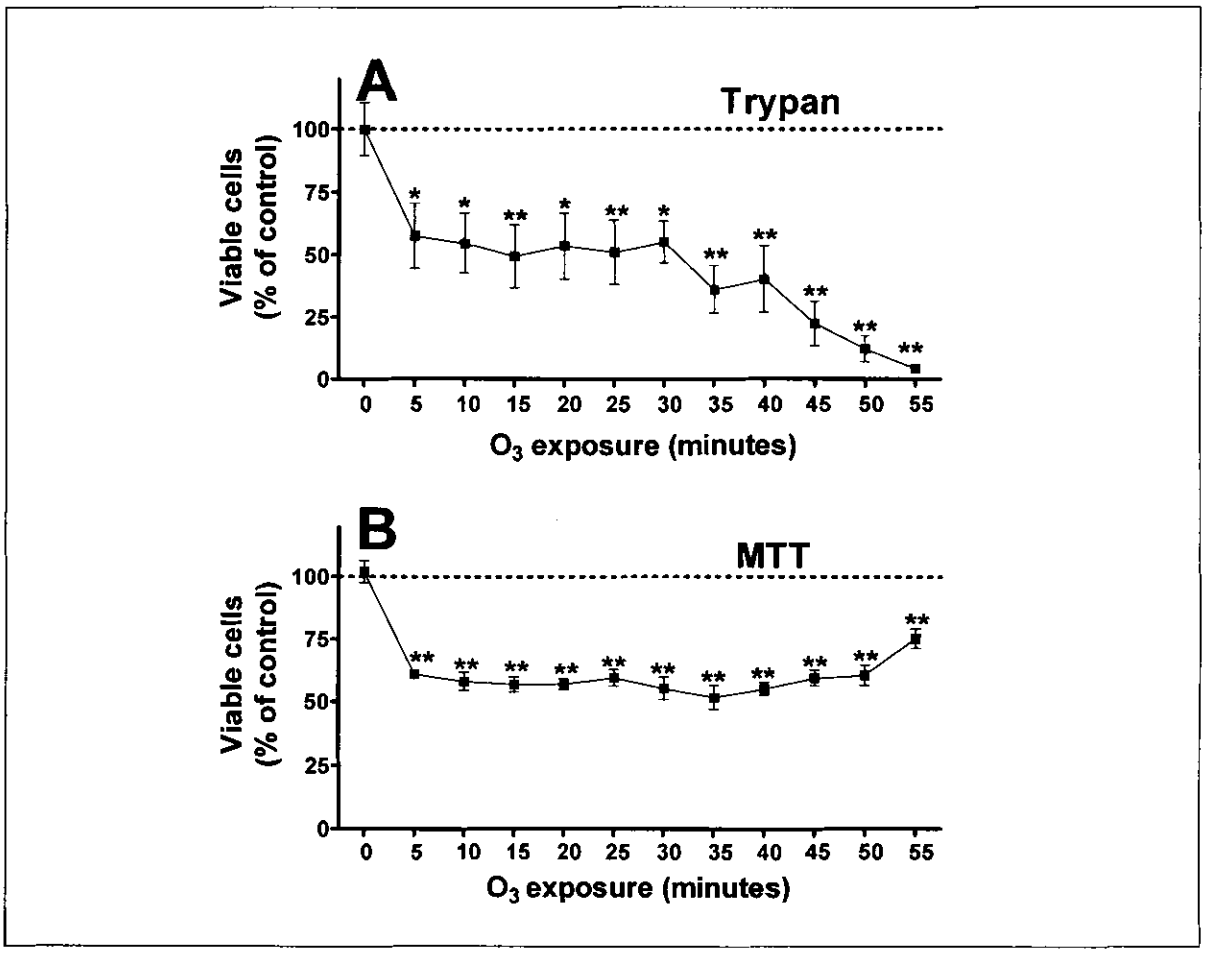


Figure 4-4 The effect of a 0 to 55 minute acute exposure to ozone-saturated KH solution on the viability of HeLa cells as measured by using A.) trypan blue and B.) MTT cell viability assays. Data points are averages ± standard error of the mean of triplicate measurements from independent and comparable experiments (n = 25 for the trypan blue assay and n = 9 for the MTT assay) and are expressed as percentage of control. The data were analysed statistically by performing a one-way ANOVA and then implementing the Dunnett's post-test (comparing values to the control), with * indicating $p < 0.05$ and ** indicating $p < 0.01$.

In Figure 4-4 A the significant decrease in cell membrane integrity (as measured using the trypan blue cell viability assay) can be seen during the 55 minute exposure period. Within the first 5 minutes of ozone exposure the cell membrane integrity decreases significantly (42.3 ± 13.0%, $p < 0.05$), with a further time-dependent decrease over 55 minutes. In contrast, the mitochondrial function (Figure 4-4 B) as measured by using the MTT cell viability assay is maximal already at 5 minutes (39.0 ± 1.7%, $p < 0.01$), remaining relatively constant until 50 minutes, whereafter a partial recovery in mitochondrial activity is observed. This uncharacteristic observation in the MTT assay warranted further investigation, especially to establish whether this reflects a rapid effect of ozone on mitochondrial activity, or whether it reflects a toxic effect of a putative reaction product of glucose in KH solution with ozone. The experiment was therefore repeated in gf-KH solution and the results compared with that obtained in the normal glucose containing KH solution. The results are depicted in Figure 4-5.

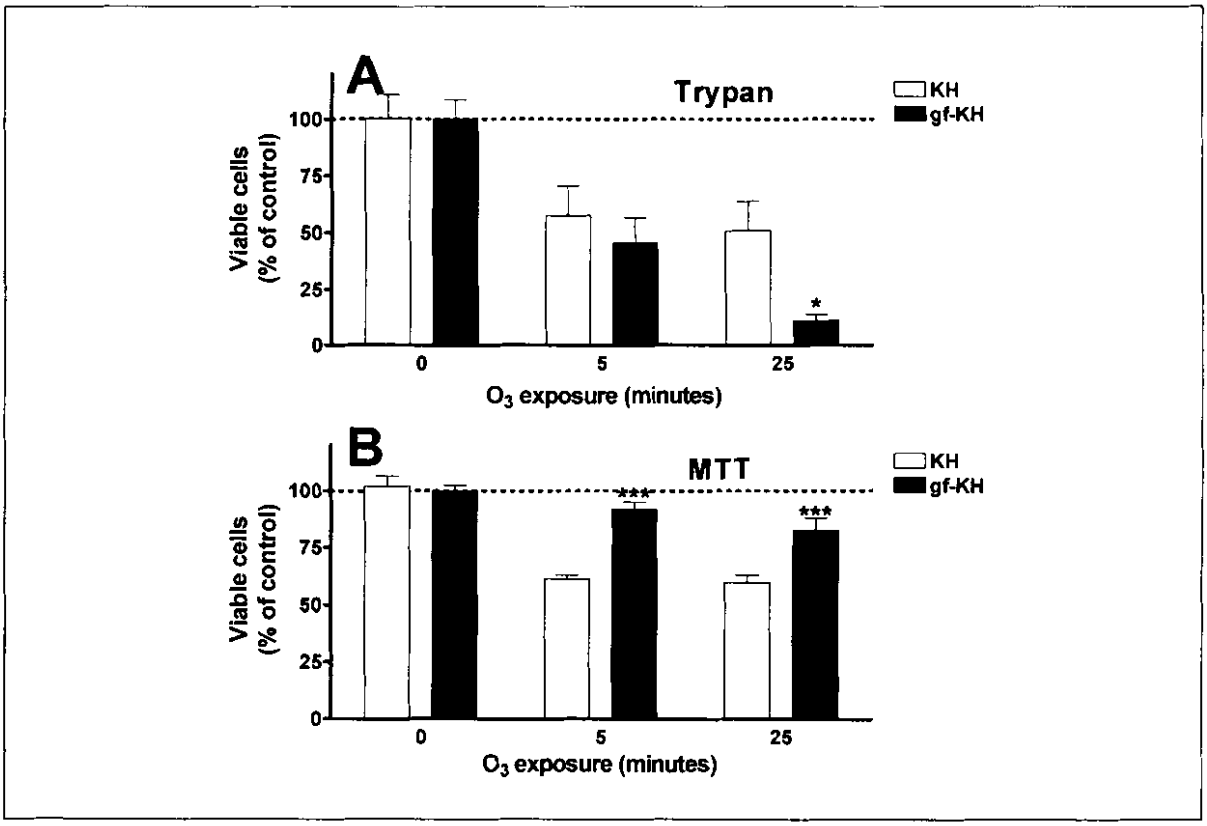


Figure 4-5 Comparison of the decrease in HeLa cell viability after ozone exposure in KH and gf-KH solution at 0, 5 and 25 minutes as measured by using A.) trypan blue and B.) MTT cell viability assays. Data are averages ± standard error of the mean of triplicate observations from independent and comparable experiments (n = 25 for KH and n = 9 for gf-KH) and are expressed as percentage of control. The data were analysed statistically by performing a two-tailed Student *t* test (comparing two values), with * indicating $p < 0.05$.

In both the trypan blue and MTT assay results we can observe the significant effect that glucose has on cell viability. Although cell membrane integrity (Figure 4-5 A) was not significantly affected by glucose after 5 minutes, there was a trend to decreased cell membrane integrity in HeLa cells incubated in gf-KH (54.3 ± 11.0%, $p > 0.05$). After 25 minutes of ozone exposure, the cell membrane integrity was however significantly decreased in the gf-KH solution (89.0 ± 3.0%, $p < 0.01$). This suggests that ozone may damage the cellular membrane more significantly in the absence of glucose.

The opposite trend was observed in HeLa mitochondrial function (Figure 4-5 B). When glucose was excluded from the physiological solution, very significant differences ($p < 0.001$) were observed in HeLa cells incubated in KH and gf-KH solution. Therefore, mitochondrial function of cells incubated in KH solution was more significantly affected by ozone than cells incubated in gf-KH solution.

The question on why glucose has such a great effect on these cell viability experiments was investigated. Another study from our laboratories (Lotriet, 2003) investigated the concentration of ozone present in individual solutions of the Krebs-Henseleit salts after bubbling ozone through the solution for a 30 minute period (to ensure ozone-saturation) and reported very low ozone concentrations in the glucose solution. These results suggest that ozone does react with glucose possibly forming toxic products that may enter the mitochondria and affect mitochondrial function. This may explain our results showing that cell membrane integrity is preserved (as ozone reacts with the glucose instead of the HeLa cell membranes), but mitochondrial function is decreased (as toxic products may be produced in the reaction of glucose with ozone). For this reason, it was decided to use the gf-KH solution as ozone administration vehicle in the remainder of the *in vitro* ozone exposure investigations.

