Investigations on the respiratory effects of ozone in the rodent

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“Poison is in everything, and no thing is without poison. The dosage makes it either a poison or a remedy”

– Paracelsus

Abstract

Ozone, being an unstable molecule, is believed to be one of the strongest oxidant agents known to man. Rapid growth in the application of ozone — both as disinfectant and as form of alternative medicine — led to questions about the effects of uncontrolled ozone exposure and inhalation, whether intentional or unintentional, on the human body.

This study specifically focussed on examining, identifying and substantiating the respiratory effect of acute exposure (10 min or less) to considerably higher ozone concentrations than reported on before (19.5 ± 0.5 ppm). Respiratory tissue of rodents (Duncan-Hartley guinea pigs of both sexes and Male Wistar rats) was subjected to ozone by utilising three distinctly diverse models of ozone introduction: (a) *in vitro* exposure, (b) *in vivo* exposure, and (c) *ex vivo* by employing an isolated lung perfusion model which allows for real-time, breath-by-breath data acquisition of ozone's effect on respiratory mechanics. The effect of ozone on the isolated trachea in the presence of various drugs with well-known effects, including methacholine, isoproterenol and ascorbic acid was also examined.

The results found in this study identified two direct effects on the isolated trachea due to ozone exposure: (1) a definite contraction of the isolated trachea immediately after exposure to ozone, and (2) a clearly visible and significant hyper responsiveness of the isolated trachea to irritants, e.g. methacholine. Although ozone has a negative effect on the trachea, it was concluded that ozone has no adverse effect on muscarinic acetylcholine receptors. An apparent EC_{50} value of ozone on the trachea was established by two different methods as (2.77 ± 0.02) x 10^{-3} M and (2.10 ± 0.03) x 10^{-3} M, respectively.

Ozone furthermore displayed an attenuation of the beneficial pharmacological response of β-sympathomimetic drugs (i.e. isoproterenol), while isoproterenol itself has a relaxing effect on the ozone-induced contraction of the isolated trachea. Indomethacin pre-treatment of isolated tracheal tissue significantly (77%) reduced the ozone-induced contraction of tracheal smooth muscle, suggesting that COX-products of arachidonic acid play a prominent role in the development of pulmonary function decrements consequent to acute high-dose ozone exposure. Ascorbic acid exhibited a meaningful prophylactic effect on ozone-induced contraction of both isolated tracheal tissue and in the isolated lung perfusion model, emphasising the major role antioxidants play in both the epithelium lining fluid (ELF) of the respiratory system and in plasma throughout the body in protecting against the destructive effects of ozone.

Surprisingly, pre-treatment with ascorbic acid did not prevent hyper responsiveness of isolated tracheal preparations to methacholine after a 10 min ozone (19.5 ± 0.5 ppm) exposure. In the lung perfusion model, the presence of ascorbic acid in the perfusion medium did, however, significantly reduce the magnitude and rate of decline in lung compliance after ozone exposure (46% decline with ascorbic acid *versus* 96% in the control study without ascorbic acid).

Examination of a lung perfusion model exposed to ozone (19.5 ± 0.5 ppm O_3; 5 seconds) presented a significant decline in lung compliance (95.6% within 2 min), tidal volume (70%) and maximum inspiratory flow (71.2%), with an ensuing reduction in lung elasticity and severely hampered breathing pattern.

Microscopic examination after acute high-dose inhalation studies did not display any significant cellular damage, oedema or inflammation after acute high-dose ozone exposure. This suggests that significant cellular injury and inflammation is possibly not the causative factor of early breathing difficulty experienced after acute high-dose ozone inhalation, as these symptoms and particularly the result of inflammatory precursors, is believed to probably only set in at a later stage.

Although the potential advantages of ozone in certain fields of medicine are not disputed, ozone, depending on its concentration and cumulative dose, can be either

therapeutic or toxic. Observations in this study emphasised that even short bursts of high-dose ozone inhalation have deleterious effects on respiratory health and care should be taken not to jump to conclusions regarding ozone's medical application without relevant scientific evidence. It must be stressed that high-dose inhalation of ozone should be avoided at all cost - especially by those with existing airway diseases.

Keywords: Isolated trachea · Ozone · Hyper responsiveness · Methacholine · Isoproterenol · Ozone concentration-response curve · Isolated lung perfusion model · *in vivo* · *in vitro* · *ex vivo*

Uittreksel

Osoon is 'n onstabiele molekule, en is een van die sterkste oksideermiddels bekend aan die mens. ‘n Geweldige toename in die aanwending van osoon — beide as ontsmettingsmiddel en as vorm van alternatiewe medisyne — het gelei tot vrae oor die gevolge van ongekontroleerde osoonblootstelling en inaseming op die menslike liggaam, hetsy per abuis of opsetlik.

Hierdie studie het spesifiek gefokus op die ondersoek, identifikasie en stawing van die respiratoriese effekte van akute blootstelling (10 min of minder) aan aansienlike hoër osoon-konsentrasies (19.5 ± 0.5 dpm) as dit waarop voorheen in die literatuur berig is. Lugweg-weefsel van knaagdiere (Duncan-Hartley marmotte van beide geslagte en manlike Wistar-rotte) was onderwerp aan osoon deur middel van drie diverse modelle van osoonblootstelling: (a) *in vitro* blootstelling, (b) *in vivo* blootstelling, en (c) *ex vivo* deur die gebruik van 'n geïsoleerde longperfusiemodel wat voorsiening maak vir regstreekse dataverkryging van osoon se effek op respiratoriese meganika na elke asemteug. Die effek van osoon op die geïsoleerde tragea in die teenwoordigheid van verskeie geneesmiddels waarvan die effekte op die tragea bekend is, onder meer metacholien, isoproterenol en askorbiensuur, is ook ondersoek.

Die resultate wat tydens hierdie studie na vore gekom het, het twee direkte gevolge van osoonblootstelling op die geïsoleerde tragea geïdentifiseer: (1) 'n definitiewe sametrekking van die geïsoleerde tragea onmiddellik na blootstelling aan osoon, en (2) 'n duidelik waarneembare en beduidende hiperreaktiwiteit van die geïsoleerde tragea tot allergene, bv. metacholien. Alhoewel osoon 'n negatiewe uitwerking op die tragea het, is daar wel bevind dat osoon geen nadelige uitwerking op muskariniese asetielcholien reseptore het nie. ‘n Oënskynlike EC_{50}-waarde van osoon op die tragea is deur twee verskillende metodes bepaal as onderskeidelik $(2.77 \pm 0.02) \times 10^{-3}$ M en $(2.10 \pm 0.03) \times 10^{-3}$ M.

Osoon het ook 'n beduidende afname in sensitiwiteit teenoor die farmakologiese effek van β-simpatomimeties middels (isoproterenol) vertoon, terwyl isoproterenol self 'n verslappende uitwerking op die osoon-geïnduseerde sametrekking van die geïsoleerde tragea het. Voorafbehandeling van trageale weefsel met indometasien verminder die osoon-geïnduseerde sametrekking van trageale gladdespier beduidend (77%), en dit dui dui moontlik daarop dat siklo-oksigenase produkte van aragidoonsuur 'n prominente rol speel in die ontwikkeling van longfunksie-afname weens akute hoëdosis osoonblootstelling.

Voorafbehandeling met askorbiensuur het 'n betekenisvolle inkorting van osoon-geïnduseerde sametrekking in beide geïsoleerde trageale weefsel en binne ‘n geïsoleerde longperfusiemodel vertoon — Dit beklemtoon die belang van anti-oksidante binne beide die epiteelwand-vloeistof (ELF) van die asemhalingstelsel, sowel as binne plasma regdeur die liggaam in die beskerming teen die vernietigende gevolge van osoon.

Dit was wel verrassend dat voorafbehandeling van geïsoleerde trageale weefsel met askorbiensuur nie die ontwikkeling van hiperreaktiwiteit teenoor metacholien na 'n 10 min osoonblootstelling (19.5 ± 0.5 dpm O_3) kon voorkom nie. In die longperfusiemodel het die teenwoordigheid van askorbiensuur in die perfusie-medium egter wel die tempo en mate van die afname in respiratoriese meganiese-maatstawwe na osoonblootstelling beduidend verlaag (46% daling met askorbiensuur teenoor 96% in die kontrole-studie sonder askorbiensuur).

Studies op 'n longperfusiemodel wat blootgestel is aan osoon (19.5 ± 0.5 dpm O_3 vir ‘n duur van 5 sekondes) het 'n beduidende afname in long-vervormbaarheid (95.6% binne 2 min), gety-volume (70%) en maksimum inspiratoriese vloei (71.2%) getoon, met 'n daaropvolgende vermindering in long-elastisiteit en duidelik waarneembare bemoeiliking van asemhaling.

Mikroskopiese ondersoek kort na akute hoëdosis osooninaseming het geen beduidende sellulêre skade, edeem of inflammasie vertoon nie. Dit dui daarop dat die aansienlike sellulêre besering en inflammasie wat in die literatuur beskryf word, moontlik nie die oorsaak is van vroeë asemhaling-bemoeiliking wat waargeneem

word kort na akute osoon inaseming nie. Daar word voorgestel dat hierdie simptome, en veral inflammasie, waarskynlik eers op ‘n later stadium hul verskyning maak en dat die aanvanklike waargenome effekte van hoëdosis osooninaseming waarskynlik deur inflammatoriese-voorgangers veroorsaak word.

Alhoewel die potensiële voordeligheid van osoon in verskeie mediese velde nie betwis kan word nie, kan osoon afhangende van die konsentrasie en kumulatiewe dosis daarvan, óf terapeuties óf giftig wees. Waarnemings in hierdie studie beklemtoon dat selfs kort sarsies van hoëdosis osooninaseming skadelike effekte op respiratoriese gesondheid mag hê. Sorg moet veral geneem word om nie té vinnig uitlatings te maak rakende osoon se mediese toepassing sonder dat die nodige wetenskaplike bewyse die veiligheid en effektiwiteit daarvan bevestig het nie. Ten slotte moet dit beklemtoon word dat hoëdosis-inaseming van osoon ten alle koste vermy moet word - veral deur diegene met bestaande lugwegsiektes.

Sleutelwoorde: Geïsoleerde tragea · Osoon · Hiperreaktiwiteit · Metacholien · Isoproterenol · Osoon konsentrasie-reaksie kurwe · Geïsoleerde longperfusiemodel · *in vivo* · *in vitro* · *ex vivo*

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Chapter 1
Aim and Objective

Rapid growth in the application of ozone as form of alternative medicine — unfortunately often by uninformed “ozone-therapists” unaware of the risk associated with ozone therapy — led to the question being asked what effect uncontrolled ozone exposure and inhalation, whether intentional or unintentional, has on the human body (Labuschagne, *et al.*, 2009; Bocci, 2010).

1.1 Introduction

Ozone, being an unstable molecule, is believed to be the third strongest known oxidizing agent after fluorine and persulphate — a fact that explains its high reactivity and toxic effects (Gottschalk *et al.*, 2010; Bocci, 2010). Because of its powerful anti-bacterial properties and the widespread availability of ozone generators which often generate ozone at unknown and uncontrolled concentrations, it is not surprising that ozone is increasingly being used as disinfectant in industry, as well as in offices and at home.

However, ozone is also a major air pollutant and has been known to cause toxic pulmonary effects in animals and man for decades (Kosmider *et al.,* 2010; Stokinger, 1965). Inhalation of moderate doses can induce rapid damage of epithelial cell membranes in the pulmonary airways. Various adverse sequelae of ozone exposure have been documented, including increased airway hyper responsiveness, bronchoconstriction, epithelial sloughing, and neutrophil influx in the airways (Kosmider *et al.,* 2010; Schelegle *et al.,* 1991; Hyde *et al.,* 1992; *Park et al*., 2004).

Calculations have suggested that the high reactivity of ozone and its low solubility in water would prevent it from passing through the lung epithelial lining fluid, reducing the likelihood of it reacting directly with the underlying epithelial cells (Cvitaš

et al., 2005; Pryor, 1992). The mechanism by means of which ozone causes cell injury is linked to its powerful oxidative capacity and involves the peroxidation of cell membrane components (Ciencewicki *et al.,* 2008; Wright and Wheeler, 1990). Once inhaled, ozone triggers in lungs the production of reactive oxygen species, induces the release of inflammatory factors such as prostaglandins, and stimulates the sensory afferents. These secondary products of ozone — messenger species derived from reactions that occur between inhaled ozone and epithelial-lining fluid (ELF) or lung tissue — are suspected to mediate ozone toxicity throughout the body (Kafoury *et al.,* 1999; Kosmider *et al.,* 2010; Ciencewicki *et al.,* 2008 Escalante-Membrillo, 2005; Cvitaš *et al*., 2005; Rivas-Arancibia *et al.,* 2003).

The objective of this study is to examine, identify and substantiate the pulmonary effects of acute exposure to considerably higher ozone concentrations (19.5 ± 0.5 ppm) than reported on before, with the direct and modulatory effects of ozone in the respiratory tract being primary research objectives.

Respiratory tissue will be subjected to ozone by utilising three distinctly diverse models of ozone introduction: *In vitro* exposure*, in vivo* exposure*,* and by employing an *ex vivo* isolated lung perfusion model which allows for breath-by-breath and real-time data acquisition of ozone's effect on respiratory mechanics. The intent with utilising three models of ozone introduction is to confirm whether certain experimental results are repeatable across multiple experimental platforms.

1.2 Problem statement

The upsurge in laymen utilization of ozone amplifies the importance of identifying potential safety risks associated with exposure to ozone. It is of importance to recognize possible pharmacological and toxicological effects, and to determine whether even short episodes of high-dose ozone exposure has any toxic or irreversible adverse effects.

Should this be the case, governmental health departments, manufacturers and marketers of ozone generating equipment, employers utilising ozone application,

ozone therapists and the public, especially those with pre-existing respiratory conditions, should be cautioned of the potential risks that accompany ozone application. Both responsible marketing and use is of utmost importance, and the quality of ozone generating equipment need to be strictly regulated — A prospect that, since the ozone industry in recent times became quite a lucrative one, can be expected to be met with much resistance.

1.3 Objective and approach

1.3.1. Objectives

The objectives set for this study are:

1.3.1.1. Primary objective

- To examine, identify and substantiate the pulmonary effects of acute exposure to considerably higher ozone concentrations (19.5 ± 0.5 ppm) than reported on before, with the direct and modulatory effects of ozone in the respiratory tract being the primary research objecvtives.

1.3.1.2. Secondary objectives

- The design, development and successful implementation of an experimental method to measure ozone concentration, solubility and decay in the experimental environments employed during this study;

- The design, development and successful implementation of an experimental model that will enable *in vitro* ozone exposure and related pharmacological studies on isolated tracheal tissue. This furthermore include:

  a. *The design, development and successful implementation of an experimental model to determine the effect of glucose omission from the physiological solution on ozone induced tracheal contraction,*

  b. *The design, development and successful implementation of an experimental model to obtain a concentration-response curve of ozone — something that is, to the best of our knowledge, not available in current*

scientific literature — from which the effect of ozone on isolated tracheal tissue can accurately be predicted, and

c. *The design, development and successful implementation of an experimental model to determine the effect of calcium omission from the physiological solution on ozone induced tracheal contraction.*

- The design, development and successful implementation of an experimental model that will enable *in vitro* ozone exposure and/or related pharmacological studies on isolated tracheal tissue after initial *in vivo* ozone treatment. This furthermore includes:

  The microscopic evaluation of tissue samples collected immediately after acute in vivo ozone inhalation.

- The design, development and successful implementation of an experimental model that will enable ozone inhalation simulation and measurement of lung function parameters in a lung perfusion (*ex vivo)* model,

1.3.2. Approach

In vitro experiments and studies on isolated tracheal tissue were performed in the molecular pharmacology laboratory of the North-West University. The isolated Duncan-Hartley guinea pig trachea was the organ of choice on which the majority of experiments were performed.

Ex vivo lung perfusion experiments were performed at the perfusion laboratory of Professor James Syce at the University of the Western Cape. During these experiments the isolated lung of the rat — an exception — was used due to availability and familiarity of technical staff with the procedure on this particular species.

All experimental procedures performed in this study were in accordance with the regulations stipulated by the Ethical Committees of both the North-West University and the University of the Western Cape, complying with national legislation

and in accordance with the guidelines of the National Institutes of Health guide for the care and use of laboratory animals.

In vivo exposure to ozone was performed under the meticulous supervision of qualified and competent personnel at the North-West University's laboratory animal research centre. After *in vivo* exposure, tissue samples were collected by trained personnel in accordance with the protocols of research centre. Morphological studies were performed on these collected samples at the Department of Morphology at the University of the Free State.

All studies on respiratory tissue were performed with medicinal substances of which the effect on respiratory tissue is known. The study compared the effects of these substances before and after exposure to ozone, thereby determining whether ozone modulates the effects of any of these substances.

1.4 Conclusion

In view of the information on ozone referred to earlier in this chapter, it is suggested that ozone does possess the ability to evoke adverse health effects. Despite these warnings uncertainty still prevails regarding the safety of ozone as a medicine. The objective of this study therefore is to examine, identify and substantiate the pulmonary effects of acute exposure to considerable higher ozone concentrations than reported on before, to report on its effect on the efficacy of, and reaction to certain pharmacological agents and their receptors, and to identify potential histological and morphological changes brought on as a result of acute ozone exposure.

Chapter 2
Literature Review

Velio Bocci recently published the book, *Ozone. A New Medical Drug*. The author was quick to acknowledge that the success of ozone therapy depends on using small and safe ozone dosages. "*In these doses, ozone enables stimulation of biochemical pathways responsible for the activation of the natural healing capacity*" (Bocci, 2010).

This "*natural healing capacity*" is postulated to present as a result of activation of several biochemical pathways within the body (Bocci and Aldinucci, 2006). It includes adaptation to chronic oxidative stress on the basis of a hypothesis by Calabrese (2008) which states that "*the exposure of an organism to a low level of an agent, harmful at high levels, induces an adaptive and beneficial response*". This may be of particular importance in specific pathologies such as chronic infections, neurodegenerative, and autoimmune diseases in which an imbalance between overproduced oxidants and depleted antioxidant defence mechanisms may lead to cell degeneration (Victor *et al.*, 2006).

Unfortunately "*unscrupulous quacks*" — many without any medical qualification — applied research on the use of ozone in medicine, together with other often unproven scientific claims, to strengthen excessive assertions that ozone can cure almost anything (Bocci, 2006), which in itself is a disturbing trend. Even in South Africa, self proclaimed "*ozone therapists*" lacking proper training are opening practices at an ever increasing rate, many without any idea what dangers the improper use of ozone use holds.

It is true that ozone does hold a number of demonstrated advantages in certain fields of medicine, and single ozone doses can be therapeutically used in selected human diseases without any toxicity or side effects. Moreover, the versatility and amplitude of beneficial effect of ozone applications have become evident in

orthopaedics, cutaneous, and mucosal infections as well as in dentistry (Bocci *et al,* 2009).

It should however be noted that ozone, depending on its concentration and cumulative dose — as is the case with any other drug — can be either therapeutic or toxic (Bocci *et al.,* 2009). In the publication *The poison paradox; chemicals as friends and foes* Timbrell (2005) states that "*the essential facts are that first it is the dose that makes a chemical toxic, and second and more important, toxicity results from the interaction between chemical and biological defences*". The subtlety and complexity of biological systems may indeed defy the concept that ozone is always toxic. (Bocci *et al,* 2009).

Care should however be taken not to jump to conclusions regarding ozone's medical application without relevant scientific evidence — a miscalculated leap which is unfortunately made much too often in the field of ozone therapy. Claims, such as the false allegation that direct IV gas administration could cure HIV infection, are frequent and may sound attractive to the uninformed. This is a great cause for worry and has added to the stigma of ozone therapy being labelled as dangerous quackery (Bocci, 2006).

With the increased use of ozone in various other fields besides medicine, the question arose as to what the exact effect of ozone exposure — and more importantly, ozone inhalation — whether intentional or unintentional, is on the human body.

The literature often emphasises the possible effects of ozone on respiration, and much research on the topic have been done before (Mustafa, 1990; Mudway and Kelly, 2000). Concern is however mounting as ozone-therapy is becoming a highly fashionable form of both cosmetic and alternative medical treatment, often in institutions were the exact yield of the applied ozone generators are unknown.

The United States Food and Drug Administration (FDA) requires ozone output of indoor medical devices to be no more than 0.05 ppm. However, after discussing the yield of their generators, many South African suppliers of ozone generators for

indoor use are not aware of the ozone output produced by their respective products. The actual concentration of ozone produced by an ozone generator depends on many factors. Concentrations will be higher if a more powerful device or more than one device is used, if a device is placed in a small space rather than a large space, if interior doors are closed rather than open, if the room has fewer rather than more materials and furnishings that adsorb or react with ozone and, provided that outdoor concentrations of ozone are low, if there is less rather than more outdoor air ventilation. The proximity of a person to the ozone generating device can also affect one's exposure - The concentration is highest at the point where the ozone exits from the device, and generally decreases as one moves further away. Manufacturers and vendors advise users to size the device properly to the space or spaces in which it is used (EPA, 2010(b)).

Unfortunately, some manufacturers' recommendations about appropriate sizes for particular spaces have not been sufficiently precise to guarantee that ozone concentrations will not exceed public health limits. Further, some literature distributed by vendors suggests that users err on the side of operating a more powerful machine than would normally be appropriate for the intended space, the rationale being that the user may move in the future, or may want to use the machine in a larger space later on. Using a more powerful machine increases the risk of excessive ozone exposure (EPA, 2010(b)).

In one study (Shaughnessy and Oatman, 1991), a large ozone generator recommended by the manufacturer for spaces "up to 3,000 square feet," was placed in a 350 square foot room and run at a high setting. The ozone in the room quickly reached concentrations that were exceptionally high - 0.50 to 0.80 ppm which is 5-10 times higher than public health limits.

Since many industrial strength ozone generators are freely available, the question has been asked within this laboratory, what the effects of above average ozone concentrations will be if accidentally applied in one of the above mentioned settings. The decision was therefore taken to investigate the effect of accidental exposure to high doses of ozone, i.e. exposure of the respiratory system to above average concentrations of ozone for short periods of time, and whether the effect of

pharmacological agents previously employed in respiratory studies will differ in subjects exposed to excessive concentrations of ozone.

Very little research has been done *in vitro* to determine the effect of above average ozone concentrations on isolated organs, or what the effect of these higher ozone concentrations are if applied in an isolated lung perfusion model, emphasising the importance of this study.

The purpose of this study is not to reflect negatively on ozone, but rather to identify the potential risks associated with improper use thereof. The objective was to determine the pharmacological effect of above average ozone concentrations on the respiratory system by means of *in vitro* and *in vivo* exposure, as well as by means of an *ex vivo* lung perfusion model, and to compare the results found to form an overall picture.

Ozone is both a source of protection and risk for all species. In the stratosphere, where the majority of atmospheric ozone is found, ozone plays an important role in preventing harmful ultraviolet radiation from reaching the surface of the earth. In contrast, ozone present within the lower troposphere (from ground level up to 10 km), is detrimental to health (Bocci, 2006; Mudway and Kelly, 2000). Ozone generated by ozone-generators with uncontrolled output and applied by the uninformed, falls within this category and therefore holds the same risks.

It is these harmful effects of ozone that is the subject of this study.

2.1 Ozone's mechanism of action

Exposure to ozone has been shown to cause *both* airway epithelial damage and physiological changes in distant systemic locations, including peripheral and central regions (Pryor *et al.,* 1995; Soulage *et al.,* 2004; Bocci, 2010).

The toxic effect of ozone is often first seen in the respiratory tract. These often detrimental effects noted with ozone on the respiratory system, are commonly as a result of direct oxidation of sensitive tissue at the airway-air interface, and involve the peroxidation of cell membrane components (Soulage *et al.,* 2004; Bocci, 2010).

However, because ozone is a very reactive molecule, evidence suggest that ozone is entirely consumed as it passes through the first layer of tissue it encounters at the airway-air interface and therefore cannot penetrate very far into the cells that line the airways (Mudway and Kelly, 2000; Pryor *et al.,* 1995). This first layer of contact layer includes the very thin layer of epithelium lining fluid (ELF) and, where the ELF is thin or absent, the membranes of the epithelial cells that line the airways (Wynalda and Murphy, 2010; Bocci, 2010; Cvitaš *et al.*, 2005; Pryor *et al.,* 1995).

But how does ozone exert a remote effect, albeit in deeper parts of the pulmonary system or on distant organs systems, if it is entirely consumed by this first layer?

The answer lies in the fact that ozone is believed to react directly with polyunsaturated fatty acids (PUFA), antioxidants and proteins in this layer. It is well documented that the primary target is thought to be these unsaturated fatty acids. Its ozonation has been shown to release a variety of biochemical mediators including H_20_2 and aldehydes with reactive oxygen species (ROS) as intermediates (Bocci, 2010; Cvitaš *et al.*, 2005; Pryor *et al.,* 1995; Mustafa, 1990).

It is likely that these biochemical mediators are the messenger species that relay many of ozone's pulmonary and all non-pulmonary toxic effects to more distant

organs (Escalante-Membrillo *et al.*, 2005; Bocci, 2010; Cvitaš *et al*., 2005; Pryor *et al.,* 1995).

2.2 The pulmonary and extrapulmonary effects of ozone

Ozone has pulmonary and extrapulmonary effects (Pryor *et al.,* 1995; Soulage *et al.,* 2004; Bocci, 2010). Although ozone's effect on the respiratory system is the major focus point of this thesis, accumulating evidence does suggest that ozone is also able to produce extrapulmonary effects, including effects on the cardiovascular, reproductive and central nervous system (Silva *et al.,* 2009; Srebot *et al.,* 2009; Sokol *et al.*, 2006; Bhalla *et al.,* 1999; Escalante-Membrillo *et al.,* 2005; Soulage *et al.,* 2004).

As mentioned in § 2.1 it is hypothesized that the reaction of ozone with, amongst others, PUFA's in the first layer of contact — the very thin layer of epithelium lining fluid — release a number of biochemical messenger species which relay many of ozone's pulmonary and all non-pulmonary toxic effects to more distant organs (Escalante-Membrillo *et al.*, 2005; Bocci, 2010; Cvitaš *et al*., 2005; Pryor *et al.,* 1995).

2.2.1. The impact of ozone exposure on pulmonary function

Ozone is a powerful and unstable gaseous oxidant that should never be deliberately inhaled (Bocci, 2006). However, if inhaled, its primary target is the mucous membranes and airway surface tissue (Soulage *et al.,* 2004; Bocci, 2010).

Inhaling even slightly elevated concentrations of ozone may result in a variety of respiratory symptoms. These may include a decrease in lung function and increased airway hyper-reactivity. Moreover, those with pre-existing conditions such as asthma and chronic obstructive pulmonary disease (COPD), generally experience an exacerbation of their symptoms (Mudway and Kelly, 2000; Bocci, 2006; Lotriet *et al.,* 2007).

Exposure to ozone has been shown to precipitate acute falls in FEV_1, FVC, total lung capacity (TLC), inspiratory capacity (IC) and a decrease in pulmonary compliance (Katzung, 2007; Mudway and Kelly, 2000). These decrements are dependant on ozone-dose and ozone-exposure duration, and do, in most ozone introduction models, resolve back to what is observed as normal pulmonary function within 12 - 24 hours after withdrawal of exposure (Mudway and Kelly, 2000). This may already, at this early stage of this study, imply that the effects of ozone on the respiratory system may be reversible, even at higher doses of exposure.

Mild ozone exposure produces upper respiratory tract irritation and inflammatory reactions as well as rapid, shallow breathing, whilst high-dose exposure may initiate deep lung irritation. Exposure to high concentrations may even lead to death from pulmonary oedema or respiratory paralysis (Schelegle *et al.,* 2001; Katzung, 2007; Bocci, 2010).

Exposure to doses as low as 0.1 ppm for 10-30 min has been found to cause lacrimation and irritation of upper respiratory tract (Gottschalk *et al.,* 2010; Bocci, 2010; Katzung, 2007). A dose above 1 ppm has been suggested to affect visual activity and may initiate bronchial spasm, retrosternal pain, cough, headache, occasional nausea, pain and dyspnoea (Gottschalk *et al.,* 2010; Bocci, 2010; Katzung, 2007). Acute tracheobronchial epithelial injury, possibly resulting in bronchitis, bronchiolitis, fibrosis and emphysematous changes, have furthermore been reported in test subjects, amongst others the rhesus monkey (Hyde *et al.,* 1992), exposed to concentrations as low as 0.96 ppm (Katzung, 2007; Hyde *et al.*, 1992). These results reiterate the potential danger associated with ozone inhalation.

Increased airway responsiveness to muscarinic agonists is an important consequence of exposure to ozone (Lotriet *et al.*, 2007). An elevated airway responsiveness to methacholine challenge furthermore indicates that the airways are predisposed to bronchoconstriction induced by a variety of stimuli (e.g., specific allergens, sulphur dioxide, cold air, etc. (Katzung, 2007)).

It has also been observed that acute exposure to ozone activates lung macrophages and type II epithelial cells to release cytotoxic and proinflammatory mediators – factors that may contribute to the pathopysiologic effects observed in the lung (Laskin *et al.*, 1994).

Table 2.1 Toxic effects of gaseous ozone in humans (Gottschalk *et al.,* 2010 (*and references cited therein*); Bocci, 2010 *(and references cited therein)*).

Ozone concentration in air (ppm)	*Toxic effect*
0.1	Lacrimation and irritation of upper respiratory airways
1.0 – 2.0	Rhinitis, cough, headache, occasional nausea and retching
	Predisposed subjects may develop asthma
2.0 – 5.0	Progressively increasing dyspnoea, bronchial spasm, retrosternal pain
5.0 (60 minutes)	Acute pulmonary oedema and occasional respiratory paralysis
10.0	Death within 4 hours
50.0	Death within minutes

The toxic effects of ozone in humans are presented in Table 2.1 as compiled by Bocci (2010) and Gottschalk *et al. (2010)*. According to the data presented in this table, the severity of symptoms and pathological changes after prolonged breathing of ozone-contaminated air, are directly in relation to ozone concentration and the exposure time. This supports earlier concern expressed regarding ozone exposure in humans (Chapter 1).

Although Katzung (2007) states that there is no specific treatment for acute ozone intoxication, Bocci (2010) suggested a treatment protocol that should be implemented after exposure to dangerously high concentrations of ozone: It is recommended that an intoxicated patient lie down and, if possible, breath humidified oxygen. A slow intravenous administration of ascorbic acid and reduced glutathione in 5% glucose may limit oxidative damage. Bocci (2010) furthermore postulates that Ascorbic acid, vitamin E and N-Acetylcysteine can also be administered by oral route, but this type of treatment is more rational as a preventative than curative measure.

