THE EFFECT OF MATERNAL NICOTINE EXPOSURE ON CELLULAR SENESCENCE IN THE LUNGS OF THE OFFSPRING

YUSRAH SALIE

A thesis submitted in partial fulfilment of the requirements for the degree of Magister Scientiae in the Department of Medical Biosciences, University of the Western Cape.

Supervisor: Professor G.S. Maritz

November 2012
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Lung Development; Fetal Programming; Tobacco Smoking; Nicotine; Maternal Exposure; Cell Senescence; Premature Aging; Emphysema; Anti-Oxidant; Lycopene; Alveoli; Gestation
ABSTRACT

THE EFFECT OF MATERNAL NICOTINE EXPOSURE ON CELLULAR SENESCENCE IN THE LUNGS OF THE OFFSPRING

Yusrah Salie

M. Sc thesis, Department of Medical Biosciences, University of the Western Cape.

Several studies conducted in laboratories at the University of the Western Cape has demonstrated an interference with the parenchymal lung tissue of the offspring when exposed to nicotine (smoking cigarettes and/or Nicotine Replacement Therapy [NRT]), maternally i.e. during gestation and lactation. This in turn, decreases the amount of air sacs (alveolar number) resulting in a reduced surface area available for efficient gas exchange in the offspring. Since the foetus and offspring are only exposed to nicotine during gestation and lactation, emphysema-like lesions appear to develop after nicotine withdrawal in the foetus.

It has been proposed that during lung development in utero, a change in the “program” that controls the maintenance of lung integrity will occur in the long term due to the initial maternal nicotine exposure. Therefore, animals that were exposed to maternal nicotine resemble lungs that have undergone rapid, premature aging caused by cellular senescence. Furthermore, energy metabolism
and structural changes in the glycolytic pathways appear irreversibly slower compared to animals that were not exposed to nicotine via the mother during gestation and lactation, resulting in a reduction in the anti-oxidant capacity of lung development. Previous studies have also shown that strong anti-oxidants supplemented by smoking mothers during gestation and lactation could possibly resist change in the “program” which controls lung development and integrity of the offspring in the long term. Lycopene – as a strong anti-oxidant supplementation have shown to decrease the alveolar volume and increase the alveolar surface area for better gas exchange after the offspring has been exposed to maternal nicotine.

In this study I have treated pregnant wistar rats with nicotine, tomato juice (containing lycopene among other phytonutrients), and a combination of nicotine and tomato juice during gestation, to determine various changes in the lung structure and signs of premature aging in the lungs of the offspring. I have also performed various staining techniques such as H&E, connective tissue and β-galactosidase staining which indicated whether maternal nicotine exposure indeed induced premature cellular senescence in the lungs of the offspring.

November 2012
DECLARATION

I declare that *The Effect of Maternal Nicotine Exposure on Cellular Senescence in the Lungs of the Offspring* is my own work, that it has not been submitted for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

Yusrah Salie

November 2012

Signed: ...............................................

UNIVERSITY of the WESTERN CAPE
DEDICATIONS

I dedicate this Master of Science thesis to my supervisor, Professor G.S. Maritz, in hopes of gaining more enlightenment on the harmful effects of nicotine, and discovering effective strategies in preventing the adverse effects of nicotine to the mother and her unborn child in the long term. And I also dedicate this thesis to my dearest parents, Abdullah Salie, Sharifa Mostert, and Jasmine Bassier, who raised me with unconditional love and support throughout the years, and always allowed me to follow my dreams.

I sincerely thank my husband, Adiel Bassier, from the bottom of my heart, for his utmost understanding, encouragement, inspiration, support, and endless love throughout this project. Thank you to my dearest family and friends, for their well wishes, and sincere prayers, it will always be appreciated.

I would like to express my utmost gratitude to Thee Almighty, Our Creator, for granting me this wonderful opportunity. He made difficult situations easy by allowing me to persevere, and protected my health throughout this project. Most importantly, I thank Him for blessing my life with such wonderful people. All Praise Is Due To You, Lord Of The Worlds.
ACKNOWLEDGEMENTS

I would like to give thanks to the National Research Foundation for funding and the Research Committee of the University of the Western Cape for funding this project.

I would also like to express my appreciation to my supervisor, Professor G.S. Maritz, for all his mentoring, guidance, and dedication in making this research project successful. And also, thank you to my colleague, Ms M. Mutemwa, for all her inspiration and setting an excellent academic example.