4.1.5. EFFECT OF ACUTE OZONE EXPOSURE ON CELL VIABILITY

This experiment was conducted to determine the exposure periods for subsequent studies involving repetitive ozone exposure and the extent of damage inflicted upon HeLa cells when exposed to ozone for a period of 0 to 55 minutes. The following graphs depict the effect of ozone exposure (in gf-KH) on the cell membrane integrity and mitochondrial function:

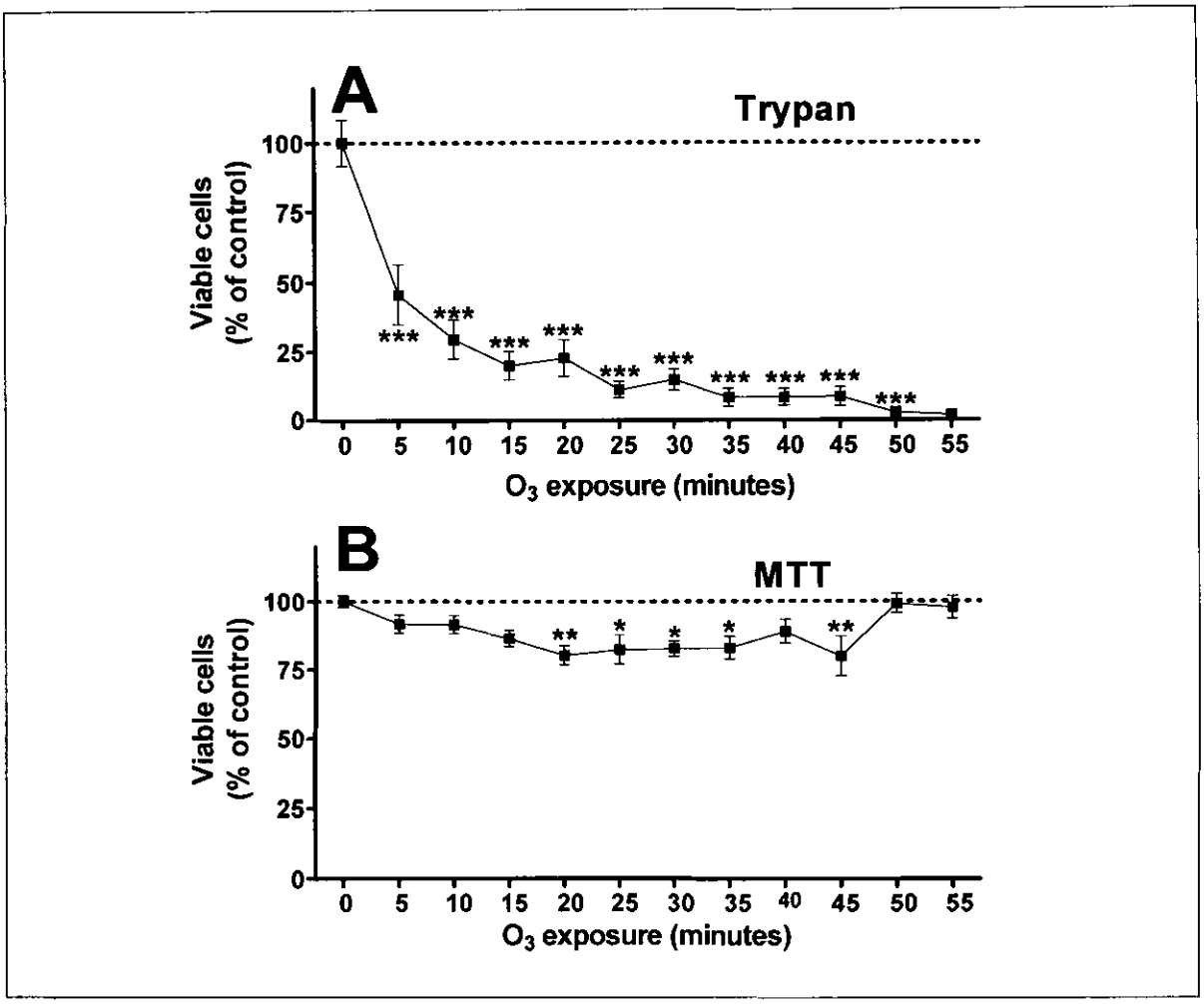


Figure 4-6 The effect of a 0 to 55 minute ozone exposure period on the viability of HeLa cells as measured by A.) trypan blue and B.) MTT cell viability assays. Data points are averages ± standard error of the mean of triplicate measurements from three independent and comparable experiments (n = 9) and are expressed as percentage of control. The data were analysed statistically by performing a one-way ANOVA and then implementing the Dunnett's post-test (comparing values to the control), with * indicating $p < 0.05$, ** indicating $p < 0.01$ and * indicating $p < 0.001$.**

In Figure 4-6 A a significant (54.3 ± 11.0%, $p < 0.001$) decrease in cell viability was observed after the first 5 minutes of ozone exposure. This decrease in cell membrane integrity continues for the remainder of the 55-minute exposure period until cell viability, as described by the trypan blue assay, is virtually decreased to zero. The results suggest that ozone causes extensive, time-dependent damage to the cellular membrane being significant already within the first 5 minutes of ozone exposure. This correlates with other related studies (Bhalla & Crocker, 1986; Bhalla & Hoffman, 1997; Yu *et al.*, 1994).

In contrast with the cell membrane integrity (trypan blue) results, changes in the mitochondrial function (MTT cell viability assay - Figure 4-6 B) of the HeLa cells was less pronounced. There is a statistically significant reduction in cell viability of about 20% present after 20, 25, 35 and 45 minutes of ozone exposure. Interestingly, mitochondrial function is restored to control values after approximately 50 minutes, suggesting an adaptation of the mitochondrial function.

These results therefore suggest that the HeLa cellular membrane is more susceptible to ozone damage after acute exposure than the mitochondrial function. While a significant effect is observed in membrane integrity after only 5 minutes of ozone exposure, between 20 and 45 minutes of exposure may be necessary to ensure substantial reduction in both membrane integrity and mitochondrial activity. For subsequent studies involving repetitive ozone exposure, exposure durations of 0, 5 and 25 minutes were therefore selected.

4.2. EFFECT OF REPEATED OZONE EXPOSURES ON CELL VIABILITY

There are suggestions and evidence in literature of changes in functional, biochemical and cellular responses to ozone after repeated ozone exposures. In animal models these changes occur after several days (see § 2.6.1.2), while it may be different in *in vitro* cultured cells. This phenomenon of apparent recovery is termed "adaptation". Adaptive responses to ozone referred to include forced expiratory volume (FEV), forced vital capacity (FVC), symptoms of respiratory irritation, bronchial reactivity, exercise performance and inflammatory markers (Hackney *et al.*, 1977; Horvath *et al.*, 1981; Devlin *et al.*, 1997; Frank *et al.*, 2001). It was therefore necessary to investigate whether repeated low and high doses of ozone may also induce adaptation in membrane integrity and mitochondrial function of cultured HeLa cells.

HeLa cells were repeatedly exposed to ozone over a period of two days. On day 1 the cells received four times 5-minute doses of control, oxygen or ozone every four hours (denoted pre-exposure or **P**). Thereafter the cells were incubated in normal growth medium overnight for a period of 16 hours. The incubation was followed by a single 25-minute dose (denoted re-exposure or **R**) of control, oxygen or ozone. Again the cells were incubated in cell culture medium for 0, 4, 8 or 12 hours, whereafter the trypan blue or MTT cell viability assays were performed after various treatments with oxygen and ozone (where **(+)** in front of **P** or **R** indicates exposure, while **(-)** indicates no exposure).

cell seeding $\xrightarrow{5h}$ 4 x 5 min exposure 4 hourly $\xrightarrow{16h}$ 1 x 25 min exposure $\xrightarrow{0, 4, 8 \text{ or } 12h}$ cell viability assay

4.2.1. TRYPAN BLUE TEST FOR CELL VIABILITY

The trypan blue assay results from the various time-dependent experiments conducted after repeated ozone exposures are depicted in Figure 4-7.