2.2.2. Extrapulmonary effects of ozone inhalation

Exposure to ozone has been shown to elicit a wide spectrum of pulmonary responses. However, the potential damage induced by ozone does not end with reduced pulmonary function — a number of experimental studies show that prolonged exposure by inhalation of ozone also damages extrapulmonary organs (Bocci, 2006).

Although this study only focuses on the pulmonary effect of ozone, many other remote effects of ozone have been reported as a result of biochemical messengers the exerts ozone toxic effect in remote organ systems. During exposure to ozone these toxic compounds flow continuously into the blood and reach vital organs complicating the pulmonary damage (Bocci, 2006).

Some of the extrapulmonary effects of ozone include (but are not limited to) effects on the cardiovascular, reproductive, endocrine, sensory, hepatic, and central nervous system (Srebot *et al.,* 2009; Bocci, 2006; Sokol *et al.*, 2006; Bhalla *et al.,* 1999; Escalante-Membrillo *et al.,* 2005; Soulage *et al.,* 2004).

2.3 The physical-chemical properties of ozone

To understand how ozone exerts its biological effect, it is necessary to have a good understanding of the structure and resonance of ozone.

2.3.1. The structure of ozone

Ozone is an unstable molecule that consists of three oxygen atoms that are in an unstable state. These unstable oxygen atoms tend to revert to the more stable state of diatomic oxygen (0_2) by releasing one of the oxygen atoms. The latter release of an oxygen atom explains ozone's strong oxidising capacity, while it also explains the short half-life ($t_{½}$) of ozone in the atmosphere and in aqueous solutions, and its inability to reach high concentrations systemically in biological systems as an intact molecule. It is also believed to be responsible for most of the side effects associated with ozone (Van Niekerk, 2008; Pretorius, 2005; Kloos, 2001).

Ozone is furthermore not inherently static and exists as a resonance structure. The resonance structure of ozone defines the electrophylic nature of its chemical reaction with other molecules. The proposed resonance structure for ozone is shown in Figure 2.1.

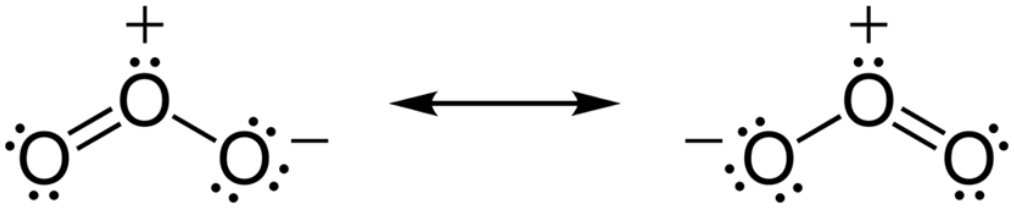

Figure 2.1. The proposed resonance structure of ozone (Kloos, 2001).

Conversion between the two resonance structures occur at such a rapid pace that the observed structure of ozone appears as a combination of the two structures depicted in Figure 2.1 (Kloos, 2001).

2.3.2. Physical properties

Although ozone is a natural allotrope of molecular oxygen (O_2), it does differ significantly from oxygen in terms of solubility and physical properties (Kloos, 2001).

Table 2.2 presents the difference between the physical properties of ozone compared to that of molecular oxygen. Since the solubility of ozone in the Krebs-Henseleit physiological solution will be investigated in this study, it is of particular interest to note that the solubility (β[1]) in water (at 0º C) of either ozone or oxygen is either 0.64 or 0.049 (thirteen-fold lower), respectively.

Consequently the solubility of ozone in water allows its immediate reaction with any soluble compounds and biomolecules present in biological fluids (Bocci, 2010).

Table 2.2. Physical properties of ozone and oxygen (MKS, 2002)

Property	Ozone (O_3)	Oxygen (O_2)
Color	Gas: blue colored	Gas: colorless
	Dissolved in water: purple blue in concentration > 20 ppm	Dissolved in water: light blue
Molecular weight, g/mol	48	32
Boiling Point, °C (K)	-112 (161.3)	-183 (90)
Density, kg/m³	2.144	1.429
Solubility in water at 0 °C	0.64	0.049
Electrochemical potential, V	2.08 (Hydroxyl radical OH° 2.80)	1.23

[1] β =Bunsen coefficient derived from Henry's law

2.3.3. The solubility of ozone in an aqueous medium

Ozone is unstable in water and, as seen in Table 2.3, the solubility of ozone in water is temperature dependent. A higher temperature will therefore lead to a lower solubility of ozone in aqueous media (Gottschalk *et al.*, 2010; Bocci, 2010).

Table 2.3. Solubility of ozone in water as a function of temperature (Gottschalk *et al.*, 2010).

Solubility (β)	*Temp (ºC)*
0.64	0
0.50	5
0.39	10
0.31	15
0.24	20
0.19	25
0.15	30
0.12	35

If the data presented in Table 2.3 is plotted on a graph (Figure 2.2), the solubility of ozone in water at 37 ºC, the temperature of the physiological solution at which experiments are performed, may be predicted by means of extrapolation.

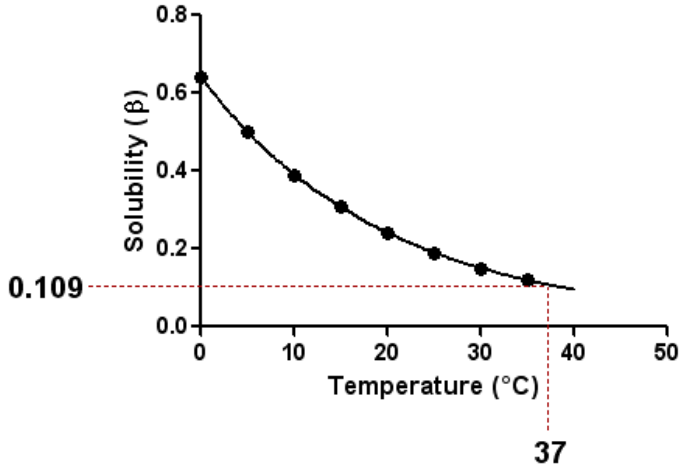

Figure 2.2. Determining the solubility of ozone in water at 37 ºC by extrapolating data presented in Table 2.3.

From the extrapolation depicted in Figure 2.2, the solubility of ozone at 37 ºC is estimated to be 17% (0.109 (β)) of the noted value at 0 ºC (0.64 (β)). Preliminary experiments in which the solubility of ozone was measured suggest that, depending on the method used to measure solubility, experimental results correlates with this extrapolated value.

If the Indigo Colorimetric method (see § 2.4) is employed to determine solubility, ozone appear to be better soluble (as much as 63%) compared to what is suggested in Table 2.3. However, when the UV spectrophotometric (see § 2.4) method is used, the measured result at 37 ºC agrees with that predicted by extrapolation.

It is hypothesized that results obtained by means of the Indigo Colorimetric method may be time-delayed and this is believed to explain the better and apparent incorrect solubility observed when this method is employed.

2.4 Methods for determining ozone concentration in an aqueous medium

The literature describes several analytical methods for determining the concentration of ozone dissolved in a liquid phase. Gottschalk *et al.* (2010) compared several of these methods. Taking available resources into consideration, three of these — the iodometric, indigo colorimetric, and UV absorption methods — were believed to be the most suitable options for application during this study.

2.4.1. **The Iodometric Method** (Gottschalk *et al.*, 2010)

When applying the iodometric method, a water sample containing ozone is mixed with potassium iodide. The iodide I^- is oxidized by ozone. The reaction product, iodine I_2, is titrated immediately with sodium thiosulphate ($Na_2S_20_3$) to a pale yellow colour. With a starch indicator the endpoint of titration can be intensified to a deep blue. The ozone concentration can be calculated by the consumption of $Na_2S_20_3$.

This method was, however, found to be time consuming, and since iodide is oxidized by substances with an electrochemical potential higher than 0.54 eV, another negative is the fact that this method is not very selective. Interference may occur with CI_2, Br^-, H_20_2, Mn-components and organic peroxides.

2.4.2. **Indigo colorimetric method** (Gottschalk *et al*., 2010; Abad *et al*., 2002)

The indigo colorimetric method determines the concentration of aqueous ozone by the decolourisation of indigo trisulphate (λ = 600 nm). This method must however be performed in an acidic environment. The method is stoichiometric and extremely fast. The indigo molecule contains only one C=C double bond which is expected to react directly with ozone (with little chance of interference) and with a very high reaction rate.

One mole ozone decolorizes one mole of aqueous indigo trisulphate at a pH less than four. Hydrogen peroxide and organic peroxides react very slowly with the indigo reagent and, as long as ozone is measured in less than six hours after adding the reagents, hydrogen peroxide will not cause any interference.

The ozone concentration (mg O_3/l) in an aqueous medium is calculated using the equation:

$$[O_3]^* = \frac{V_T \times \Delta A}{f \times b \times V} \qquad (3.1)$$

where:

ΔA	=	difference in absorbance between sample and blank
V_T	=	Total volume of sample and indigo solution in ml
b	=	Optical path length of cell in cm
V	=	Volume of ozonated sample in ml
f	=	0.42
$[O_3]$	=	Mg O_3/l

2.4.3. **UV absorption method** (Gottschalk *et al.*, 2010)

The UV absorption method is based on absorbance of the ozone aqueous solution at 254 nm. The molar absorptivity used is 3000 $M^{-1}cm^{-1}$, which is the recommended value by the International Ozone Association.

The major advantage of this method is that it is very easy and simple, whilst continuous measurement is possible, making it ideal for measurements related to ozone decay. It should, however, be noted that aromatic pollutants in water absorb UV radiation at $\lambda = 254$ nm and can interfere with the measurement.

2.4.4. Conclusion

In conclusion, when measuring the concentration of ozone in an aqueous medium, the indigo colorimetric method is believed to be the more reliable method, whilst the UV absorption method appears to be the most useful when determining ozone's half-life and decay as this method can be followed in real-time.

2.5 Safety standards

The FDA (2001) states that "*ozone is a toxic gas with no known useful medical application in specific, adjunctive or preventative therapy*". In order for ozone to be effective as a germicide, it must be present in a concentration far greater than which can be safely tolerated by man and animals."

Ozone exposure has been associated with increased susceptibility to respiratory infections, medication use by asthmatics, doctor visits, and emergency department visits and hospital admissions for individuals with respiratory disease. Ozone exposure may also contribute to premature death, especially in people with heart and lung disease (EPA, 2010(a)). As a result, various organisations in the USA have set standardised levels for the safe application of ozone. To date, the USA seems to be the most aware of the hazards that are associated with improper ozone application (EPA, 2010(a)).

Ground-level ozone is not emitted directly into the air, but forms through a reaction of nitrogen oxides (NOx), volatile organic compounds (VOCs), carbon monoxide (CO) and methane (CH4) in the presence of sunlight. Emissions from industrial facilities and electric utilities, motor vehicle exhaust, gasoline vapors, and chemical solvents are the major man-made sources of NOx and VOCs (EPA, 2010(a)).

In this country, the *South African Bureau of Standard (SABS)* is currently determining and implementing new standards that will set acceptable levels of ozone in the workplace as well as, ultimately, output-limits for ozone generators to which manufacturers must adhere to in future once legislation is set in place. In order to take into consideration the specific formation mechanisms of ozone and the potential which exists for trans-boundary transportation of this pollutant, target values rather than limit values, are set (SABS, 2004).

Current regulations by the SABS states that measures shall be taken to ensure that concentrations of ozone in ambient air are managed with the aim of achieving the target values laid down in Table 2.4 within the time frames as determined in accordance with SABS. These target values primarily aim at the protection of human health (SABS, 2004).

Table 2.4: Target values, margins of tolerance and dates for compliance with limit values for ozone (SABS, 2004)

1	2	3	4	5
Exposure periods	Averaging period	Target Value	Margin of tolerance	Date by which target value should be complied with
Hourly limit value for the protection of human health	1 hour	200 µg/m^3 (102 ppb) (permissible frequency for exceeding limit values to be determined)	*	*
8-hourly limit value for the protection of human health	8-hourly running average calculated on 1-hourly averages	120 µg/m^3 (61 ppb)	*	*
*To be determined in accordance with the SABS				

Target values are expressed in µg/m^3 (see 2.4). The volume shall be standardised at a temperature of 25 ºC and a pressure of 101.3 kPa. Permissible frequencies for exceeding limit values, margins of tolerance and dates by which values should be compiled with, can only be determined after preliminary assessments have been undertaken in accordance with the SABS.

According to the SABS (2004) the maximum daily 8-hourly mean concentration will be selected by examining 8-hourly running averages, calculated from hourly data and updated each hour. Each 8-hourly average so calculated will be

assigned to the day on which it ends, i.e. the first calculation period for any one day will be the period from 17:00 on the previous day to 01:00 on that day; the last calculation period for any day will be the period from 16:00 to 24:00 on that day.

Chapter 3
Materials and methods

During the course of this study an array of experiments were performed to determine the pharmacological effect of ozone on the respiratory system, following both *in vitro* and *in vivo* exposure. In addition, further experiments were performed using a lung perfusion model, to assess the effect of ozone on a functional lung model. The effect of selected pharmacological agents on isolated tissue and the effect of ozone in turn on the functioning of these agents were also investigated.

In this chapter the materials, experimental design and experimental model for the experimental results presented in the latter chapters of this study are explained and discussed.

3.1. Measuring ozone concentration, solubility and decay

In order to determine the pharmacological effect of ozone, an accurate method to measure ozone concentration under controlled conditions in both air and aqueous media had to be developed. This section presents the methods employed to manufacture ozone, measure generator yield and to determine solubility of ozone.

3.1.1. Ozone preparation

Ozone was prepared by feeding ultra-high purity (UHP) 99.995% oxygen (Afrox South Africa) into a Sterizone PHP250 (PCT/ZA00/00031) ozone generator (Figure 3.1) at a flow rate constantly controlled by means of using a rotameter (Model

6AV5101BN, Dakota Instruments, USA) on which a constant flow rate of 5 l/min was maintained.

Figure 3.1. The Sterizone PHP250 ozone generator

3.1.2. Measuring generator yield

This method presents the technique applied to measure the ozone concentration produced by the ozone generator mentioned above.

The gaseous output of the Sterizone PHP250 ozone generator was kept stable at a continuous controlled flow rate, whilst a UV spectrophotometer (Unico 2800 VIS/UV Spectrophotometer) modified for the specific purpose of measuring ozone concentrations in air, was applied to measure the absorbance (at 254 nm; path length 2 mm) of the generated product.

From the spectrophotometrically measured absorbance, the concentration of ozone produced is calculated by employing an equation specifically refined for the equipment used in this study (Labuschagne, 2007):

$$[O_3]^{**} = \frac{\left(Absorbance \times 4.625^{*}\right)}{4.67^{*} \times 100} \qquad (3.1)$$

** The values 4.625 and 4.67 employed in this equation are correction factors for the specific environment in which these experiments were performed.*

** $[O_3]$ = mg O_3/l

3.1.3. The aqueous solubility of ozone

When ozone is constantly bubbled through an aqueous medium, a percentage of the gas is expected to dissolve in the medium to ultimately reach a point of saturation. The detection of ozone dissolved in liquid phase can be done using the potassium-indigo trisulfonate colorimetric method (see § 2.4.1), a simple, quantitative and selective method. The method is based on the principle that ozone rapidly decolorizes the indigo-reagent ($C_{16}H_7N_70_{11}S_3K_3$) in an acidic solution, and measures colorimetric change at 600 ± 5 nm. The decrease in absorbance is linear with increasing ozone concentration and this measurement was performed using a Shimadzu Multispec (model 1501) spectrophotometer.

Depending on the ozone content in a sample, an indigo-reagent is prepared from a stock solution and spectrophotometrically examined according to one of three methods.

3.1.3.1. Reagents

A stock solution is made up by adding 500 ml distilled water and 1 ml concentrated phosphoric acid in a 1 litre volumetric flask. Whilst stirring, 770 mg potassium indigo trisulphonate ($C_{16}H_7N_2O_{11}S_3K_3$) is added. The flask is then filled to volume with distilled water.

Indigo reagent I is prepared by adding 20 ml of the stock solution, 10 g sodium dihydrogen phosphate (NaH_2PO_4) and 7 ml concentrated phosphoric acid to a 1 litre volumetric flask. The flask is filled to volume with distilled water.

Indigo reagent II is prepared by adding 100 ml of the stock solution, 10 g sodium dihydrogen phosphate (NaH_2PO_4) and 7 ml concentrated phosphoric acid to a 1litre volumetric flask. The flask is filled to volume with distilled water.

3.1.3.2. Spectrophotometric procedure

The spectrophotometric procedure that is followed depends on the ozone content of the sample. Table 3.1 summarises the procedure followed for each concentration range.

Table 3.1. Spectrometric procedure according to concentration range for applying the Iodometric Colorimetric method when calculating ozone concentration in an aqueous medium (Gottschalk *et al.,* 2010).

Concentration	*Procedure*
0.01-0.1 mg O_3/l	Add 10 ml **indigo reagent I** to two 100 ml volumetric flasks. Fill one flask (blank) to volume with distilled water and the other with the sample. Add sample so that completely decolorized zones are eliminated quickly, but no degassing occurs.
0.05-0.5 mg O_3/l	Add 10 ml **indigo reagent II** to two volumetric flasks. Fill one flask (blank) to volume with distilled water and the other with the sample. Add sample so that completely decolorized zones are eliminated quickly, but no degassing occurs.
Larger than 0.3 mg O_3/l	Add 5 ml of the sample to 10 ml **indigo reagent II** in a 100 ml volumetric flask and fill to volume with distilled water. Make another solution, the blank, by measuring 10 ml **indigo reagent II** in a 100 ml volumetric flask and filling it to volume with distilled water.

3.1.3.3. Calculations

The calculation used to determine ozone concentration in a liquid medium is performed after absorbance of a potassium indigo trisulphonate reagent is spectrophotometrically measured at 600 nm (Abad *et al.,* 2002):

$$[O_3]^{**} = \frac{V_T \times \Delta A}{f \times b \times V} \qquad (3.2)$$

where:

ΔA = difference in absorbance between sample and blank

V_T = Total volume of sample and indigo solution

b = path length of cell in centimetre

V = Volume of sample in ml

f = 0.42*

** The factor f is based on a sensitivity factor of 20000/cm for the change of absorbance (600 nm) per mole of added ozone per litre. It was calibrated by iodometric titration. The UV absorbance of ozone in pure water may serve as a secondary standard: the factor f=0.42 corresponds to an absorption coefficient for aqueous ozone, ε = 2950/M·cm at 258 nm.*

*** [0_3] = mg O_3/l*

3.1.3.4. A note on ozone concentrations applied in experiments

Under experimental conditions, saturation of the aqueous media used in this study was reached within 10 min of continuous ozone bubbling. It should be noted that, when water-ozone solutions were employed as treatment protocol during certain experiments in this study, it was always concentrated solutions of ozone in distilled water at 20 ºC. However, since ozone decomposes rapidly (Gottschalk ***et al.,*** 2010), especially at higher temperatures, it is important to note that all ozone concentrations referred to in this study are the initial ozone concentrations at the start of each experiment.

3.1.4. The viability of glucose omission from the physiological solution

During initial *in vitro* experiments on isolated tracheal tissue, glucose was included in the modified Krebs-Henseleit solution. However, since solubility studies suggested that ozone is less soluble in a Krebs-Henseleit solution containing glucose, it was decided that subsequent exposures to ozone should be performed in a glucose-free Krebs-Henseleit medium (see data and discussion in § 4.2). The glucose-free Krebs-Henseleit solution (KH-G) was prepared (see § 3.2.1.4) as described by Patil and Jacobowitz (1968), but with omission of 11.1 mM glucose.

This method presents the technique employed to determine the viability of glucose omission.

Subsequent to the isolation and suspension of the tracheal tissue sample in the organ chamber of a jacketed organ bath (as described in § 3.2.2), a cumulative concentration-response curve was determined for methacholine (as described in § 3.2.3) in Krebs-Henseleit devoid of glucose (KH-G), thereby establishing the effect of methacholine on the isolated guinea pig trachea in the absence of glucose. KH-G was added to each organ bath prior to methacholine and ozone exposure. However, during the resting periods and for rinsing of tissue samples, KH+G was used to incur physiological haemostasis.

The resulting cumulative concentration-response curve (KH-G) was compared with the known concentration-response curve for methacholine in the presence of glucose (KH+G) (see Figure 5.1). Fluctuations from this curve may indicate increased or reduced responsiveness of isolated tracheal tissue as a result of glucose omission.

3.1.5. The effect of ozone on pH of the physiological solution

To establish whether exposure to ozone affects the pH of a KH-G solution, ozone was bubbled through a KH-G solution at 37 °C for 10 min — the time suggested needed for the physiological solution to reach saturity under experimental conditions. pH was measured immediately prior to, and right after completing ozone exposure.

As a control study, bubbling of UHP oxygen through a KH-G solution for the same length of time under the same conditions was furthermore employed.

3.1.6. The half-life of ozone in glucose-free Krebs-Henseleit

Since ozone is unstable in water and decays rapidly (Gottschalk ***et al.***, 2010), experiments were performed to establish ozone's precise half-life under the experimental conditions employed in this study.

Ozone was bubbled through a 100 ml KH-G solution at 1, 20 or 37 ºC for 10 min, whereafter a 3 ml sample was taken from the freshly prepared saturated ozone solution and sealed in a quartz cuvette before absorbance was apectrophotometrically measured (at 254 nm) employing a Shimadzu Multispec-1501 spectrophotometer at intervals of 1 second.

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

$$y = plateau + span.exp\ (-k*x) \quad (3.3)$$

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

3.2. Respiratory effects of ozone in vitro

During *in vitro* experiments, freshly prepared tracheal chains were isolated and suspended in a jacketed organ baths. Data obtained during these experiments allowed for the determination of concentration-response curves which, in turn, enabled the observation and identification of the respective effects of both ozone and other pharmacological agents in the context of each experiment.

3.2.1. Equipment, chemicals and animals

3.2.1.1. Laboratory Animals

Duncan-Hartley guinea pigs (450-550 g), obtained from the laboratory animal centre at the North-West University, were used. Animals had free access to water and food prior to experiments, whilst ultimate care was taken to prevent contamination by preventing exposure to other animals. Stress induced as a result of excessive human interference was furthermore minimised.

All experimental procedures performed in this chapter were in accordance with the regulations stipulated by the Ethical Committee (Approval number: 05D02) of the North-West University, complying with national legislation and in accordance with the guidelines of the National Institutes of Health guide for the care and use of laboratory animals.

3.2.1.2. Instruments and equipment

The following instruments and equipment were used: A small operating table and dissection equipment, a gas chamber (Department of Technical Services, North-West University), a water heating unit and pump fitted with a thermostat, 6 jacketed organ baths, a Statham UL-2 Force Displacement Transducer attached to a Metrohm Labograph (Model E-478) recorder, a Sterizone P-HP 250 (PCT/ZA00/00031) ozone

generator (see Figure 3.1), a rotameter and a Unico 2800 VIS/UV Spectrophotometer.

3.2.1.3. Chemicals

The following drugs and chemicals were used: Sodium chloride, potassium chloride, calcium chloride 2-hydrate, sodium hydrogen carbonate, glucose and potassium dihydrogen phosphate, all of analytical grade, and obtained from Holpro Analytics (Johannesburg, RSA). Methacholine, isoproterenol, indomethacin and atropine were obtained from Sigma Aldrich, while carbomer gas, carbon dioxide and UHP oxygen were obtained from Afrox South Africa.

3.2.1.4. Krebs-Henseleit Solution

A modified Krebs-Henseleit solution was made to the specifications of Patil and Jacobowitz (1968) composing of: 119 mM NaCl, 4.7 mM KCl, 1.9 mM $CaCl_2$, 0.54 mM $MgCl_2$, 24.0 mM $NaHCO_3$, 1.0 mM NaH_2PO_4 and 11.1 mM glucose. Each ingredient was added after the previous salt was dissolved, while KCl and $MgCl_2$ were added last to prevent the formation of precipitate (Van Rossum, 1963). The solution was then heated to 37 ºC and kept at this temperature throughout the experiment, whilst the pH of the solution was constantly measured and kept stable at 7.4.

3.2.2. Tissue preparation

The trachea of Duncan-Hartley guinea pigs of both sexes were used in experiments during this study. The primary advantages of the guinea pig are the similar potencies and efficacies of agonists and antagonists in human and guinea pig airways and the many similarities in physiological processes, especially airway autonomic control, the response to allergen, stability as well as suitability for sympathomimetic, sympatholitic, cholinomimetic and cholinolytic studies (Canning and Chou, 2008).

Guinea pigs of the same size and age were used throughout this study as age and mass (450-550 g) may influence the sensitivity of smooth muscle towards drugs and other stimuli (Collier, 1970).

Animals were euthanized by carbon dioxide asphyxiation, and the trachea was rapidly removed and manually dissected form connective and other tissues. Each of the isolated tracheas was cut longitudinally through the cartilaginous rings on the opposite side of the tracheal muscle according to the technique of Timmerman and Scheffer (1968). The trachea was folded open and five incisions were made on alternate sides of the tracheal muscle, dissecting the muscle and leaving the trachea, when stretched out, to form a chain that is attached only at alternate parts of the cartilage. This tracheal chain was suspended between two L-shaped stainless steel hooks and suspended in a 10 ml jacketed organ bath containing KH+G buffer solution at 37 ºC continuously aerated with O_2/CO_2 (19:1 ratio). Isometric contractions were measured with a force transducer (Statham UL-2 Force Displacement Transducer) and recorded on a polygraph (Metrohm Labograph Model E-478 recorder).

The trachea was allowed to equilibrate for at least 60 min at a resting tension equivalent to 2.0 g. During the period of stabilization, the tissue was washed with Krebs–Henseleit solution at 15 min intervals and after the relaxation period, the tension in each tracheal segment was readjusted to 2 g.

KH-G was added to each organ bath prior to drug addition or before ozone exposure. However, during the initial equilibrium phase, during resting periods and for rinsing of tissue samples, KH+G was used to incur physiological haemostasis. No glucose was therefore present in the organ baths when experiments with ozone were performed.

3.2.3. The basic methacholine concentration-response curve

Subsequent to the suspension (as described in § 3.2.2), washout and return to baseline tension of the isolated tracheal tissue sample in an organ chamber, a cumulative concentration-response curve was determined for the muscarinic agonist methacholine. This was done according to the method described by van Rossum (1963) and entailed using methacholine concentrations increasing in log increments from 1×10^{-9} M to 3×10^{-4} M in each organ bath. Each concentration of the agonist was added when the effect of the preceding addition had reached its maximum.

On a graph (X-axis: Logarithmic; Y-axis: Linear) the contractile responses to the above cumulative concentrations of methacholine were plotted as a percentage (%) of the maximal (100%) response to methacholine (E_{max}) to determine the cumulative concentration-response curve of methacholine. This basic cumulative concentration-response curve of methacholine is presented in Figure 5.1 and is generally the standard to which all *in vitro* results in this study are compared.

Concentration–response curves of methacholine in the presence of atropine (a competitive antagonist of methacholine on muscarinic acetylcholine receptors) were performed by firstly adding a single concentration atropine (1×10^{-7} M) to the organ bath wherein the trachea was suspended. After a 30 min equilibrium period, the concentration–response curve of methacholine in the presence of atropine was determined.

3.2.4. General experimental procedure

After isolating and suspending a tracheal strip (as described in § 3.2.2), two methacholine concentration-response curves (as described in § 3.2.3) were usually obtained — the second concentration-response curve was the curve used as reference.

After each concentration-response curve was determined, the isolated organ was rinsed and rested for 20 min before a stable baseline was re-established. At this point experimental treatment procedures as discussed in this chapter were performed. Once these experimental treatment procedures were concluded and contractile effect noted as a percentage of the maximal methacholine effect, the isolated tracheal sample was rinsed and rested for 20 min to allow a stable baseline to re-establish. Another, final methacholine concentration-response curve was then determined and compared to the original reference curve. Fluctuations from the reference curve may indicate increased or reduced responsiveness of isolated tracheal tissue as a result of the treatment employed.

3.2.5. The contractile effect of ozone on isolated tracheal tissue

To determine ozone's direct *in vitro* effect on the airway, the guinea pig trachea was isolated and suspended as described in § 3.2.2. A single tracheal sample was subjected to three successive concentrations of methacholine (1×10^{-4} M) at the start of the experiment. After each methacholine application the organ was rinsed and rested for 20 min before the baseline is re-established.

If the maximal effect of the last two methacholine concentrations differed less than 10%, the mean of these contractions (height of registration) was taken as the maximal (100%) effect. Ozone was next introduced to the tissue, either as a gas bubbled through the Krebs-Henseleit solution via a silicone tube, or as 1 ml of saturated water-ozone solution added directly to the organ bath. Ozone exposure was limited to 10 min which was the average time found to obtain the maximal response. The contractile effect of ozone-concentration was noted as a percentage of the maximal contractile effect to methacholine.

3.2.6. Concentration-response curve of ozone

As far it could be ascertained, no concentration-response curve for ozone has ever been determined before. *In this study a concentration-response curve of ozone was determined by employing two different methods:*

a) In the first method a single tracheal sample (suspended in a jacketed organ bath as described in § 3.2.2) was subjected to three successive concentrations of methacholine (1 x 10^{-4} M) at the start of the experiment. After each methacholine application the organ was rinsed and rested for 20 min. If the maximal effect of the last two methacholine concentrations differed with less than 10%, the mean of these contractions (height of registration) was taken as the maximal (100%) effect. After the third methacholine contraction, multiple doses of a concentrated water-ozone solution (in § 4.2 the concentration is calculated as 12.439 mg O_3/l) were added in increasing quantities to the single isolated, glucose-free tracheal preparation. Once the contractile effect of each ozone-concentration was noted as a percentage of the maximal effect, the tissue was rinsed with KH+G and allowed to equilibrate for 20 min prior to the addition of the next ozone concentration.

b) In the second method the concentration-response curve was obtained by employing a single ozone concentration per isolated trachea (suspended in a jacketed organ bath as described in § 3.2.2). The trachea was subjected to three successive concentrations of methacholine (1 x 10^{-4} M). After each methacholine application the organ was rinsed and rested. If the maximal effect of the 2^{nd} and 3^{rd} methacholine concentrations differed with less than 10%, the mean of these contractions (height of registration) was taken as the maximal (100%) effect. After the third methacholine contraction a single ozone concentration was added to the organ bath and the obtained contractile response to ozone was calculated as a percentage of the maximal methacholine response. Each tracheal preparation was treated only once with ozone whereafter it was discarded. The same procedure was repeated for different ozone concentrations, the response to each individual ozone concentration recorded, calculated in terms of the maximal response to ozone (the maximal

response to ozone was taken as 100% in this method) response, and applied to construct the concentration-response curve depicted in Figure 5.3.