The assistance of the staff of the Animal facility of the University of the Western Cape is also much appreciated.
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<tr>
<td>%</td>
<td>percentage</td>
</tr>
<tr>
<td>α</td>
<td>alpha</td>
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<td>β</td>
<td>beta</td>
</tr>
<tr>
<td>µ</td>
<td>micro</td>
</tr>
<tr>
<td>53BPI</td>
<td>p53 building protein</td>
</tr>
<tr>
<td>5-HT3 receptors</td>
<td>5 hydroxytryptamine</td>
</tr>
<tr>
<td>A1AT</td>
<td>alpha-1-antitrypsin</td>
</tr>
<tr>
<td>Ach</td>
<td>acetycholine</td>
</tr>
<tr>
<td>AIDS</td>
<td>acquired immune deficiency disease</td>
</tr>
<tr>
<td>AMP</td>
<td>adenosine monophosphate</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>AT-LPL</td>
<td>adipose tissue – lipoprotein lipase activity</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BMP-4</td>
<td>bone morphogenic protein 4</td>
</tr>
<tr>
<td>BW</td>
<td>body weight</td>
</tr>
<tr>
<td>BW/Lv</td>
<td>body weight/lung volume ratios</td>
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<tr>
<td>CC</td>
<td>chest circumference</td>
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<td>CC/CRL</td>
<td>chest circumference/crown-rump length ratios</td>
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<tr>
<td>CC/Lv</td>
<td>chest circumference/lung volume ratios</td>
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<tr>
<td>Cd</td>
<td>cadmium</td>
</tr>
<tr>
<td>Co</td>
<td>cobalt</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>COPD</td>
<td>chronic obstructive pulmonary disease</td>
</tr>
<tr>
<td>CRL</td>
<td>crown-rump length</td>
</tr>
<tr>
<td>CSC</td>
<td>cigarette smoke condensate</td>
</tr>
<tr>
<td>CSE</td>
<td>cigarette smoke extract</td>
</tr>
<tr>
<td>Cst</td>
<td>static compliance of the lung</td>
</tr>
<tr>
<td>Cu</td>
<td>copper</td>
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<tr>
<td>dH₂O</td>
<td>distilled water</td>
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<td>DMF</td>
<td>dimethylformaldehyde</td>
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<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<tr>
<td>EGF</td>
<td>endothelial growth factor</td>
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<td>F generation</td>
<td>filial generation</td>
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<tr>
<td>Fe</td>
<td>iron</td>
</tr>
<tr>
<td>FEV₁</td>
<td>forced expiratory volume in 1 second</td>
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<tr>
<td>FGF</td>
<td>fibroblast growth factor</td>
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<td>GABA receptors</td>
<td>gamma aminobutyric acid</td>
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<td>GPI</td>
<td>glucosphosphateisomerase</td>
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<td>H&amp;E</td>
<td>haematoxylin and eosin</td>
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<td>H3</td>
<td>histone 3</td>
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<td>HIF2α</td>
<td>hypoxia inducible transcription factor 2</td>
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<td>HNF-3β</td>
<td>hepatocyte nuclear factor – 3 beta</td>
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<td>IHD</td>
<td>ischaemic heart disease</td>
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<td>IUGR</td>
<td>intra-uterine growth restriction</td>
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<td>KCl</td>
<td>potassium chloride</td>
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<td>LDL</td>
<td>low density lipoprotein</td>
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<td>Abbreviation</td>
<td>Definition</td>
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<td>Lm</td>
<td>linear intercept</td>
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<td>lysyl oxidase</td>
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<td>Lv</td>
<td>lung volume</td>
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<td>MREII</td>
<td>metal response element (DNA)</td>
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<td>Na</td>
<td>alveolar number</td>
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<tr>
<td>Na$_2$HPO$_4$</td>
<td>disodium hydrogen phosphate</td>
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<td>NAC</td>
<td>N-Acetylcysteine</td>
</tr>
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<td>NaCl</td>
<td>sodium chloride</td>
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<td>NE3</td>
<td>neutrophil elastase 3</td>
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<tr>
<td>Ni</td>
<td>nickle</td>
</tr>
<tr>
<td>NO$_2$</td>
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<td>nicotine replacement therapy</td>
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<td>PAH</td>
<td>polycyclic aromatic hydrocarbons</td>
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<tr>
<td>PAI</td>
<td>plasminogen activator inhibitor</td>
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<td>PBS</td>
<td>phosphate-buffered saline</td>
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<td>PGM</td>
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<tr>
<td>PRb</td>
<td>retina blastoma</td>
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<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
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<td>SAHF</td>
<td>senescence associated heterochromatic foci</td>
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<tr>
<td>SA-β-gal</td>
<td>senescence associated β-galactosidase</td>
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<td>SK-hep1</td>
<td>human hepatocellular carcinoma cell</td>
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<tr>
<td>SLPI</td>
<td>secretory leukocyte peptidase inhibitor</td>
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<td>SO$_2$</td>
<td>sulphur dioxide</td>
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TB  tuberculosis
TJC  tomato juice consumption
TTF-1 transcription factor
Va  alveolar air volume density
Valv alveolar volume
VEGF vascular endothelial growth factor
Vt  tissue volume density
WHO world health organization
Xga  5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside
Zn  zinc
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CHAPTER ONE

Literature Review

1.1 LUNG DEVELOPMENT

The process of lung development occurs mainly in utero for most mammalian species. It begins early in embryogenesis and proceeds until the final stages of development are reached postnatally. Subsequently, postnatal lung development occurs within a very different, physical environment than in utero, thus the transition to air breathing must have a major impact on lung development. A change in lung expansion is allowed mainly by tissue stretch, and this stimulus influences cellular proliferation, cell differentiation, and the three-dimensional tissue structure of the terminal gas-exchange units. The absence of this tissue stretch stimulus would result in the impediment of lung growth and structural development (Hooper et al, 2006).

1.2 PHASES IN LUNG DEVELOPMENT

In humans lung development begins at 3-4 weeks of gestation, whereas in rats it occurs from 11.5 days post-conception (Warburton et al., 2005).

The phases of lung development can be divided into two groups, namely, prenatal and postnatal periods, whereby each group consists of various phases. All the phases of lung development show the same structural characteristics in all mammals.
1.2.1 Prenatal Period

It is increasingly evident that conditions *in utero* during lung development influences lung growth, and that any abnormality will be carried out into adulthood (Barker, 1995). The primary goal of lung development is to produce a large area for gas exchange within a relatively small thorax, which allows for branching morphogenesis of the airways and the formation of a vascular system (Hislop, 2008). The prenatal period can be divided into the:

1.2.1.1 Embryonic Phase

Embryonic Phase (1-7 weeks gestation in humans): During this phase initial budding and branching of the lung buds form from the primitive foregut. This phase is all under programmed or genetic control. There is also interaction between pulmonary blood vessels and airways. Airways grow by dichotomous branching from the hilum towards the periphery. The first division takes places at the left and right bronchus. Terminal buds are formed by each division and have a similar appearance throughout this period. A similar appearance is seen in other species such as mice, rats, and pigs (Merkus et al, 2006). The surrounding mesenchyme and ectodermally derived buds is essential for normal branching morphogenesis and mesenchyme is also required for the final pattern of branching (Alescio and Cassini, 1962).

The initial induction of the lung is signalled from the matrix surrounding the foregut of fibroblast growth factor 7 (FGF-7), which is stimulated via the
transcription factor TTF-1 and HNF-3β of the ectoderm. Further branching is induced by FGF 10 via FGF receptor 2 on the epithelium. Cleft formation is also involved in division via BMP-4 and fibronectin with the inhibition of FGF 10 (Weaver et al., 2000). Epithelial growth factor (EGF) found in epithelial cells induces proliferation and differentiation whereas TGFβ acts as an inhibitor of epithelial proliferation (Maeda et al., 2007).