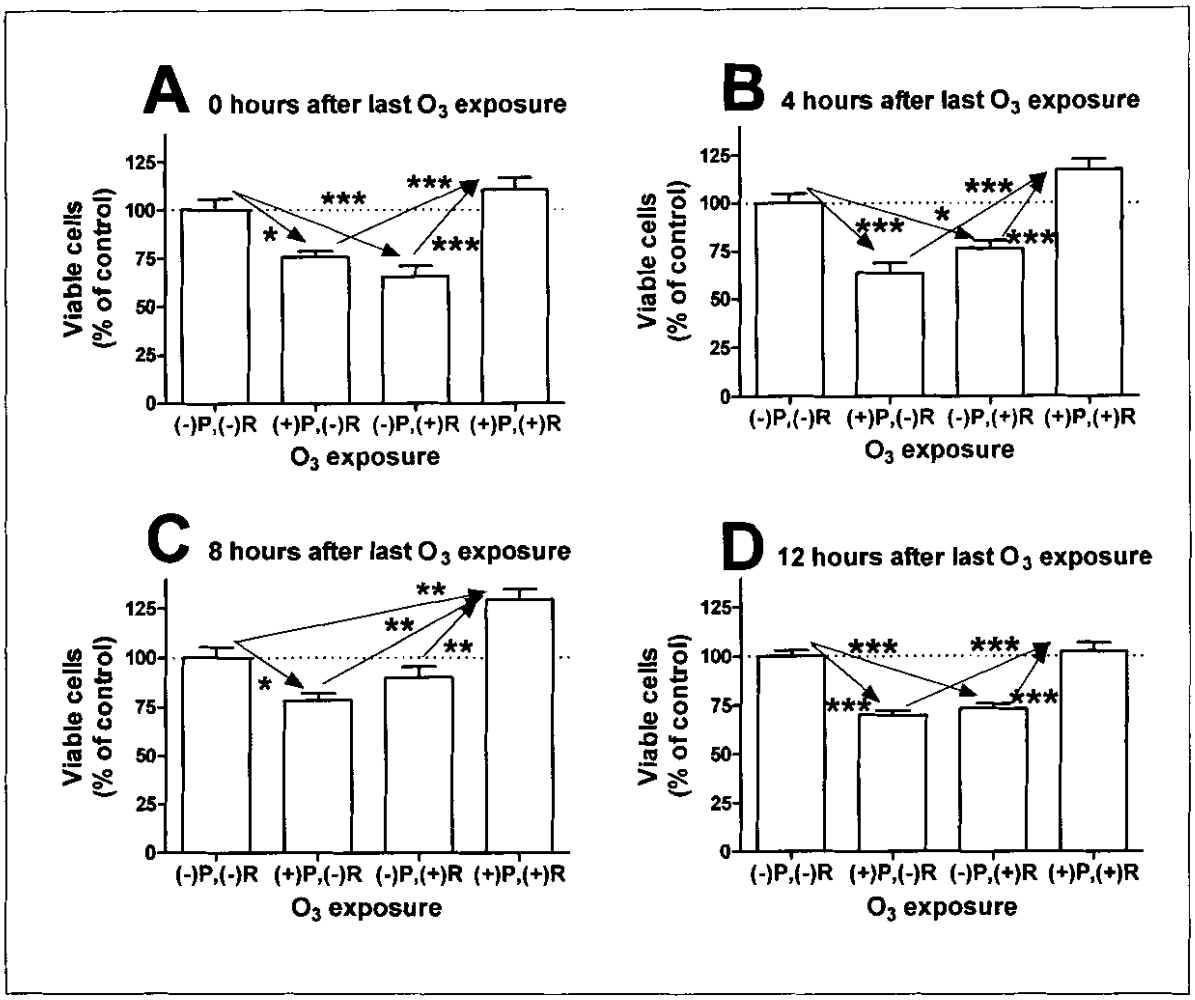


Figure 4-7 The effect of repeated ozone exposures on cell membrane integrity A.) 0 hours after re-exposure, B.) 4 hours after re-exposure, C.) 8 hours after re-exposure and D.) 12 hours after re-exposure as measured by the trypan blue cell viability assay. Data are averages ± standard error of the mean of triplicate measurements from three independent and comparable experiments (n = 9) and are expressed as percentage of control. The data were analysed statistically by performing a one-way ANOVA and then implementing the Tukey-Kramer post-test (comparing all values), with * indicating $p < 0.05$, ** indicating $p < 0.01$ and * indicating $p < 0.001$.**

It can be seen in Figure 4-7 A that pre-exposure **P** or re-exposure **R** to ozone alone reduces HeLa cell membrane integrity (42.5 ± 3.1%, $p < 0.05$ and 34.6 ± 5.6%, $p < 0.001$ respectively). However, a combination of pre-exposure **P** and re-exposure **R** to ozone restores membrane

integrity to the control value. The reduction in membrane integrity after the 25 minute re-exposure **R** was expected, as this corresponds with the acute ozone exposure, where similar results were obtained (see § 4.1.5).

The same trends observed in Figure 4-7 A are also present after the 4 hour recovery period (Figure 4-7 B). The most noteworthy observation, however, was made when observing membrane integrity after both pre-exposure **P** and re-exposure **R** to ozone, followed by 8 hours recovery (Figure 4-7 C), when cell membrane integrity was significantly increased from control (29.5 ± 5.3%; $p < 0.01$). Importantly, this increase in cell membrane integrity only after an 8 hour incubation, suggests that an adaptive (pre-conditioning) response to ozone is time-dependent, as well as dependent on the treatment regime with ozone (i.e. repeated exposure to low doses of ozone, followed by a single high dose exposure).

In Figure 4-7 D it is observed that after 12 hours cell membrane integrity after both pre-exposure **P** and re-exposure **R** to ozone returns to pre-treatment values. Therefore, the increase in cell membrane integrity as observed after 8 hour recovery (Figure 4-7 C) is reversed and the trends are similar to those observed in Figure 4-7 A and B. This reversal of the effect on cell membrane integrity may be explained by two possible contributing factors, namely increased cell multiplication or a slow reversal of the cellular adaptation.

Other studies have also suggested that new proteins may be synthesised after pre-conditioning against oxidative stress (Sun *et al.*, 1996). In these studies it was postulated that the proteins synthesised included heat shock proteins and antioxidant enzymes, although other proteins may also be synthesised. Larini *et al.* (2004) showed that levels of caspase-3, superoxide dismutase, glutathione peroxidase and glutathione reductase are increased after 4-hydroxynonenal (HNE) treatment (also see § 2.1.3.3.5). HNE, a secondary aldehyde formed during ozone exposure, also promotes the inactivation of Akt (Nakashima *et al.*, 2003) and induces apoptosis (Hamilton *et al.*, 1998; Kirichenko *et al.*, 1996). Other oxidative substances such as hydrogen peroxide (H_2O_2) also induce apoptosis, but when cells were pre-conditioned with H_2O_2, the cells are protected against apoptosis (Tang *et al.*, 2003).

4.2.2. MTT TEST FOR CELL VIABILITY

These experiments were conducted using the same procedures, time-points and abbreviations as for the trypan blue cell viability tests (see § 4.2.1). Figure 4-8 depicts the results of the MTT cell viability assays after various treatments with ozone.

Figure 4-8 A illustrates the results of the MTT assay conducted immediately after the last 25 minute re-exposure **R**. No significant decrease in mitochondrial function is observed directly after the last ozone exposure. The results obtained from the MTT assay 4 hours after the last exposure is depicted in Figure 4-8 B. Only HeLa cells exposed to ozone for 25 minutes (**R**) and cells exposed to both the short, repeated (**P**) (10.4 ± 3.4%) and acute (**R**) (16.8 ± 2.8%) doses of ozone had significantly decreased mitochondrial function. Results from the MTT assay performed 8 hours after the last ozone exposure can be seen in Figure 4-8 C. Mitochondrial function was not significantly decreased or increased after the 8 hour incubation period. Note that no adaptive response is evident after the 8 hour recovery period.

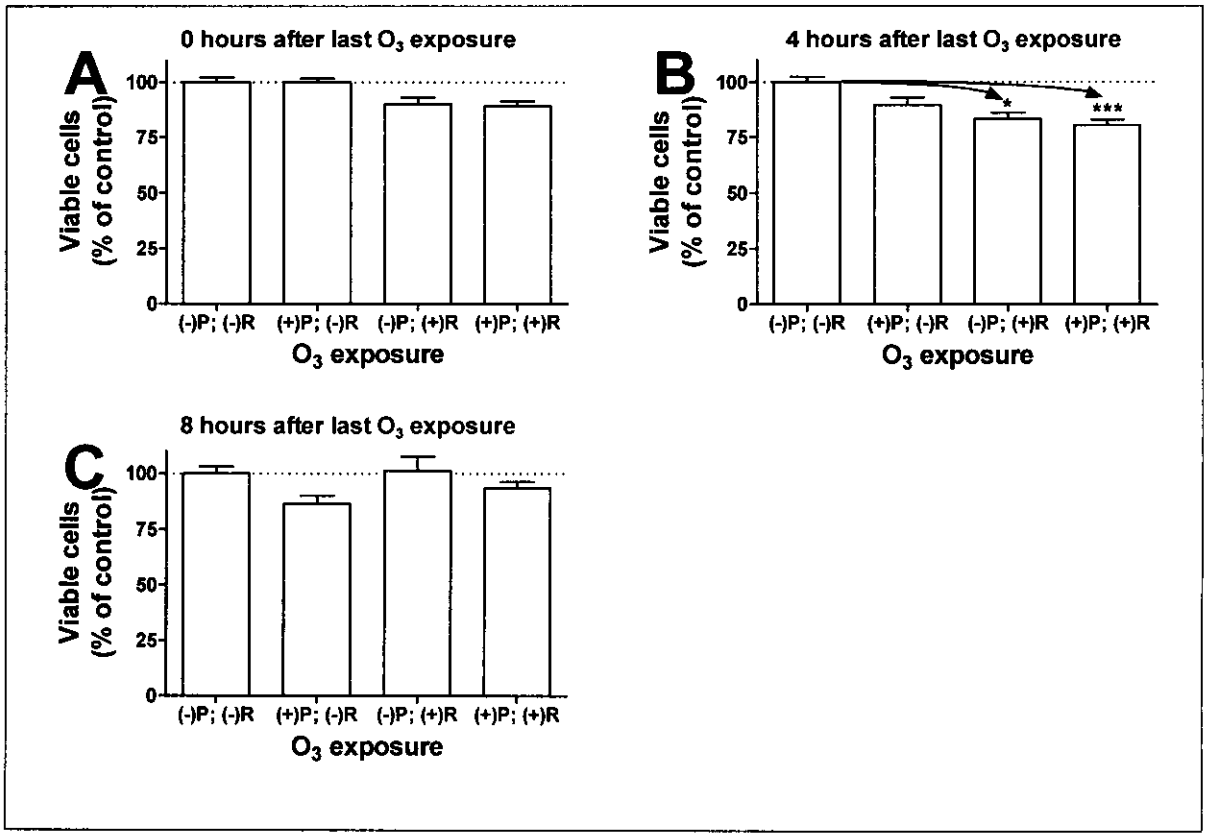


Figure 4-8 The effect of repeated ozone exposures on mitochondrial function A.) 0 hours after re-exposure, B.) 4 hours after re-exposure and C.) 8 hours after re-exposure as measured by the MTT cell viability assay. Data are averages ± standard error of the mean of triplicate measurements from three independent and comparable experiments (n = 9) and are expressed as percentage of control. The data were analysed statistically by performing a one-way ANOVA and then implementing the Tukey-Kramer post-test (comparing all values), with * indicating $p < 0.05$, ** indicating $p < 0.01$ and * indicating $p < 0.001$.**

The results obtained suggest that mitochondrial function is not affected as significantly as the cellular membranes and that no adaptation of mitochondrial function is induced after repeated

ozone exposures. These results differ significantly from the results obtained from the trypan blue cell viability assays after repeated ozone exposures. Ozone exposure may therefore have various effects on HeLa cell viability, depending on the dose of ozone and the duration of exposure and these effects differ with regard to membrane integrity and mitochondrial activity.

4.2.3. EFFECT OF REPEATED OXYGEN EXPOSURE ON CELL VIABILITY

It is important to note that the ozone generator was sourced with ultra-pure oxygen to generate ozone and therefore oxygen may also be present when performing subsequent experiments (Eriksson, 2005). Therefore, the potential contribution of any remaining oxygen to the observed effects had to be accounted for. In order to investigate this, gf-KH solution was exposed to ultra-pure oxygen under the same conditions as to ozone. HeLa cells were then exposed to oxygen-saturated gf-KH according to the following treatment regime: on day 1 the cells received four times 5-minute doses of ultra-pure oxygen every four hours (denoted pre-exposure or **P**). Thereafter the cells were incubated in normal growth medium overnight for a period of 16 hours. The incubation was followed by a single 25-minute dose (denoted re-exposure or **R**) of ultra-pure oxygen. Again the cells were incubated in cell culture medium for 0, 4, 8 or 12 hours, whereafter the trypan blue or MTT cell viability assays were performed after the various treatments (where **(+)** in front of **P** or **R** indicates oxygen exposure, while **(-)** indicates no exposure).