3.2.7. The effect of ozone on tracheal responsiveness

When examining the effect of ozone on tracheal responsiveness, tracheal segments were isolated and suspended in a jacketed organ bath as described in § 3.2.2, washed with fresh KH+G solution after the reference methacholine control concentration-response curve was determined (as described in § 3.2.4), and rested for 20 min before the baseline is re-established. At this point ozone was introduced to the tissue either as a gas bubbled through the Krebs-Henseleit solution via a silicone tube, or as 1 ml of saturated water-ozone solution (§ 4.2 the concentration is calculated as 12.439 mg O_3/l) added directly to the organ bath. Ozone exposure was limited to 10 min which was the average time found to obtain the maximal response. After exposure to ozone, the isolated tracheal tissue was rinsed with fresh Krebs-Henseleit solution and rested for 20 min to allow a stable baseline to re-establish. The final methacholine concentration-response curve (as described in § 3.2.4) was then determined and compared to the original reference curve. Fluctuations from the reference curve may indicate increased or reduced responsiveness of isolated tracheal tissue as a result of exposure to ozone.

3.2.8. The effect of ozone on the responsiveness of the isolated trachea to isoproterenol

Isoproterenol, a β-sympathomimetic, was employed during *in vitro* studies to relax airway smooth muscle contraction. Freshly isolated tracheal preparations, suspended inside jacketed organ baths containing KH-G according the method described in § 3.2.2, was maximally contracted with a single addition of a high concentration of methacholine (1×10^{-4} M) and then relaxed by addition of a single concentration isoproterenol (1×10^{-4} M). The average time it took for the single isoproterenol concentration to induce the maximal observed relaxation was recorded,

whereafter the same experiment was repeated on a set of tracheal preparations which was first exposed to a maximal concentration of ozone (as per the method described in § 3.2.5). The average time needed to induce the maximal observed relaxation of the ozone-treated tracheal tissue with isoproterenol was compared to that measured in a control group not exposed to ozone.

3.2.9. The effect of indomethacin on ozone-induced tracheal contraction

Freshly isolated tracheal preparations, suspended inside a KH-G containing jacketed organ baths according to the method described in § 3.2.2, was maximally contracted with a single addition of a high concentration of methacholine (1×10^{-4} M). The isolated tracheal preparations were then thoroughly rinsed with KH+G and allowed to equilibrate for 20 min in a KH+G solution prior to the addition of a maximal ozone concentration ozone (as per the method described in § 3.2.5). One group of randomly allocated isolated tracheas was pre-treated with a single dose of 8 µg indomethacin immediately prior to ozone exposure, and the effect of ozone on this indomethacin-treated group was compared to a group isolated tracheas not treated with indomethacin.

3.2.10. The influence of ascorbic acid on the responsiveness of the isolated trachea to ozone

Freshly isolated tracheal preparations, suspended inside jacketed organ baths containing KH-G according to the method described in § 3.2.2, was maximally contracted with a single addition of a high concentration of methacholine (1×10^{-4} M).

The tracheal preparations were thereafter thoroughly rinsed with KH+G and allowed to equilibrate for 20 min in a KH+G solution prior to pre-treatment with ascorbic acid (30 mg as a single dose). After treatment with ascorbic acid (duration of exposure = 30 min) the tracheal preparations were thoroughly rinsed with KH+G and allowed to equilibrate for a further 20 min in a KH+G solution before the isolated

tracheal tissue was exposed to a maximal ozone concentration ozone (as per the method described in § 3.2.5). The effect of ozone on the control group — a set of suspended isolated tracheal strips not treated with ascorbic acid — was compared to the ascorbic acid treated group.

3.3. The in vitro respiratory effects of in vivo ozone exposure

To determine the *in vitro* respiratory effect of *in vivo* ozone exposure, experiments were conducted by applying a model where Duncan-Hartley guinea pigs (450-550 g) were exposed to relatively high concentrations of ozone for short periods of time (10 min).

3.3.1. Equipment, chemicals and animals

3.3.1.1. Laboratory Animals

Duncan-Hartley guinea pigs (450-550 g) of both sexes obtained from the laboratory animal centre at the North-West University, was used. Animals had free access to water and food, and a week before commencing the experiment, the guinea pigs were acclimatised to the experimental area. Prior to the start of the actual experimental procedure, test subjects were introduced to the exposure chamber and allowed a period of 20 min to adapt to their surroundings. To avoid stress, excessive human interference was minimised.

3.3.1.2. Instruments and equipment

The following instruments and equipment were used: A small operating table and dissection equipment, a gas chamber and an Plexiglas ozone exposure chamber (Department of Technical Services, North-West University), a water heating unit and pump fitted with a thermostat, 6 jacketed organ baths, a Statham UL-2 Force Displacement Transducer attached to a Metrohm Labograph (Model E-478) recorder, a Sterizone P-HP 250 (PCT/ZA00/00031) ozone generator (see Figure 3.1) , a rotameter and a Unico 2800 VIS/UV Spectrophotometer.

3.3.1.3. Chemicals

The following drugs and chemicals were used: Sodium chloride, potassium chloride, calcium chloride 2-hydrate, sodium hydrogen carbonate, glucose and potassium dihydrogen phosphate, all of analytical grade, and obtained from Holpro Analytics (Johannesburg, RSA). Methacholine, isoproterenol and atropine were obtained from Sigma Aldrich (South Africa), while carbomer gas, carbon dioxide and UHP oxygen were obtained from Afrox South Africa.

3.3.1.4. Krebs-Henseleit Solution

The modified Krebs-Henseleit solution was made to the specifications of Patil and Jacobowitz (1968) composing of: 119 mM NaCl, 4.7 mM KCl, 1.9 mM $CaCl_2$, 0.54 mM $MgCl_2$, 24.0 mM $NaHCO_3$, 1.0 mM NaH_2PO_4 and 11.1 mM glucose. Each ingredient was added after the previous salt was dissolved, while KCl and $MgCl_2$ were added last to prevent the formation of precipitate (Van Rossum, 1963). The solution was then heated to 37 ºC and kept at this temperature throughout the experiment, whilst the pH of the solution was constantly measured and kept stable at 7.4.

3.3.2. Experimental setup

Thirty Duncan-Hartley guinea pigs (450-550 g) of both sexes were randomly allocated into three groups (*n* = 10 each). Prior to the start of the actual experimental procedure, test subjects were introduced to the exposure chamber and allowed a period of 20 min to adapt to their surroundings. During this period normal airflow was circulated throughout the chamber.

After this period of introduction, test subjects were exposed to (a) ozone, (b) pure air containing 0 µg O_3/ml, or (c) ultra-high purity oxygen for 10 min. The concentration of ozone produced was measured and constantly monitored spectrophotometrically using a Unico 2800 VIS/UV spectrophotometer to ensure that

the level of ozone introduced to the exposure chamber remained constant at all times. Following the 10-minute exposure to ozone, animals were euthanized by carbon dioxide asphyxiation, and the trachea was rapidly removed and manually freed form connective and other tissues. Subsequently the tracheal tissue sample was isolated and suspended in an organ chamber as described in § 3.2.2.

3.3.3. The basic methacholine concentration-response curve

To determine the effect of *in vivo* ozone exposure on maximal attainable *in vitro* methacholine-induced isolated tracheal smooth muscle contraction, guinea pigs were exposed to relatively high concentrations of ozone (19.5 ± 0.5 µg/ml) for short periods of time (10 min). Animals were subsequently euthanized by carbon dioxide asphyxiation and tracheal tissue were isolated and suspended in a jacketed organ bath as described in § 3.2.2.

A cumulative concentration-response curve was thereafter determined for the muscarinic agonist methacholine. This was done according to the method described by van Rossum (1963) and entailed using methacholine concentrations increasing in log increments from 1×10^{-9} M to 3×10^{-4} M in each organ bath. Each concentration of the agonist was added when the effect of the preceding addition had reached its maximum. On a graph (X-axis: Logarithmic; Y-axis: Linear Y-axis) the contractile responses to the above cumulative concentrations of methacholine are plotted as a percentage (%) of the maximal (100%) response to methacholine (E_{max}) to determine the cumulative concentration-response curve of methacholine. This basic cumulative concentration-response curve of methacholine is presented in Figure 5.1 and is generally the standard to which all *in vitro* results in this study are compared.

3.3.4. Experiments where calcium has been excluded

Roux *et al*. (1996) postulated that *in vitro* exposure to ozone increases responses to agonists that, as part of their mechanism of action, release intracellular calcium ions. As a result, a series of experiments were conducted to establish whether calcium omission will have any significant impact on the *in vitro* determined concentration-response curves of methacholine after *in vivo* ozone exposure. Experiments were performed using the same method as described § 3.3.3, but with the omission of calcium from the Krebs-Henseleit solution.

3.4. The lung perfusion model

The primary objective of this study was to establish the effect of ozone inhalation on an isolated perfused rat lung model. To realize this objective it was necessary to set up an isolated perfused lung model that would permit ozone inhalation and the measurement of dynamic mechanical lung function, and use the above mentioned model to investigate the effect of ozone-induced lung injury on, and more specifically, lung compliance (CL).

3.4.1. Equipment, chemicals and animals

3.4.1.1. Animals

Male Wistar rats obtained from the University of Cape Town (Animal Unit), South Africa, were used. Prior to experiments the animals were kept on a 12-hour light/12 hour darkness cycle in the well ventilated laboratory animal room of the Department of Pharmacology at the University of the Western Cape. The animals had free access to water and food until surgery.

Experiments were performed in accordance with the protocol approved by the Animal Ethics Subcommittee of the University of the Western Cape Senate Research Committee.

3.4.1.2. Chemicals

The following drugs and chemicals were used: Sodium chloride, potassium chloride, calcium chloride 2-hydrate, sodium hydrogen carbonate, glucose and potassium dihydrogen phosphate, all of analytical grade, were obtained from Holpro Analytics (Johannesburg, RSA).

Bovine Serum Albumin (Fraction V) was obtained from Sigma Aldrich (RSA), while Heparin Sodium injections (5000 units/ml) were obtained from Fresenius Kabi (Cape Town, RSA).

3.4.1.3. Instruments and Equipment.

The following instruments and equipment were used: A small operating table, tracheal canula, pulmonary artery canula and left atrium canula were obtained from Medical Bioscience (Kent, UK). A roller pump (model MS-4 REGLO/8-100), PLUGSYSTM modular central electronic system (type 603), ventilation control module (VCM, type 681), timer counter module (TCM, type 868), external input module (EIM, type 673), analogue digital converter (ADC, type 663), Validyne pressure transducer (model DP45-24), differential pressure transducer (model SP 2040 D), HSE DC bridge amplifier, glass artificial thorax chamber (with Perspex lid), perfusate reservoir, pneumotachometer, Pulmodyn™ software and analogue-digital PC-card, Intel Pentium 4 PC, ventilation pressure limiter and a pressure equilibrium vessel were all obtained from Hugo Sachs Elektronik (HSE, Germany).

Other equipment included a digital pH meter (model PHM82; Radiometer, Denmark), chart recorder (model R-03; Rikadenki Kogyo, Japan), vacuum pressure gauge (model 7769; Control Instrument Products (RSA), universal pressure transducer (P 23DB; Gould Statham, USA), carrier demodulator (model CD12; Validyne Engineering, USA), ozone generator (PHP250; Sterizone, RSA) and water pump (model 02PH623) obtained from Heto (Denmark).

3.4.2. Experimental setup

Experiments in this study were based on the isolated perfused lung (IPL) model of Uhlig *et al.* (1997). A schematic diagram of the experimental model is shown in Figure 3.2.

A water-jacketed artificial glass thorax chamber (m) supplied by Hugo Sachs Elektronik was used for the perfusion and ventilation of the isolated rat lungs. The thorax chamber was sealed with a Plexiglas lid (e), which had a central opening

(organ holder) on one side, while the pneumotachometer (b) was connected to the other side. The lung (g) was attached to the organ holder via the tracheal canula (h). In addition the Plexiglas lid also had openings for perfusate inflow (pulmonary arterial canula, k), and perfusate outflow (perfusate venous canula, j). These canulas were connected to glass tubes in the lid.

The lung chamber had additional connections for a negative pressure venture nozzle (f) and pressure transducer (d) needed for the generation of negative pressure and the measurement of trans-pulmonary pressure (chamber pressure), respectively. Warm water (37ºC) was circulated through the lung chamber via tubing from a water pump (i) fitted with a thermostat. Before each experiment a small amount of water (m) was added at the bottom of the lung chamber to humidify the air and a custom made plastic stopper was used to block the drain hole.

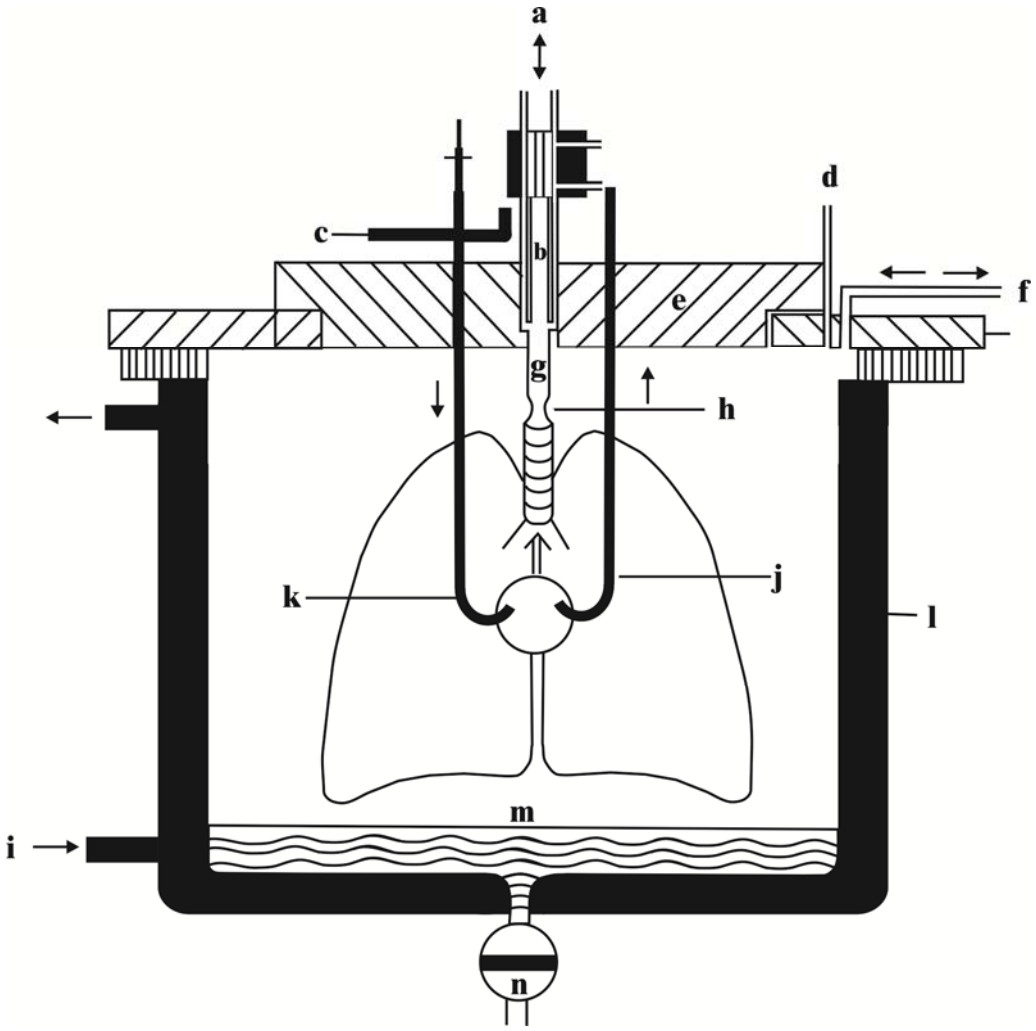

Figure 3.2. Diagram of the artificial thorax chamber. (a) Airflow; (b) pneumotachometer;(c) arterial inflow; (d) to pressure transducer; (e) Plexiglas lid; (f) to venture nozzle; (g) organ holder; (h) tracheal canula; (i) thermostated water inlet; (j) pulmonary venous canula; (k) pulmonary arterial canula; (l) water-jacketed artificial thorax chamber; (m) water; (n) drain. (Uhlig, et al., 1997)

3.4.3. Ventilation

Lungs were ventilated with either positive or negative pressure. To achieve ventilation a rotary vane compressor pump (VCM) was used. The VCM was connected to the trachea via a tube. A valve was inserted between the lung and the VCM.

For positive pressure ventilation, air was pumped directly from the VCM into the lung. Switching of the valve allowed the change of airflow direction through the Venturi gauge, thereby transforming positive pump pressure and creating a negative (with respect to ambient atmosphere) chamber pressure (negative pressure ventilation). The venture gauge was mounted within the chamber wall.

Depending on the experimental protocol, end-inspiratory and deep breath pressures were preset via the VCM. The latter was also set to achieve a breathing frequency of 80 breaths/min and the duration of inspiration equal to 50% of the breathing cycle. Deep breaths were initiated automatically every 30 seconds using the VCM.

A pneumotachometer was used to measure the airflow passing through the lung. The device was connected to the trachea through the central opening in the Perspex lid. The signal from the pneumotachometer was passed though a differential pressure transducer, the amplifier and the external input module (EIM) of the PLUGSYS™ electronic unit to the computer. The respiratory volume was derived from the airflow signal by using the PULMODYN™ computer program.

To measure the trans-pulmonary pressure the two arms of the differential pressure transducer was connected to the inside and outside of the chamber an the signal obtained amplified, and passed on to the computer. The negative pressure gauge was connected to the chamber and continuously displayed the chamber pressure. Atmospheric air was used (via VCM) to ventilate the lung.

3.4.4. Perfusion

A peristaltic pump were used to perfuse the lung and recirculate the perfusion medium at a constant flow rate of 25 ml/min. This perfusion medium provides the lung with the required nutrients necessary to survive and function within the chamber.

Before the start of each experiment the perfusion medium was heated for 15 min at 37 ºC in a water jacketed reservoir. A roller pump was used to pump perfusate from the reservoir through a short perfusion line to and through the lung vasculature. The perfusate entered the lung via the pulmonary artery and left it via the left ventricle back to the reservoir. A bubble trap was placed before the pulmonary arterial canula prior to the point where the perfusion line entered the inside of the thorax chamber. A thin plastic tube was connected to the bubble trap, and extended to a three-way stopcock through which a bolus administration or infusion of a drug could be made. The thin plastic tube was also connected to a pressure transducer for the measurement of pulmonary artery pressure.

After the perfusate exited the chamber it passed through the pressure equilibrium chamber, which, when a negative pressure ventilation protocol was used, was connected to the ventilation chamber. By doing this, venous pressure changes followed the pleural pressure changes during negative pressure ventilation; a situation, which has been suggested to be the most physiological way to perfusate isolated lungs and help minimize oedema formation.

Because negative pressure and positive pressure has the same effect on interstitial pressure relative to pleural pressure, the pressure difference between the interstitial and intra-vascular pressures (trans-mural pressure) was lower if the intravascular pressure was connected to the chamber (pleural) and not atmospheric pressure. As indicated above, the pulmonary artery pressure (perfusion pressure) was also continuously monitored with a pressure transducer connected via a thin plastic tube to the pulmonary arterial canula.

3.4.4.1. Perfusion medium

For each experiment the perfusion medium used consisted of 400 ml of Krebs-Henseleit buffer containing 2% bovine serum albumin (BSA), d-glucose 5.6 mM, Calcium chloride 2.54 mM, magnesium sulphate 1.2 mM, sodium chloride 118 mM, potassium chloride 4.7 mM, potassium dihydrogen phosphate 1.2 mM and sodium bicarbonate 25 mM.

After addition of all the components the medium was filtered before use. The medium was also heated to 37 ºC in a water-jacketed glass reservoir before use. Throughout each experiment the perfusion medium was, at regular intervals, gassed with carbon dioxide to keep the pH between 7.3 and 7.4.

3.4.5. Surgical removal of the lung

Specific pathogen free male Wistar rats were used to obtain the lungs required. Each rat was aneasthetised with sodium pentabarbitone (100 mg/kg i.p) and assessed for the optimum depth of anaesthesia by testing for the pain reflex (e.g. by briefly clamping the animals front paw with forceps), before the surgical procedure was commenced.

The anaesthetized animal was placed on its back on the operating table and its spread paws tied with custom made elastic bands to the side of the table. Using scissors, the skin was incised ventrally in the median line from the upper abdomen to the neck. The trachea was exposed using blunt dissection and a thread drawn through underneath the trachea and loosely looped around it. The trachea was then cut about 2/3 though its width with small string scissors, the tracheal canula inserted and carefully tied in place.

The canula was connected to the organ holder situated in the bottom side of the lid of the chamber, and thereby, the pressure limiter which had been mounted to its position in the lid beforehand. Immediately thereafter the ventilation pump (VCM)

was switched on and the lung ventilated at positive pressure of about 10 cm H_20 and 80 breaths/min with no periodic deep inhalation.

Next, the two femoral arteries were cut to exsanguinate the animal. The abdominal wall was incised and the incision extended laterally on both sides to the lower extremities. The diaphragm was carefully resected from the thorax wall, the thorax then opened by median sternotomy and the thorax halves held open by using two thorax-hooks.

Heparin sodium (2000 IU/kg) was injected into the right ventricle and the thymus removed with scissors. The heart was lifted, a curved forceps inserted between the left heart auricle and the left ventricle (behind the aorta and the pulmonary trunk), a thread of cotton drawn through behind the two large vessels and a loose ligature placed around it. Using a small scissors the right ventricle was then incised 5 mm before the start of the pulmonary trunk in the region of the outflow path.

The arterial canula, pre-filled with perfusate, was now inserted through the ventricular incision into the pulmonary trunk and tied with the pre-looped thread. The thread was positioned in such a way so that it simultaneously tied off the aorta, thus preventing any uncontrolled outflow of the perfusate.

After using a forceps to raise the heart's apex, another loose ligature was placed around the middle part of the heart and a longitudinal incision made in the left ventricle, close to the heart's apex and parallel to the inter-ventricular septum. The pulmonary venous canula was then inserted the ventricular incision into the left atrium. Care was take to apply only limited force to pass the canula through the mitral valve without damaging the extremely thin pulmonary vein. The canula was secured with the pre-looped thread, but not too tightly. Set at an initial flow water of 5 ml/min, the roller pump was then switched on and the perfusion of the lung started.

Next, the inferior vena cava, thoracic aorta, the two vagal trunks, oesophagus and the upper part of the trachea were carefully removed. The lower part of the trachea and the lung was dissected free by cutting step-by-step in the cranio-caudal direction.

Finally, the isolated lung tied to the lid via the tracheal and the two blood vessel canulas was lifted free of the animal carcass, placed in the artificial thorax chamber and secured with the lid in position.

After the isolated lung was placed in the chamber, the positive pressure ventilation was stopped and negative pressure ventilation commenced by simply switching the changeover stopcock. The chamber pressure fluctuated between an end-inspiratory pressure of -10 to -16 cmH_20 and an end-expiratory pressure of -2 cmH_20. Now the perfusate flow rate was slowly increased to 25 ml/min by means of adjusting the pump. The pressure limiter was removed from the central opening of the chamber and replaced with the pneumotachometer to measure the airflow rate.

Finally, the Timer Counter Module (TCM) of the PLUGSYS™ was adjusted to produce a hyperinflation (-16 cmH_20) every 30 seconds.

Under these conditions a tidal volume of 1.5 ± 1.0 ml and a lung compliance of 0.1-0.2 ml/cmH_20 was attained. The lungs were then allowed to equilibrate under these conditions.

After equilibrium the lungs included in the study had (i) a homogenous white appearance with no signs of homeostasis, oedema of atelectasis, (ii) pulmonary artery and ventilation pressures in the normal range, (iii) lung compliance between 0.1 to 0.2 ml/cmH_20 and (iv) tidal volume of 1.5 ± 1.0 ml. Based on these criteria 5 to 10% of all lung preparations were deemed not suitable and discarded.

3.4.6. Ozone inhalation

Ozone was prepared by feeding ultra high purity (UHP) 99.995% oxygen (Afrox, RSA) into a Sterizone ozone generator and introduced to the model via the airflow inlet (*a* in Figure 3.2). Inhalation was limited to five seconds of exposure and this resulted in the isolated lung inhaling high concentrations of ozone (19.5 µg/ml) for a short period of time.

3.5. The morphological and histological effect of ozone on the respiratory system

Guinea pigs were exposed to high levels of ozone, whereafter samples of the trachea and lungs were collected and microscopically analyzed for histological and morphological changes as a result of ozone's effect on respiratory tissue.

3.5.1 Experimental procedure

Thirty Duncan-Hartley guinea pigs (450-550 g) of both sexes were randomly allocated into three groups (*n* = 10 each). Prior to the start of the actual experimental procedure, test subjects were introduced to the Plexiglas exposure chamber and allowed a period of 20 min to adapt to their surroundings. During this period normal airflow was circulated throughout the chamber. After the appropriate equilibration period, test subjects were exposed to (a) ozone, (b) pure air containing 0 µg O_3/ml, or (c) ultra-high purity oxygen for 10 min. The concentration of ozone produced was measured and constantly monitored spectrophotometrically using a Unico 2800 VIS/UV spectrophotometer to ensure that the level of ozone introduced to the exposure chamber remained constant at all times.

Following the 10-minute exposure to ozone, animals were euthanized by carbon dioxide asphyxiation and the trachea and lungs were rapidly removed and manually freed form connective and other tissues, whereafter samples were fixated in 10% formalin for morphological and histological examination.

3.6. Statistical analysis

All statistical analyses and curve fitting were done with Graphpad Prism (Version 5.01). Data were obtained as 6-fold observations from at least 3 separate and independent experiments, and expressed as percentage of control, unless otherwise stated, in which case data are presented as the mean ± SEM. Results were statistically analysed using Student's t test for unpaired samples. Differences in mean values were considered significant at $p<0.05$.

For the purpose of this thesis, selective statistical significance with regards to concentration-response curves is presented in terms of (a) maximal observed response, and (b) half maximal effective concentration (EC_{50}). Non-linear curve-fitting was done with the Hill slope set at 1.

A Dunnett's Multiple Comparison Test was furthermore performed on the data presented in Table 4.2 to indicate statistical significance ($p<0.05$) between ozone and each individual salt in a Krebs-Henseleit physiological solution.

Chapter 4

Results and discussion: Measuring ozone concentration, solubility and decay

In order to determine the pharmacological effect of ozone, an accurate method to measure ozone concentration under controlled conditions in both air and aqueous media had to be developed.

Studies in which isolated organs were exposed to ozone via bubbling of the gas directly (through the physiological solution in which the tissue was suspended), reiterated the importance of employing a method which will accurately determine the ozone concentration applied.

The experiments discussed in this chapter were performed to determine the ozone concentration, solubility and decay of ozone. Experiments were performed to determine:

(a) The ozone concentration produced by the specific ozone generator used throughout this study;

(b) The solubility of generated ozone in aqueous media (water and Krebs-Henseleit physiological solution) at various known and controlled temperatures;

(c) The effect of ozone on the pH of an aqueous medium (certain methods employed to measure ozone concentration in aqueous media are pH dependant – See § 3.1.3), and

(d) The half-life ($t_{½}$) of ozone in an aqueous medium at various known and controlled temperatures.

4.1. Measuring generator yield

In this experiment the amount of ozone produced by the ozone generator used in this study was measured to accurately calculate the ozone concentration used in each experiment.

Throughout this study ozone was prepared by passing UHP[1] oxygen (99.995%) through a Sterizone P-HP 250 ozone generator at a constantly controlled flow rate. A spectrophotometer (Unico 2800 VIS/UV spectrophotometer) modified for the specific purpose of measuring ozone concentrations in air, was used to calculate the ozone concentration manufactured by the ozone generator used.

Under controlled conditions a constant absorbance of 0.2019 was achieved at 254 nm. The concentration ozone produced was calculated by applying the equation (Labuschagne, 2007):

$$[O_3]^* = \frac{(Absorbance \times 4.625)}{4.67 \times 100} \qquad (4.1)$$

$[O_3]$ = µg/ml O_3

By employing this equation, the generator output was calculated to be 19.5 ± 0.5 µg/ml O_3 (n=10).

[1] Utra-High Purity

4.2. The aqueous solubility of ozone

Since experiments on isolated tissue samples were performed in aqueous media, a model for exposing tissue to ozone, and a method for measuring ozone concentration in aqueous media had to be developed.

When ozone (19.5 ± 0.5 µg/ml O_3) is constantly bubbled through an aqueous medium, a percentage of the gas is expected to dissolve in the medium to ultimately reach a point of saturation.

The experiment discussed in this section was performed to confirm the solubility of ozone in an aqueous medium, and to determine the concentration of ozone dissolved in both a saturated water-ozone and a Krebs-Henseleit-ozone physiological solution. Experiments were performed at 1, 20 and 37 °C to determine the effect of temperature on the aqueous solubility of ozone, whilst the duration of exposure of the aqueous medium to ozone and the time between discontinuation of ozone exposure and spectrophotometric measurement of ozone concentration within the relevant media, was constant at 10 min and 15 seconds respectively. The results from this experiment are presented in Table 4.1.

Table 4.1. The solubility of ozone in water compared to a Krebs-Henseleit solution ($p<0.05$; $n=6$).

Temperature (°C)	**Solubility of ozone (µg/ml)**	
	Water	***Krebs-Henseleit***
1 °	17.72 ± 0.16	2.39 ± 0.17
20 °	12.43 ± 0.2	0.78 ± 0.12
37 °	2.91 ± 0.11	0.08 ± 0.02

From the data in Table 4.1 it can be confirmed that

(a) Ozone was better soluble at lower temperatures, and

(b) Ozone is less soluble in Krebs-Henseleit than in water.

Findings (a) is in accordance with that of Gottschalk *et al.* (2010) who states *that the solubility and stability of ozone decreases rapidly with temperature, causing a fast decay of dissolved ozone concentrations at a higher temperature.* It also concurs with Carrel Morris' review of the aqueous solubility of ozone (1988).

The results shown in Table 4.1 furthermore suggest that ozone seems to be significantly (up to 97.35%) less soluble in Krebs-Henseleit than in water. As a result an experiment was performed to determine whether certain chemical ingredients of the Krebs-Henseleit solution are responsible for this apparent reduced solubility and why solubility in these two mediums differ. In this experiment the solubility of ozone in a solution of each individual component of Krebs-Henseleit was examined. The results are presented in Table 4.2.

The results in Table 4.2 indicates a marked contrast between the solubility of ozone in a glucose solution (2.88 ± 014 µg O_3/ml) compared to its solubility in individual solutions of the other chemical components within a Krebs-Henseleit solution. These solutions displayed an average ozone concentration of 16.53 µg O_3/ml (with the exception of $CaCl_2$ in which a higher ozone concentration was observed). From this it can be assumed ozone reacts with glucose in the perfusion medium, resulting in a lower concentration of ozone in this solution.