1.2.1.2  Pseudoglandular Phase

Pseudoglandular Phase (5-17 weeks in humans), where further branching of the duct system generates the presumptive conducting portion of the respiratory system up to the level of the terminal bronchioles. Blood vessel growth occurs by vasculogenesis in the mesenchyme surrounding the lung buds, which is induced by vascular endothelial growth factor (VEGF) produced by the epithelial cells. VEGF stimulates endothelial cell and Type II cell growth survival by binding VEGF R2 (Tuder et al., 2006, Healy et al., 2000). This is an obligatory lung maintenance factor throughout lung development. Failure of blood vessel development will be due to the absence of VEGF receptor (Shalaby et al., 1995).

1.2.1.3  Canalicular Phase

Canalicular Phase (16-26 weeks in humans): The onset of this phase is characterized by extensive angiogenesis within the mesenchyme that surrounds the more distal reaches of the embryonic respiratory system to form a dense capillary network. The diameter of the airways increases with a consequent decrease in epithelial thickness similar to that of a cuboidal structure. The
terminal bronchioles branch to form several orders of respiratory bronchioles. Differentiation of the mesenchyme progresses down the developing tree, giving rise to condrocytes, fibroblasts and myoblasts (Silverman and Kuehni, 2007).

The respiratory airways produced are formed by branching, but the epithelium is at this point much thinner in the peripheral airways, and air spaces are larger. Detection of surfactant is present and Type I and Type II pneumocytes allows for epithelial differentiation to take place. Studies have shown that underlying capillaries appears to be responsible for the thinning and stretching of the epithelium which in turn leads to differentiation (Gutierrez et al., 1999). There is also an increase in VEGF production and the conversion of glygogen to surfactant in the Type II cells, which is controlled by the transcription factor HIF2α (Compernolle et al., 2002).

1.2.1.4 Terminal Phase

Terminal Phase (24-38 weeks in humans), allows for more branching and growth of the terminal sacs or primitive alveolar ducts to occur. Continued thinning of the stroma brings the capillaries into apposition with the prospective alveoli.

1.2.2 Postnatal Period

The postnatal period can be divided into the:
1.2.2.1  

**Alveolar Phase**

Alveolar Phase: In some mammals alveolarisation starts before birth and proceed postnatally, whereas in humans, alveolarisation begins at 36 weeks of gestation and lasts 1-3 years postnatally. Although a certain degree of maturation of the lung is associated with the appearance of fully mature alveoli at 36 weeks, new alveoli will continue to form for approximately three years after birth. Alveolar formation is closely linked to the deposition of elastin in the saccular lung (Grant et al., 2001). At birth, a newborn infant would have developed approximately 50 million alveoli and has the potential to expand it in number to about 250 million alveoli with an increase in surface area from approximately 3 to 70m² (Grant et al., 2001, Langston et al., 1984).

In rats and mice, alveoli form entirely after birth (Hislop, 2005). At postnatal day 4, cells of the alveolar walls show high proliferation rates. The inter-airspace walls consist of double capillary layers and a cellular sheet of connective tissue which is the basis for rapid alveolar formation from postnatal days 4 to 13, resulting in an increased capillary volume as well as a larger alveolar-capillary surface area (Bolle et al., 2008). Angiogenesis is promoted by VEGF, whereas the inhibition of VEGF receptor will result in a decrease in alveolar number (Jakkula et al., 2000).
1.2.2.2  Microvascular Maturation

Microvascular Maturation (human birth – 2-3 years/adulthood), where double capillary layers of the immature alveolar septa is reduced to a single capillary layer (Roth-Kleiner et al, 2003). In rats, the finalization of septal restructuring is complete at around postnatal day 21 (Bolle et al., 2008). The double capillary network that merges into a single layer is an important event in postnatal lung growth as angiogenesis and alveolarization goes hand in hand (Joshi and Kotecha, 2007).

1.2.2.3  Stage of Late Alveolarization

Stage of Late Alveolarization (3-5 years to young adult age in humans): Alveolar formation appears to continue by peripheral (perhaps repetitive) septation at a slow pace (Burri, 2006).

1.3  WINDOWS OF PLASTICITY DURING LUNG DEVELOPMENT

The respiratory system consists of approximately 40 different cell types with multiple functions. Common air pollutants such as airborne particles, oxidant gases, and environmental tobacco smoke exposure affects the fetus and impacts on target cells, which are likely to affect critical signals or mediators expressed during distinct stages of lung development. Consequently, it impacts the pre- and postnatal periods of life in the offspring (Pinkerton and Joad, 2006). Studies have shown that the anti-oxidant capacity of the developing lung is low during most of
its development, but rapidly increases just before birth (Barker, 1995). There is also a natural neonatal susceptibility to environmental pollutants due to direct or indirect hits on a number of cell types to influence cell differentiation, proliferation and/or maturation (Pinkerton and Joad, 2006). This indicates that exposure to foreign substances, in utero such as nicotine, at the critical periods of lung development, can alter the normal “program” that controls lung growth and maturation. This could result in the offspring becoming more prone to diseases such as cardiovascular disease, and type II diabetes mellitus in their adult life, as well as inadequate lung maintenance (Barker, 1995). An example of the effect of a change in the in utero environment is that inadequate nutrient supply in utero leads to metabolic changes in the developing fetus that enhance their chance of postnatal survival in a supposedly deprived environment. These adaptions are generally beneficial only after birth (Somm et al., 2009). However, if the fetus is programmed during pregnancy to survive in an environment of low nutrient supply, but is born into an environment of abundant nutrient supply, they become more prone to adult obesity (Eriksson and Forsen, 2002). Maternal undernutrition, vascular diseases, drug abuse and placental pathology are of the major causes resulting in reduced oxygen and/or nutrient supply to the developing fetus and the consequent adjustment of metabolism in the offspring. Studies have shown that the degree and type of nutrient restriction, as well as gestational timing, affects the final structure of the fetal lungs. Experiments done in sheep showed that they had fewer, larger alveoli with thicker septa and blood-gas barriers in their adult life when intra-uterine growth restriction (IUGR) was induced. This was due to an inadequate nutrient supply from the mother during
gestation (Maritz and Harding, 2011). It is therefore, plausible that changes in the in utero environment due to maternal smoking or nicotine replacement therapy (NRT) may interfere with the development of the offspring and increase susceptibility to disease.