When comparing the results in Figure 4-9 A and B, it is clear that cell membrane integrity is not affected by ultra-pure oxygen (measured with the trypan blue viability assay 8 hours after the last exposure). In contrast, mitochondrial function (Figure 4-9 B) of HeLa cells receiving ultra-pure oxygen is statistically significantly reduced by all treatment regimes, although the reduction is relatively small (roughly between 84.1 and 91.5%). When these results are compared to the results obtained after repeated ozone exposures (see § 4.2.1 and 4.2.2) the increase in cell membrane integrity is only evident after repeated ozone exposures. Therefore we can conclude that all results obtained from repeated exposure to ozone, may only be attributable to ozone and that residual oxygen molecules did not affect the results.

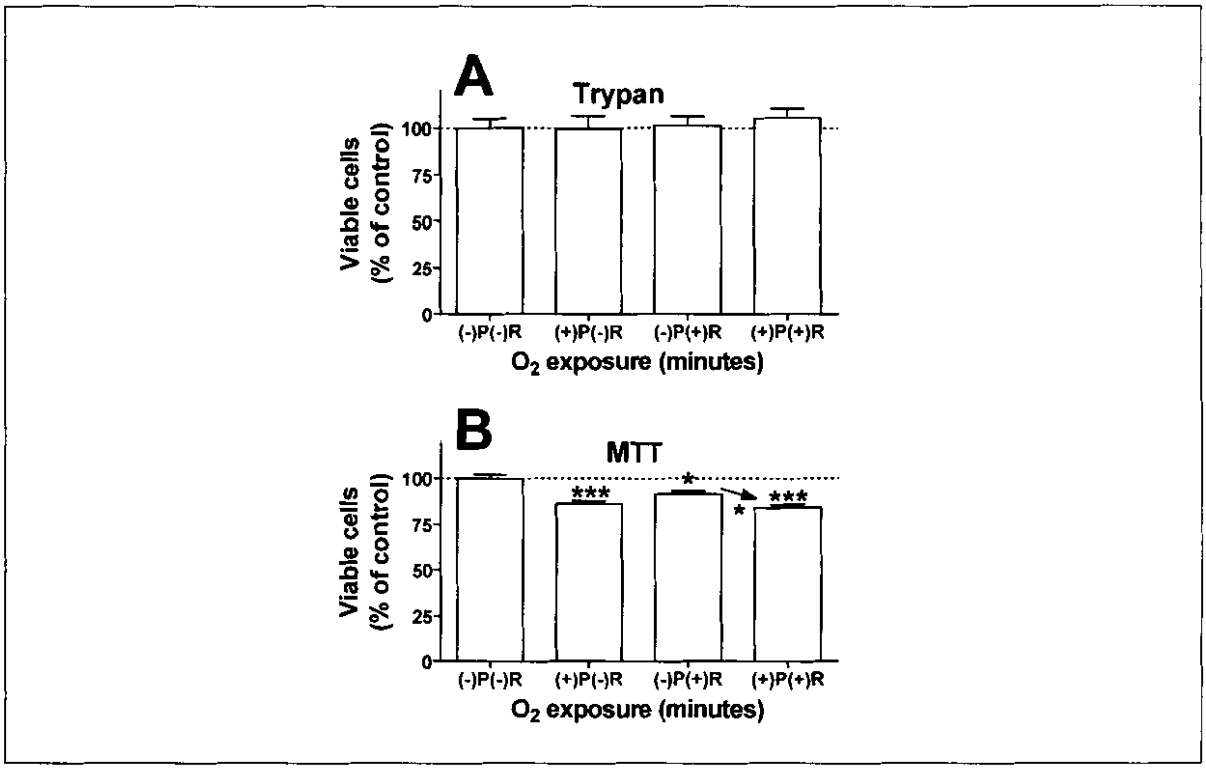


Figure 4-9 The effect of repeated exposure to ultra-pure oxygen on cell viability as measured by A.) trypan blue and B.) MTT cell viability assays. Data are averages ± standard error of the mean of triplicate measurements from three independent and comparable experiments (n = 9) and are expressed as percentage of control. The data were analysed statistically by performing a one-way ANOVA and then implementing the Tukey-Kramer post-test (comparing all values), with * indicating $p < 0.05$ and * indicating $p < 0.001$.**

4.2.4. EFFECT OF ENZYME INHIBITION INTERVENTION ON REPEATED OZONE EXPOSURES

To investigate the mechanism by which ozone affects cellular plasticity (i.e. whether apoptotic and/or pro-apoptotic pathways are involved in the adaptive response of cell membranes to repeated ozone exposure **(+)P,(+)R** versus control), three enzyme inhibitors were introduced into the protocol. These enzyme inhibitors included the caspase-3 and –6 inhibitor, Z-DQMD-FMK, the Akt inhibitor, (-)-deguelin and the xanthine oxidase and NFκβ inhibitor, ME10092 (see § 3.4.3). Cells were treated with the inhibitors during the 8 hours incubation period after ozone exposures **P** and **R**, whereafter cell viability tests were performed.

Figure 4-10 A and C depict the results obtained from the trypan blue cell viability assay. As with the results discussed in § 4.2.1, the HeLa cells show significantly increased cell membrane integrity after repeated exposure to ozone. Importantly, the inclusion of all three enzyme inhibitors reversed the adaptation in cell membrane integrity to repeated ozone exposure,

resulting in significant reductions in cell membrane integrity after repeated ozone exposures (for deguelin a decrease from 100% to 16.2 ± 1.4%, $p < 0.001$; for Z-DQMD-FMK an increase from 100% to 148.1 ± 4.1%, $p < 0.001$ and for ME10092 a decrease from 100% to 61.6 ± 5.5%, $p < 0.001$ – see Figure 4-10 A). (-)-Deguelin induced the most pronounced decrease (80 ± 1.9%, $p < 0.001$ when compared to the control, and Z-DQMD-FMK and $p < 0.01$ when compared to ME10092 – see Figure 4-10 C) on cell membrane integrity after repeated ozone exposure, followed by the xanthine oxidase and NFκβ inhibitor, ME10092 (57 ± 6.2%, $p < 0.001$ when compared to the control and Z-DQMD-FMK and $p < 0.01$ when compared to deguelin – see Figure 4-10 C). HeLa cell viability decreased significantly when repeatedly exposed to ozone and treated with Z-DQMD-FMK (12 ± 1.82%, $p < 0.001$ when compared to the control, deguelin and ME10092 – see Figure 4-10 C) as can be expected during the inhibition of apoptosis.

The results from the MTT cell viability assay can be seen in Figure 4-10 B and D. As with the results discussed in § 4.2.2, control cells show no significant change in mitochondrial function when they are repeatedly exposed to ozone. Although there was a trend towards decreased mitochondrial function after treatment with the three enzyme inhibitors after repeated ozone exposures, only two of the results reached statistical significance. (-)-Deguelin and ME10092 had the most significant effect on mitochondrial function (a decrease of 44 ± 5.4%, $p < 0.001$ and 45 ± 4.4%, $p < 0.001$, respectively, when compared to the control and Z-DQMD-FMK – see Figure 4-10 D). These results correlate well with the results from Figure 4-10 A. Repeated ozone exposure did not statistically significantly decrease mitochondrial function in the presence of Z-DQMD-FMK ($p > 0.05$ when compared to the control – see both Figure 4-10 B and D). This observation also correlates with the observations made in Figure 4-10 A.

These results suggest that caspase-3, Akt, xanthine oxidase and NFκβ are involved in the pre-conditioning effect of ozone, although the anti-apoptotic enzymes had a more pronounced effect on HeLa cell viability. These enzymes also play a more significant role in the cellular membrane than in the mitochondria. Inhibition of Akt and NFκβ (which are essential for cellular survival) decrease both the cell membrane integrity and mitochondrial function of HeLa cells, while inhibition of caspase-3 (an initiator of apoptotic cell death) increases cell viability.