In addition, the results in Table 4.2 also revealed that ozone is significantly (22.3%) more soluble in a $CaCl_2$ solution (21.08 µg O_3/l) compared to the mean of the other solutions in Table 4.2 (16.53 µg O_3/l). Although further investigation into this occurrence is needed, the apparent favourable conditions for ozone solubility in a calcium solution may be partly explained by the fact that $CaCl_2$ does not contain any ozone-depleting substances (ODS), and this compound is interestingly enough also promoted by the US Environmental Protection Agency's (EPA) clean air act as a

preferred chlorofluorocarbon (CFC) replacement in the preservation of atmospheric ozone.

Table 4.2. Ozone concentration in solutions of each individual component of Krebs-Henseleit at 1 °C after 10 min of ozone bubbling (*n=6; p<0.05).*

Component	***Concentration of chemical component in both Krebs-Henseleit and individual solution (g/l)***	***Ozone concentration at 1 ℃ (µg/ml)***
H_2O	-	17.72 ± 0.16
NaCl	6.95	16.77 ± 0.16
KCl	0.34	16.96 ± 0.14
$CaCl_2.2H_20$	0.25	21.08 ± 0.26
$MgCl_2.6H_20$	0.10	15.81 ± 0.13
$NaHCO_3$	2.02	16.05 ± 0.17
NaH_2PO_4	0.12	16.29 ± 0.19
Glucose	**1.87**	**2.88 ± 0.14**

A Dunnett's Multiple Comparison test (See Addendum C) indicate that there is a significant statistical difference difference (p<0.05) between ozone and each individual salt in a Krebs-Henseleit solution.

Since calcium impact nearly every aspect of cellular life and therefore plays a pivotal role in the physiology and biochemistry of organisms and cells (Clapham, 2007), the apparent link between Ca^{2+} and ozone concentration as observed in Table 4.2 may possibly be influential in some of the *in vivo* and *in vitro* effects observed with ozone as discussed in some of the later chapters of this study.

It is expected that, if the experiments performed to obtain the results displayed in Table 4.1 is repeated in the absence of glucose (KH-G[2]), the concentration of ozone in the physiological solution (KH-G) will be similar to that found in distilled water. The results of this experiment are presented in Table 4.3.

Table 4.3. The solubility of ozone in distilled water compared to KH-G ($p<0.05$; $n=6$).

Temperature (°C)	**Solubility (µg/ml)**	
	Water	***Krebs-Henseleit***
1°	17.72 ± 0.16	17.29 ± 0.18
20°	12.43 ± 0.2	11.98 ± 0.14
37°	2.91 ± 0.11	2.78 ± 0.07

The results displayed in Table 4.3 confirm earlier assumptions that ozone reacts with glucose in the perfusion medium, resulting in a lower concentration of ozone in this solution. The omission of glucose from the physiological solution resulted in differences of ozone solubility in water compared to its solubility in KH-G of 5.6%, 3.76% and 4.68% respectively. When compared to the difference in solubility observed in Table 4.1 (up to 97.35%), the difference is significant.

[2] Krebs-Henseleit devoid of glucose

4.3. The viability of glucose omission from the physiological solution

In view of the fact that multiple experiments in this study will be performed using an isolated, perfused trachea model, experiments were done to investigate the possibility of performing these perfusion model experiments in the absence of glucose. Because glucose acts as an energy source for cell maintenance (Krebs & Henseleit, 1932), the question needed to be asked whether glucose omission from the perfusion medium would have an effect on experimental results.

To determine the viability of glucose omission, cumulative concentration-response curves were determined, comparing the effect of methacholine on the isolated guinea pig trachea in the absence of glucose (KH-G). The result was compared with known results obtained from experiments performed in the presence of glucose (KH+G[3]) (Figure 4.1). This basic cumulative concentration-response curve is the standard to which all *in vitro* results throughout this study are compared, and fluctuations from this curve may indicate increased or reduced responsiveness of isolated tracheal tissue.

KH-G was added to each organ bath prior to methacholine and ozone exposure. However, during the resting periods and for rinsing of tissue samples, KH+G was used to incur physiological haemostasis.

[3] Krebs-Henseleit containing glucose

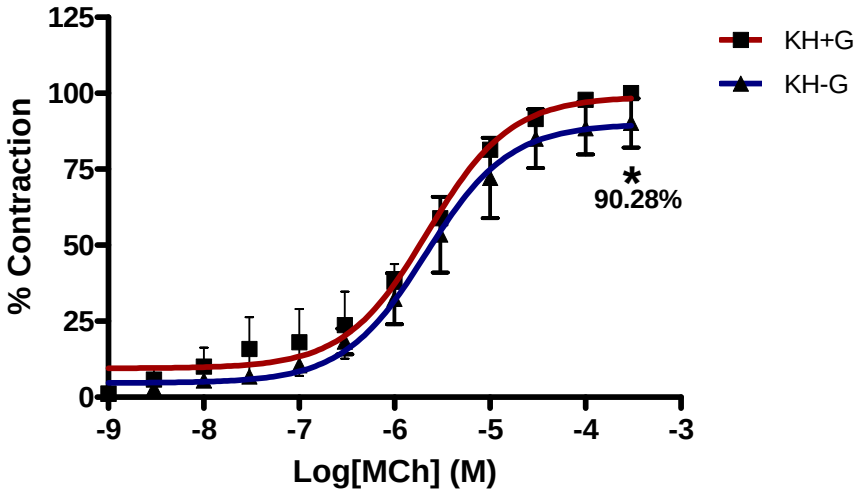

Figure 4.1. The effect of glucose omission from the Krebs-Henseleit (KH-G) solution on methacholine-induced contraction of isolated tracheal smooth muscle. These concentration-response curves depicts the response of isolated tracheal tissue to cumulative methacholine doses when suspended in a Krebs-Henseleit solution that (a) contains, or (b) is devoid of glucose. (*p>0.05, n=6, Student's t test). No statistical difference (p>0.05) exists between the EC_{50} values obtained from these curves (2.214 ± 0.098) x 10^{-6} M (KH+G) vs. (2.139 ± 0.116) x 10^{-6} M (KH-G).

The result seen in Figure 4.1 suggests that glucose may indeed be omitted from the physiological solution as there are no significant difference (p>0.05, n=6) between results obtained in the absence and presence of glucose.

This critical result, established during the preliminary phase of this study, was later, based on this finding, successfully supported and utilized by other studies performed within this department (Brink *et al.*, 2008; Van Niekerk, 2008; Pretorius, 2005).

One such study, a series of experiments performed in our laboratory on HeLA cells (Henrietta Lacks cervical carcinoma epithelial cells), have confirmed that the absence of glucose from the Krebs-Henseleit solution had no significant detrimental

effect on either mitochondrial activity or membrane integrity after the cells were exposed to KH-G for at least 55 min (Brink *et al.*, 2008).

4.4. The effect of ozone on pH of the physiological solution

The experiment discussed in this section was performed to establish whether bubbling of ozone (19.5 µg/ml) through KH-G at 37 °C for 10 min (the time determined to be necessary for the physiological solution to reach saturation with ozone under experimental conditions) will affect the pH of the solution.

After ozone exposure, a significant increase in pH from 7.36 ± 0.003 to 8.19 ± 0.008 (p<0.0001, n=3) was noted. This observation can be explained by the displacement of CO_2 (which is necessary for buffer action) from the physiological solution as a result of bubbling with ozone, thereby increasing pH. Bubbling of pure oxygen as a control study gave rise to a similar, indistinguishable, increase of pH from 7.36 ± 0.009 to 8.22 ± 0.029 (p<0.0001, n=3) confirming the hypothesis of CO_2 displacement.

These results concur with those of Brink *et al.* (2008) in which it was found that the changes in pH of ozone-treated KH-G (30 min continuous ozone bubbling at 37 °C) were indistinguishable from the pH changes of oxygen-treated KH-G.

To verify that any changes observed in the measured parameters of perfusion model experiments after ozone exposure are indeed a result of ozone-exposure and not due to an exposure of tissue to increased pH or remnant oxygen that were not converted to ozone by the generator, an oxygen control was introduced in subsequent experiments where appropriate. In preliminary *in vivo* and isolated lung perfusion studies (of which the results are not presented in this study), it was found that oxygen-control studies did not differ significantly from the standard controls employed throughout this study with respect to tracheal contractility and hyperreactivity to muscarinic agonists. During *in vivo* studies oxygen-control studies were however performed.

4.5. The half-life of ozone in glucose-free Krebs-Henseleit

Ozone is unstable in water and decays rapidly (Gottschalk *et al.*, 2010). In this experiment the decay of ozone in KH-G at 1, 20 and 37 °C was spectrophotometrically studied. The results from this study are presented in Figures 4.2, 4.3 and 4.4.

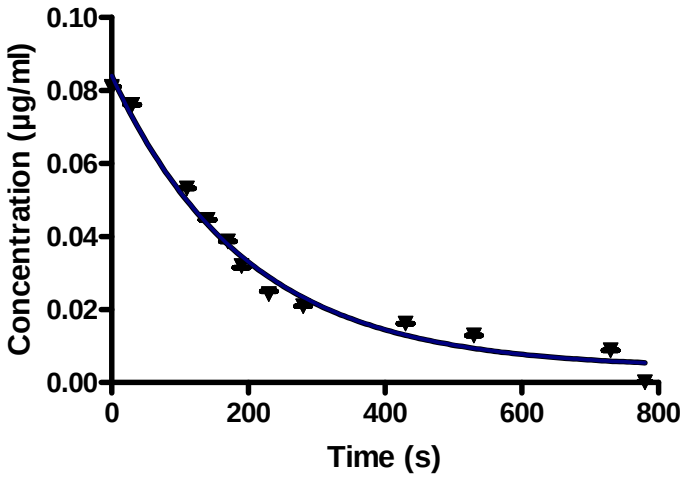

Figure 4.2. The decay of ozone at 37 °C in KH-G. Each sample was taken from a freshly prepared saturated ozone solution (initial ozone concentration=2.78 µg/ml) and sealed in a quartz cuvette before absorbance was spectrophotometrically measured at 254 nm at intervals of 1 second ($p<0.05$, $n=6$).

On average, $t_{½}$ of ozone in KH-G is suggested to be 137 seconds (Figure 4.32). When the same experiment was performed at 20 °C and 1 °C, $t_{½}$ was found to be 536 seconds (Figure 4.3) and 1.056×10^4 seconds (Fig. 4.4) respectively.

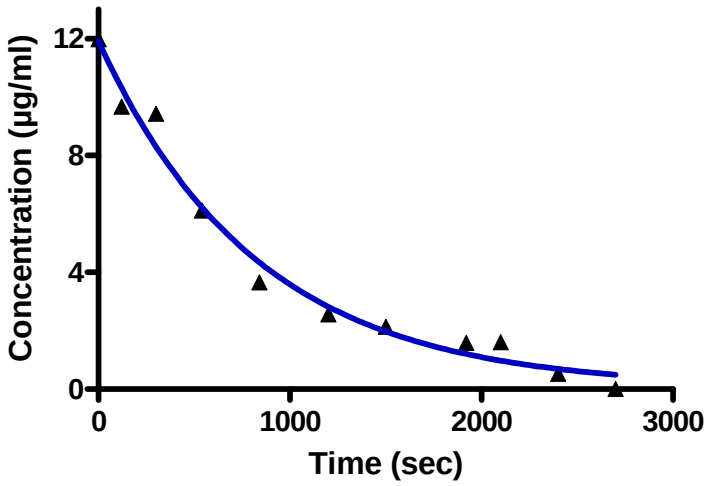

Figure 4.3. The decay of ozone at 20 °C in KH-G. Each sample was taken from a freshly prepared saturated ozone solution (initial ozone concentratio*n*=11.98 µg/ml) and sealed in a quartz cuvette before absorbance was spectrophotometrically measured at 254 nm at intervals of 1 second (p<0.05, n=6).

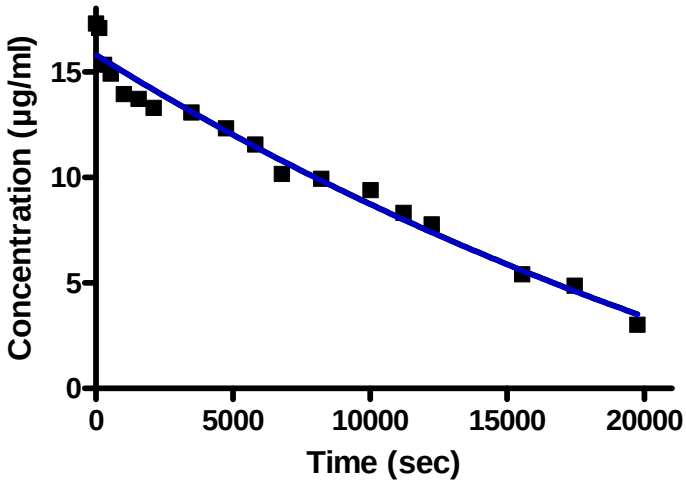

Figure 4.4. The decay of ozone at 1 °C in KH-G. Each sample was taken from a freshly prepared saturated ozone solution (initial ozone concentratio*n*=17.29 µg/ml) and sealed in a quartz cuvette before absorbance was spectrophotometrically measured at 254 nm at intervals of 1 second (p<0.05, n=6).

From the results seen in Figures 4.2, 4.3 and 4.4 it can be confirmed that

(a) the decay-rate of ozone is temperature-dependent, and

(b) that a higher temperature of the physiological solution will lead to a faster decay of ozone which, in turn, will result in a smaller $t_{½}$ (Gottschalk *et al.*, 2010).

4.6. Summary

In summary, from the experiments discussed in this chapter, the following were found:

a) The yield of the ozone generator used throughout this study was calculated to be 19.5 ± 0.5 µg O_3/ml;
b) the solubility of ozone is temperature dependent and was found to be 17.72 ± 0.16, 12.43 ± 0.2 and 2.92 ± 0.11 µg/ml at 1, 20 and 37 °C respectively in distilled water;
c) ozone reacts with glucose in the Krebs-Henseleit perfusion medium, resulting in a lower solubility of ozone in the perfusion medium;
d) glucose may be omitted from the perfusion medium without having any noticeable effect on isolated tracheal tissue function under experimental conditions;
e) the solubility of ozone was found to be 17.29 ± 0.18, 11.98 ± 0.14 and 2.78 ± 0.07 µg/ml at 1, 20 and 37 °C respectively in KH-G;
f) ozone appeared to be on average 22.3% more soluble in a calcium solution;
g) the changes in pH of ozone-treated KH-G were indistinguishable from the pH changes of oxygen-treated KH-G, and
h) the half-life of ozone was found to be 137 s, 537 s and 1.056 x 10^4 s in KH-G at 37, 20 and 1 °C respectively.

These experimental results, even though the majority of it has previously not been published, was the precursor of, and laid the foundation for all ozone-related analytical and pharmacological studies performed at this department.

Chapter 5
Results and discussion: Respiratory effects of ozone *in vitro*

The detrimental effect of ozone on the respiratory tract is often emphasized, and much research on the topic has been done before. Very few studies have however been performed *in vitro* to determine what effect above average ozone concentrations have on isolated respiratory tissue, or what effect these higher ozone concentrations will have if applied in an isolated lung perfusion model (Chapter 6).

The objective of this chapter was to determine the pharmacological effect of acute ozone exposure on the isolated tracheal tissue by employing *in vitro* techniques, to identify potential risks associated with acute exposure to relatively high concentrations of ozone, and to elaborate on the clinical importance of ozone application within safe concentration and dosage ranges.

Freshly isolated tracheal tissue was suspended inside jacketed organ baths containing KH+G[1]. The organ baths were continuously aerated with 95% oxygen and 5% CO_2 and maintained at 37 °C according to the specifications of Collier (1970) as described in the method chapter (see § 3.2.2). In Chapter 4 it was reported that the presence of glucose in the physiological solution negatively affects ozone concentration (see § 4.2). It was concluded that glucose omission from the perfusion medium during experiments in this study would be viable. KH-G[2] was added to each organ bath prior to the addition of pharmacological agents or before ozone exposure. However, during the resting periods and for rinsing of tissue samples, KH+G was

[1] Krebs-Henseleit solution containing glucose

[2] Krebs-Henseleit solution devoid of glucose

used to incur physiological haemostasis. No glucose was therefore present in the organ baths when experiments with ozone were performed.

5.1. The basic methacholine concentration-response curve

The basic cumulative concentration-response curve of methacholine, a muscarinic acetylcholine receptor agonist, is the standard to which all *in vitro* results in this study are compared. Contractile responses to cumulative concentrations methacholine are expressed as a percentage (%) of the maximal (100%) response to methacholine, E_{max}. The basic cumulative concentration-response of methacholine is presented in Figure 5.1. The EC_{50} of methacholine under the experimental conditions were calculated at EC_{50}= (4.338 ± 0.028) x 10^{-6} M.

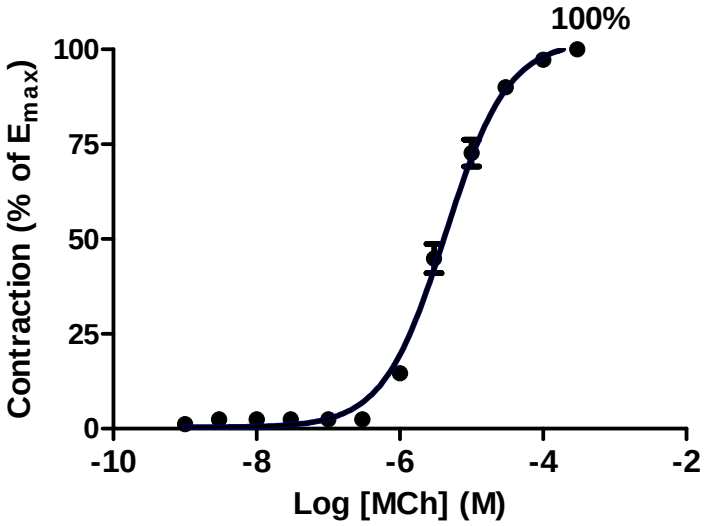

Figure 5.1. A cumulative concentration-response curve of methacholine, indicating the maximal (100%) contractile response of isolated tracheal tissue to methacholine. (EC_{50}= (4.338 ± 0.028) x 10^{-6} M ; *n*=6).

Any pharmacological effect that affects contractility or responsiveness of respiratory tissue after treatment with a substance or biotoxin, albeit as a result of interaction with specific receptors or from physiological damage, should reflect as a notable fluctuation from this standard curve.

5.2. The contractile effect of ozone on isolated tracheal tissue

In this experiment isolated tracheal tissue was exposed to a maximal ozone concentration (19.5 ± 0.19 μg/ml) to determine if ozone itself has any significant effect on tracheal smooth muscle contraction.

After determining the maximal contractile response (100%) that can be obtained with a single high concentration methacholine (1 x 10^{-4} M), ozone was introduced to the isolated tissue. The maximal concentration of ozone in the organ bath was maintained by continuously bubbling ozone (19.5 ± 0.19 μg/ml) through the KH-G solution in which the trachea was suspended. The results from this experiment are shown in Figure 5.2.

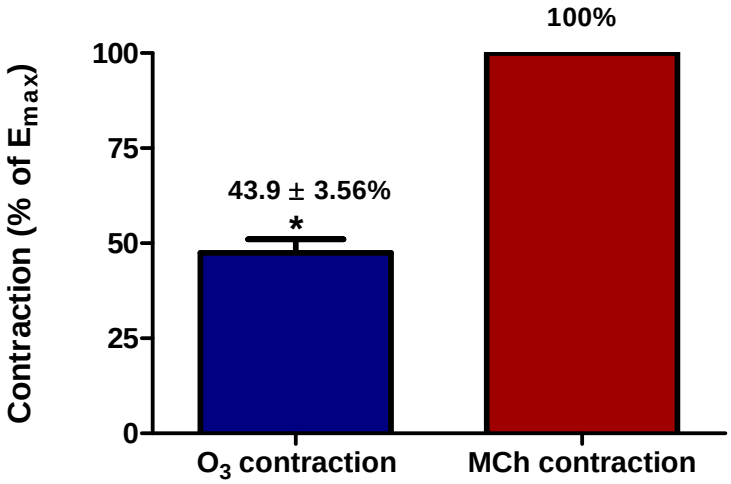

Figure 5.2. *In vitro* comparison between ozone (19.5 ± 0.19 μg/ml) and methacholine (1x10^{-4} M) -induced tracheal contraction (* p<0.05; n=9; Student's t test).

Shortly after ozone exposure was initiated, a noticeable contraction of tracheal tissue in response to contact with ozone was observed. Figure 5.2 compares the maximal possible contraction (43.9 ± 3.56%; $p<0.05$; $n=9$) elicited by a maximal ozone concentration to the maximal response (100%) that can be obtained with a single high concentration methacholine ($1x10^{-4}$ M). The maximal response to ozone is maintained as long as the ozone concentration in the organ bath is maintained at the maximum level.

This observed contraction may explain symptoms such as rapid shallow breathing shortly after ozone inhalation (Schelegle *et al.*, 2001).

If the tracheal preparation is washed with KH-G solution, and allowed to rest for 20 min, the trachea returns to its original ground state, i.e. the baseline returns to zero. This result possibly suggests that acute ozone exposure did not result in an irreversible pharmacological effect.

5.3. Concentration-response curve of ozone

In current scientific literature no concentration-response curves are available that illustrate the effect of ozone on airway responsiveness as a result of *in vitro* ozone exposure. In the experiment discussed in this section, a concentration-response curve for ozone was determined by employing two different methods (see also § 3.2.6):

(a) In the first method a single tracheal sample was subjected to multiple doses of a concentrated water-ozone solution (12.439 ± 0.011 mg O_3/l) was added to the single isolated (suspended in KH-G) tracheal preparation in increasing quantities. After the effect of each ozone concentration was noted as a percentage of the maximal (100%) response to methacholine in the same experimental setup (E_{max}), the tissue was rinsed with KH-G and allowed to equilibrate for 20 min prior to the addition of the next ozone concentration.

(b) In the second method the concentration-response curve was obtained by employing a single ozone concentration per trachea. Each tracheal preparation was treated only once with ozone and discarded. The same procedure was repeated for different ozone concentrations. The response to each individual ozone concentration was recorded and used to construct the corresponding concentration-response curve. The maximal response to ozone was taken as 100% response.

Figure 5.3 presents the resulting concentration-response curve of ozone.

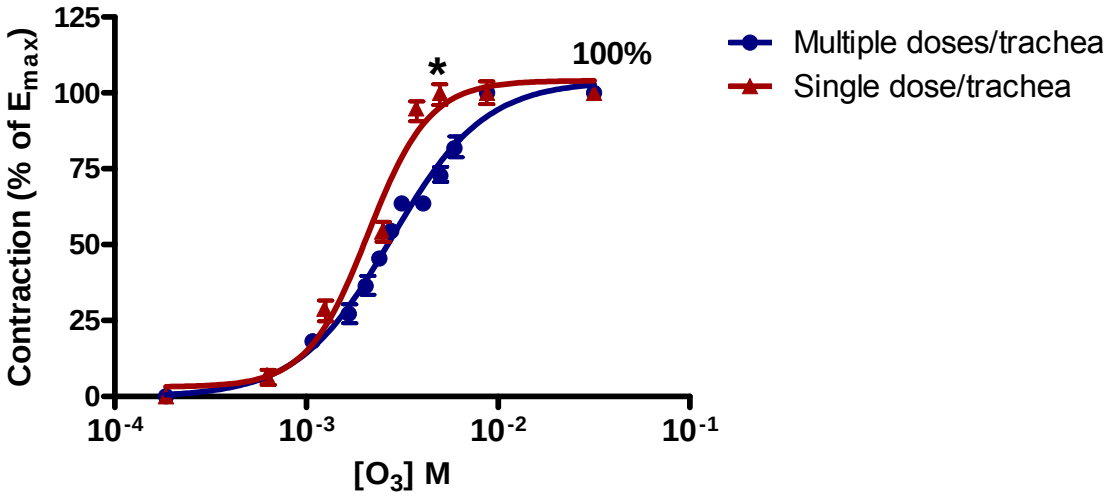

Figure 5.3. Concentration-response curves of ozone (**p*<0.05; *n*=6; Students t test). All ozone concentrations are the initial concentrations (12.439 ± 0.011 mg O_3/l). No statistical difference (*p*>0.05) exists between the EC_{50} values obtained from these curves (2.77 ± 0.02) x 10^{-3} M vs. (2.10 ± 0.03) x 10^{-3} M). No statistical difference (*p*>0.05) was furthermore found between the pEC_{50} values of these concentration-response curves.

From the results illustrated in Fig. 5.3, the apparent EC_{50} [3] value of ozone is respectively (2.77 ± 0.02) x 10^{-3} M when multiple ozone concentrations are used on a single trachea, and (2.10 ± 0.03) x 10^{-3} M when a single concentration is added per trachea. In comparison, the EC_{50} value of methacholine on the isolated trachea is (4.338 ± 0.028) x 10^{-6} M (Figure 5.1). It follows from these EC_{50} values that methacholine is on average about 5.6 x 10^{2} times more potent than ozone, whilst the maximal response is about 43.9% that of methacholine (see Figure 5.2).

It should be noted that the ozone concentrations given in Figure 5.3 are the initial concentrations. As ozone decomposes, this initial concentration will decline at a steady rate due to ozone decomposition (see § 4.5) and, therefore, the observed response for a specific concentration will actually be at a lower concentration.

The observed onset of the ozone-induced contraction was almost immediate, and the maximal response was generally obtained within 36 seconds. If the half-life of ozone ($t_{½}$ = 137 seconds; see § 4.5) in KH-G at 37 °C was taken into consideration, then in the time span of an average experiment, the ozone concentration would decline by about 13% from the initial concentration.

It is interesting to note that the two logarithmic dose-response curves in Figure 5.3 coincide at low concentrations, although they were determined by using different methods. It seems that the series of lower O_3 concentrations employed on a single organ had no detrimental effects on the trachea. At higher concentrations, however, the trachea became more unresponsive, i.e. an ozone concentration of 2.4 x 10^{-2} M induced a response of 68.39 ± 0.31% (first method) whilst the same concentration induced a response of 89.06 ± 0.22% (second method). This result possibly suggests fatigue of smooth muscle within the isolated tissue when exposed to ozone according to the first method – a method where a single organ is subjected and possibly fatigued by a series of O_3 treatments – potentially as a result of reduction in or

[3] The concentration that provokes a response half-way between the minimal and the maximal response

modification of Ca^{2+} in the sarcoplasmic reticulum (SR) of airway cells exposed to ozone. This smooth muscle fatigue does not repeat in the second method where each individual O_3 treatment is performed on a separate, fresh organ.

Ozone has a direct action on airway smooth muscle by changing Ca^{2+} mobilization - Ca^{2+} refilling via a Ca^{2+} pump and Ca^{2+} release via Ca^{2+} channels in the SR were increased, while Ca^{2+} extrusion via the plasma membrane Ca^{2+} pump was unchanged (Yoshida *et al.,* 2002). The mechanism of O_3-induced increase in airway smooth muscle responsiveness is likely to involve an enhancement in intracellular Ca^{2+} release (Roux *et al.,* 1996). The reduced responsiveness seen in isolated tracheal tissue after repeated ozone exposure (method one, Figure 5.3) does however indicate a deviation from this finding. According to Favero (1999) it is understood that precise control of intracellular Ca^{2+} is central to optimal performance of skeletal muscle and that a reduction in SR Ca^{2+} content or release will primarily compromise tension development through reduced Ca^{2+} transients. Inadequate or suboptimal delivery of Ca^{2+} to myofilaments of smooth muscle *via* SR Ca^{2+} release could occur as a result of reductions in or modification of SR Ca^{2+} content. In support of the earlier hypothesis that muscle fatigue resulting from a reduction in SR Ca^{2+} causes tracheal smooth muscle to be less reactive with repeated ozone exposure, Favero (1999) states that it is possible for SR Ca^{2+} to be diminished when considering that the catalytic activity of SR Ca^{2+}-ATPase is inhibited by a single bout of muscle activity.

5.4. The effect of ozone on tracheal responsiveness

Exposure of the airway to methacholine produces bronchoconstriction and methacholine challenge is often employed to evaluate respiratory hyper responsiveness (Hargreave *et al.,* 1986). Ozone, in return, is known to affect airway responsiveness (Fedan, 2000) and increase respiratory response to bronchoconstrictors and allergens in asthmatic and non-asthmatic subjects (Wagner *et al.,* 2007).

The objective of the experiment discussed in this section was to establish whether *in vitro* ozone exposure affects the maximal bronchoconstrictive effect of methacholine on isolated tracheal tissue.

In experiments pertaining to Figures 5.4 and 5.5 the control curves (a cumulative logarithmic concentration-response curve of methacholine) was firstly determined, whereafter the isolated tracheal tissue was thoroughly rinsed with KH+G, before the organ was subjected to 1.0 ml of an ozone-water solution (12.439 ± 0.011 mg O_3/l) plus 9 ml KH-G solution. Once a maximal response was obtained, the organ was again thoroughly rinsed with KH+G to ensure that all traces of ozone are removed. Finally, the second cumulative logarithmic dose-response curve of methacholine was determined.

Results shown in Figure 5.4 were obtained by employing KH+G as physiological solution, whilst the results shown in Figure 5.5 were obtained by employing a Krebs-Henseleit (KH-G) physiological solution.

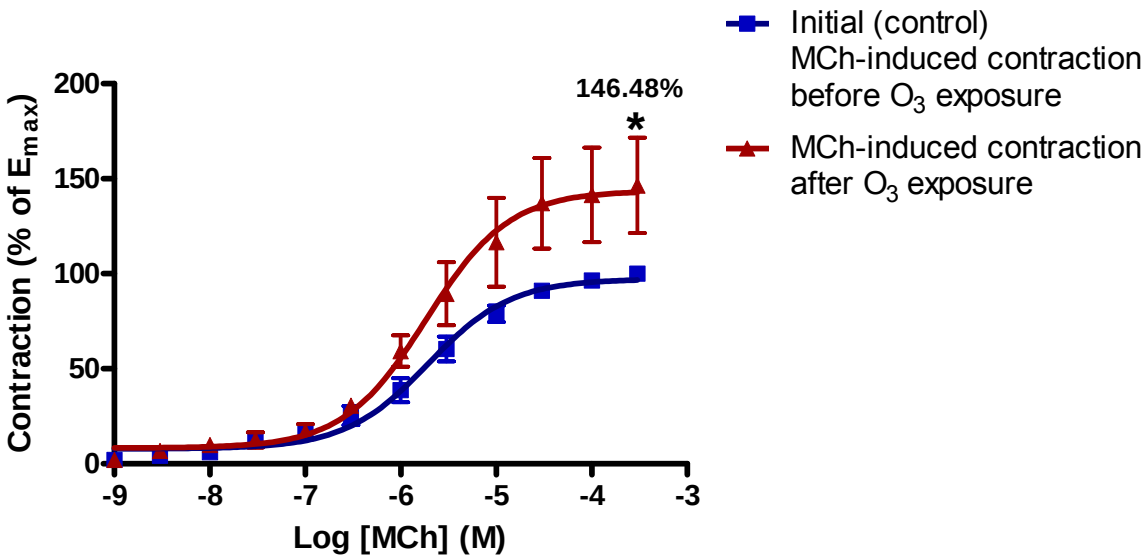

Figure 5.4. Cumulative concentration-response curves of methacholine illustrating the increased response of isolated tracheal tissue to methacholine after *in vitro* exposure to ozonated water in a Krebs solution (KH+G) Initial O_3 concentration=12.439 ± 0.011 mg O_3/l; *n=7; *p<0.05;* Student's t test). No statistical difference (p>0.05) exists between the EC_{50} values obtained from these curves (1.93 ± 0.07) x10^{-6} M (control) vs. (1.862 ± 0.163) x 10^{-6} M).