1.4 THE ROLE OF LYSYL OXIDASE DURING LUNG DEVELOPMENT

Lysyl Oxidase (LO), for example, functions by initiating the cross linking of collagen and elastin by oxidizing specific peptidyl lysine residues, stabilizing collagen or elastin fibres in the extracellular matrix, thereby playing a central role in lung morphogenesis and tissue repair. A LO reaction produces hydrogen peroxide which may possibly regulate gene expression and cell behavior. It is also considered a tumor suppressor gene. The molecular structure of LO is synthesized by the fibrogenic cells as a 46 preproenzyme. LO is a metalloenzyme that requires copper II (Cu II) as a cofactor for enzymatic function. Cu (Raunio et al.) binds at its active site: one Cu per molecule of the enzyme. Loss of Cu results in total inactivation of LO. Studies have shown that other divalent metals such as Cd, Fe, Co, Zn and Ni cannot replace Cu as a cofactor for LO (Chen et al., 2005). Therefore, animals that received a diet with reduced Cu showed in induction of lythyritic manifestation due to the reduction of elastin deposition in the lung which resulted from suppression in LO activity (Harris, 1986). Studies have also shown that the catalytic activity of LO may be suppressed by cigarette smoke condensate (CSC) and cadmium (Cd), which are major components of cigarette smoke. It has indeed been shown that smoke induced emphysema in chick lungs
was due to Cu deficiency, causing a disruption in lung structure (Li et al., 2011). This suggests that Cu is essential for the functioning of LO and thus normal lung growth, maintenance and aging and that a lack of adequate intake will compromise lung development.

1.5 FACTORS AFFECTING THE DEVELOPING LUNG

1.5.1 Chronic Obstructive Pulmonary Disease (COPD)

Emphysema is a major pathologic feature of COPD. It is defined as the abnormal enlargement of alveolar air spaces and the destruction of alveolar walls. It is now known that fibrosis is present even with alveolar loss, and that fibroblasts of the alveolar are central to the remodelling process of cigarette smoke-induced emphysema. The pathogenesis of smoke-induced emphysema encompasses a culmination of an imbalance between: a) proteolytic and antiproteolytic mechanisms, b) imbalance between oxidative damage and anti-oxidative repair mechanisms and, c) an imbalance between cell proliferation and apoptosis of alveolar cells. These processes are modified by cellular senescence and infection (Sirianni et al., 2006). A lung with COPD presents with an increased number of neutrophils, macrophages and lymphocytes. Recent research raises the concern whether the increased numbers of potentially protective cells are directly targeted because of cigarette smoke or if it is a secondary response to cellular and molecular alterations induced by cigarette smoke inhalation (Tuder et al., 2006).

Reactive oxygen species (ROS) and tobacco components activates a series of receptor-mediated signal transduction pathways, consequently causing an impairment of a variety of cell signalling and cytokine networks, which
subsequently leads to alveolar destruction, airway remodelling and chronic airway responses with mucus production (Yoshida et al, 2007).

Cigarette smoke is a highly complex mixture of about 4,000 compounds, including free radicals. Long-lived radicals present in aqueous extracts of cigarette smoke that is cytotoxic cause’s protein and DNA damage. DNA fragmentation and protein damage are the hallmarks of emphysema (Banerjee et al., 2008). Chronic bronchitis, which is characterized by mucus production leading to cough with expectoration, and emphysema are both classified as representatives of COPD. Both result in a lowered forced expiratory volume (FEV$\textsubscript{1}$) (Yamaya et al., 2007). Studies have shown that the FEV$\textsubscript{1}$ in smokers decrease by 50 to 100mL per year. This is significantly faster than the 20mL per year with normal aging (Ito and Barnes, 2009). COPD is the largest recognized public health problem worldwide, being the fifth leading cause of death in the world as reported by the WHO in 2002. It is a disabling condition associated with progressive airflow limitation that is largely irreversible. Many researchers, health care planners and clinicians view COPD as a self-inflicted disease by smoking. Surveys have shown that 11% of patients who have severe COPD, smoked more than 20 cigarettes per day (Viejo-Banuelos et al., 2006). Furthermore, an important aspect of patients with COPD is the lack of knowledge regarding this disease which could either be due to them being reluctant to consult their physician for respiratory problems or are unconscious of their condition (Viejo-Banuelos et al., 2006).
1.5.1.1 Epithelial and Endothelial Cells

Pulmonary alveolar walls are composed of Type I and Type II epithelial cells, endothelial cells and fibroblasts. This exceptional structure becomes susceptible to potential mechanisms for pathogenic dysfunction when exposed to cigarette smoke, consequentially compromising all alveolar septal cells (Tuder et al., 2006). Studies on patients with COPD showed that they have a reduced proliferation rate of alveolar epithelial and endothelial cells, which indicates that cigarette smoke not only causes injury to the lungs, but also impairs the healing process. It has been hypothesized that cigarette smoke induces fibroblast senescence, which is a biological state related to cell longevity but has a reduced ability to proliferate (Tsuji et al., 2004).

1.5.2 Pathogenesis of Emphysema

Emphysema is a distinctive feature of the lung-destructive process which is associated with the spectrum of chronic obstructive pulmonary diseases. The major characteristics of emphysema involve an abnormal enlargement of the alveoli of the lung distal to terminal bronchioles, associated with the destruction of the alveolar walls. Two major forms of emphysema have been identified: 1.) panacinar emphysema, which involves airspace enlargement throughout the acinus which arises from a deficiency in synthesis or secretion of \(a_1\)-proteinase inhibitor, and 2.) centroacinar emphysema, which develops in the central portions of the acinus in close proximity to respiratory bronchioles. Centroacinar emphysema is the most frequent observed form of emphysema and is predominantly associated with prolonged exposure to cigarette smoke (Vlahovic
et al., 1999). In the United States approximately 4-5 million people suffer from emphysema. The major challenge is that there are currently no effective treatments aimed at this irreversibly fatal disease (Tuder et al., 2006).