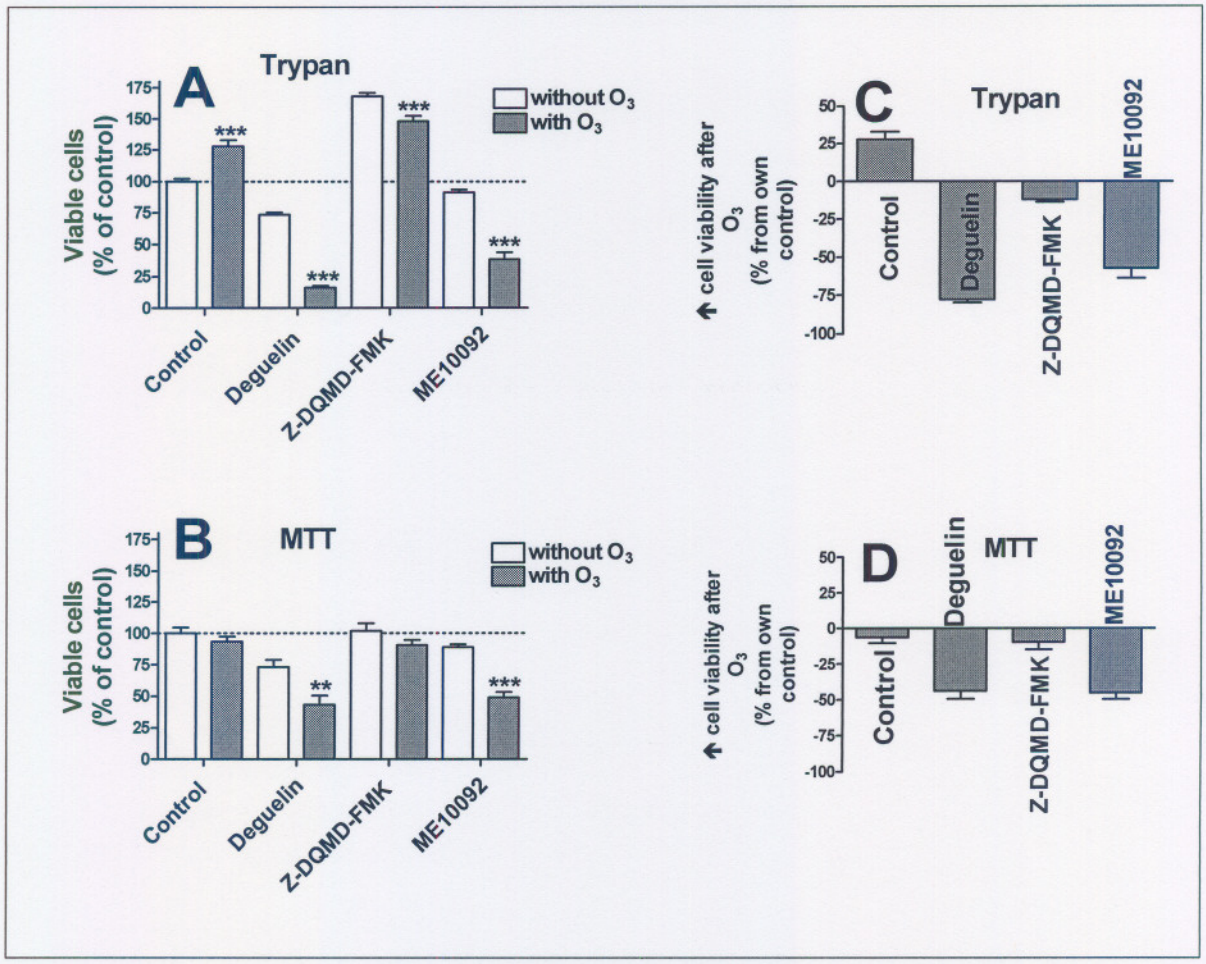


Figure 4-10 The effect of treatment with the enzyme inhibitors ME10092, (-)-deguelin and Z-DQMD-FMK on cell viability after repeated ozone exposure as measured by A.) trypan blue and B.) MTT cell viability assays, respectively, as well as the percentage of increase in cell viability (i.e. after repeated ozone exposure in comparison to without ozone exposure) as calculated from the results obtained from the C.) trypan blue and D.) MTT cell viability assays, respectively. Data are the averages ± standard error of the mean of triplicate measurements from three independent and comparable experiments (n = 9) and are expressed as percentage of control. The data were analysed statistically by performing the two-tailed Student *t* test (comparing two values) in A & B, with ** indicating $p < 0.01$ and * indicating $p < 0.001$ and the Tukey-Kramer post-test (comparing all columns) in C & D.**

4.2.5. EFFECT OF ENZYME INHIBITION INTERVENTION ON ACUTE OZONE EXPOSURE

To investigate the effect of various enzyme inhibitors on the mechanism of acute ozone damage, HeLa cells were treated with the three enzyme inhibitors (-)-deguelin, Z-DQMD-FMK and ME10092 prior to a 25 minute acute ozone exposure, whereafter the MTT and trypan blue

cell viability assays were performed. The results of this experiment are depicted in Figure 4-11 A and B.

The same trend was observed in the untreated, ozone exposed HeLa cells as observed in the results of the acute ozone exposure (Figure 4-11 A) described in § 4.1.5. Cell membrane integrity decreased dose-dependently over the 25 minute exposure period **(R)**, while the mitochondrial function did not decrease significantly during ozone exposure ($p > 0.05$). When HeLa cells were pre-treated with the enzyme inhibitors the following observations were made:

All HeLa cells treated with the enzyme inhibitors also tended towards a dose-dependent decrease (which was not always significant) in cell membrane integrity. Treatment with ME10092 (the xanthine oxidase and NFκβ inhibitor) significantly decreased the cell membrane integrity ($49.9 \pm 25.4\%$, $p < 0.01$ at 5 minutes, $62.0 \pm 2.8\%$, $p < 0.01$ at 10 minutes and $80.0 \pm 1.5\%$, $p < 0.01$ at 25 minutes), but not mitochondrial function ($p > 0.05$) of HeLa cells. Pre-treatment with Z-DQMD-FMK (the capase-3 and -6 inhibitor) initially increased the cell viability of the HeLa cell significantly in the absence of ozone exposure. The cell membrane integrity ($13.9 \pm 7.7\%$, $p < 0.01$ at 5 minutes, $23.3 \pm 3.6\%$, $p < 0.01$ at 10 minutes and $79.9 \pm 1.5\%$, $p < 0.01$ at 25 minutes) and mitochondrial function (from $21.3 \pm 5.63\%$, $p < 0.05$ after 25 minutes of ozone exposure) decreased dose-dependently with ozone exposure. Treatment with (-)-deguelin (the Akt inhibitor) affected the cell membrane integrity of HeLa cells significantly ($p < 0.05$), but did not affect the mitochondrial function when compared statistically to the deguelin-treated, unexposed HeLa cells.

ME10092 and (-)-deguelin had a more pronounced decreasing effect on cell viability when comparing the results to that of the control cells. In contrast, cell viability was significantly increased with the introduction of the caspase inhibitor Z-DQMD-FMK. Although cells treated with this enzyme inhibitor also showed marked decreases in cell viability, these decreases tended to be less significant as with the other two enzyme inhibitors.

These results suggest that NFκβ and xanthine oxidase play a significant role in the prevention of apoptosis in ozone-exposed cells. Once these enzymes are inhibited, the cell viability significantly decreases when cell are exposed to ozone. The same conclusion can be made from (-)-deguelin. Since cell viability is significantly decreases when Akt is inhibited by (-)-deguelin, Akt may play a pivotal role in cellular survival and may protect cells when oxidative stress is induced after ozone exposure.

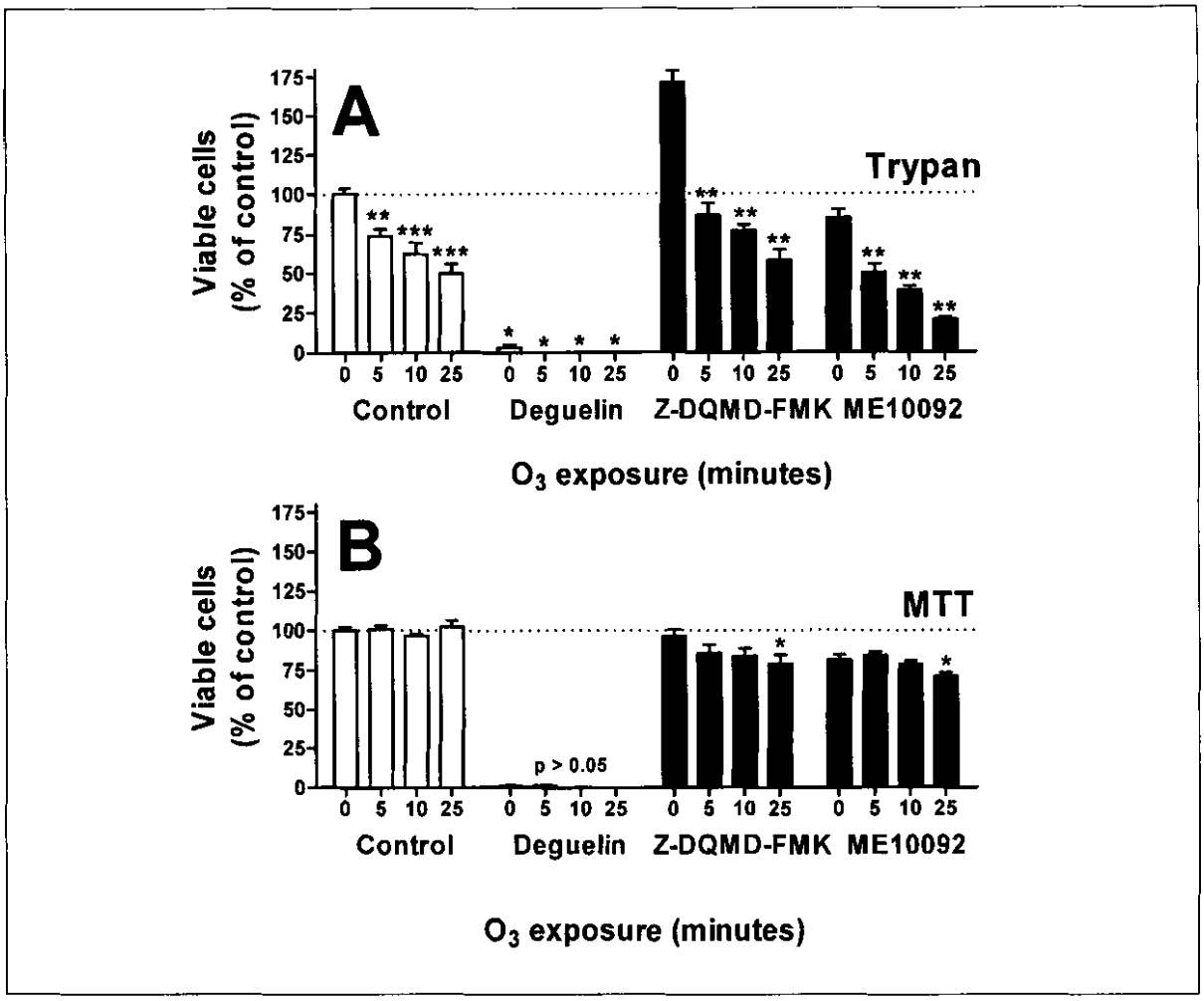


Figure 4-11 The effect of treatment with the enzyme inhibitors (-)-deguelin, Z-DQMD-FMK and ME10092 on cell viability after acute ozone exposure as determined with A.) trypan blue and B.) MTT cell viability assays. Date are the averages ± standard error of the mean of triplicate measurements from three independent and comparable experiments (n = 9) and are expressed as percentage of control. The data were analysed statistically by performing a one-way ANOVA and then implementing the Dunnett's post-test (comparing values to the corresponding control), with * indicating $p < 0.05$, ** indicating $p < 0.01$ and * indicating $p < 0.001$.**

On the other hand, treatment with Z-DQMD-FMK significantly increases cell viability in the absence of ozone, but once the cells are exposed to ozone, cell viability also decreases. Caspase-3 may therefore play a very important role in cellular protection, even in the absence of ozone-induced oxidative stress. Once this enzyme is inhibited, cells tend to have greater resistance towards ozone exposure, but as the exposure to ozone increases, the cellular survival decreases.

These three enzymes may therefore play a significant role in the mechanism by which ozone induces cellular death.

4.3. DNA INTEGRITY AND REPAIR AFTER OZONE EXPOSURE

4.3.1. DNA INTEGRITY AFTER REPEATED OZONE EXPOSURES

It was investigated whether repeated ozone exposure affects DNA integrity. Cells were repeatedly exposed to ozone (as was described in § 4.2) and the comet assay was performed 8 hours after the last ozone exposure (previously denoted control, **P**, **R** and **P + R** in § 4.2). Images and the results from the investigation into DNA integrity after repeated ozone exposure are represented in Figure 4-12 and Figure 4-13 below.