From the results depicted in both Figures 5.4 and 5.5 it can be seen that *in vitro* exposure to an ozone-water solution (12.439 ± 0.011 mg O_3/l) significantly increased the mean maximal response of tracheal tissue to methacholine when compared that of unexposed tissue (see Table 5.1). The EC_{50} values of the methacholine curves in each of these Figures do not differ significantly (1.93 ± 0.07) x10^{-6} M (control) vs. (1.862 ± 0.163) x 10^{-6} M (n=7; p>0.05) and (3.43 ± 0.07) x 10^{-6} M (control) vs. (2.32 ± 0.16) x 10^{-6} M (n=6; p>0.05)), indicating that no shift (EC_{50}-fold shift) of the experimental curves have occurred.

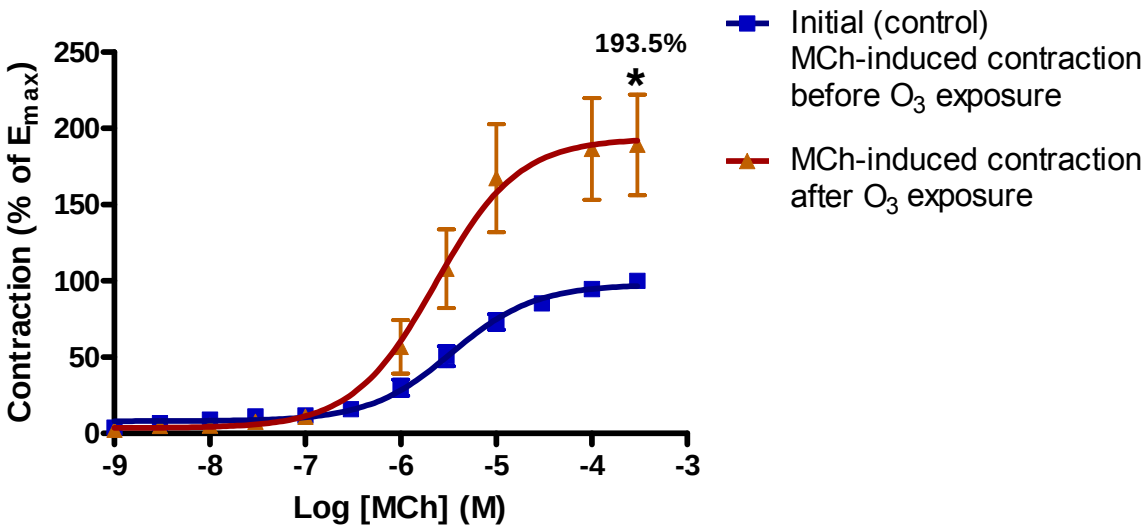

Figure 5.5. Cumulative concentration-response curves of methacholine illustrating the increased response of isolated tracheal tissue to methacholine after *in vitro* exposure to ozonated water in a KH-G. (Initial O_3 concentration = 12.439 ± 0.011 mg O_3/l; *n=6; *p<0.05;* Student's t test). No statistical difference (p>0.05) exists between the EC_{50} values obtained from each of these curves (3.43 ± 0.07) x 10^{-6} M (control) vs. (2.32 ± 0.16) x 10^{-6} M).

As can be seen in Table 5.1, ozone had practically no effect on the EC_{50} values of methacholine, indicating that ozone possibly does not influence the interaction of methacholine and its receptors. It therefore appears that the muscarinic acetylcholine receptors have possibly not been affected by ozone with respect to the binding of methacholine to these receptors.

Table 5.1. Maximal responses and EC_{50} values obtained from Figures 5.4 and 5.5

	EC_{50} values of methacholine (M)		**Maximal response of methacholine (%)**	
	Before ozone incubation	After ozone incubation	Before ozone incubation	After ozone incubation
Figure 5.4	(1.93 ± 0.07) $x10^{-6}$	(1.862 ± 0.163) x 10^{-6}	100	146.48*
Figure 5.5	(3.43 ± 0.07) x 10^{-6}	(2.32 ± 0.16) x 10^{-6}	100	193.5*

**A significant statistical difference (*p<0.05) exists between the maximal response to methacholine obtained after ozone incubation in Figure 5.4 and Figure 5.5 respectively.*

The finding that the maximal response seen in Figure 5.4 (KH+G) only increased by 46.48%, whilst the maximal response seen in Figure 5.5 (KH-G) increased by 93.5%, can be ascribed to the reaction of ozone with glucose. This can be explained by data from Table 5.1, suggesting that the ozone concentration in KH+G (Figure 5.4) decreases at a much faster rate than the ozone concentration in KH-G (Figure 5.5). By performing experiments in KH+G, the trachea is subjected to lower ozone concentrations than with KH-G, resulting in a lesser degree of responsiveness to methacholine. Therefore, it is suggested that the degree of the observed tracheal sensitivity to methacholine depends on the concentration of ozone to which the trachea was subjected.

Figure 5.6 presents the cumulative logarithmic concentration-response curves for methacholine before and after treatment of the isolated tracheal preparation with a maximal ozone concentration. In this experiment the ozone concentration in the organ bath in which the tracheal tissue was suspended, was kept constant by continuously bubbling ozone through the KH-G solution, and thus subjecting the trachea for 10 min to the maximal ozone concentration (see also § 3.2).

As a result of this exposure model, the isolated tracheal tissue is not only exposed to the saturated ozone-KH-G solution (2.78 ± 0.07 mg O_3/l) inside the organ bath, but also directly to the high concentrations of ozone which was constantly

bubbled through the physiological solution (KH-G; 19.5 ± 0.5 mg O_3/l). It can be expected that the actual amount of ozone interacting with the isolated tracheal tissue in this model is much higher than with experiments pertaining to Figures 5.4 and 5.5. The observed effect on the exposed airway tissue is therefore expected to be more prominent.

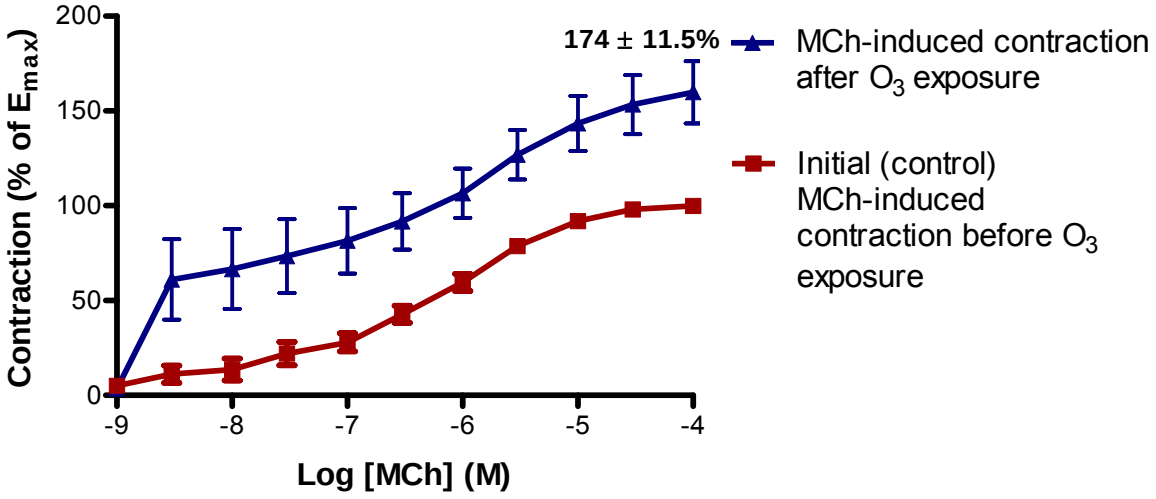

Figure 5.6. Cumulative concentration-response curves of methacholine illustrating the increased response of isolated tracheal tissue to methacholine after constant *in vitro* exposure to ozone gas in KH-G (Constant O_3 concentration = 19.5 ± 0.5 mg O_3/l; *n*=6; *p<0.05 for all points on graph;* Student's t test). No statistical difference (*p*>0.05) exists between the EC_{50} values obtained from each of these curves (1.23 ± 0.03) x 10^{-7} M (control) vs. (4.06 ± 0.05) x 10^{-7} M).

Figure 5.6 illustrates a much larger maximal response of the isolated tracheal tissue when treated with methacholine after ozone exposure (174 ± 11.5%) as compared to the methacholine control curve (100%), demonstrating the possible development of airway hyper responsiveness. As with Figures 5.4 and 5.5 ozone had, once again, practically no effect on the observed EC_{50} values of methacholine (1.23 ± 0.03) x 10^{-7} M before ozone incubation vs. (4.06 ± 0.05) x 10^{-7} M after ozone

incubation), accentuating the possibility that ozone influences neither the interaction of methacholine and its receptors, nor the muscarinic acetylcholine receptors itself.

To verify that the muscarinic acetylcholine receptors are still intact, the pA_2 values was determined for the muscarinic antagonist, atropine, on a normal (pA_2 = 8.52 ± 0.25) and ozonated (pA_2 = 8.42 ± 0.36) tracheal preparation. This finding that the affinity values of atropine for muscarinic acetylcholine receptors, located in the mentioned tracheal preparations, are practically identical underlines the notion that ozone has no effect on these receptors.

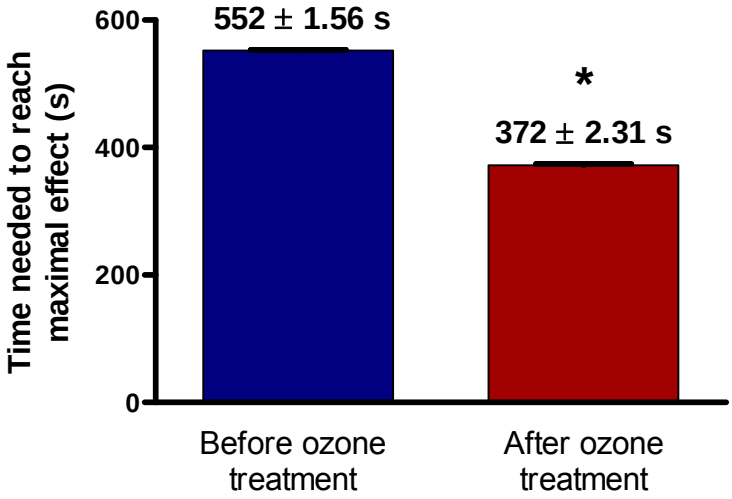

Figure 5.7. Hyper responsiveness of the isolated trachea to methacholine as a function of time. A graphical comparison of the time needed to determine the cumulative concentration-response curves of methacholine as shown in Figure 5.5. The initial methacholine-induced contraction reached a maximum after 552 ± 1.56 seconds, while the same level was reached within 372 ± 2.31 seconds after ozone exposure (19.5 ± 0.5 mg O_3/l; n=6; *p<0.001; Student's t test).

Figure 5.7 depicts the average hyper responsiveness of the tracheal preparation to methacholine as a function of time (contraction before and after ozone exposure). The time stated in Figure 5.7 is the time it takes to determine the cumulative concentration-response curves for methacholine as shown in Figure 5.6, *i.e.* the time that is required to reach maximal response after cumulative addition of the methacholine to the organ bath. In the control experiment 552 ± 1.56 seconds total time was required to determine the concentration-response curve (from 0 to 100% response). After the trachea was exposed to ozone, the same experiment was completed in 372 ± 2.31 seconds (n=6; *p<0.001) – the rate at which the ozonated trachea contracts (in seconds) increased with 32.2%. The latter increase is indicative of the hyper responsiveness of the ozone treated tracheal preparation.

It was found that the ozone-induced hyper responsiveness shown in Figures 5.4, 5.5, 5.6 and 5.7 declined with time and the tracheal preparation eventually returned to its normal state. If the ozonated tracheal preparation is thoroughly rinsed with ozone-free KH+G and rested for 2 hours, the tracheal preparation maintained an appreciable, although reduced, sensitivity to methacholine. After a 2-hour resting period the maximal observed tracheal response to methacholine was still about 120%. This gradual return to the ground state, as can be seen in the sensitivity towards methacholine, is in agreement with the findings of Fedan *et al.* (2000) who noted that *in vitro* reactivity of the isolated trachea to methacholine after *in vivo* ozone exposure is reversible over time.

The results pertaining to Figures 5.4, 5.5, 5.6 and 5.7 suggests that the observed hyper responsiveness is caused by changes in the tracheal preparation brought about by the reaction of ozone with the trachea. The mechanism of action by which this is achieved is however still poorly understood. As potential explanation, Fedan *et al*. (2000) suggest that the increase in tracheal reactivity to methacholine after ozone exposure involves a reversible increase in epithelial permeability resulting in greater accessibility of the drug through the mucosa to the smooth muscle. This hypothesis is supported by the finding of Murlas *et al.* (1990) that the increased tracheal reactivity to methacholine after ozone exposure was not observed in

tracheae devoid of epithelium, indicating that the epithelium was the principle site of ozone effects and was primarily involved in the increase in responsiveness to methacholine (Matsubara *et al.,* 1995; Fedan, 2000). Based on these findings ozone-induced airway hyper responsiveness may be linked to non-cyclooxygenase mucosa-derived factors (Murlas *et al.,* 1990) or by loss of normal epithelial cell functions, but not by infiltration of inflammatory cells into the airway (Matsubara *et al.,* 1995).

In a recent study Wu *et al*. (2008) observed that ozone-induced airway hyper responsiveness is mediated partly by Substance P (SP) release from nerve terminals of intrinsic airway neurons. It was noted that Interleukin (IL)-1 increases the synthesis and release of SP from intrinsic airway neurons. Neutrophils, alveolar macrophages and tracheal epithelial cells produce IL-1, suggesting that the airway epithelium may provide an important source of IL-1 since macrophage and neutrophil influx would not occur extensively in an *in vitro* experiment. It was concluded that it is possible that epithelial release of IL-1 initiates the early responses subsequent to ozone exposure, promoting neural and smooth muscle responses (Wu *et al*., 2008).

Roux *et al*. (1996) furthermore suggest that since *in vitro* exposure to ozone increases responses to agonists that, as part of their mechanism of action, release intracellular calcium ions, and since this effect is maintained in calcium free physiological solution, the mechanism of ozone-induced increase in airway smooth muscle responsiveness is likely to involve an enhancement in intracellular calcium release.

5.5. The effect of ozone on the responsiveness of the isolated trachea to isoproterenol

β-adrenergic receptor agonists are potent relaxants of airway smooth muscle, capable of rapidly relieving bronchoconstrictive respiratory tract conditions and are the most potent and effective bronchodilators known at present (Hui, 1994; Merck Veterinary Manual, 2010). Since multiple studies were previously performed in this

laboratory employing isoproterenol, a non-selective β-adrenergic receptor agonist, and due to the availability of an abundance of reference data available as a result of these studies, the decision was made to employ isoproterenol, as the bronchodilator of choice throughout this study. Future studies is suggested in which treatment with a clinically selective β_2-adrenergic receptor agonist is employed in comparison with isoproterenol.

The objective of the experiment discussed in this section was to establish whether *in vitro* treatment of tracheal preparations with ozone (19.5 ± 0.5 mg O_3/l) has any effect on isoproterenol function.

Freshly isolated tracheal preparations, suspended inside jacketed organ baths containing KH-G according the method described in Chapter 3 (see § 3.2.2.), was maximally contracted with a single addition of a high concentration of methacholine (1 x 10^{-4} M) and then relaxed by addition of a single concentration isoproterenol (1 x 10^{-4} M). This isoproterenol concentration was chosen because it would give maximal relaxation under normal experimental conditions (Timmerman & Scheffer, 1968).

The average time it took for the single isoproterenol concentration to induce the maximal observed relaxation was recorded, whereafter the same experiment was repeated on a set of tracheal preparations which was first exposed to a maximal concentration of ozone. The maximal concentration of ozone in the organ bath was maintained by continuously bubbling ozone (19.5 ± 0.5 mg O_3/l) through the KH-G solution in which the trachea was suspended. The result from this experiment is presented in figure 5.8.

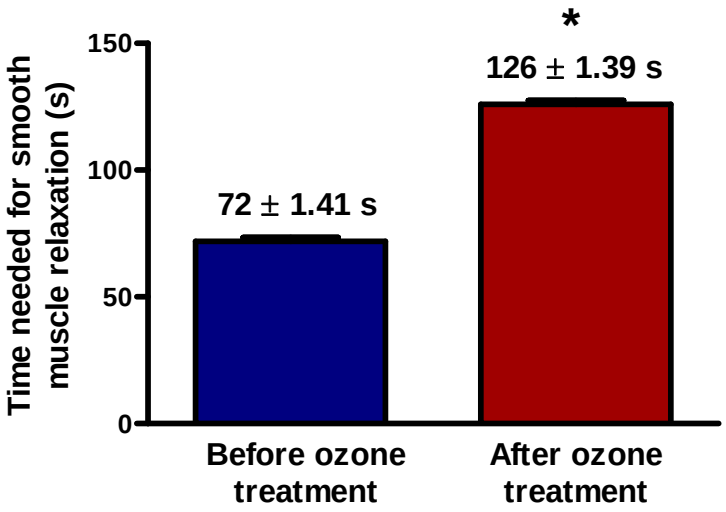

Figure 5.8. The response of ozone on isoproterenol function. It took longer to relax a methacholine-induced contraction of the trachea if the trachea was first exposed to ozone (19.5 ± 0.5 mg O_3/l; *n*=6*;* **p*<0.001; Student's t test).

Figure 5.8 illustrates that, in the control experiment, the single concentration isoproterenol relaxed the trachea within 72 ± 1.41 seconds, whereas a tracheal preparation which was first exposed to ozone reacted much slower and the maximal response was obtained after only 126 ± 1.39 seconds (*n*=6; *p*<0.001). Ozone exposure of the trachea therefore increased the normal time required to relax the methacholine-induced contraction of the trachea by 75%.

This result points to the possibility that the effectiveness of β-sympathomimetics (used by asthmatics) may be reduced when it is used by an asthmatic during an asthma attack caused by an allergen shortly after inhalation of ozone.

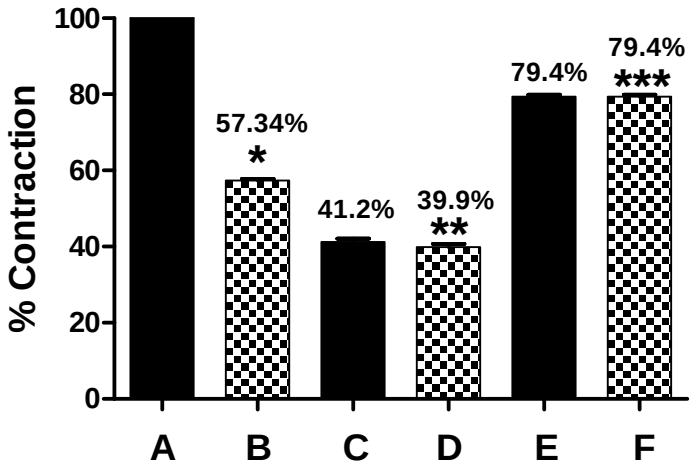

Figure 5.9. Graphical representations of the responses of single concentrations isoproterenol (1 x 10^{-4}) and methacholine (1 x 10^{-4}). *A* Initial (control) single concentration methacholine-induced contraction of the trachea. *B* Relaxation of the methacholine-induced contraction with a single concentration isoproterenol (*n*=6*; *p*<0.05, Student's t test). *C* Ozone-induced contraction of the trachea (19.5 ± 0.5 mg O_3/l). *D* Relaxation of the ozone-induced contraction of the trachea with a single concentration isoproterenol (*n*=6*; **p*>0.05; Student's t test). *E* Contraction of the trachea with a single concentration methacholine post ozone-exposure. *F* Relaxation of the methacholine-induced contraction (curve *E)* with a single concentration isoproterenol (*n*=6*; ***p*>0.05; Student's t test).

When comparing the relaxing effect of isoproterenol (1 x 10^{-4} M) on both a methacholine and ozone-induced contraction (Figure 5.9) of the tracheal preparation respectively, it is interesting to note that isoproterenol relaxes the observed methacholine-induced contraction by only 58.8% (curve B, 39.9 ± 0.91%), whilst the observed ozone-induced tracheal contraction (Curve C, 41.2 ± 0.91%; *n*=6; *p*<0.001; Dunnett's multiple comparison test) was relaxed by 96.84% (curve D). This may indicate that the mechanism leading to the observed contraction of tracheal smooth muscle after exposure to ozone is different from that leading to tracheal contraction noted with methacholine treatment.

The relative resistance of the methacholine-induced tracheal contraction to relaxation by β-adrenergic receptor agonists (i.e. isoproterenol), especially at (supra) maximal contractile concentrations, is largely determined by its higher potency in inducing intracellular Ca^{2+} changes generated by muscarinic type 3 acetylcholine receptor (M3 AChR) stimulation (Hoiting *et al.,* 1996; Ostroms, 1999). Stimulation of the M3 AChR subtype mediates contraction of isolated strips of tracheal tissue after exposure to a muscarinic agonist by coupling of the M3 AChRs to G-proteins of the G_q family, which stimulates direct calcium mobilization (Ostroms, 1999; Candell *et al*., 1990).

Ozone also stimulates calcium mobilization (Yoshida *et al.,* 2002*),* although by means of a different mechanism, most probably directly related to ozone-induced epithelial damage. A possible example of this is the epithelial release of Interleukin (IL)-1 following ozone exposure, which increases the synthesis and release of Substance P (SP) from intrinsic airway neurons (Wu *et al.,* 2008). The SP receptor is a G-protein coupled molecule and SP (or other agonists interacting with the SP receptor) is thought to initiate an elevation of intracellular Ca^{2+} in some cell types (Marriott & Bost, 2001).

β-adrenergic receptor agonists cause relaxation of airway smooth muscle mediated by a reduction in the concentration of intracellular Ca^{2+} and also Ca^{2+} sensitisation (Oguma *et al.,* 2006). Since M3 AChRs stimulation is thought to be associated with a decrease in isoproterenol potency, these results indicated that ozone possibly has no effect on the M3 AChR.

Figure 5.9 (curve E) depicts the tracheal preparations, after relaxation of the initial methacholine-induced tracheal contractions with isoproterenol, being once more contracted maximally with a single addition of a high concentration methacholine (1×10^{-4} M). This is done to establish whether the trachea is still functioning. Interestingly, this second methacholine-induced contraction (79.4 ± 0.44%; *n*=6) of the tracheal preparation which was first exposed to a maximal concentration of ozone (19.5 ± 0.5 mg O_3/l), did not display any hyper

responsiveness of the trachea towards methacholine. Considering the observed hyper responsiveness of tracheal preparations pre-treated with ozone to methacholine seen in Chapter 4, this result possibly suggests that isoproterenol mediates resistance to tracheal muscarinic agonist hyper responsiveness, possibly as a result of reduced Ca^{2+} sensitization and a reduction in intracellular Ca^{2+} associated with β-adrenergic agonist action on airway smooth muscle (Oguma *et al.*, 2006)

Surprisingly, as can be seen in Figure 5.9 (curve F), this second contraction (79.4 ± 0.44%) of the organ with 1 x 10^{-4} M methacholine could not be relaxed with isoproterenol (1 x 10^{-4} M). The observed unresponsiveness of the trachea can only be ascribed to the effect of ozone on the trachea, since it is known that isoproterenol relaxes successive methacholine-induced contractions of the trachea (Timmermann & Scheffer, 1968). Although it has been reported that the effects of β-sympathomimetics on methacholine-induced contraction gradually decreases after repeated application of these agents for 45-60 min (Barnes, 1995; Kume & Takagi, 1997), it is not expected that the β-sympathomimetics would desensitize β-adrenergic receptor agonists in the time course of the *in vitro* experiments conducted in this study. However, experiments in this study have also confirmed experimentally that repeated successive methacholine contractions and subsequent relaxations of the trachea with isoproterenol had no detrimental effect on the responsiveness of the trachea for either methacholine or isoproterenol. This indicates that the trachea possibly became desensitized to isoproterenol after the initial action of isoproterenol.

5.6. The effect of indomethacin on ozone-induced tracheal contraction

Indomethacin is a non-steroidal anti-inflammatory drug (NSAID) and is thought to exert its chemical effects by inhibiting prostaglandin synthesis. Its primary site of action is the cyclooxygenase enzyme which catalyzes the conversion of arachidonic acid to prostaglandin and endoperoxide.

Since several studies have implicated products of the cyclooxygenase metabolism in the mediation of ozone induced effects (Schelegle *et al.,* 1987) and because ozone is said to cause significant airway-inflammation (Joad, 1998; Tepper *et al.,* 1989), *in vitro* experiments were conducted to establish whether treatment with this NSAID will have any influence on the observed ozone-induced increase in responsiveness of tracheal tissue to methacholine.

Freshly isolated tracheal preparations, suspended inside jacketed organ baths containing KH-G solution according to the method described in Chapter 3 (see § 3.2.2.), was maximally contracted with a single addition of a high concentration of methacholine (1×10^{-4} M), whereafter the tracheal preparations were thoroughly rinsed with KH+G and allowed to equilibrate for 20 min in a KH+G solution prior to the addition of a maximal ozone concentration. The maximal concentration of ozone in the organ bath was maintained by continuously bubbling ozone (19.5 ± 0.5 mg O_3/l) through the glucose-free physiological solution (KH-G) in which the isolated tracheal preparation was suspended.

It was found that the addition of 8 μg indomethacin (an amount found not to have any adverse effect on tracheal functioning under experimental conditions) to the organ bath immediately prior to exposure of the tracheal preparation to ozone, significantly (77%; $p<0.05$, n=6) reduced the ozone-induced contraction of tracheal smooth muscle when compared to a control experiment in which no indomethacin was used.

This agrees with the finding of Insel (1996) and Schelegle *et al.,* (1987) that pre-treatment with a prostaglandin synthetase inhibitor reduces ozone induced pulmonary function decrements. This suggests that cyclooxygenase products of arachidonic acid, which are sensitive to indomethacin inhibition – including the broncho-constrictive prostaglandins Prostaglandin D2 and F2 – may play a role in the development of pulmonary function attenuation consequent to acute ozone exposure.

5.7. The influence of ascorbic acid on the responsiveness of the isolated trachea to ozone

It is believed that some of the effects of ozone observed in isolated tracheal preparations are mediated through oxidant damage to cell structures (Pryor, 1995). The experiment discussed in this section was performed to investigate whether antioxidant compounds present may serve to reduce ozone-induced lung injury by counteracting oxidative stress.

Freshly isolated tracheal preparations, suspended inside jacketed organ baths containing KH-G according to the method described in Chapter 3 (see § 3.2.2.), was maximally contracted with a single addition of a high concentration of methacholine (1×10^{-4} M), whereafter the tracheal preparations were thoroughly rinsed with KH+G and allowed to equilibrate for 20 min in a KH+G solution prior to pre-treatment with ascorbic acid (30 mg as a single dose), a potent antioxidant that has been reported to play an important role in pulmonary health. After treatment with ascorbic acid (duration of exposure = 30 min) the tracheal preparations were thoroughly rinsed with KH+G and allowed to equilibrate for a further 20 min in a KH+G solution prior to the addition of a maximal ozone concentration. The maximal concentration of ozone in the organ bath was maintained by continuously bubbling ozone (19.5 ± 0.5 mg O_3/l) through the glucose-free physiological solution in which the isolated tracheal preparation was suspended.

The results from this experiment are presented in figure 5.10.

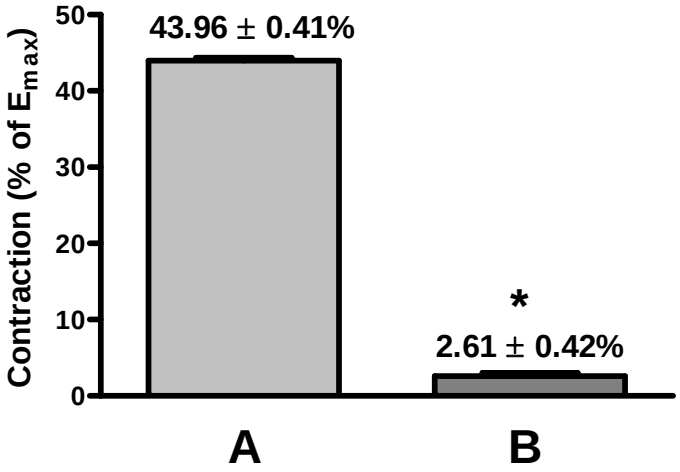

Figure 5.10. Ozone-induced contractions of the tracheal tissue. The physiological solution was either pre-treated with ascorbic acid (**B**), or not (**A)** prior to ozone exposure (19.5 ± 0.5 mg O_3/l; *n*=6; **p=0.0082;* Student's t test*).*

Figure 5.10 illustrates the protective effect provided by an antioxidant (ascorbic acid) in a physiological solution pre-treated with ascorbic acid against ozone-induced contraction of isolated tracheal tissue compared to the control (2.61 ± 0.42%; *n*=6; **p*=0.0082 *vs.* 43.96 ± 0.41%). The significantly larger response observed in the control group is possibly the result of oxidative damage from ozone exposure. This result is in accordance with that of Samet *et al.* (2001) who concluded from *in vivo* experiments that dietary antioxidants protects against ozone-induced pulmonary function decrements in humans.

Although Yeadon *et al*. (2002) found that pre-treatment with ascorbic acid prevented hyper responsiveness to bronchoconstrictors (e.g. methacholine) induced by a 30 minute *in vivo* exposure to ozone (3 ppm), results from the current *in vitro* study found the contrary: Pre-treatment with ascorbic acid did not successfully prevent hyper responsiveness of isolated tracheal preparations to methacholine induced by a 10 minute ozone (19.5 ± 0.5 mg O_3/l) exposure, and an effect equivalent to that noted in Figure 5.6 was observed.

Apart from the fact that the higher ozone concentration may speed up antioxidant depletion in the isolated tracheal preparations, this result may also, in part, be explained by the finding of Samet *et al*., (2001) who concluded that, although antioxidants exhibits a protective effect to ozone-induced decrements in pulmonary function, the accompanying inflammatory response caused by acute exposure to a moderate level of ozone was not affected. This suggests that the inflammatory response – and the inflammatory mediators responsible for this response - may play a significant role in the development of airway hyper responsiveness after exposure to ozone.