1.5.2.1 Apoptosis and Cell Proliferation

Apoptosis is known as ‘programmed cell death’, which plays a role during developmental stages as well as aging to maintain cell populations in tissues, thereby contributing to homeostasis. Damaged cells caused by diseases or noxious agents are also controlled by apoptosis, whereby it acts as a defence mechanism (Norbury and Hickson, 2001). Recently the role of pulmonary emphysema has been highlighted. Studies have shown that emphysematous lungs are associated with increased levels of apoptosis in alveolar epithelial and endothelial cells (Yokohori et al., 2004). As a result, the integrity of the alveolar structure in the lungs decreases and therefore needs to be maintained due to the loss of alveolar cells by apoptosis. To compensate for alveolar loss, cell proliferation will consequently increase (Tsuji et al., 2006). Supporting studies showed that patients with emphysematous lungs had high levels of cell proliferation than in patients with normal lungs (Imai et al., 2005). For that reason, there is a direct correlation between apoptosis and cell proliferation in the pathogenesis of emphysema. However, cellular and molecular mechanisms of pathophysiology of emphysematous lung damage remain enigmatic.
1.5.2.2  **Phenotypic Changes in Fibroblasts**

Fibroblasts play an important role in the maintenance of alveolar structure, and a change in fibroblast phenotype could be involved in the pathogenesis of emphysema (Muller et al., 2006). Studies have shown that lung fibroblasts from patients with emphysema show a reduced proliferation rate, altered growth factor response, and a lower number of population doublings in long-term culture (Holz et al., 2004). Together with clinical observations, these findings lend support to the hypothesis that premature aging of fibroblasts is involved in the pathogenesis of emphysema. Senescent cells not only lose their ability to divide and respond to mitogenic stimuli, but also display alterations in morphology and metabolic profile (Bird et al, 2003). This phenotype can be induced by oxidative stress, in association with epigenetic changes in gene expression. As fibroblasts provide part of the integrity of the lung, a senescent phenotype could affect tissue microbalance and structural maintenance (Muller et al., 2006).

1.5.2.3  **Proteinase-Antiproteinase Balance**

As mentioned, the pathogenesis of emphysema is also largely due to an imbalance between proteinase enzymes released from inflammatory cells and protective proteins, i.e. antiproteinases found in the interstitial and extracellular spaces of the lung. This occurs when an antiproteinase, known as A1AT (alpha-1-antitrypsin) is inactivated by substances contained in tobacco smoke and exposed to lung tissue. The alveolar structures of the lower respiratory tract in normal individuals are protected from endogenous proteinases such as neutrophil elastase, cathepsin G and proteinase-3 (Knight et al., 1997).
However, in the smoking individual, A1AT is synthesized in the liver and then transported to the lungs via the bloodstream simultaneously with inflammatory leukocytes such as neutrophils and macrophages. It is usually identified with an early onset of emphysema. SLPI (secretory leukocyte peptidase inhibitor) and elafin are small molecules also playing roles as proteinase inhibitors, where it is closely associated with elastin fibres in the extracellular matrix of the lung, and functions to protect the connective tissue matrix against surface-bound neutrophil elastase 3 (NE3), a proteinase of the lung due cigarette exposure (Knight et al., 1997). Interference with the synthesis and function of these molecules will thus result in impaired maintenance of the lung structure. Studies have, for example, shown that cigarette smokers with an A1AT deficiency experience a greater rate of deterioration in lung function (Knight et al., 1997, Janus et al., 1985).

1.6 OXIDANT-ANTIOXIDANT CAPACITY OF THE LUNG

1.6.1 Tobacco Smoking as a Source of Oxidants

Each puff of cigarette smoke contains 4000 toxic compounds, with $10^{15}$ oxidants in the gas phase and $10^{18}$ per gram of tar. Amongst these are potent oxidants such as hydrogen peroxide, hydroxyl anion and organic radicals (Tuder et al., 2006). Oxygen is a highly reactive atom capable of becoming part of potentially damaging molecules known as ‘free radicals’. Free radicals are electrically charged molecules with an unpaired electron and attacks healthy body cells causing loss of structure and function. This can be neutralized and controlled by antioxidants, which stabilizes or deactivates free radicals before attacking cells and is critical for maintaining optimal cellular and systemic health and well being.
Types of reactive oxidant species (ROS) include hydroxyl radical, superoxide anion, hydrogen peroxide, and various lipid peroxides (Callahan et al., 2001).

An increased oxidant load can be induced by chronic inflammation, infections, allergens exposure, presence of ‘leaky gut’ syndrome, exposure to drugs or toxins such as cigarette smoke, pollution, pesticides and insecticides (Halliwell, 1994). An antioxidant overload may impair cell metabolism, function and characteristics. It has been shown that a common cause for aging and emphysema is oxidative stress. It has been noted that complex I and complex III in the mitochondria of the cell is a potential source of free radicals, of which 90% is produced in vivo. Glutathione and enzymes such as superoxide dismutase, glutathione peroxidase and peroxiredoxins, a set of antioxidant defenses, maintains homeostasis by reducing free radicals (Tuder et al., 2006). When the ability of the body to maintain the oxidant/anti-oxidant balance is compromised through, for example, exposure to large quantities of oxidants via the air we inhale, or the food we eat, or when the anti-oxidant levels are reduced, it may result in a gradual deterioration of the lungs.

ROS, is the major harmful substances found in cigarette smoke. There is a wide variety of chemicals found in cigarette smoke, the most significant of which are mutagens/carcinogens, PAHs (polycyclic aromatic hydrocarbons), aromatic amines, N-nitrosamines and aldehydes. These substances cause DNA damage by inducing strand breaks. ROS, are the primary causes of DNA lesions which is
crucial in cancer development in various organs such as cancers of the lung, oral cavity, nasal cavity, paranasal sinuses, larynx, stomach, pancreas, liver and kidney (Weng et al., 2009).

Cells affected with ROS grow slower than normal. Thus, a potent antioxidant response of a normal, fully functional lung epithelial cell should effectively protect it against ROS. However, increased ROS might cause a transformation in epithelial cells if the antioxidant defences are overwhelmed substances, such as hydroquinone and catechol, present in high concentrations in tobacco smoke (Dennis et al., 2005).