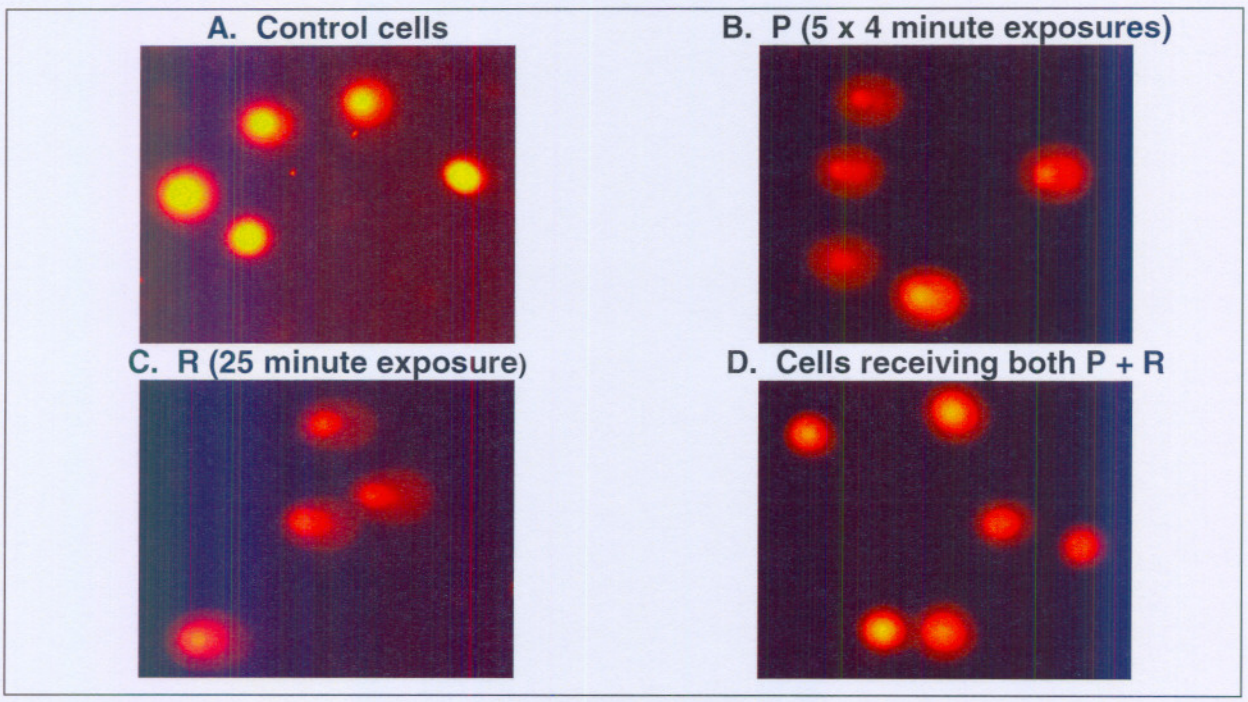


Figure 4-12 DNA integrity of HeLa cells after repeated ozone exposures.

The images in Figure 4-12 depict the effect of ozone exposure on DNA integrity. Unexposed HeLa cells (as can be seen in Figure 4-12 A) showed no DNA damage (round spots), while HeLa cells pre-exposed, **P** (Figure 4-12 B) and re-exposed for 25 minutes, **R** (Figure 4-12 C) presented with "comet tails" suggesting DNA fragmentation. The most extensive damage occurred at 25 minute ozone exposures (**R**), while the **P + R** cells (i.e. receiving both the 5 and 25 minute ozone exposures - Figure 4-12 D) did not show any DNA integrity damage (i.e. round spots as control).

Figure 4-13 represents the quantification of the results obtained from the images in a bar graph. HeLa cells treated for only the repeated 5 minute ozone exposures (**(+)P;(-)R**) did not show any

significant change in DNA integrity, although there was a trend towards decreased DNA integrity. The 25 minute acute ozone exposures (**(-)P;(+)R**) significantly ($p < 0.05$) decreased DNA integrity, while HeLa cells exposed to both ozone exposures (**(+)P;(+)R**) had DNA integrity comparable with that of the control. Therefore, cells repeatedly exposed to ozone may have no damage to DNA, as observed with cells exposed for only the 5 minute of 25 minute periods.

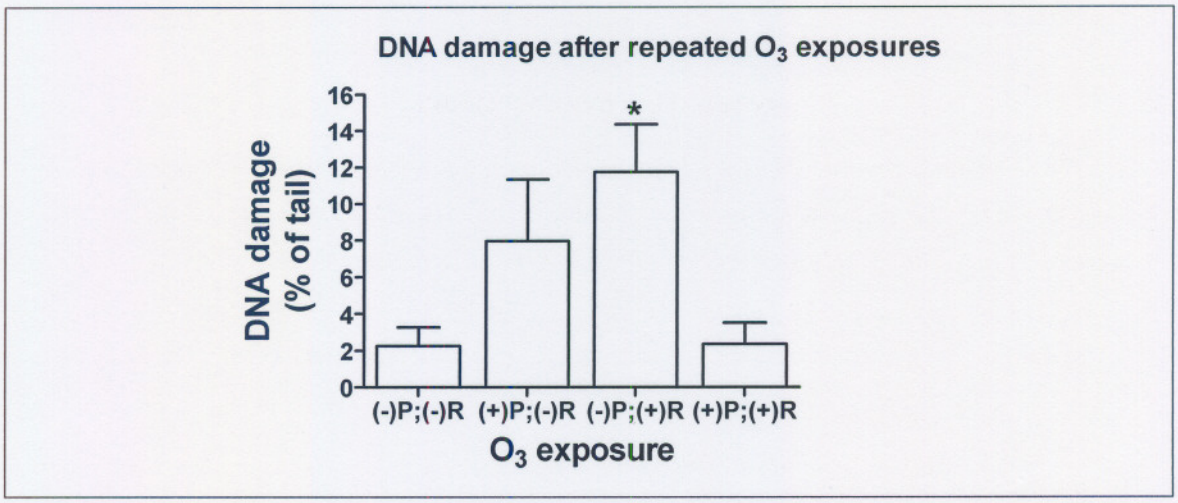


Figure 4-13 The effect of repetitive ozone exposure on DNA integrity in HeLa cells. Data are the averages ± standard error of the mean of triplicate measurements from three independent and comparable experiments (n = 9) and expressed as % DNA damage in the tail. The data were analysed statistically by performing a one-way ANOVA and then implementing the Dunnett's post-test (comparing values to the corresponding control), with * indicating $p < 0.05$.

Other studies have also shown that ozone induces single stranded DNA breaks and cytosolic DNA fragments (Hamilton *et al.*, 1998). With repeated ozone exposures, however, the DNA repair enzyme poly(ADP-ribose) synthetase and the free radical scavenging enzyme, superoxide dismutase are induced (Hussain *et al.*, 1985). This results in cellular DNA repair.

4.3.2. DNA REPAIR AFTER REPEATED OZONE EXPOSURES

To investigate whether repeated ozone exposure may cause HeLa cells to adapt also with regard to DNA repairing capacity, the unexposed and pre-conditioned (**P + R**) were incubated in 100 μM H_2O_2 solution for a period of 40 minutes. After this period, the cells were incubated in normal growth medium (DMEM + FBS) at 37 °C to allow recovery. Images of the cells were taken directly after H_2O_2 exposure and 40 minutes after the recovery period was initiated. These images are shown in Figure 4-14.

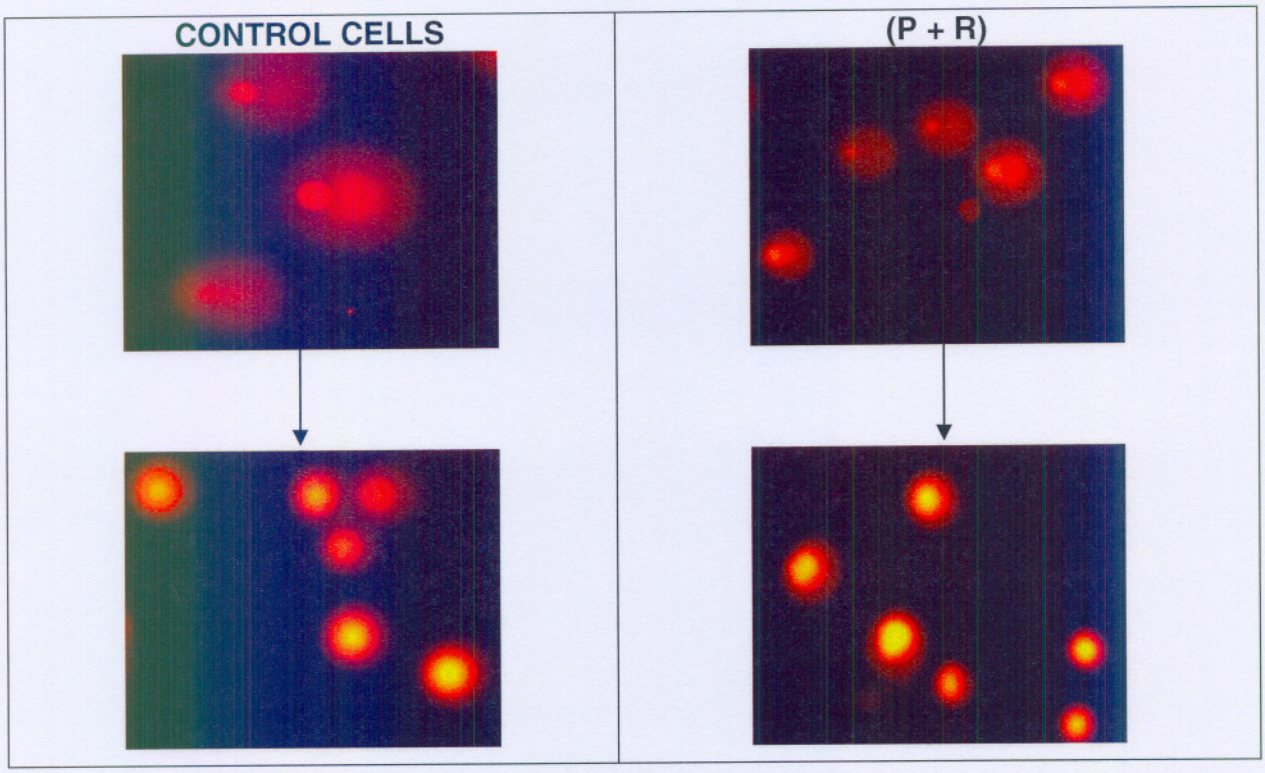


Figure 4-14 DNA repair of control and repeatedly (P + R) exposed HeLa cells after a 0 and 40 minute incubation period in H_2O_2.