Chapter 6

Results and discussion: The *in vitro* respiratory effects of ozone after *in vivo* exposure

Little is known about the effect that *in vivo* ozone inhalation will have when post-exposure experiments are performed *in vitro* on isolated tracheal tissue. One question in particular arose: Will respiratory hyper responsiveness similar to that observed during experiments where *in vitro* ozone exposure was employed, be displayed?

The objective of this chapter is to:

(a) Determine the effect that *in vivo* ozone exposure will have on maximal attainable *in vitro* methacholine-induced isolated tracheal smooth muscle contraction;

(b) Determine the effect that *in vitro* ozone exposure following initial *in vivo* ozone exposure will have on maximal attainable *in vitro* methacholine-induced isolated tracheal smooth muscle contraction;

(c) Assess the effect that *in vitro* methacholine treatment after initial *in vivo* ozone exposure will have on maximal attainable isolated tracheal smooth muscle contraction in the absence of calcium from the physiological solution, and to

(d) Compare the results obtained in *(c)* to those observed after additional *in vitro* ozone exposure, or to experiments performed in the presence of calcium.

6.1 The effect of ozone on tracheal responsiveness

As observed in Chapter 5 (see § 5.4), *in vitro* stimulation of muscarinic acetylcholine receptors with methacholine after *in vitro* ozone exposure resulted in a significant (94% in KH-G[1]) increase in maximal attainable tracheal smooth muscle contraction.

To determine the effect of *in vivo* ozone exposure on maximal attainable *in vitro* methacholine-induced isolated tracheal smooth muscle contraction, Guinea pigs were exposed to relatively high concentrations of ozone (19.5 ± 0.5 µg/ml) for short periods of time (10 min) before tracheal tissue were isolated and suspended in a jacketed organ bath containing KH+G[2] at 37 ºC according to the specifications of Collier (1970) as described in Chapter 3 (see § 3.3). This allowed for changes in tracheal smooth muscle tension in response to methacholine *in vitro* after *in vivo* ozone exposure (as well as secondary *in vitro* exposure to ozone) to be isometrically recorded and compared to results found in Chapter 5 (see § 5.4).

The data presented in Figure 6.1 indicates whether *in vitro* methacholine treatment following *in vivo* ozone exposure will result in respiratory hyper responsiveness as seen with *in vitro* ozone treatment (see Figure 5.6). In Figure 6.1 the basic cumulative concentration-response curve depicting *in vitro* methacholine (Figure 5.1) treatment of isolated tracheal tissue not exposed to ozone was taken as the control group, and its maximal attainable contraction was taken as 100%.

[1] Krebs-Henseleit devoid of glucose

[2] Krebs-Henseleit containing glucose

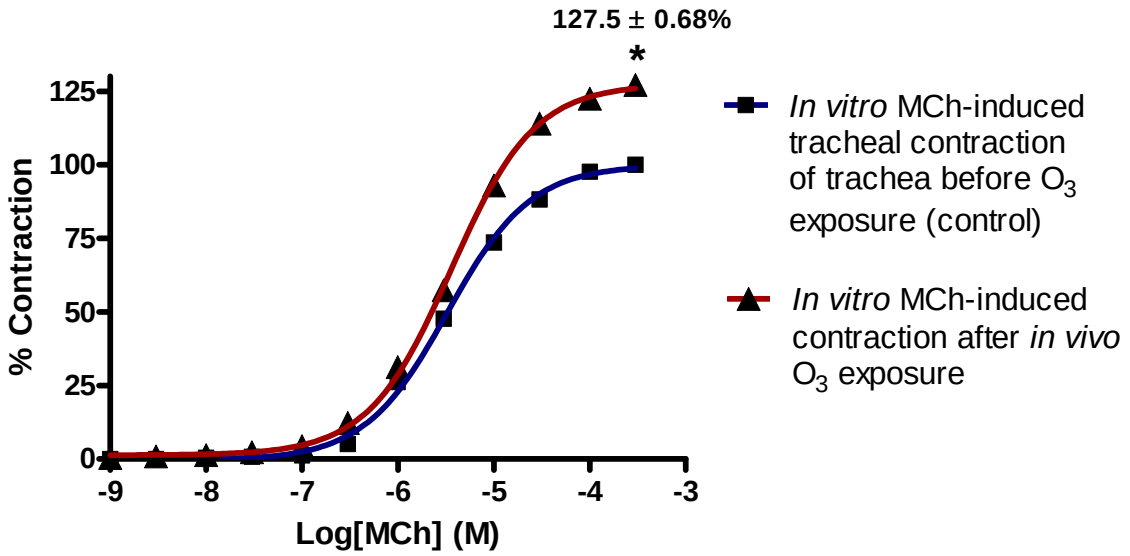

Figure 6.1. Cumulative concentration-response curve of *in vitro* methacholine treatment (in KH-G at 37 ºC) indicating hyper responsiveness of an isolated tracheal tissue sample after *in vivo* exposure to ozone (19.5 ± 0.5 µg/ml O_3) compared to a standard methacholine cumulative concentration-response curve (in KH-G at 37 ºC) of isolated tracheal tissue not exposed to ozone (n=8; *p<0.05; Stuent's t test). No statistical difference (p>0.05) exists between the EC_{50} values obtained from these curves (3.28 ± 0.019) x 10^{-6} M *vs. (*3.60 ± 0.01) x 10^{-6} M).

The result observed in Figure 6.1 indicates a clear and statistical significant (p<0.05; n=6) increase in maximal attainable contraction of the tracheal smooth muscle sample after stimulation of muscarinic acetylcholine receptors with methacholine. In the tracheal preparation exposed to ozone *in vivo,* a maximal contraction of 127.5 ± 0.68 % was recorded *in vitro*. This 27.5 ± 0.68 % increase in maximal attainable contraction of tracheal smooth muscle is indicative of hyper responsiveness.

Although the degree of hyper responsiveness is not as significant as that observed with methacholine treatment after *in vitro* exposure to ozone (174 ± 11.5%, Fig. 5.6), the result is nevertheless still significant and correlates with the findings of §

5.4. This result furthermore supports the finding of van Hoof *et al.* (1997) who noted a 55% increase in maximal attainable contraction of tracheal smooth muscle *in vitro* to a muscarinic agonist after *in vivo* ozone exposure under the experimental conditions used in that particular study.

The smaller effect noted in Figure 6.1 compared to that observed in Figure 5.5 (a 45.6 ± 6.32% difference in maximal attainable contraction of tracheal smooth muscle), can possibly be attributed to a defense mechanism of the body against oxidative damage that is either not present in the isolated tracheal tissue samples used, or which is more substantial in the *in vivo* method of exposure. This mechanism of defense is potentially related to antioxidant status within the body. Pryor *et al.* (1995) hypothesized that ozone does not penetrate airway cells upon inhalation, but oxidizes available antioxidants present at the air-epithelium lining fluid (ELF). Bocci (2006) furthermore asserts that the antioxidant capacity present in human ELF indicates only average values and that, although different portions of the respiratory tract may have different antioxidant levels, these are mostly irrelevant in comparison to the amount of antioxidants in the blood which easily neutralizes ozone reactivity.

This author's hypothesis may, to some extent, be supported by performing an experiment in which the isolated tracheal tissue which has already been exposed to ozone *in vivo,* is once again exposed to ozone. This second exposure to ozone is performed *in vitro* to eliminate a possible influx of antioxidants via blood as proposed by Bocci (2006).

In this experiment the isolated tissue is subjected to 1 ml of an ozone-water solution (12.439 ± 0.11 mg O_3/l, 20 ºC) added to 9 ml KH-G solution (20 ºC) which is then introduced to the organ bath (See § 3.3). The result of this experiment can be seen in Figure 6.2.

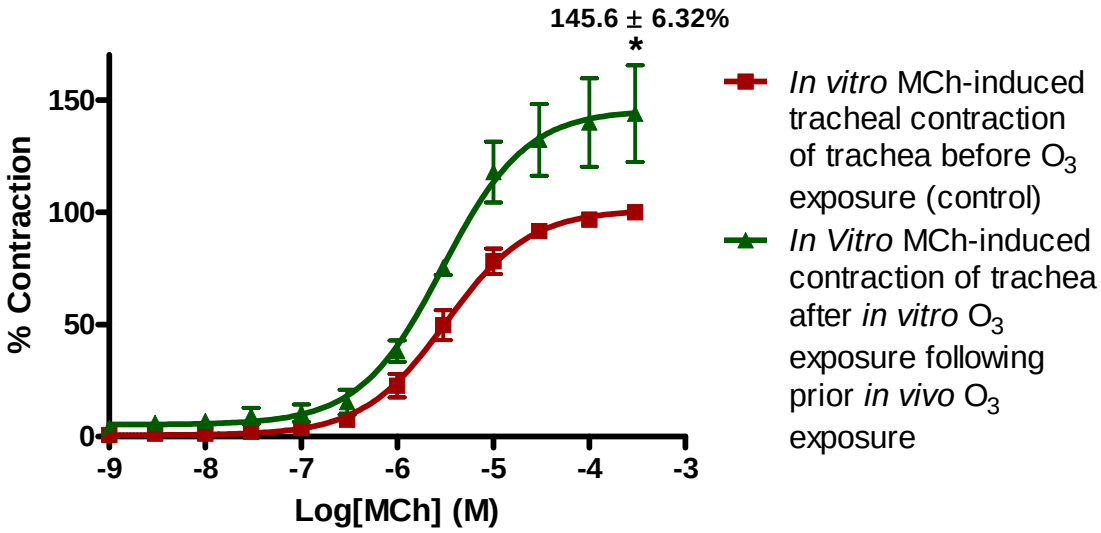

Figure 6.2. Cumulative concentration-response curves of *in vitro* methacholine treatment (in KH-G at 37 ºC) indicating hyper responsiveness of the isolated trachea (pre-exposed to 19.5 ± 0.5 µg/ml ozone *in vivo)* to methacholine after further *in vitro* exposure to ozone (12.439 ± 0.11 mg O_3/l) in comparison to a standard methacholine cumulative concentration-response curve (in KH-G at 37 ºC) of isolated tracheal tissue not exposed to ozone. The basic cumulative concentration-response curve depicting *in vitro* methacholine treatment of isolated tracheal tissue not exposed to ozone was taken as the control group, and its maximal attainable contraction was taken as 100% (**p*<0.05; *n*=6; Student's t test). No statistical difference (*p*>0.05) exists between the EC_{50} values obtained from these curves (3.22 ± 0.05) x 10^{-6} M vs. (2.99 ± 0.11) x 10^{-6} M).

From the result shown in Figure 6.2 it can be seen that the isolated tracheal tissue (which has already been exposed to ozone *in vivo)* shows an additional response of 45.6 ± 6.32% (173.1% (100% + 27.5% + 45.6%) *vs.* 127.5%) in the maximal attainable contraction to methacholine in response to further *in vitro* exposure to ozone. The combined response of 173.1% is nearly identical to the 174 ± 11.5% maximal response seen after *in vitro* ozone exposure (Figure 5.6), as compared to the 100% response obtained with the control (methacholine-treated tracheal tissue not exposed to ozone) experiment.

This additional response in the maximal attainable contraction to methacholine may potentially be explained by the findings of Yoshida *et al*. (2002), who noted that more Ca^{2+} seems to be taken back into intracellular storage sites after the first administration of a muscarinic agonist, leading to more contraction at the second and third administrations. Increased functioning of the sarcoplasmic reticulum (SR) to effect refilling with Ca^{2+} is thus suggested.

It is possible that the increased maximum attainable tracheal contraction to methacholine noted when ozone treatment is repeated *in vitro* after initial *in vivo* treatment (Figure 6.2), may be related to this suggested increased refilling of the SR with Ca^{2+}.

This result furthermore supports the hypothesis that the difference (45.6 ± 6.32%) in maximal attainable contraction to methacholine after *in vitro* ozone exposure (following prior *in vivo* ozone exposure), as compared to the contraction observed after a single bout of *in vivo* ozone exposure (27.5 ± 0.68%), is potentially as a result of the antioxidant defense-system against oxidative damage within the body of living test subjects, being more effective.

6.2 The effect of ozone on intracellular calcium mobility

Research suggests that calcium mobilization is one of the most important factors affecting airway smooth muscle tension as vertebrate smooth muscle contractility is known to be initiated by agents that increase intracellular calcium (Yoshida *et al.,* 2002). It is therefore speculated that exposure to ozone may directly affect intracellular Ca^{2+} mobilization in muscle cells.

In the experiments discussed in this section, changes in maximal attainable *in vitro* contraction of isolated tracheal tissue samples in response to methacholine as a result of *in vivo* ozone exposure, were studied in a KH-G solution devoid of Ca^{2+} (see § 3.3). The result of this study is presented in Figure 6.3.

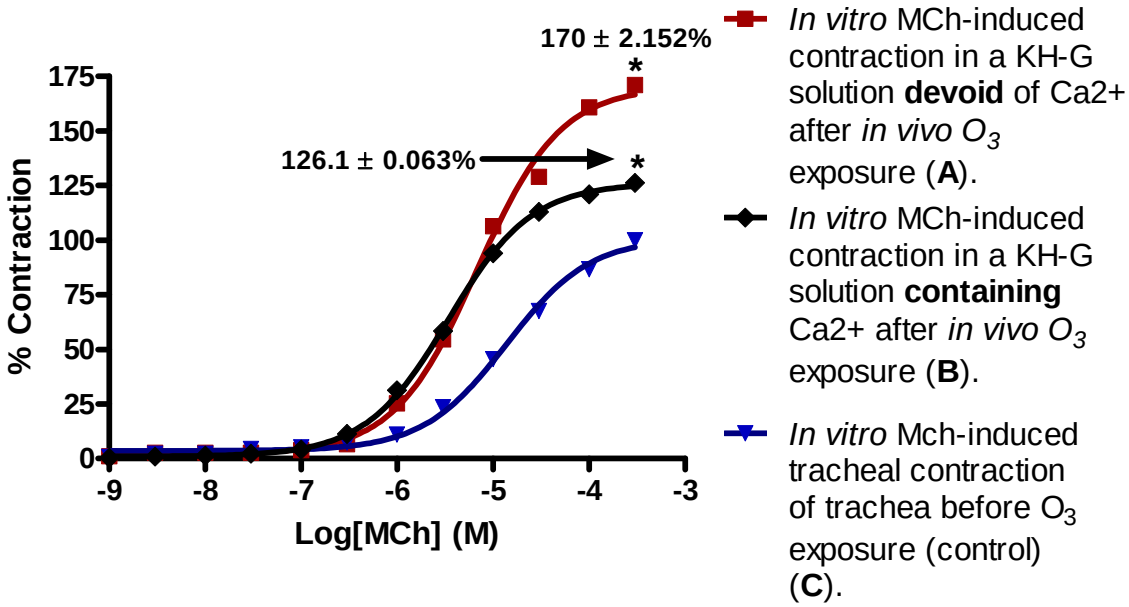

Figure 6.3. Cumulative concentration-response curves of methacholine depicting the *in vitro* effect of methacholine on the isolated trachea in the *absence* of calcium after *in vivo* exposure to ozone (19.5 ± 0.5 µg/ml) as compared to *in vitro* ozone exposure (12.439 ± 0.11 mg O_3/l). The basic cumulative concentration-response curve depicting *in vitro* methacholine treatment of isolated tracheal tissue not exposed to ozone was taken as the control group, and its maximal attainable contraction was taken as 100% (*p<0.05; n=6; Student's t test). No statistical difference (p>0.05) exists between the EC_{50} values ontained from these curves (6.81 ± 0.41) x 10^{-6} M (A) & (3.42 ± 0.01) x 10^{-6} M (C) vs (1.378 ± 2.154) x 10^{-5} M (B).

From the result shown in Figure 6.3 it can be seen that the maximum attainable *in vitro* contraction of tracheal smooth muscle in response to methacholine in KH-G devoid of Ca^{2+} after *in vivo* ozone exposure (19.5 ± 0.5 µg/ml for 10 *min*), increased significantly (70.4 ± 3.19% in a Ca^{2+}-free solution as compared to 26.1 ± 0.66% in a solution containing Ca^{2+}; p<0.05; n=6;).

This result is in accordance with that of Yoshida *et al.* (2002) and suggests that Ca^{2+} release from the SR increases after ozone exposure, causing an increase in intracellular Ca^{2+} mobilization and muscle contractility.

From the hypotheses that (a) maximal attainable tracheal contraction in response to *in vitro* methacholine treatment after *in vitro* ozone exposure following initial *in vivo* ozone exposure is larger than *in vitro* methacholine treatment of tracheal tissue exposed only *in vivo* to ozone, possibly as a result of increased antioxidant status and influx within the body during *in vivo* experiments, and (b) that more Ca^{2+} is taken into the SR after the first (*in vivo*) ozone exposure, leading to larger concentrations observed with the second (*in vitro*) ozone administration, a larger contraction to *in vitro* methacholine in KH-G devoid of Ca^{2+} is expected.

The cumulative methacholine concentration-response curve (in KH-G devoid of $Ca^{2+)}$ determined after *in vitro* ozone exposure following initial *in vivo* ozone exposure is shown in Figure 6.4.

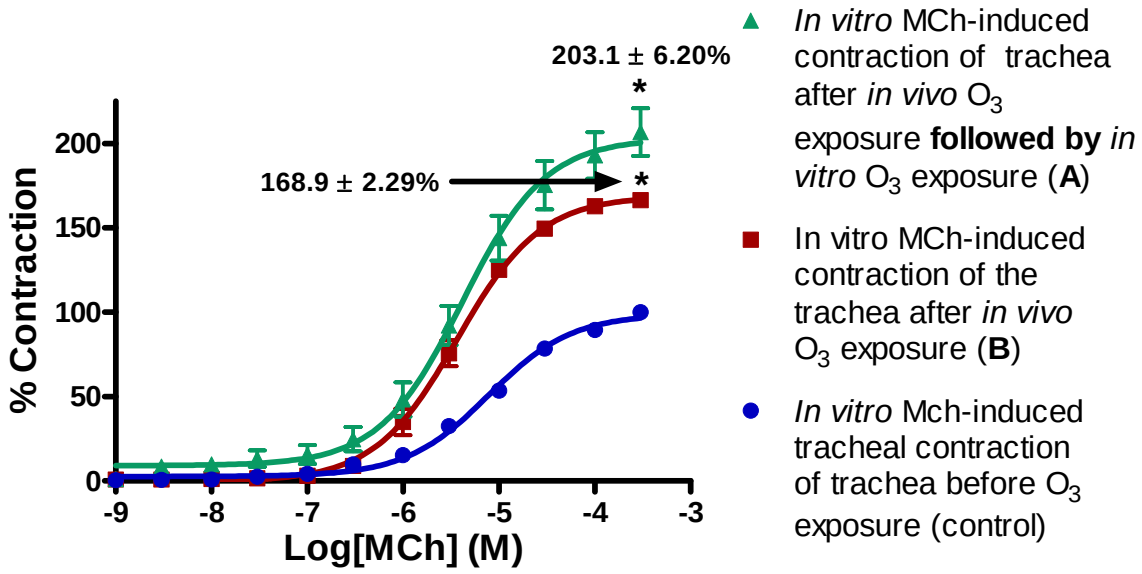

Figure 6.4. Cumulative concentration-response curve of *in vitro* methacholine treatment (in KH-G devoid of Ca^{2+} at 37 ºC) indicating hyper responsiveness of the isolated trachea (pre-exposed to 19.5 ± 0.5 µg/ml ozone *in vivo)* to metcholine after further *in vitro* exposure to ozone (12.439 ± 0.11 mg O_3/l), compared to a methacholine cumulative concentration-response curve (in KH-G devoid of Ca^{2+} at 37 ºC) of isolated tracheal pre-exposed to ozone *in vivo*, and a standard methacholine cumulative concentration-response curve (in KH-G at 37 ºC) of isolated tracheal tissue not exposed to ozone. The basic cumulative concentration-response curve depicting *in vitro* methacholine treatment of isolated tracheal tissue not exposed to ozone was taken as the control group, and its maximal attainable contraction was taken as 100%. (**p*<0.05; *n*=6; Student's t test). No statistical difference (p>0.05) exists between the EC_{50} values obtained from these curves (4.16 ± 0.07) x 10^{-6} M (A) vs. (3.70 ± 0.03) x 10^{-6} M (B) *vs.* (7.815 ± 0.047) x 10^{-6} M (Control).

From the result shown in Figure 6.4 a slight, but statistical significant increase in the maximal attainable contraction to methacholine was observed (34.2%). This result supports the hypotheses mentioned above.

To summarize, the results of the study discussed in this section suggests that ozone exposure results in increased recycling and release of Ca^{2+} in tracheal smooth

muscle cells. This is thought to be the result of increased activity of the Ca^{2+} refilling pump and the Ca^{2+} releasing channel on the SR respectively. Ca^{2+} release from the storage site is of importance in smooth muscle contraction by an agonist, especially at its initiation (Yoshida *et al.*, 2002).

In vivo ozone exposure may therefore affect muscle contraction, particularly on initiation, which can lead to instability of muscle tension. This suggested instability is suspected to have an important role in airway hyper responsiveness, and the observation that ozone increases Ca^{2+} refilling of the SR may therefore be an important mechanism of ozone-induced hyper responsiveness.

Chapter 7

Results and discussion: The effect of ozone on an isolated lung perfusion model

In human ozone toxicology, reversible decrements in pulmonary function (including difficulty breathing as deeply and vigorously as normal) are a common complaint (Bocci, 2010; Funabashi *et al.,* 2004; Yamauchi *et al.*, 2002).

In the study discussed in this chapter, an isolated lung perfusion model was developed and employed to establish the effect of ozone inhalation on respiratory mechanics. Isolated lung perfusion models allow for real-time data collection and analysis of lung mechanical function, and offer the distinct advantage of breath-by-breath data aquisition of ozone's effects on lung fuction.

In comparison to *in vivo* studies where tissue samples are isolated and examined after exposing test subjects to ozone, tissue in an isolated lung perfusion model remains part of the intact organ and offers the advantage that the lung is isolated from other organs and their possible effects. The isolated lung perfusion model may furthermore be used to deliver substances such as ozone, by way of inspired air (Bernard *et al.,* 1997).

The objective of the study discussed in this chapter was to develop a method that will incorporate ozone inhalation into an isolated lung perfusion model to allow for real-time data collection and analysis of lung mechanical function (See § 3.4). Lung mechanical parameters that were monitored included (a) lung compliance, (b) tidal volume, (c) inspiratory flow and (d) expiratory flow.

This experiment is the first, as far as could be established, in which ozone was introduced to an isolated lung perfusion model by way of inspired air and is therefore a truly unique study.

7.1 The effect of ozone on lung compliance

Lung compliance is a measurement of the stiffness of the lung and is therefore a good indicator of lung elasticity – high lung compliance indicates good lung elasticisty, while low lung compliance indicates severely impaired lung elasticity (Moussavi, 2007).

Since it was determined that the risk of developing asthma increased with outdoor activity in communities with high ozone concentrations (McConnell *et al*., 2002), the study discussed in this chapter was performed to investigate the effect of ozone inhalation on lung compliance. An isolated perfused lung was exposed to ozone (19.5 ± 0.5 µg/ml) for 5 seconds where after ozone exposure was stopped. During the 60 minutes which followed this short period of ozone exposure, the isolated lung perfusion model was observed for signs of recovery. The result from this experiment (which is presented in Figure 7.1), was compared to an isolated lung perfusion model exposed to pure air (the control study).

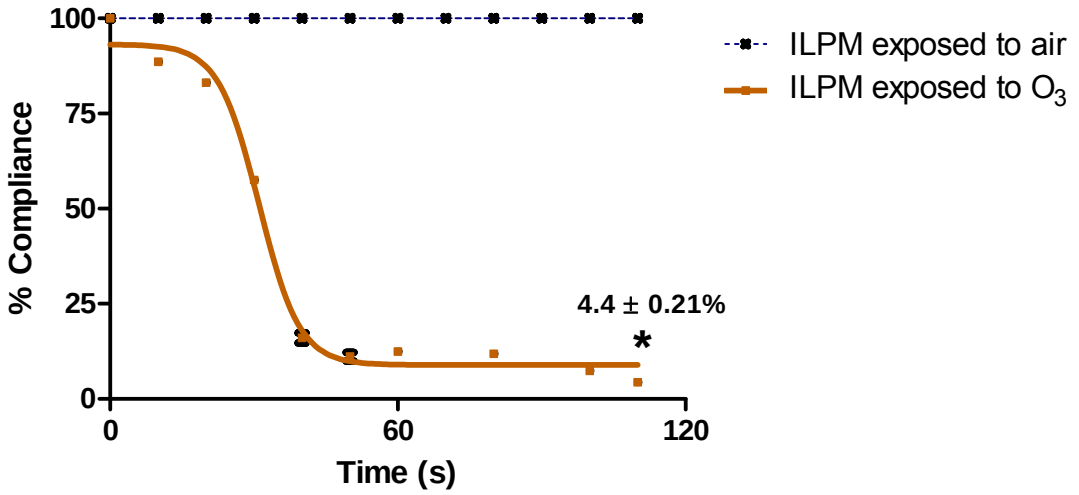

Figure 7.1. The effect of ozone (19.5 ± 0.5 µg/m) after a 5 second exposure in an isolated lung perfusion model (ILPM) on lung compliance (*n*=6; **p*<0.05; Student's t test).

From the result shown in Figure 7.1 the effect of ozone inhalation in an isolated lung perfusion model on lung compliance, and therefore lung elasticity, can be noted. After ozone (19.5 ± 0.5 µg/ml) was introduced to an isolated lung perfusion model by way of inspired air (5 second exposure), a notable decrease in lung compliance was observed almost immediately. Within 40 seconds lung compliance dropped by 85%, and by 95.6% after 110 seconds. Furthermore, no recovery in lung compliance was observed over the next 60 minutes, and after significant pulmonary oedema set in, the organ was presumed dead.

The deterioration in lung fuction observed in Figure 7.1 is believed to be indicative of acute lung injury as a result of oxidative damage, possibly due to a lack of antioxidants within the isolated lung perfusion model. Since it is postulated that the effects of ozone are mediated through oxidant damage to cell structures (Ballinger *et al*., 2005; Kafoury *et al.,* 1999), it has been hypothesized that antioxidant compounds present in the lung may serve to reduce ozone-induced lung injury by counteracting oxidative stress (Samet *et al.*, 2001). Earlier *in vitro* studies with ascorbic acid confirmed this (see § 5.7) and suggest that antioxidants are effective in blunting the pulmonary toxicity of ozone.

Figure 7.2 illustrates the effect of ascorbic acid (10 mg/ml) on the ozone-induced reduction in lung compliance if added to the lung perfusion medium (for the duration of the experiment) at a flow-rate of 1.0 ml/min prior to ozone inhalation.

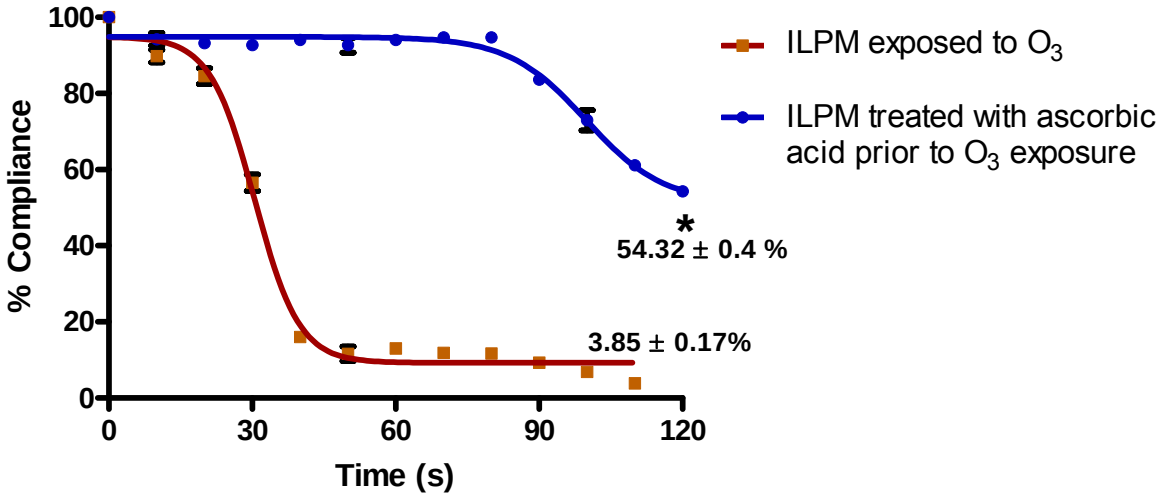

Figure 7.2. The influence of ascorbic acid (10 mg/ml), introduced to the perfusate at a flow-rate of 1.0 ml/min, on the ozone-induced (19.5 ± 0.5 µg/ml; 5 second exposure) reduction in lung compliance as seen in an isolated lung perfusion model (ILPM) (*n*=6; **p*<0,05; Student's t tetst).

The result observed in Figure 7.2 suggests that the addition of ascorbic acid (10 mg/ml) to the perfusion medium of the isolated lung perfusion model notably reduces the magnitude (45.68% in presence of ascorbic acid *versus* 96.15% in absence of ascorbic acid) and rate of decline in lung compliance after ozone exposure (19.5 ± 0.5 µg/ml; 5 second exposure). This concurs with the finding in § 5.7 as well as the hypothesis of Samet *et al.* (2001), and confirms that antioxidant components present in the lung reduce ozone-induced lung injury by counteracting oxidative stress.

7.2 Ozone's effect on other mechanical lung parameters

Lung compliance was not the only lung function parameter monitored during this study. Other mechanical parameters that were monitored included tidal volume, maximum inspiratory flow and maximum expiratory flow.

In comparison to an isolated lung perfusion model exposed to clean air, a 19.5 ± 0.5 µg/ml ozone exposure (5 second exposure) lead to a 70% decline in tidal volume, a 71.2% decrease in maximum inspiratory flow, and an 87.9% increase in maximum expiratory flow.

These results confirm the occurrence of breathing difficulty and rapid-shallow breathing in subjects exposed to relatively high levels of ozone, and it is suggested that the observed pulmonary function decrements in response to ozone exposure is a progression of epithelial cell injury and subsequent inflammatory response (Mautz, 2003).

Mautz (2003) furthermore asserts that the terminal bronchioles and proximal alveoli are prominent target tissues for cellular injury from ozone, but remarks that the larger bronchi and the trachea also show ozone-induced tissue injury. In addition, Schelegle *et al.* (1993) claims that airway irritants such as ozone act on lung C-fibers, producing a vagus nerve-mediated reflexive shift in breathing pattern, whilst Currie *et al.* (1998) concluded that leakage of plasma proteins into the airway lumen was probably the main reason for surfactant dysfunction, which may contribute to an altered breathing pattern.