1.6.2 Nutritional Supplementation as a Source of Antioxidants

The anti-oxidant capacity of organs, such as the lungs can become depleted when the dietary supply is inadequate, especially when the individual is exposed to chronic oversupply of oxidants such as smoking. This can be prevented by supplementing the diet with:

1.6.2.1 Vitamin C

Vitamin C is a water-soluble antioxidant; it occurs in extracellular fluids and is known as ascorbic acid. We depend on ascorbic acid for many aspects of our biochemical functioning. Humans, unlike many animals, cannot produce their own supply of vitamin C. This can cause a Vitamin C deficiency resulting scurvy, if it is not obtained in our diet such as fruits and vegetables. Vitamin C helps the
immune system to fight off foreign invaders and tumour cells; it also supports the cardiovascular system by facilitating fat metabolism and protecting the tissues from free radical damage (Becher and Winsel, 1989). Vitamin C "scavenges" aqueous peroxyl radicals before it can cause damage to the cell membranes. It works along with vitamin E, a fat-soluble antioxidant, and the enzyme glutathione peroxidase to stop free radical chain reactions (Becher and Winsel, 1989).

Vitamin C also protects the DNA of the cells from the damage caused by free radicals and mutagens. It may be especially important in this day and age of widespread environmental pollution because it combats the effects of many such toxins, including ozone, carbon monoxide, hydrocarbons, pesticides and heavy metals (Becher and Winsel, 1989).

It appears that vitamin C fights off these pollutants by stimulating various enzymes in the liver that detoxify the body. In several studies, vitamin C reduced chromosome abnormalities in workers exposed to pollutants such as coal tar, styrene, methyl methacrylate and halogenated ethers. Another way in which vitamin C protects us is by preventing the development of nitrosamines, the cancer-causing chemicals that stem from the nitrates contained in many foods. It also prevents free radical damage in the lungs and may even help to protect the central nervous system from damage (Becher and Winsel, 1989).

When considering smokers, it is a well accepted fact that cigarette smoke has a negative impact on the metabolism of vitamin C. According to Schectman and
colleagues (1991), people who smoke have a much lower level of ascorbic acid in the blood than do non-smokers. Research done by Maritz and van Wyk (1997), demonstrated that vitamin C protects against the some of the harmful effects that nicotine has on lung development in the rat model (Maritz, 1993, Maritz and van Wyk, 1997). It has been recommended that smokers consume 100 mg of vitamin C a day; they may need 200 mg or more to maintain the same concentration of serum ascorbate as a nonsmoker who gets 60 mg of vitamin C per day (Schectman et al., 1991). Vitamin C occurs in large quantities in the extracellular lining fluid of the lung and serves as a first line of protection against inhaled oxidants (van der Vliet and Cross, 2000). Exposure to nicotine result in an almost 90% decrease in lung vitamin C content which explains why the vitamin C intake of smokers or NRT (nicotine replacement therapy) must be so high. Apart from chilli peppers, guavas and fresh herbs containing the highest quantities of vitamin C, tomatoes are also an important source of vitamin C.

### 1.6.2.2 Vitamin E

Vitamin E is an important lipid-soluble antioxidant found in high quality whole grain foods and properly extracted vegetable oils. It protects membrane fatty acids from lipid peroxidation (Elsayed, 1987). Vitamin E is a naturally occurring antioxidant that is virtually free of toxicity in man. Its antioxidant properties reflect its ability to neutralize free radicals, including toxic oxygen intermediates, thereby preventing peroxidation of unsaturated lipids. Numerous animal studies have demonstrated that vitamin E is an important determinant of the lung's susceptibility to injury by various oxidants, including hyperoxia, nitric oxide,
ozone, paraquat, nitrofurantoin, and other oxidizing agents (Yamaoka et al., 2008). Oxidizing substances in cigarette smoke also produce lipid peroxidation and various studies have shown that vitamin E-deficient rats exposed to cigarette smoke die prematurely compared with control animals (Pacht et al., 1986). Vitamin E and vitamin C interact in the lungs of animals to ensure optimal protection against oxidants in inhaled air (Maritz and Harding, 2011).

1.6.2.3 Lycopene

Lycopene, an anti-proliferative carotenoid in the same family as beta-carotene, is what gives tomatoes, pink grapefruit, apricots, red oranges, watermelon, rosehips, and guava their red colour. This acyclic carotenoid, which is a powerful antioxidant, contains 11 conjugated double bonds arranged in an all-trans configuration, whereby the presence of these conjugated double bonds plays an important role in quenching a singlet oxygen, as well as in trapping peroxyl radicals, thereby conferring protection against prostate cancer, breast cancer, atherosclerosis, and helps reduce cholesterol levels in the blood (Gitenay et al., 2007). This polyene molecule undergoes cis-trans isomerization by light, thermal energy and chemical reactions. However, lycopene obtained from plants exist in all-trans configuration, the most thermodynamically stable form. In human plasma, lycopene is present in an isomeric mixture, with 50% as cis isomers. Although lycopene has no provitamin A activity, is reveals other biological properties such as the induction of gap-junction communication, suppress growth factor induced proliferation, and inhibit neoplastic transformation of normal human and mouse cells (Ferreira et al., 2000, Liu et al., 2003).
It has been shown that lycopene decrease DNA damage, as well as the susceptibility to oxidative stress in lymphocytes. It also decreases peroxidation or low density lipoprotein (LDL). However, data regarding the antioxidative effects of lycopene alone in biological systems are limited (Mein et al., 2008). Studies reported a significant inhibition of N-methyl-N-nitrosourea-testosterone induced carcinogenesis in male wistar-unilever rats following consumption of tomato powder (13 mg/lycopene/kg diet), whereas no effects were observed with lycopene supplementation per se (161 mg lycopene/kg diet) (Basu and Imrhan, 2007). Another study demonstrated that purified lycopene was shown to act synergistically with other natural antioxidants like the flavonoid glabridin, the phenolics rosmaniric acid and carnosic acid, in inhibiting LDL oxidation in vitro. This suggests that lycopene in association with other natural oxidants, such as in tomatoes, may be more potent in inhibiting lipid peroxidation, than lycopene itself (Liu et al., 2006).

Lycopene is a stronger growth inhibitor than β-carotene, and has been recently associated with an inhibition of tumor invasion, cell proliferation, and angiogenesis in the lungs of nude mice injected with SK-hep1 human hepatoma cells. Furthermore, studies have illustrated that due to cell proliferation inhibition, the growth inhibitory effect of lycopene may also be attributed to the induction of apoptosis. Subsequently, studies revealed that lycopene (0.5-2µM) inhibited the growth of cigarette smoke condensate-exposed immortalized RAT-1 fibroblast cells by arresting cell cycle progression and inducing apoptosis (Mein et al., 2008). Studies have shown that oxidative stress is also involved in the aging
In experimental studies, it has been confirmed that tomato juice given concomitantly with chronic tobacco smoke exposure completely protected SAMP1 mice from smoke-induced emphysema (Kasagi et al., 2006).