Both the control and ozone-exposed (**P + R**) HeLa cells had significant DNA integrity damage after the H_2O_2 incubation (oxidative stress). In both cases the DNA damage was repaired within 40 minutes of incubation in normal growth medium. The graphic representation of the quantified results is depicted in Figure 4-15 below.

Results obtained from the bar graph suggest that both unexposed and repeatedly exposed HeLa cells were damaged by H_2O_2. Within 40 minutes the DNA damage was repaired in both the unexposed and repeatedly exposed HeLa cells and DNA integrity was unimpaired by the H_2O_2. Wang *et al.* (2000) investigated the mechanisms involved in the activation of Akt during cellular response to oxidant injury. They used H_2O_2 as a model oxidant and found that Akt activity was stimulated by H_2O_2 treatment. Maximum levels of Akt were seen 15 - 30 minutes after addition of H_2O_2. Therefore, it may be possible that the H_2O_2 challenge activated the anti-apoptotic protein Akt and this may explain the significant repair in control and exposed cells observed in our results.

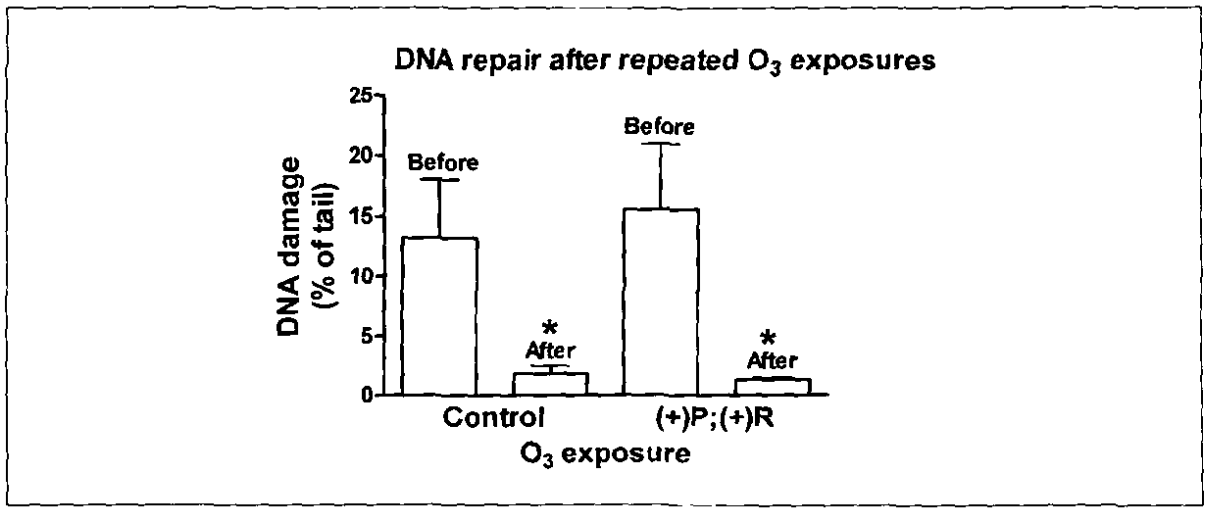


Figure 4-15 DNA repair of control and HeLa cells repeatedly exposed to ozone (P + R) at 0 min and 40 min after a single H_2O_2 challenge. Data are the averages ± standard error of the mean of triplicate measurements from three independent and comparable experiments (n = 9) and are expressed as percentage of control. The data were analysed statistically by performing one-tailed Student *t* test (comparing two values), with * indicating $p < 0.05$.

4.4. SYNOPSIS

In conclusion our results suggest that ozone may have both damaging and protective properties, depending on the specific exposure conditions. Acute ozone exposure affects cell membrane integrity of HeLa cells extensively. Pre-conditioning HeLa cells with repeated short (5 minute) doses and one acute (25 minute) dose, induces adaptation in HeLa cell membrane integrity, suggesting protective effects to oxidative stress. This may suggest an upregulation of antioxidant mechanisms and needed to be further investigated. Mitochondrial function is not affected by pre-conditioning ozone exposures.

When investigating the mechanism by which ozone induces this cell membrane integrity increase, it was found that four enzymes may play a pivotal role in this pathway. When Akt, caspase-3, NFκβ and xanthine oxidase are inhibited, the pre-conditioning effect of ozone on HeLa cells is reversed and cell viability decreases, suggesting that apoptotic and anti-apoptotic pathways play an important role in the adaptation process. Another important factor that may be involved in the adaptive response to ozone is an increase of DNA integrity. Repeatedly exposed HeLa cells showed the same DNA integrity as control cells and repaired any damage inflicted upon the DNA within the same period as the control cells.

SUMMARY, CONCLUSIONS & PROSPECTIVE STUDIES

CHAPTER 5

5.1. SUMMARY

The current study aimed to establish treatment conditions and regimes for *in vivo* ozone treatment of cultured human epithelial (HeLa) cells and to investigate the biological effects of acute and repeated ozone exposure on the viability of these cells. For the latter, two cell viability assays (trypan blue and MTT tests), as well as DNA integrity and repair capacity assays (comet analysis) were utilised.

From the control experiments to establish treatment conditions and regimes the following results were obtained:

- **Vehicle for ozone administration**
  Krebs-Henseleit physiological solution can sustain HeLa cells for a duration of 1 hour, yielding about 50% membrane integrity and about 70% mitochondrial activity of the cell in comparison with cells incubated in normal DMEM growth medium. However, data suggest that ozone may interact with glucose to form a cytotoxic product. Since 1 hour incubation of HeLa cells in glucose-free Krebs-Henseleit (gf-KH) also yields sufficient remaining viable cells, it was chosen as administration vehicle for ozone to HeLa cells.
- **Half-life of ozone in aqueous medium at 37° C**
  The half-life of ozone in Krebs-Henseleit at 37 °C was found to be relatively short (i.e. 5.6 minutes). Therefore, during ozone treatments of cells, the solution was replaced every 5 minutes to ensure a sufficient ozone concentration during the duration of ozone exposure.

After initial establishment of experimental conditions, the effect of acute and repeated ozone exposure of HeLa cells to ozone was investigated and the following results were obtained:

- **Acute ozone exposure**
  Acute ozone exposure affected cell membrane integrity of the HeLa cells significantly by increasing the cellular membrane permeability, while the mitochondrial function of HeLa cells remains relatively unchanged during the exposure period.
- **Repeated ozone exposure**
  By exposing HeLa cells repeatedly to ozone for short periods, followed by an acute

exposure the following day, cell membrane integrity is significantly increased. This pre-conditioning effect observed in the cell membrane integrity was not present at mitochondrial level and mitochondrial function was relatively unaffected by these repeated ozone exposures. This suggests that HeLa cells may be able to "adapt" to the ozone by increasing cell membrane integrity and this adaptation or pre-conditioning is only initiated after very specific ozone exposure conditions.

- **Role of apoptotic and antiapoptotic pathways**

  For investigating the mechanism of the HeLa pre-conditioning effect observed after repeated ozone exposures, three enzyme inhibitors were introduced. These enzymes either play a role in apoptotic pathways (caspase-3 and -6 and xanthine oxidase) or initiate anti-apoptotic pathways (Akt and NFκβ). The inhibitors were added to the HeLa cells prior to acute ozone exposure or during the 8 hours incubation period after the final re-exposure to ozone. The enzyme inhibitors did not protect the HeLa cells against acute ozone exposure damage and reversed the upregulation of cell membrane integrity observed after repeated ozone exposures. This suggests that these enzymes play a pivotal role in the response observed after repeated ozone exposure and that apoptotic and anti-apoptotic pathways may be involved in initiating the ozone damage in HeLa cells.

- **DNA integrity and repair**

  When the DNA integrity of the HeLa cells repeatedly exposed to ozone was determined, it was found that DNA integrity and repair capacity are comparable to control. Although these data do not suggest improved cellular viability after repeated ozone exposure, it supports the finding of restoration of cellular viability after repeated ozone exposure.

5.2. CONCLUSIONS

The current study suggest that gf-KH solution is a suitable vehicle for ozone administration, as it does not contain any amino acids or glucose (that may interact with ozone), delivers a sufficient dose of ozone to the cultured cells and is able to sustain HeLa cells for the periods of ozone exposure. The problem with the relative short half-life of ozone in the aqueous gf-KH solution was overcome by replacing both the ozonated and unexposed gf-KH solution in all the cell-containing wells every 5 minutes, ensuring an effective supply of ozone to the cells at near-to-saturated concentrations. This short half-life of ozone may have implications for future *in vivo* and clinical ozone experimentation, as this may limit applications and dosing regimes. There are also a multitude of biological molecules that may interact with ozone and decrease the half-life of the gas even more significantly. For the current experimentation, it was decided to exclude glucose from the Krebs-Henseleit solution, as results indicated that ozone may interact with glucose to form toxic products that in turn may interfere in the viability studies conducted.

This study therefore succeeded in the objective to establish the most favourable conditions for *in vitro* ozone exposure and associated experimental investigations.

Although the injurious effects of ozone on biological molecules have been extensively researched and reported, many questions still remain unanswered regarding the mechanism by which repeated ozone exposures may induce cellular protection. Pryor (1991) suggested that once ozone is inhaled into the airways, it immediately reacts with the polyunsaturated fatty acids (PUFAs) contained in the lung lining fluid. Although most of the inhaled ozone immediately react with these PUFAs, very small amounts of ozone may penetrate into the deeper tissues (such as airway epithelial cells), reacting with the lipids of the cellular membranes. With this lipid peroxidation, secondary reaction products are produced that are able to penetrate further and reach the systemic circulation. The current study support this hypothesis, as the cellular membranes of HeLa cells showed the most significant damage after acute ozone exposures, while the mitochondrial function reduction was less pronounced and partially restored after the acute exposure. In addition, the current study suggests that, when epithelial cells are repeatedly exposed to ozone, some protective and adaptive changes may be induced, including the increase in cell membrane integrity. The current study suggests that apoptotic (caspase-3 and -6 and xanthine oxidase), anti-apoptotic enzymes (Akt and NFκβ) and reparative DNA processes may be involved in this pathway (see Figure 5-1 below).