Breathing pattern may also be changed by reflex responses to the irritating properties of inhaled toxic compounds. Depending on rate of inhalation (i.e. relaxed inhalation *versus* exercise-induced inhalation), such reflexes may consist of a shift to more rapid and shallow breathing in response to lower airway irritation or to slow and deep breathing in response to upper airway irritation (Mautz, 2003).

In summary, the isolated lung perfusion model exposed to ozone in this study displayed a significant decrease in lung compliance, tidal volume and maximum inspiratory flow, whilst an increase in maximum expiratory flow was noted. These changes in lung mechanics resulted in a reduction in lung elasticity and an altered breathing pattern.

Chapter 8

Results and discussion: The pulmonary effects of acute high-dose ozone inhalation

The mechanism of ozone-induced cellular lung cell injury is poorly understood, and since much of the inhaled ozone during ozone exposure reacts with the lining of the conducting airways, the question remains how ozone exerts most of its pulmonary effects. One hypothesis is that ozone induces lipid peroxidation and that these peroxidated lipids produce oxidative stress and DNA damage (Kosmider *et al.,* 2010).

At high doses, ozone inhalation is, however, believed to also induce acute lung injury as a result of rapid oxidant damage (Ballinger *et al.,* 2005; Soulage *et al*., 2004; Kafoury *et al.,* 1999).

The objective of the experiments performed in this chapter was to identify the histological effect of acute high-dose ozone inhalation (19.5 ± 0.5 µg/ml for 10 min), and to determine whether ozone at this dose does indeed exert its effect as a result of rapid oxidant damage.

All experimental procedures performed in this chapter were in accordance with the regulations stipulated by the Ethical Committee of the North-West University, complying with national legislation and in accordance with the guidelines of the National Institutes of Health guide for the care and use of laboratory animals.

8.1 Histological examination of respiratory tissue after acute ozone inhalation

During the experiments discussed in this section, the impact of inhaling an excessive dose of ozone (19.5 ± 0.5 µg/ml for 10 min) on the morphology and histology of respiratory tissue was investigated. A purpose-built, airtight exposure-chamber was developed for this purpose and ensured ozone exposure at consistent, controlled concentrations throughout this study.

Thirty Duncan-Hartley guinea pigs (450-550 g) of both sexes were randomly allocated into three groups (*n* = 10 each). Before commencing the experiment, the guinea pigs were acclimatised to the experimental area a week before experiments, and prior to the start of the actual experimental procedure, test subjects were introduced to the exposure chamber and allowed a period of 20 min to adapt to their surroundings. During this period normal airflow was circulated throughout the chamber. After the initial period of introduction, test subjects were exposed to (a) ozone (19.5 ± 0.5 µg O_3/ml), (b) pure air containing 0 µg O_3/ml, or (c) ultra-high purity oxygen for 10 min. The concentration of ozone produced was measured and constantly monitored spectrophotometrically using a Unico 2800 VIS/UV spectrophotometer to ensure that the level of ozone introduced to the exposure chamber remained constant at all times. After exposure, the test subjects were killed by carbon dioxide asphyxiation for 5 min and the tissue samples (lung and tracheal tissue) were removed within 3 min of death, whereafter samples were fixated in 10% formalin for morphological and histological examination.

During the acute exposure phase, breathing difficulty (rapid, shallow, laboured breathing) was observed in all test subjects. This observation suggests that the finding of Schelegle *et al.* (2001) who noted a characteristic pattern of rapid, shallow breathing during the first two days of a five day episode of ozone exposure (1 µg O_3/ml for 8 hours per day), is also applicable to an acute 10 minute exposure session to higher doses of ozone.

Hiccups were observed in 70% of ozone treated subjects during the exposure phase, whilst clear and significantly visible mucous membrane irritation was also noted in all ozone exposed subjects after a mean acute ozone inhalation period of 6 min. These results are believed to be related to increased airway resistance, possibly due to a swelling of the airway wall and surfactant dysfunction as a result of plasma protein leakage into the airway lumen, thereby influence breathing (Currie *et al.*, 1998). The mucus membrane irritation that was observed is believed to be the result of ozone's direct oxidative effect (Funabashi *et al.,* 2004).

At this point of the experimental procedure, tissue samples were subjected to optical microscopic evaluation. The effect of ozone on ciliated cells, the predominant cell type in airway epithelium, can be seen in Figure 8.1.

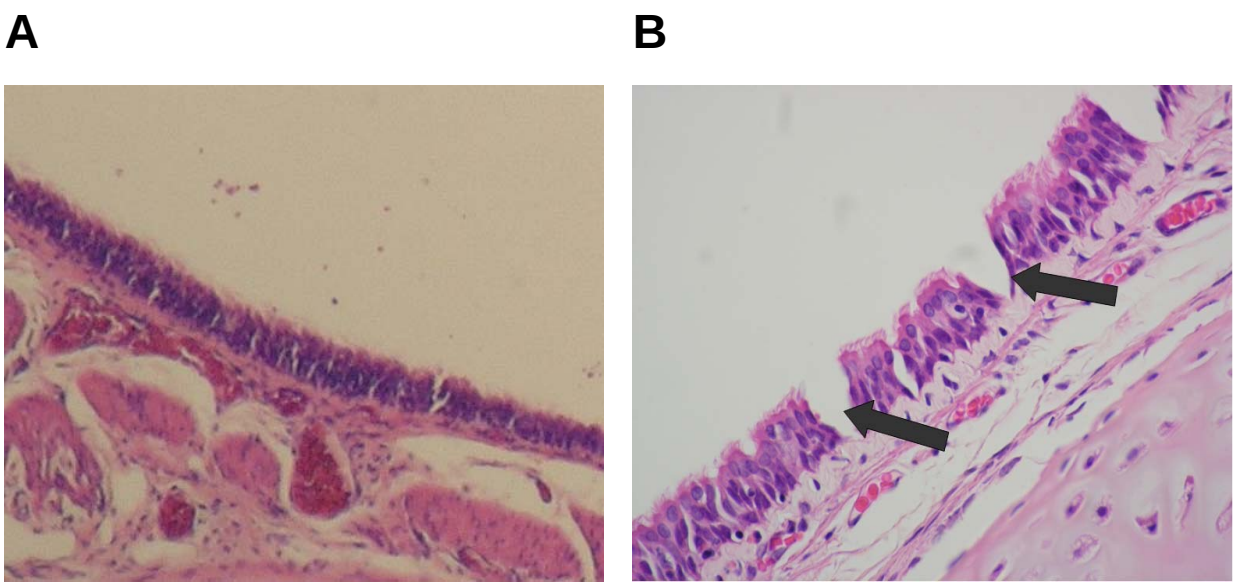

Figure 8.1. High magnification optical microscopic view of the respiratory epithelium lining of the trachea after ozone (19.5 ± 0.5 µg O_3/ml) inhalation (B) compared to an optical microscopic view of the respiratory epithelium lining of the trachea not exposed to ozone (A, control) . The loss of cilia and ciliated cells on the surface in B are clearly visible.

It is widely believed that ozone inhalation can cause epithelial injury and interstitial as well as intraluminal inflammation (Schelegle *et al.,* 2001). In Figure 8.1 the effect of acute high-dose ozone inhalation on ciliated cells is clearly visible and confirms that ciliated cells appear to be especially vulnerable to direct ozone-induced deciliation (Schelegle *et al.,* 2001; Bromberg and Koren, 1995).

Optical microscopic evaluation furthermore suggests that ozone-exposed lungs appeared mildly hyper-inflated compared to the control groups. Interestingly, microscopic evaluation of isolated tissue samples also noted small, waxy plugs consisting of homogenous pink material and cells in the lumen of bronchioles. These were only noted in ozone exposed subjects and are shown in Figure 8.2.

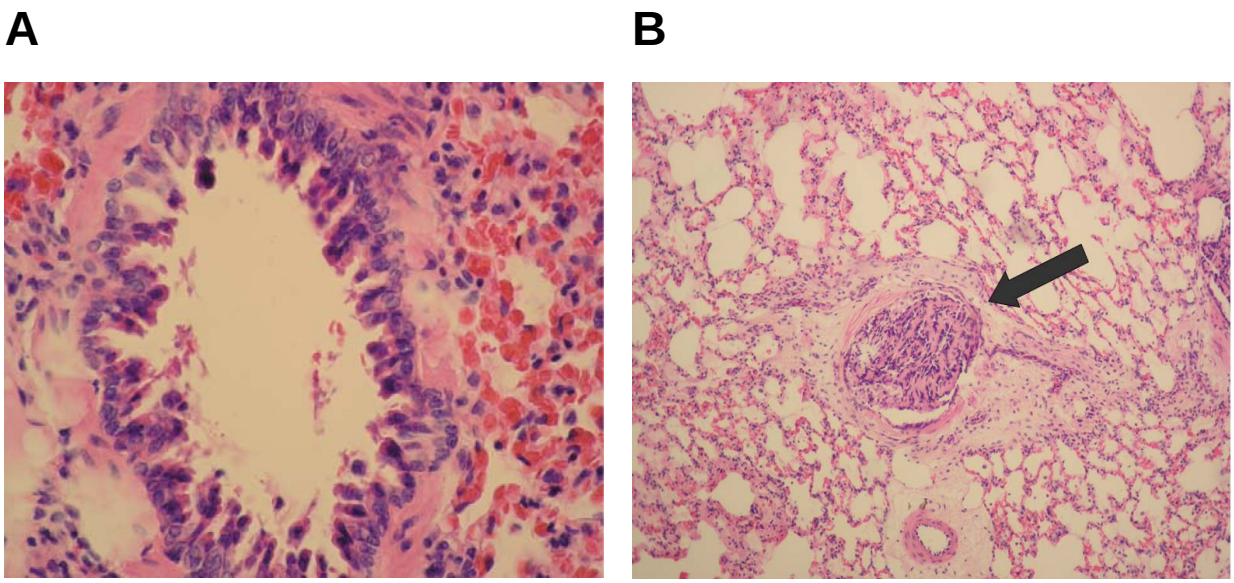

Figure 8.2. High magnification optical microscopic view of the unidentified plug observed after ozone inhalation within the lumen of a bronchiole (B), compared to the unexposed bronchiole with no plug in the lumen (A, control)

The precise nature of the plug as seen in Figure 8.2 could not be determined by optical microscopy and electron microscopy was performed on the lung tissue to try to identify the nature of these bronchiole plugs. In Figure 8.3 an electron microscopy image is shown.

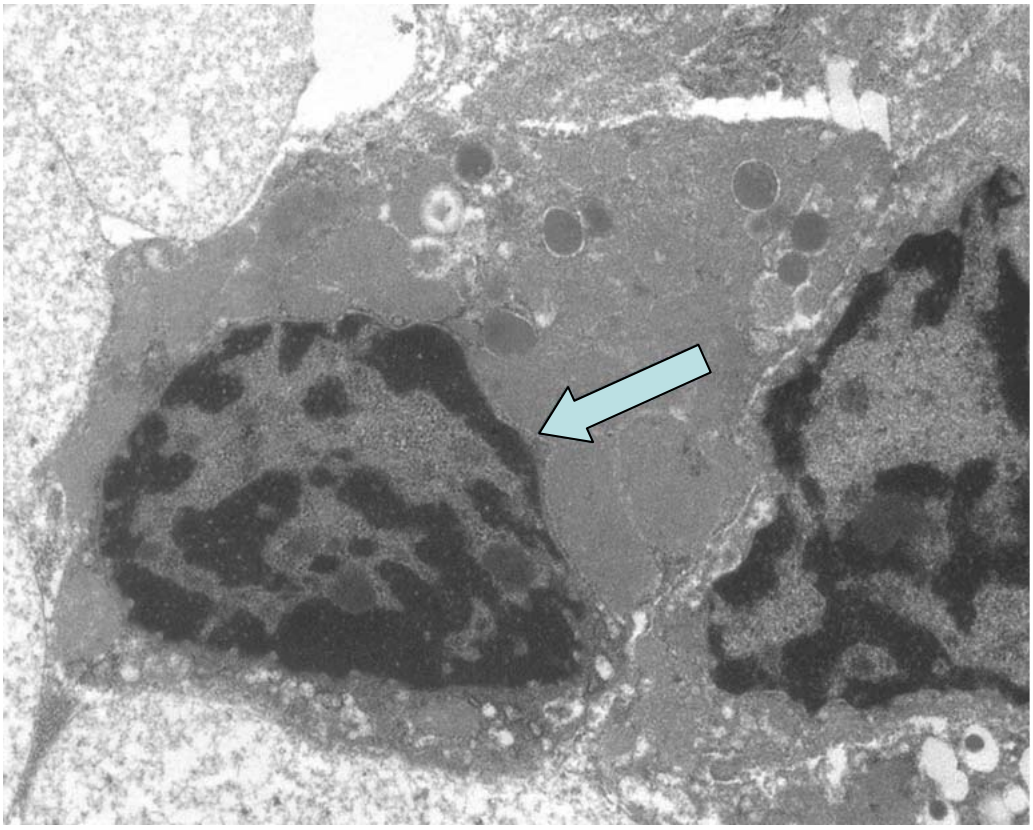

Figure 8.3. Electron microscopic view of the unidentified plugs noted in the bronchioles.

Electron microscopic examination of the lung tissue obtained from the wax plug shows cells in the lumen of the bronchiole. The cells do not show any ultra-structural differentiation and the precise nature of the cells could unfortunately not be identified.

However, as these plugs were only noted in guinea pigs exposed to ozone, they may be of significance and may warrant further investigation in another study. Based on the finding of Schelegle *et al.* (2002) that the epithelium surfaces of

exposed rats contained large numbers of exfoliated cells and cell debris, it is possible to suggest that these plugs are possibly degenerated epithelium cells.

It is widely believed that ozone cause airway inflammation and rapid damage of epithelial cells, and Van Bree *et al.,* (2002) reported results to confirm that even short-term ozone exposure (0.4 µg O_3/ml for 12 hours) does indeed cause significant airway inflammation and rapid damage of epithelial cells.

However, against expectations, all tracheal examples studied in this experiment showed the contrary. Apart from mild damage to ciliated cells as seen in Figure 8.1, all tracheal samples were found to be microscopically normal, and did not show any other cellular damage, oedema or inflammation after acute high-dose ozone exposure. This can possibly be explained by the fact that the duration of exposure (10 min), albeit at a much higher concentration than that employed by Van Bree *et al.*, was much shorter than experiments mentioned in the literature and insufficient for inflammation and ed cellular damage to develop. This is believed to be as a result of antioxidant defence-systems of the body not being overcome.

In summary, the results from this experiment propose that significant cellular injury and inflammation is possibly not the causative factor of breathing difficulty experienced after acute high-dose ozone inhalation as these, and particularly inflammation, is believed to probably only set in at a later stage.

Chapter 9
Summary and Conclusion

Ozone, a potent oxidant, in view of its interaction with biological material, can exhibit potential toxic effects (Kosmider *et al.,* 2010; Gottschalk *et al.*, 2010; Bocci, 2010; Lotriet *et al.,* 2007). It is clear that time and dose regimens of ozone administration are critical components to differentiate between potential beneficial and toxic effects. These aspects are of particular importance in view of the increased worldwide use of ozone as an alternative or a complimentary medicine.

The objective of this study was to examine, identify and substantiate the pulmonary effects of acute ozone exposure to fairly high doses (19.5 ± 0.5 µg/ml O_3), utilising three distinctly diverse models of ozone introduction: *In vitro* exposure*, in vivo* exposure*,* and an *ex vivo* isolated lung perfusion model which allows for breath-by-breath and real-time data acquisition of ozone's effect on respiratory mechanics.

The experimental model of an elevated ozone dose (to the extent of that applied during this study) being introduced to the respiratory system for only a brief duration of exposure is believed to be the first of its kind.

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9.1 Summary

In order for this study to be of analytical value and before any experiments could be carried out, an accurate method of measuring and validating ozone concentration in both air and aqueous media had to be developed and applied. Once this objective was realized, the yield of the ozone generator used in this study could be assessed (see § 4.1). In addition, the solubility (see § 4.2) and half-life ($t_{½;}$ see §

4.5) of ozone in aqueous media (water and Krebs-Henseleit physiological solution) at various known and controlled temperatures could be accurately calculated.

From these initial analytical studies, it was confirmed that the decay-rate of ozone in an aqueous medium is temperature-dependent, and that a higher temperature of the physiological solution will lead to a faster decay of ozone which, in turn, will result in a smaller $t_{½}$. The foremost findings from these studies were, however, the finding that ozone reacts with glucose in the Krebs-Henseleit physiological solution which results in a lower solubility of ozone (see § 4.2), and that glucose may be omitted from the perfusion medium without having any noticeable effect on the functioning of isolated tracheal tissue (see § 4.3). This critical result was, based on this finding, successfully supported and utilized by other studies performed within this department (Brink *et al.*, 2008; Van Niekerk, 2008; Pretorius, 2005).

As mentioned earlier, three diverse models of ozone introduction were utilised. In the first of these models *in vitro* experiments (see Chapter 5) were carried out on isolated tracheal tissue and demonstrated, beyond any doubt, the potential impact of acute high-dose ozone exposure on the respiratory system. Under experimental conditions, ozone itself set off a considerable contraction of the isolated tracheal tissue that was noticeable almost immediately following introduction to ozone (see § 5.2). A significant hyper responsiveness (an increase in the mean maximal contraction) of ozone-treated isolated tracheal tissue to subsequent methacholine treatment was also been observed (see § 5.4). This perceived hyper responsiveness of the isolated trachea to methacholine, although gradual, was found to be fully reversible.

The beneficial effects of β-sympathomimetic drugs (isoproterenol, etc.) on ozone-treated tracheal samples (of which muscarinic acetylcholine receptors were subsequently stimulated by means of exposure to methacholine), were attenuated by ozone treatment with respect to both time and extent of tracheal relaxation. Results from this study (see § 5.4) do however suggest that ozone does not have any

negative impact on muscarinic acetylcholine receptors, and therefore potentially excludes ozone-induced muscarinic acetylcholine receptor damage. These results, indicating potential attenuated β-sympathomimetic drug efficacy, warrants caution in the use of ozone in patients with pre-existing pulmonary conditions.

Further *in vitro* experiments carried out on isolated tracheal tissue indicated that an antioxidant (ascorbic acid was used in this study) may be of particular significance in the body's defence against the harsh oxidative effect of ozone on the respiratory system. *In vitro* pre-treatment of tracheal smooth muscle with ascorbic acid managed to considerably limit immediate ozone-induced contraction of these isolated tissue samples (see § 5.7). This result substantiates hypotheses that antioxidant components present in the lung reduce ozone–induced lung injury by counteracting oxidative stress. However, antioxidants do not provide adequate defence to avert the development of hyper responsiveness in ozone-treated tracheal preparations to muscarinic agonist stimulation.

By applying the same *in vitro* ozone introduction model, it was furthermore noted that indomethacin, a non-steroidal anti-inflammatory drug, reduced the *in vitro* ozone-induced contraction of tracheal smooth muscle (see § 5.6) if added to the organ bath immediately prior to exposure of the tracheal preparation to ozone. This has been suggested to be as a result of indomethacin's anti-inflammatory properties and its inhibition of bronco-constrictive prostaglandins, thereby supporting the belief that ozone exerts its deeper pulmonary (and non-pulmonary effects) not only by means of direct oxidative damage, but also by producing ozone-induced oxidative mediators which are though to induce a state of oxidative stress throughout the entire body.

In the second model of ozone introduction, acute ozone-inhalation studies on a lung perfusion model emphasized the destructive effect of ozone-inhalation on lung tissue. Lung elasticity, a factor affecting both inspiratory and expiratory volume, were significantly lowered after ozone exposure (see § 7.1), indicating that inhalation of high concentrations of ozone, even for short periods of time, may contribute to

irreversible emphysema-like symptoms. In the lung perfusion model it was furthermore established that antioxidants (ascorbic acid) markedly reduces the extent and rate of decline in lung compliance which is observed with ozone inhalation.

In the third and final model of ozone introduction, *in vivo* experiments revealed that acute high-dose ozone inhalation induce breathing difficulty (including rapid, shallow and laboured breathing), hiccups and mucous membrane irritation in the majority of animals exposed (see § 8.1).

If *in vitro* experiments are performed after initial *in vivo* ozone inhalation, a significant increase in maximal attainable contraction of the isolated tracheal smooth muscle after stimulation of muscarinic acetylcholine receptors with methacholine (see § 6.1) are noted. Although significant, the degree of hyper responsiveness observed is considerable smaller compared to that observed with methacholine treatment after *in vitro* exposure to ozone (see § 5.2). It is believed that this reduced response to a muscarinic agonist may be attributed to a defence mechanism of the body against oxidative damage which is more pronounced within living animals (and therefore more noticeable with *in vivo* ozone exposure) and is suspected to be related to antioxidant status within the body.

By performing an experiment in which the isolated tracheal tissue (which has already been exposed to ozone *in vivo*) is once more exposed to ozone *in vitro*, the possible influx of antioxidants via the blood to replenish antioxidant status within the respiratory system (one suggested method of action of the body's defence mechanism against oxidative damage) can be discounted. Results from this study confirms that the maximal attainable contraction of the isolated tissue to methacholine shows an additional response which, when added to the initial contraction, adds up to a combined response which is virtually identical to the maximal response seen after *in vitro* ozone exposure (see § 6.1). This result supports the above mentioned hypothesis that the difference in maximal attainable contraction to methacholine after *in vitro* ozone exposure as compared to *in vitro* ozone exposure

following initial *in vivo* ozone exposure, is potentially as a result of a more prominent *in vivo* antioxidant defence-system against oxidative damage.

By utilizing the same introduction model (*in vitro* ozone exposure following initial *in vivo* ozone), experiments carried out in a physiological solution devoid of calcium furthermore confirmed that ozone is likely to initiate airway smooth muscle contraction by directly affecting the recycling and release of intracellular Ca^{2+} in airway smooth muscle cells (see § 6.2).

Immediately after *in vivo* acute high-dose ozone inhalation, a series of tissue samples from the airways of test subjects were furthermore isolated and fixated in 10% formalin for microscopic evaluation. Optical microscopy revealed minor pulmonary oedema with mild ciliar damage to tracheal tissue (see § 8.1), whilst unidentified small, waxy plugs were noted in the lumen of bronchioles of guinea pigs exposed to ozone. Surprisingly, no ozone-induced inflammation or serious cellular damage was noted with microscopy. From this result is can be suggested that cellular injury and inflammation are probably not the causative factor of breathing difficulty experienced after acute high-dose ozone inhalation as these, and particularly inflammation, are believed to only set in at a later stage.

9.2 Conclusion

It may be concluded that the majority of findings in this study are in all probability the result of oxidative damage inflicted by ozone, either due to direct oxidation, or as the result of the formation of Reactive Oxidative Species (ROS) which rapidly diffuses throughout the respiratory system and possibly the whole body (Prior *et al.,* 1995), triggering generalised oxidative stress. The role of ozone on Ca^{2+} mobilization in smooth muscle should also not be discounted.

Based on these results, it should be emphasised that ozone concentrations in commercially available ozone generators and applications should be strictly controlled, especially in light of the various ozone related products and treatments

randomly entering the market at present without any form of legislation. The importance of ozone exposure in the workplace can furthermore not be emphasised enough, since chronic exposure to untoward levels may bring about irreversible adverse health effects.

Ozone does hold potential medical value, but if used irresponsibly, or by naïve and uninformed members of the public or alternative healthcare providers, the risk associated with ozone therapy definitely outweighs any potential benefit.

Index of Appendices

A. Publications and Conference Proceedings

Publications

THE PHARMACOLOGICAL EFFECTS OF OZONE ON ISOLATED GUINEA PIG TRACHEAL PREPARATIONS. Lotriet, C.J., Venter, D.P., Oliver, D.W. Innovative & Current Technologies, 22-25 August 2005. *Proceedings*: 17th World Congress & Exhibition, Ozone & Related Oxidants, August 2005

THE PHARMACOLOGICAL EFFECTS OF OZONE ON ISOLATED GUINEA PIG TRACHEAL PREPARATIONS. Lotriet, C.J., Oliver, D.W., Venter, D.P. *Archives of Toxicology* (2007) 81:433-440

Presentations

International:

THE PHARMACOLOGICAL EFFECTS OF OZONE ON ISOLATED GUINEA PIG TRACHEAL PREPARATIONS. Lotriet, C.J., Venter, D.P., Oliver, D.W. 17th World Congress & Exhibition, Ozone & Related Oxidants, Innovative & Current Technologies, 22-25 August 2005, Strasbourg, FRANCE

TIME AND DOSE INVESTIGATION ON THE CELLULAR ACTIONS OF OZONE. Oliver, D.W., Brink, C.B., Venter, D.P., Pretorius, A., Lotriet, J.C. 14-17 November 2006, 15th Alexandria International Dental Congress, Alexandria, EGYPT

OZONE IN MEDICINAL RESEARCH. Oliver, D.W., Lotriet, J.C., Venter, D.P., Brink, C.B. Research Lecture: North-Eastern University School of Medicine and Pharmacy, 22-27 July 2008, Acron Ohio, USA

OZONE IN MEDICINAL RESEARCH AND APPLICATION: Invited Speaker: Oliver, D.W., Brink, C.B., Venter, D.P., van Niekerk, B.P.J., Pretorius, A., Lotriet, J. 5th International Symposium on Computational Methods in Toxicology and Pharmacology: Integrating Internet Resources, 4-8 July 2009, Istanbul, TURKEY

National:

OZONE IN MEDICINE. Lotriet, C.J., Oliver, D.W., Venter, D.P. School of Pharmacy, Workshop on Ozone, North-West University, 14-15 November 2005, POTCHEFSTROOM

MOLECULAR PHARMACOLOGY AND OZONE TRACEAL INTERACTIONS (Lecture). Lotriet, C.J., Oliver, D.W., Venter, D.P., North-West University (Potchefstroom Campus), School of Pharmacy, Post Graduate Workshop on Drug Research and Development in Veterinary Sciences, 8 September 2005, POTCHEFSTROOM

EFFECT OF OZONE ON THE RAT TRAGEA. Lotriet, C.J., Oliver, D.W., Venter, D.P., 37th Annual Congress of the South African Pharmacology Society, Tygerberg Campus, University of Stellenbosch, Sept 2003, Tygerberg, BELLVILLE

B. Published Article

Arch Toxicol
DOI 10.1007/s00204-006-0159-0

ORGAN TOXICITY AND MECHANISMS

The pharmacological effects of ozone on isolated guinea pig tracheal preparations

C. J. Lotriet · D. W. Oliver · D. P. Venter

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Abstract Ozone is a potent oxidizing agent with a variety of potential uses, including its antimicrobial and deodorising properties. The recent increased use of ozone led to questions regarding its safety in humans. This study specifically focussed on the in vitro effect of ozone on isolated guinea pig tracheal tissue as well as its effect on the isolated trachea in the presence of various drugs with well-known effects, including methacholine and isoproterenol. The results found in this study identified two direct effects on the isolated trachea due to ozone exposure: (1) a definite contraction of the isolated trachea immediately after exposure to ozone, and (2) a clearly visible and significant hyper responsiveness of the isolated trachea to irritants, e.g. methacholine. Although ozone has a negative effect on the trachea, it was concluded that ozone has no adverse effect on muscarinic receptors. We found that ozone has a significant desensitizing effect on the pharmacological response of β sympathomimetics (isoproterenol), while isoproterenol itself has a relaxing effect on the ozone-induced contraction of the isolated trachea. Observations in this in vitro study further emphasised that ozone does have a negative effect on respiratory health. It is underlined that the inhalation of ozone should be avoided by workers who are often in contact with the gas, and especially by those with existing airway diseases. An apparent EC_{50} value of ozone on the trachea was established by two different methods as 5.71 and 9.78 × 10^{-3} M, respectively.

Keywords Isolated trachea · Hyper responsiveness · Methacholine · Isoproterenol · Ozone concentration–response curve

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Introduction

Ozone is a major air pollutant that has been known to cause toxic pulmonary effects in animals and man for decades (Stokinger 1965). Inhalation of ozone can induce rapid damages of epithelial cell membranes in the pulmonary airways. Because of its powerful anti-bacterial properties, and the availability of ozone generators, ozone is increasingly being used as disinfectant in industry as well as in offices and at home. Various adverse sequelae of ozone exposure have been documented including increased airway hyper responsiveness, epithelial sloughing, and neutrophil influx in the airways (Schelegle et al. 1991; Hyde et al. 1992; Park et al. 2004). Calculations suggest that the high reactivity of ozone and its low solubility in water would prevent it from passing through the lung epithelial lining fluid to act directly with the underlying epithelial cells (Pryor 1992). The mechanism by which ozone causes cell injury is linked to its powerful oxidative capacity and involves the peroxidation of cell membrane components (Wright and Wheeler 1990). Once inhaled, ozone triggers, in lungs, the production of reactive oxygen species, induces the release of inflammatory factors such as prostaglandins, and stimulates the sensory afferents (Kafoury et al. 1999).

In this study, we focused on the effects of ozone on the isolated trachea and its ability to cause bronchoconstriction.

Materials and methods

Tissue preparations

Duncan Hartley Guinea pigs (450–550 g) of both sexes were killed by carbon dioxide asphyxiation for 5 min, and the tracheas were removed within 3 min of death. The trachea was rapidly removed and manually freed from connective and other tissues. Each of the isolated tracheas was cut in length from top to bottom through the cartilaginous rings on the opposite side of the tracheal muscle according to the technique of Timmerman and Scheffer (1968). The trachea was folded open and five incisions were made on alternate sides of the tracheal muscle, dissecting the muscle and leaving the trachea when stretched out to form a chain that is attached only at alternate parts of the cartilage. This tracheal chain was then suspended between two L-shaped stainless steel hooks and suspended in a 10 ml jacketed organ bath containing Krebs–Henseleit buffer solution. The Krebs–Henseleit solution was made to the specifications of Patil and Jacobwitz (1968) composing of 119 mM NaCl, 4.7 mM KCl, 1.9 mM $CaCl_2$, 0.54 mM $MgCl_2$, 24.0 mM $NaHCO_3$, 1.0 mM NaH_2PO_4, and 11.1 mM glucose.

Organ chambers were maintained at 37°C and continuously aerated with 95% oxygen and 5% CO_2 to maintain pH 7.4. The tracheal segments were initially set to 2 g of tension and were allowed to equilibrate for at least 60 min before drug additions. During the period of stabilization, the tissue was washed with Krebs–Henseleit solution at 15-min intervals and after the relaxation period, the tension in each tracheal segment was readjusted to 2 g before the experiment began. Isometric contractions were recorded using a Statham UL-2 force displacement transducer attached to a Metrohm Labograph (Model E-478) recorder.

Ozone preparation

Ozone was prepared by feeding ultra high purity (UHP) 99.995% oxygen (Afrox South Africa) into a Sterizone P-HP 250 (PCT/ZA00/00031) ozone generator.