1.7 TOBACCO SMOKING AND RESPIRATORY HEALTH

1.7.1 Maternal Smoking and its Effect on Infancy

Two hundred and fifty million women smoke worldwide and over 700,000 children are born to female smokers and were thus exposed to components of cigarette smoke each year in the United States (Li et al., 2012). In North America, less than one-third of female smokers stop smoking during pregnancy. Numerous compounds in cigarette smoke, such as carbon monoxide, cyanide, ammonia, vinyl chloride and polycyclic aromatic hydrocarbons are known to be harmful, for both mother and fetus. However, nicotine, the main stimulant and dependence-forming alkaloid found in tobacco is most studied (Somm et al., 2009).

In 1957, the associations between maternal smoking and retarded fetal growth were first described. Today, maternal cigarette smoking during pregnancy is even more prevalent and varies widely across countries and socio-economic groups. It is well known that the impact of maternal smoking on the developing fetus is complex. Tobacco smoke may impinge on the fetus in several ways: inhaled nicotine causes the induction of vasoconstriction of the uteroplacental vasculature, which leads to uteroplacental-underperfusion, in turn, having a decreased flow of oxygen and nutrients to the fetus. Maternal smoking increases the levels of carboxyhemoglobin which reduces the tissue oxygenation of the fetus. Nicotine in
tobacco smoke also suppresses the mother’s appetite which results in a lesser energy intake by the mother, leading to a reduced energy supply to the fetus (Polanska and Hanke, 2004). Furthermore, nicotine causes alterations in the cellular growth and activity of the peripheral and central nervous systems. All these complications increases the risk of abruption placenta; placenta previa; stillbirth; spontaneous abortion; preeclampsia; preterm delivery; intra-uterine growth restriction; sudden infant death syndrome and congenital malformations in the offspring (Polanska and Hanke, 2004).

Statistics show that approximately 15-20% of women smoke during pregnancy. Smoking is responsible for 15% of all preterm births, 20-30% of all infants are of low birth weight, and a 150% increase in perinatal mortality. It was proven that women who stopped smoking during pregnancy are at a lower risk for most of the above mentioned pathologies. There is no doubt that cigarette smoking is one of the most important and modifiable risk factors associated with adverse perinatal outcomes (Andres and Day, 2000). According to South African statistics between 1993 and 2000, the average cigarette use for smoking women was 10.5% which is 1.7% higher than males who smoke. Smoking amongst women occurs more prevalently in the white race (16.6%), and occurs least in the African race (6.3%). Women of coloured and Indian decent smoked on average 8.8% and 9.9% cigarettes between 1993 and 2000, respectively (van Walbeek, 2002).
1.7.2 Nicotine Metabolism

Nicotine is the tobacco plants’ natural protection from being eaten by insects. It is a super toxin that is more lethal than a diamondback rattlesnake’s venom, and three times deadlier than arsenic (Caron et al., 2005). Yet, this natural insecticide’s chemical structure is similar to the neurotransmitter Ach (acetylcholine). Within eight seconds of inhalation, one experiences cough and dizziness. Nicotine then arrives in the brain where a large dose of dopamine is generated and this immediately results in a relaxing sensation for the individual (Caron et al., 2005).

Neuronal nicotinic receptors are members of the family of neurotransmitter-gated ion channels that includes GABA receptors; glycine receptors; and 5-HT3 receptors (Karlin, 2002). Neuronal nicotinic receptor subtypes arise from different subunits. Eight subunits (α2-α4; α6-α10) contribute to the acetylcholine binding site, and four subunits (α5; β2-β4) have a structural function but contribute to the binding site. In rodents, ±90% of the high-affinity nicotine binding sites in the brain contains α4 and β2 subunits (Zoli et al., 1998). Hence, α4β2 receptors are the neuronal nicotinic receptor subtype most likely to participate in nicotine addiction, although other receptors that contain α6 or α3 subunits may also be involved (Parker et al., 2004).

Prolonged exposure to nicotine increases binding in the rat and human brain, a process termed “upregulation”, which has been linked with nicotine addiction. Upregulation occurs in cells heterologously expressing α4β2 receptors and has
been further characterized in a variety of expression systems. Furthermore, a functional response of α4β2 receptors expressed in *Xenopus* oocytes decreased after long exposure to nicotine. Recent studies also reported that functional responses of α4β2 receptors expressed in mammalian cells and midbrain neurons is increased after long-term nicotine exposure (Vallejo et al., 2005).

Nicotine addiction is the reason why people continue to smoke. Each cigarette puff taken by a pregnant woman allows nicotine to be absorbed in the bloodstream which readily crosses the placenta, affecting the normal developmental processes of the fetus. Studies have shown that certain quantities of nicotine that enters the fetus via the placenta lands up in the amniotic fluid via fetal urine and also returns to the maternal circulation for excretion (Maritz and Harding, 2011).

### 1.7.3 Glucose Metabolism: Effects of Nicotine

Glycolysis is a process whereby glucose is converted into lactic acid in an animal cell. Cancer cells differs from normal cells in that it is able to maintain high levels of glycolysis under aerobic conditions, even under high oxygen levels. This is due to the fact that tumours generally outgrows its blood supply, which results in hypoxia, in turn increasing glycolysis which is the standard response to a lack of oxygen. Enhanced glycolysis is a distinctive marker for cancer progression. Observations suggest that there is a correlation between oncongenes (ras, myc and src) and enhanced glycolysis (Kondoh et al., 2005b).
Glycolysis taking place under anaerobic conditions is inefficient as it produces only two ATP molecules per glucose mole. Therefore, the increase in glycolysis could be a way how tumour cells counteract deleterious effects of oxidative stress by reducing ROS accumulation. Cumulative ROS is now recognized as a feature of many pathological conditions such as neurodegenerative disease, atherosclerosis, cancer and premature aging (Kondoh et al., 2005b). Studies furthermore suggest that expression of PGM or GPI (glucosphosphateisomerase), a glycolytic enzyme, can enhance glycolysis and bypass senescence by depletion of PGM or GPI to shorten the cellular lifespan (Kondoh et al., 2005b).