Some anecdotal research has suggested that ozone may be utilised to treat various diseases, but significant scientific research in this field is still required. Only limited scientific investigations into claims of therapeutic efficacy have been conducted and contradicting findings are reported. The most important conclusion that may be drawn form the current study is that very specific ozone exposure conditions may induce protecting effects on especially epithelial cellular membranes and DNA integrity. It is therefore necessary for scientists and researchers to specify the specific ozone exposure conditions with every therapeutic claim made and to vigorously verify these claims with supportive evidence from pre-clinical and clinical studies.

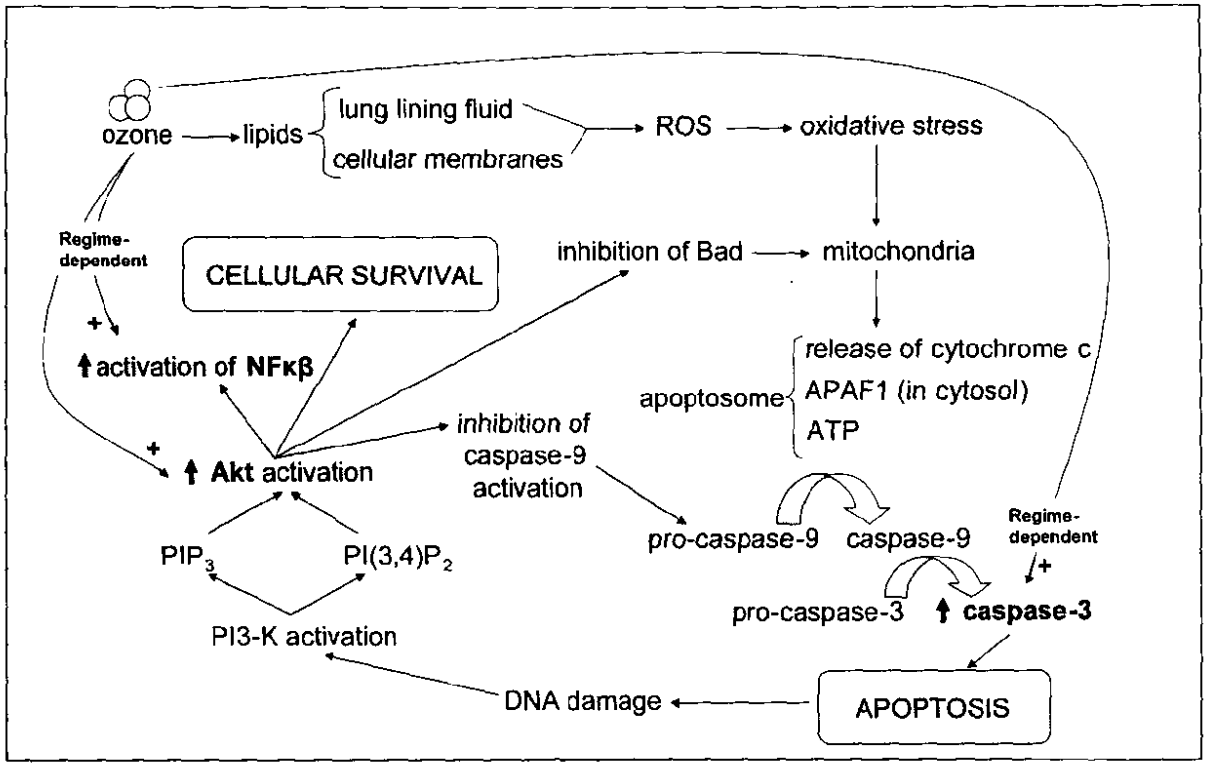


Figure 5-1 Pathways possibly involved in the effect of ozone on cellular plasticity.

5.3. PROSPECTIVE STUDIES & RECOMMENDATIONS

The current study provided several valuable cues for further investigations. While the data suggest that the cellular adaptation in cell membrane integrity after repeated ozone exposure is associated with effects on apoptotic and anti-apoptotic pathways, this needs to be confirmed and the mechanism elucidated by mRNA and protein analyses of the enzymes involved in these pathways. Therefore, other apoptotic and anti-apoptotic enzymes may be included in future mechanistic investigations. Differentiation between apoptotic and necrotic cell death after ozone exposure may also become an essential part of future studies. Furthermore, many other proteins of physiological and/or pharmacological significance may also be influenced by ozone. In this regard prospective studies may focus on the effect of ozone treatments on G protein-coupled receptor function and signalling pathways.

In addition, investigations into the biological effects of ozone may be extended to cell lines other than the HeLa cell line, for example haematological cells that are directly exposed to ozone in some therapeutic strategies of alternative health care and cancerous cell lines (as ozone is also being utilised in treating cancer patients (see § 2.1.2.2)).

Our laboratory has already conducted other *in vitro* studies on isolated organs (in this case guinea pig trachea). These studies suggest that ozone may have very significant

pharmacological effects on all tissues and organs. Future studies may employ animal models to verify the *in vivo* relevance of the *in vitro* findings (in this regard findings of the current study on HeLa cells suggesting that the treatment regime to induce adaptive effects may be very important) especially on the repeated ozone exposures experiments conducted, while clinical studies may also be utilised during the later stages of these investigations to determine the relevance of these biological effects of ozone for the therapeutic setting.

Furthermore, since ozone inhalation in rodents has been shown to be associated with systemic (even central) effects of secondary reaction products of ozone, it may be important to extend the current studies to the investigation of the effects and mechanisms of reactive oxygen species (formed by ozone) on biological tissues. This may include both *in vivo* animal studies and *in vitro* studies on animal tissue.

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. Ozone should be viewed as a pharmacologically active substance and scientific experimentation regarding its therapeutic applications should be subject to regulations as for any other therapeutic drug. Indeed, any therapeutic claim and application should be based on sound scientific evidence, ensuring responsible, accountable and professional practices.

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APPENDIX I

ABBREVIATIONS

A

Ach	Acetylcholine
AGC subfamily	Arabidopsis thaliana gene family
AHT	Autohaemotherapy
AIF	Apoptosis-inducing factor
AMA	American Medical Association
APAF-1	Apoptosis protease activating factor
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate

B

BAL	Bronchoalveolar lavage
BIR	Baculoviral IAP repeat

C

CAD	Caspase-activated deoxyribonuclease
Caspase	Cysteinyl aspartate specific proteinases
Cdk	Cyclin-dependent kinase
CFC's	Chlorofluorocarbons
CNS	Central nervous system
CO	Carbon monoxide
CO_2	Carbon dioxide
CRE	Cyclic AMP-response element
CREB	Cyclic AMP response element binding protein
Cys	Cysteine
Ca^{2+}	Calcium

D

ddH_2O	Demineralised and double distilled water
DFF	DNA fragmentation factor
DIABLO	Direct IAP-binding protein with low pI
DISC	Death-inducing signalling complex
DMEM	Dulbecco's Modified Eagles Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxiribonucleic acid
DNase	Deoxyribonuclease

E

EDTA	Ethylenediaminetetra acetic acid di-sodium salt
EEG	Electroencephalogram
EMEM	Minimum Essential Media with Earle's Base
EPA	U.S. Environmental Protection Agency's

F

FAK	Focal adhesion kinase
FBS	Foetal bovine serum
FCS	Foetal calf serum
FDA	Food and Drug Administration
FEV	Forced expiratory volume
FVC	Forced vital capacity

G

gf-KH	Glucose-free Krebs-Henseleit solution
GSH	Glutathione

H

H_2O_2	Hydrogen peroxide
HC	Hydrocarbons

HMPA	High melting point agarose
HNE	4-hydroxy-2-nonenal
HO_x	Odd hydrogen species
t½	Half-life

I

IAPs	Inhibitor of apoptosis proteins
IC_{50} values	Inhibitory concentration values
ICAD	Inhibitor of caspase-activated deoxribonuclease
ICE	Interleukin-1β-converting enzyme
IFNγ	Interferon gamma
IKKa	Iκβ kinase
IL-1α	Interleukin-1 alfa
IL-6	Interleukin-6
IL-8	Interleukin-8

K

kD	kiloDalton
KH	Krebs-Henseleit solution

L

LDH	Lactate dehydrogenase
LLF	Lung lining fluid
LMPA	Low melting point agarose
LOPs	Lipid peroxidation products

M

mAChRs	Muscarinic acetylcholine receptors
MBP	Major basic protein
ME10092	N-(3,4-dimethoxy-2-cholorobenzylideneamino)-guanidine
mRNA	Messenger ribonucleic acid

MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

N

NADP	Nicotinamide adenine dinucleotide phosphate
NADP(H)	Reduced form of NADP
NaH_2PO_4	Sodium dihydrogen phosphate
NFκβ	Nuclear factor kappa beta
NO	Nitric oxide
NO_2	Nitrogen dioxide
NOS	Nitric oxide synthase
NO_x	Nitric oxides

O

• OH	Hydroxyl radical
$^1O_2$	Singlet oxygen
O_2	Oxygen
O_2-	Superoxide anion radicals
O_3	Ozone
OSHA	Federal Occupational Safety and Health Administration

P

PAF	Platelet activating factor
PAK2	p21-activated kinase 2
PAMs	Pulmonary alveolar macrophage
PARP	Poly (ADP-ribose) polymerase-1
PARS	PolyADPR synthetase
PBS	Phosphate-buffered saline
PCD	Programmed cell death
PDKs	3-phosphoinositide-dependent protein kinases
PH	Pleckstrin homology
PI 3-K	Phosphoinositide 3-OH kinase
$PI[3,4]P_2$	Phosphatidylinositol-3,4-bisphosphate
PIP_3	Phosphatidylinositol-3,4,5-triphosphate

PKB	Protein kinase B
PMNs	Polymorphonuclear leukocytes
ppb	Parts per billion
ppm	Parts per million
PUFAs	Polyunsaturated fatty acids

R

RNA	Ribonucleic acid
RO_2	Peroxy radicals
ROS	Reactive oxygen species

S

Ser	Serine
Smac	Second mitochondria-derived activator of caspases
SOD	Superoxide dismutase

T

Thr	Threonine
TNF	Tumour necrosis factor
TNF-R2	Tumour necrosis factor receptor type 2

U

UVB	Ultraviolet B light/middle wave ultraviolet light

V

VOC	Volatile organic compound

W

λ	Wavelength

X

XIAP	X-linked inhibitor of apoptosis protein

Z

Z-DQMD-FMK	Z-Asp(OMe)-Gln-Met-Asp(OMe) fluoromethyl ketone