Ozone solutions

The concentration of dissolved ozone in both Krebs–Henseleit and distilled water was measured using the Indigo Colorimetric Method (American Public Health Association 1995), which is based on the principle that ozone rapidly decolorizes indigo in an acidic solution. It was calculated that a Krebs–Henseleit solution saturated with ozone contains 0.0770 mg O_3/l at 37°C and 0.7771 mg O_3/l at 20°C, while saturated distilled water contained 12.439 mg O_3/l at 20°C and 2.910 mg O_3/l at 37°C and a mean atmospheric pressure of 658 mmHg. Water–ozone solutions used in experiments were always concentrated solutions of ozone in distilled water at 20°C (12.439 mg O_3/l). Under our experimental conditions saturation was reached within 10 min of continuous ozone bubbling. Since ozone decomposes rapidly, especially at higher temperatures, it is important to note that all ozone concentrations referred to (Figs. 3, 4, 5) are the initial ozone concentrations.

A series of experiments, in which each component of the Krebs–Henseleit buffer was individually subjected to ozone, indicated that the significant difference between ozone concentration in a saturated water–ozone solution and Krebs–Henseleit was due to the presence of glucose in the Krebs–Henseleit solution. This is to be expected since ozone oxidises glucose. The maximal concentration of ozone in Krebs–Henseleit, devoid of glucose, was similar to that found for distilled water. It was clear, therefore, that the glucose in Krebs–Henseleit solution adversely affects the ozone concentration and the experimental results. The only possibility was to conduct pharmacological experiments with glucose-free Krebs–Henseleit. In view of our concern that the absence of glucose may adversely affect the experiments on the isolated organs, we performed pharmacological studies on the isolated guinea pig trachea in the absence and presence of glucose. The result shown in Fig. 1 indicates clearly that, for the time period in which our experiments were conducted, there is no statistical difference between pharmacological results obtained in the absence and presence of glucose. Experiments performed in our laboratory on HeLA cells (human epithelial cells lining the small peripheral airways) have also shown that the absence of glucose from the Krebs–Henseleit solution had no significant detrimental effect on either mitochondrial activity or membrane integrity after the cells were exposed to glucose-free Krebs–Henseleit solution for at least 55 min (Pretorius 2005).

Glucose-free Krebs–Henseleit solution was added to each organ bath prior to methacholine and ozone exposure (Figs. 1, 3, 5). During the resting periods and for rinsing of tissue samples ‘normal’ (glucose containing) Krebs–Henseleit was used to incur physiological haemostasis.

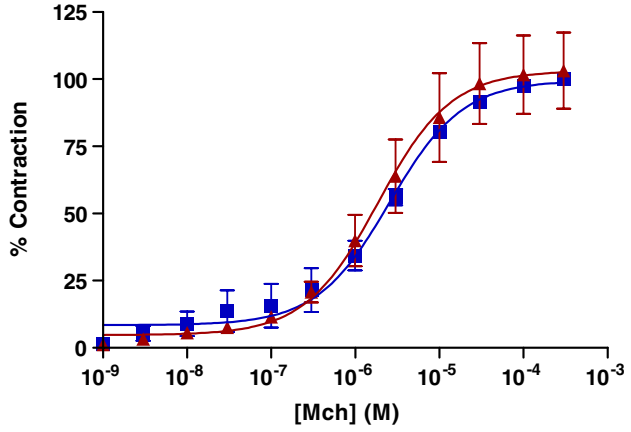

Fig. 1 Concentration–response curves of methacholine. Effect of glucose omission from Krebs–Henseleit solution on methacholine induced contraction. *Filled square* Krebs containing glucose; *filled triangle* Krebs without glucose

Concentration–response curve of methacholine

Cumulative concentration–response curves were determined according to the method described by van Rossum (1963). After the isolated trachea was suspended in the organ bath containing Krebs–Henseleit solution at 37°C, the tissue was allowed to equilibrate for 60 min prior to the beginning and 20 min between each set of experiments. Contractile responses to various concentrations are expressed as a percentage (%) of the maximal (100%) response to methacholine, E_{max}.

Concentration–response curves of methacholine in the presence of atropine (a competitive antagonist of methacholine on muscarinic receptors) were performed by firstly adding a single concentration atropine (1×10^{-7} M) to the organ bath wherein the trachea was suspended. After a 30 min equilibrium period, the concentration–response curve of methacholine in the presence of atropine was determined. The muscarinic blocking activity can be expressed in terms of a pA_2 ($-\log K_B$) value calculated according to the procedures described by van Rossum (1963).

Concentration–response curve of ozone

Concentration–response curves of ozone on the trachea were obtained by employing two different methods: In the first method multiple doses of a concentrated water–ozone solution (12.439 mg O_3/l) were added to a single isolated glucose-free tracheal preparation in increasing quantities. After the effect of each ozone-concentration was noted, the tissue was rinsed with Krebs–Henseleit solution containing glucose and allowed to equilibrate for 20 min prior to the addition of the next ozone concentration.

In the second method the concentration–response curve was obtained by employing a single ozone concentration per trachea. The trachea was first subjected to three successive concentrations of methacholine (1×10^{-4} M). If the maximal effect of the last two methacholine concentrations differed by less than 10% then the mean of these contractions (height of registration) was taken as maximal (100%). After each methacholine application the organ was rinsed and rested. After the third methacholine contraction a single ozone concentration was added to the organ bath and the obtained response of ozone was calculated as a percentage of the maximal methacholine response. A specific tracheal preparation was treated only once with ozone and discarded. The same procedure was repeated for different ozone concentrations. The response to each individual ozone concentration was recorded and used to construct the concentration–response curve shown in Fig. 3. The maximal response to ozone was taken as 100% response.

Experiments involving ozone

In a typical experiment the tracheal segments were washed with fresh Krebs–Henseleit solution and after the first control concentration–response curve was determined, the trachea was rested for 20 min and the baseline re-established. Ozone was then introduced to the tissue either as a gas bubbled through the Krebs–Henseleit solution, or as 1 ml of saturated water–ozone solution (12.439 mg O_3/l) added directly to the organ bath. Ozone exposure was limited to 10 min, which was the average time needed to obtain the maximal response. After treatment with ozone the tissue was rinsed with fresh Krebs–Henseleit solution, rested for 20 min and the second control cumulative concentration–response curve of methacholine was obtained.

Experiments involving ozone and isoproterenol

Isoproterenol (1×10^{-4} M), a β sympathomimetic used to relax smooth muscle contraction, was added to the organ bath in three variations of the experiment: (1) after the initial methacholine-induced contraction, (2) after ozone exposure, and/or (3) after the second methacholine-induced contraction (Fig. 9). Isoproterenol was freshly prepared prior to the start of each experiment.

Half-life of ozone

The half-life ($t_{½}$) of ozone at 37°C in glucose-free Krebs–Henseleit was obtained by measuring the decay

of ozone on a Shimadzu Multispec-1501. On average, $t_{½}$ was found to be 137 s.

Materials and reagents

The following drugs and chemicals were supplied by Sigma Chemical Co: methacholine, isoproterenol, and potassium indigo trisulphonate used for the indigo colorimetric method. Solutions were made using Milli-Q water (Millipore Pty Ltd, USA).

Statistical analysis

Data were analysed statistically by the unpaired Student's t test. If $P < 0.05$, the results were considered to be significant. The number of experiments performed (n) was never less than six.

Curve fitting

Graphpad Prism (version 4) was used to fit curves.

Results and discussion

Effect of ozone on the trachea

Figure 2 compares the maximal possible contraction (43.9%) elicited by a maximal ozone concentration to the maximal response (100%) that can be obtained with a high concentration methacholine (1×10^{-4} M). The maximal concentration of ozone in the organ bath was maintained by continuously bubbling ozone through the Krebs–Henseleit solution in which the trachea was suspended. Maximal response to ozone is maintained as long as the ozone concentration in the organ bath is maintained at the maximum level. If the tracheal preparation is washed with ozone-free Krebs–Henseleit solution, and allowed to rest for 20 min, the trachea returns to its original ground state, i.e. the baseline returns to zero.

Figure 3 shows the concentration–response curve of ozone. The apparent EC_{50} value of ozone is respectively 5.71×10^{-3} M when multiple ozone concentrations are used on a single trachea and 9.78×10^{-3} M when a single concentration is added per trachea. In comparison, the EC_{50} value of methacholine on the isolated trachea is 1.93×10^{-6} M. It follows from these EC_{50} values that methacholine is on average about 4×10^{3} times more potent than ozone, whilst the maximal response of ozone is about 44% that of methacholine (see Fig. 2).

The concentrations given in Fig. 3 are the initial concentrations. As ozone decomposes this initial concentration will decline at a steady rate and, therefore, the observed response for a specific concentration will actually be at a lower concentration. The onset of ozone contraction was almost immediate and the maximal response was usually obtained within 36 s. If the half-life of ozone ($t_{½} = 137$ seconds) in glucose-free Krebs–Henseleit at 37°C was taken into consideration, then in the time span of an average experiment the ozone concentration would decline by about 13% from the initial concentration. This decomposition of ozone is probably the reason why the concentration–response curve is hyperbolic. A satisfactory curve could not be obtained by employing the Hill's equation (variable slope sigmoidal dose response equation) and therefore the Boltzman sigmoidal equation was employed to fit the curves in Fig. 3.

It is interesting to note that the two concentration–response curves in Fig. 3 are identical at low

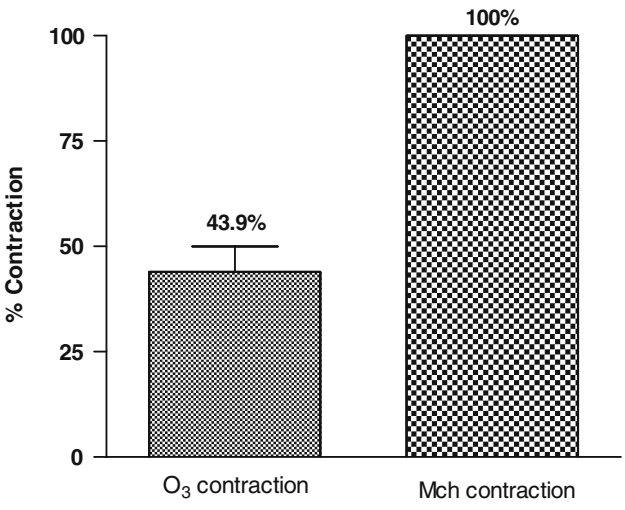

Fig. 2 In vitro comparison between ozone- and methacholine-induced tracheal contraction ($P < 0.05$; $n = 9$)

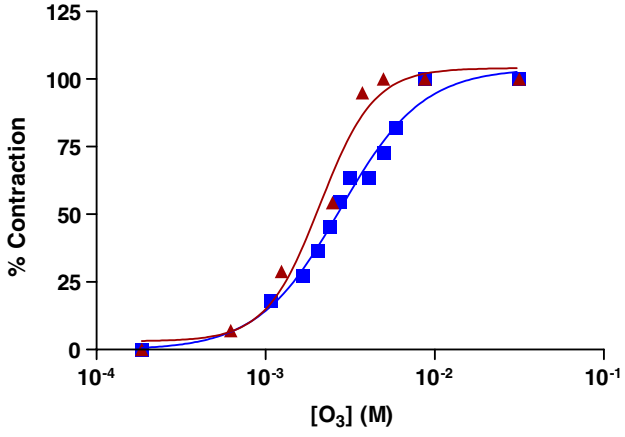

Fig. 3 Concentration–response curves of ozone. All ozone concentrations are the initial concentrations. *Filled square* Multiple doses on a single trachea; *filled triangle* single dose per trachea

concentrations, although they were determined by different methods. In the first method single concentrations were successively added to the same organ, whilst in the second method each concentration–response point was determined on a different tracheal preparation. It seems that the successive lower concentrations employed on a single organ had no detrimental effects on the trachea. At higher concentrations, however, the trachea became more unresponsive, i.e. an ozone concentration of 2.4×10^{-2} M induced a response of 94.9% (first method) whilst the same concentration induced a response of 69.7% (second method).

Effect of ozone on methacholine induced tracheal contraction

In Figs. 4 and 5 the control concentration–response curves of methacholine was firstly determined, and after thorough rinsing of the trachea with Krebs–Henseleit solution the organ was subjected to 1.0 ml of an ozone–water solution (12.439 mg O_3/l) plus 9 ml Krebs–Henseleit solution. After maximal response was obtained, the organ was again thoroughly rinsed with Krebs–Henseleit to ensure that all traces of ozone are removed. Hereafter the second concentration-response curves of methacholine was determined. Results shown in Fig. 4 were obtained by employing 'normal' Krebs–Henseleit solution, i.e. this Krebs–Henseleit solution contained glucose, whilst the results shown in Fig. 5 were obtained by employing a Krebs–Henseleit solution from which glucose was omitted.

It can clearly be seen in both Figs. 4 and 5 that the maximal responses of the second concentration–response curves, i.e. the methacholine curves determined after ozone incubation, is much larger than the maximal responses obtained with the control curves (see Table 1).

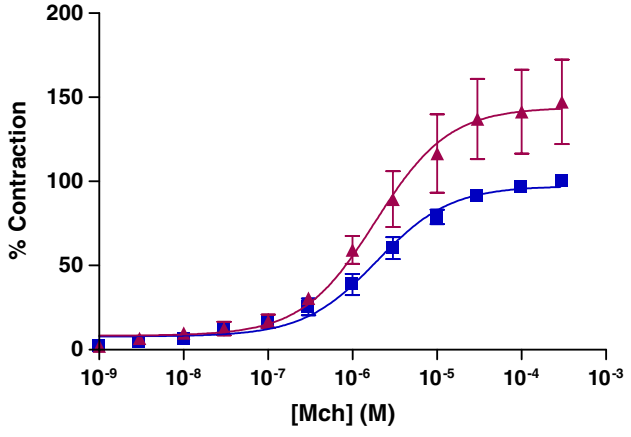

Fig. 4 Concentration–response curves of methacholine. In vitro effect of ozonated water on the trachea in a Krebs solution containing glucose. *Filled square* Initial (control) methacholine-induced contraction before ozone-exposure; *filled triangle* methacholine-induced contraction after ozone-exposure

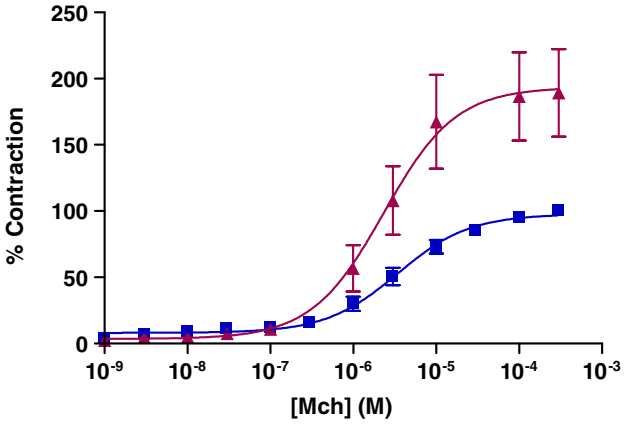

Fig. 5 Concentration–response curves of methacholine. In vitro effect of ozonated water on the trachea in a Krebs solution without glucose. *Filled square* Initial (control) methacholine-induced contraction before ozone-exposure; *filled triangle* methacholine-induced contraction after ozone-exposure

It is obvious, therefore, that the ozone sensitized the trachea leading to this observed hyper responsiveness. As can be seen in Table 1 the ozone had practically no effect on the EC_{50} values of methacholine, indicating that ozone possibly does not influence the interaction of methacholine and its receptors. It therefore appears that the muscarinic receptors have not been affected by the ozone. To verify that the muscarinic receptors are still intact, we determined pA_2 values for the muscarinic antagonist, atropine, on a normal ($pA_2 = 8.52 \pm 0.25$) and ozonated ($pA_2 = 8.42 \pm 0.36$) tracheal preparation. This finding that the affinity values of atropine for muscarinic receptors, located in the mentioned tracheal preparations, are practically identical underlines the notion that ozone has no effect on these receptors.

The finding that the maximal response seen in Fig. 4 (normal Krebs–Henseleit) increased with 44%, whilst the maximal response seen in Fig. 5 (glucose-free Krebs–Henseleit) increased by 94%, may be ascribed to the reaction of ozone with glucose. It follows that the ozone concentration in 'normal' Krebs–Henseleit (Fig. 4) decreases at a much faster rate than the ozone

Table 1 Maximal responses and EC_{50} values obtained from Figs. 4 and 5

	EC_{50} values of methacholine (M)		Maximal response of methacholine (%)	
	Before O_3 incubation	After O_3 incubation	Before O_3 incubation	After O_3 incubation
Figure 4	1.93×10^{-6}	1.86×10^{-6}	100	144
Figure 5	3.43×10^{-6}	2.37×10^{-6}	100	194

concentration in glucose-free Krebs–Henseleit (Fig. 5). By performing experiments in 'normal' Krebs–Henseleit the trachea is subjected to lower ozone concentrations than with glucose-free Krebs–Henseleit, resulting in a lesser degree of hyper responsiveness. Therefore, it seems that the degree of the observed hyper responsiveness depends on the concentration of ozone to which the trachea was subjected.

Figure 6 shows the concentration–response curves for methacholine before and after treatment of the isolated tracheal preparation with a maximal ozone concentration. In this experiment the ozone concentration in the organ bath, wherein the trachea was suspended, was kept constant by continuously bubbling ozone through the Krebs–Henseleit solution and thus subjecting the trachea for 10 min to the maximal ozone concentration. This figure also shows clearly that the ozone treated tracheal preparation was much more hyper responsive to methacholine than the untreated (control) preparation. The maximal response after ozone treatment was 174%, as compared to the 100% response obtained with the control (methacholine).

Although the observed ozone induced hyper responsiveness shown in Fig. 6 declined with time and the trachea eventually returned to its normal state, the trachea maintained an appreciable sensitivity to methacholine even after the ozonated trachea was allowed to rest for 2 h. After a 2-h resting period, the maximal response of methacholine was still about 120%. It is important to note that the observed hyper responsiveness in the trachea continued even after it was thoroughly rinsed with ozone-free Krebs–Henseleit solution. This result suggests that the hyper responsiveness is caused by changes in the trachea brought about by the reaction of ozone with the trachea.

Figure 7 shows the average hyper responsiveness of the isolated trachea to methacholine as a function of time (contraction before and after ozone exposure). The time stated in Fig. 7 is the time it takes to determine the cumulative concentration–response curves for methacholine as shown in Fig. 5, i.e. the time that is required to reach maximal response after cumulative addition of the methacholine to the organ bath. In the control experiment 9.2 min total time was required to determine the concentration–response curve (from 0 to 100% response). After the trachea was exposed to ozone the same experiment was completed in 6.2 min. It follows that the rate at which the ozonated trachea contracts (in minutes) increased with 32.2%. The latter increase is also indicative of the hyper responsiveness of the ozone treated tracheal preparation.

The effect of ozone on the responsiveness of the isolated trachea to isoproterenol

Figure 8 shows the effect of ozone on the relaxation of the trachea with a single concentration isoproterenol (1×10^{-4} M) as a function of time. The trachea was maximally contracted with a single addition of a high concentration of methacholine (1×10^{-4} M) and then relaxed by addition of a single concentration isoproterenol (1×10^{-4} M). The isoproterenol concentration (1×10^{-4} M) was chosen because it would give maximal relaxation under normal experimental conditions (Timmerman and Scheffer 1968). In the control experiment the single concentration isoproterenol relaxed

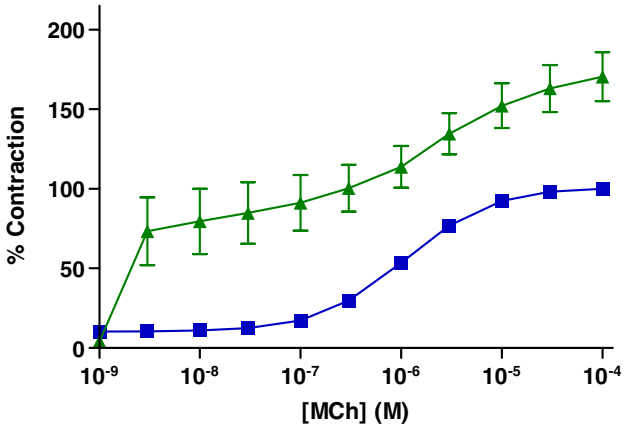

Fig. 6 Concentration–response curves of methacholine showing the hyper responsiveness of the isolated trachea to methacholine. The ozone concentration kept constant by bubbling it through the Krebs solution. *Filled square* Initial (control) methacholine-induced contraction before ozone-exposure; *filled triangle* methacholine-induced contraction after ozone-exposure ($P < 0.05$; $n = 5$)

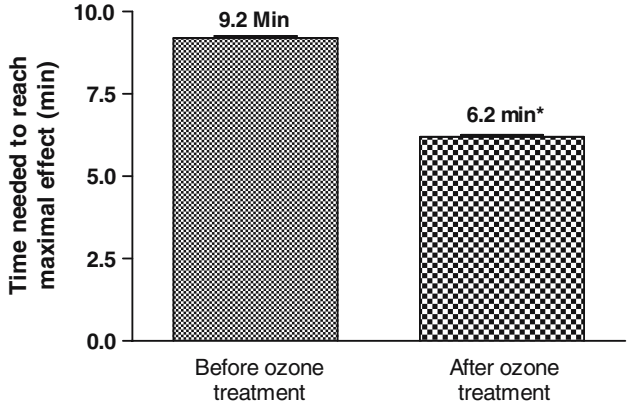

Fig. 7 Hyper responsiveness of the isolated trachea to methacholine as a function of time. A graphical comparison of the time needed to determine the cumulative concentration–response curves of methacholine as shown in Fig. 5. The initial methacholine-induced contraction reached a maximum after 9.2 min, while the same level was reached within 6.2 min after ozone exposure. *$P < 0.05$

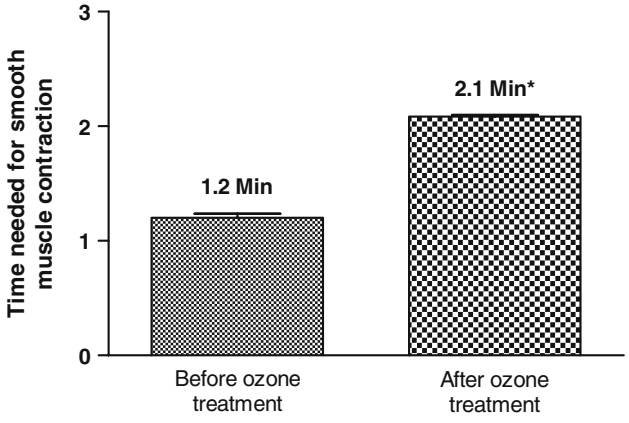

Fig. 8 The response of ozone on isoproterenol function. It took longer to relax a methacholine-induced contraction of the trachea after ozone exposure. $*P < 0.05$

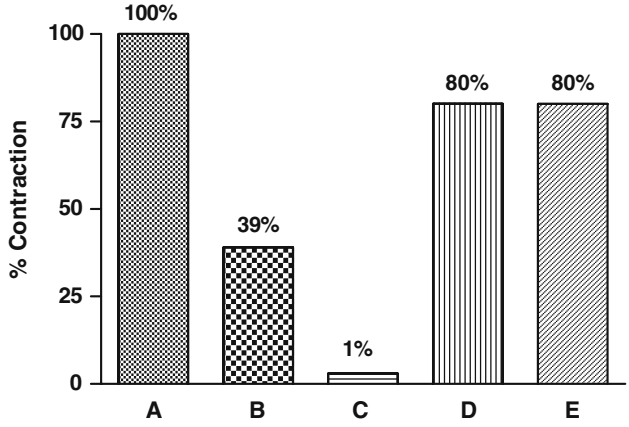

Fig. 9 Graphical representation of the responses of single concentrations of isoproterenol (1×10^{-4} M) and methacholine (1×10^{-4} M). *A* Initial (control) single concentration methacholine-induced contraction of the trachea. *B* Ozone-induced contraction of the trachea. *C* Relaxation of the ozone-induced contraction with a single concentration isoproterenol. *D* Contraction of the trachea with a single concentration methacholine. *E* After treatment of the methacholine-induced contraction (curve *D*) with a single concentration isoproterenol

the trachea within 1.2 min, whereas a tracheal preparation which was first exposed to ozone reacted much slower and the maximal response was only obtained after 2.1 min. Ozone exposure of the trachea therefore increased the normal time required to relax the methacholine-induced contraction of the trachea by 75%. This result points to the possibility that the effectiveness of β sympathomimetics (used by asthmatics) may be reduced when it is used by an asthmatic during an asthma attack caused by inhalation of ozone.

The response of isoproterenol and methacholine after ozone treatment

Figure 9 summarizes the responses of methacholine and isoproterenol on the original and ozonated tracheal preparation. In these experiments the trachea was rinsed and rested after each subsequent contraction. The contraction of the trachea with ozone (curve *B*) was relaxed with a single concentration (1×10^{-4} M) isoproterenol to afford curve *C*. The trachea was again contracted with a single concentration methacholine (1×10^{-4} M) and 80% of the initial contraction was obtained (curve *D*). The latter contraction with methacholine, a control experiment, was performed to assure that the trachea is functioning normally. Interestingly, however, this second contraction (80% contraction) of the organ with 1×10^{-4} M methacholine (curve *D*) could not be relaxed with isoproterenol (1×10^{-4} M) (curve *E*). The latter unresponsiveness of the trachea can only be ascribed to the effect of ozone on the trachea, since it is known that isoproterenol relaxes methacholine induced successive contractions of the trachea (Timmerman and Scheffer 1968). Although it has been reported that the effects of β sympathomimetics on methacholine-induced contraction gradually decreases after repeated application of these agents for 45–60 min (Barnes 1995; Kume and Takagi 1997), it is not expected that the β sympathomimetics would desensitize β receptors in the time course of the in vitro experiments conducted in this study. However, we have also confirmed experimentally that repeated successive methacholine contractions and subsequent relaxations of the trachea with isoproterenol had no detrimental effect on the responsiveness of the trachea for either methacholine or isoproterenol. This indicates that the trachea became desensitized to isoproterenol after the initial action of isoproterenol (curve *C*). The implication of this result for asthmatics is that an asthma attack that results from ozone exposure may be treated with a β sympathomimetic (isoproterenol) (also see Fig. 7), but subsequent ozone induced asthmatic attacks may be ineffective when treated with β sympathomimetics.

Conclusion

Ozone is known to have toxic pulmonary effects (Stokinger 1965) and results from this investigation indicate that these effects are also observed during in vitro experiments. Some of the effects observed include significant contraction of isolated tracheal smooth muscle after exposure to ozone. This contraction is an indication of bronchoconstrictive effects that can be observed in vivo, as reported by Linn et al. (1982).

After exposure to ozone, the isolated tracheal tissue also showed a significant hyper responsiveness towards

bronchoconstrictors such as methacholine. The maximal response observed with methacholine increased considerably and was reached in a shorter time (32.2% faster) at lower methacholine concentrations than those needed to reach a maximal response prior to ozone exposure. This observed hyper responsiveness was still present and clearly observable after thoroughly rinsing the organ and allowing it to rest for 2 h. The degree of hyper responsiveness seems to depend on the ozone concentration.

Finally, it is concluded that patients with underlying airway disease (e.g. asthma or allergic conditions) should especially be aware of the effects of ozone and the fact that the therapeutic affectivity of certain bronchodilators (especially β sympathomimetics) may decrease or even diminish after ozone exposure. The possible clinical significance of the latter finding should be confirmed by conducting appropriate in vivo studies.

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C. Dunnett's Multiple comparison Test

Table C.1. A Dunnett's Multiple Comparison Test indicting that there is a signicant ($p<0.05$) statistical difference between ozone and each individual salt in a Krebs-Henseleit solution (*n*=8).

	Mean Diff	*q*	*P <0.05?*	*95% CI of diff*
H_2O vs $NaHCO_3$	0.9500	3.879	Yes	0.2812 to 1.619
H_2O vs KCl	0.7600	3.103	Yes	0.09118 to 1.429
H_2O vs $CaCl_2.2H_2O$	-3.360	13.72	Yes	-4.029 to -2.691
H_2O vs $MgCl_2.6H_2O$	1.910	7.799	Yes	1.241 to 2.579
H_2O vs $NaHCO_3$	1.670	6.819	Yes	1.001 to 2.339
H_2O vs NaH_2PO_4	1.430	5.839	Yes	0.7612 to 2.099
H_2O vs Glucose	14.84	60.60	Yes	14.17 to 15.51

D. Glossary of Abbreviations

CL	Lung Compliance
COPD	Chronic Obstructive Pulmonary Disease
EIM	External Input Module
ELF	Epithelial Lining Fluid
E_{max}	Maximal (100%) Response to Metacholine
EPA	United States Environmental Protection Agency
FDA	United States Food and Drug Administration
FEV_1	Forced Expiratory Volume in One Second
FVC	Forced Vital Capacity
HeLA	Henrietta Lacks cervical carcinoma epitheloid cells
IC	Inspiratory Capacity
ILMP	Isolated Lung Perfusion Model
KH+G	Krebs-Henseleit containing Glucose
KH-G	Krebs-Henseleit devoid of Glucose
M	Molar
Min	Minutes
mM	Millimolar

nm	Nanometer
O_2	Diatomic Oxygen
O_3	Ozone
ODS	Ozone-Depleting Substances
ppb	Parts Per Billion
ppm	Parts Per Million
PUFA	Polyunsaturated Fatty Acids
ROS	Reactive Oxygen Species
SABS	South African Bureau of Standards
SANS	South African National Standards
SR	Sarcoplasmic Reticulum
$T_{½}$	Half-Life
TCM	Timer Counter Module
TLC	Total Lung Capacity
UHP	Ultra-High Purity
VCM	Rotary Vane Compressor Pump

E. Proof of Ethical Approval

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11 Februarie 2005

Geagte prof Oliver

GOEDKEURING VIR EKSPERIMENTERING MET DIERE

Hiermee wens ek u in kennis te stel dat u projek getiteld *"Farmakologiese effekte van osoon"* goedgekeur is met nommer 05D02.

Gebruik asseblief die nommer genoem in paragraaf 1 in alle korrespondensie rakende bogenoemde projek en let daarop dat daar van projekleiers verwag word om jaarliks in Junie aan die Etiekkomitee verslag te doen insake etiese aspekte van hulle projekte asook van publikasies wat daaruit voortgespruit het. U sal in Mei vanjaar die dokumentasie hieroor ontvang.

Goedkeuring van die Etiekkomitee is vir 'n termyn van hoogstens 5 jaar geldig (volgens Senaatsbesluit van 4 November 1992, art 9.13.2). Vir die voortsetting van projekte na verstryking van hierdie tydperk moet opnuut goedkeuring verkry word.

Die Etiekkomitee wens u alle voorspoed met u werk toe.

Vriendelike groete

Me Estelle le Roux
SEKRETARIAAT : ETIEKKOMITEE

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