Studies have shown that maternal nicotine exposure during gestation and lactation results in the suppression of glycolysis as well as glycogenolysis (Maritz, 1986, Maritz, 1987). It has also been reported that a reduced glycolytic pathway, as well as high levels of AMP (adenosine monophosphate) are associated with the premature onset of cellular senescence, consequently causing premature aging in the lungs (Zwerschke et al., 2003).

1.7.4 Nicotine Replacement Therapy (NRT)

Surveys have shown that despite the fact that 80% of smokers express some desire to stop smoking, only 30% actually make an attempt. Therefore, clearly, accessible and effective smoking cessation programs are needed (Dallery et al., 2007). Several programmes are used such as psychological programs or a combination of psychological and pharmacological programmes. NRT is another intervention prescribed by health professionals.
NRT is a way of getting nicotine in the bloodstream without smoking a cigarette. There are many types of NRT on the market and now lately electronic cigarettes specifically designed to stop or reduce the withdrawal symptoms associated with the intake of nicotine (Stead et al., 2008). It is suggested by some that NRT during pregnancy is safer than smoking. As a result NRT use is being encouraged despite the absence of direct validation for its safety. Many pregnant women are briskly resorting to alternative sources of nicotine such as smokeless tobacco products (chewing tobacco, oral and moist snuff) or pure nicotine products (nicotine patches, oral inhalers, nasal sprays, lozenges, gum and sublingual tablets). NRT usage allows for the rise in the plasma and brain nicotine levels to be slower than normal smoking, however, concentrations of nicotine in the blood also declines much slower suggesting that the lingering effect of nicotine could possibly affect the surrounding tissue. Nicotine inhaler, sublingual tablets and gum result in an estimated blood nicotine level between 5-15ng/ml, whereas nicotine patches give rise to blood nicotine levels of 10 to 20ng/ml. With nicotine patches, the absorption of nicotine occurs in the bloodstream after approximately one hour, and the deposition of the skin causes a continued absorption even after removal of the nicotine patch. Studies have shown that maternal nicotine effortlessly crosses the placental barrier and can be detected in the blood circulation of the fetus at a concentration of 15% higher compared to maternal blood concentrations, and that amniotic concentrations of nicotine in humans are 88% higher than maternal plasma (Wickstrom, 2007).
For pregnant women, nicotine patches are not as highly recommended as the other NRT options available, such as gums and inhalers. However, NRT is aimed at minimizing the exposure of the unborn baby to nicotine as well as reaching smoking cessation with time. Yet paediatric clinicians in epidemiological studies showed a high level of reluctance to NRT prescriptions (Wickstrom, 2007). Apart from exposure of the fetus to nicotine that enters the fetal circulation via the placenta, nicotine also accumulates in breast milk. Thus, during the postnatal period the offspring is exposed to nicotine via the mother’s milk, thereby lengthening the period of nicotine exposure during breastfeeding. Interestingly, studies showed that for breastfeeding women, there is no difference between cigarette smoking and NRT as it delivers the same amount of nicotine in the breast milk (Stead et al., 2008, Wickstrom, 2007). Although nicotine delays the onset of endothelial progenitor cell senescence in culture by protecting telomerase (Junhui et al., 2009), it is possible for it to induce premature cell senescence via a non-telomerase mechanism.

1.8 THE EFFECT OF INDUCED-CELLULAR SENESCENCE ON THE AGING LUNG

1.8.1 Cellular Senescence

Cellular senescence is defined as a state of permanent cell-cycle arrest induced by either replicative senescence or by telomere-independent signals. This state of irreversible growth is arrested in the G1/G0 phase of the cell cycle. Senescent cells, therefore, cannot respond to growth factors and becomes irreversibly resistant to apoptosis (programmed cell death). Structurally, they adopt distinctive
morphological changes which appear flat and enlarged. Furthermore, it allows for the induction of acidic senescence associated β-galactosidase activity (SA-β-gal).

Physiologically, these changes causes a reduction in response to growth factors, and an increase of susceptibility to toxins, drugs, irradiation, stress, an altered level of calcium, pH changes, and membrane potential, thereby reducing the overall amount of cellular respiration and energy metabolism. Molecularly, alterations in gene expression may be observed, where senescence-associated secretome bring forth changes in the surrounding cells and microenvironment. Other altered genes of senescent cells involves plasminogen activator inhibitor type 1 (PAI-1), c-Myc, p16INK4a, and p53. Changes also appear in the chromatin structure of senescent cells, and the formation of senescence associated-heterochromatic foci (SAHF) can be found. SAHF is correlated with a trimethylated lysine 9 of histone H3, heterochromatic protein 1, and high-mobility group A protein. The formation of SAHF is due to the recruitment of pRb to E2F-responsive promoters and is responsible for the stable repression in E2F genes, possibly contributing to the irreversibility of senescence. Biochemically, the decrease in the rates of protein synthesis and degradation is due to the accumulation of modified and inactivated proteins. Also, nuclear and mitochondrial DNA appears to have increased levels of oxidative damage (Kong et al., 2011).
1.8.2 Types of Cellular Senescence

Cellular senescence can either be induced dependent or independent of telomeres known as telomere-dependent replicative senescence, and telomere-independent premature senescence, respectively.

Telomeres are structures composed of specialized terminal DNA sequence repeats (TTAGGG/CCCTAA) complexed with telomere binding proteins. It is located at the end of every human chromosome and becomes shorter during cell division. The senescence stage is reached when telomere shortening disrupts telomere structures (Tsuji et al., 2006).

1.8.2.1 Telomere-Dependent Replicative Senescence

In telomere-dependent replicative senescence, telomeres get shortened with each cell division, causing the inability of DNA polymerases to replicate DNA at the very ends of linear chromosomes. As a result, critically shortened telomeres lose protection of telomere-binding proteins which leads to telomere “uncapping”. DNA damaged foci, containing multiple DNA damage-response proteins such as 53BP1, γH2AX, MDC1 and MRE11, are found in telomeres of senescent cells. Therefore, uncapped telomeres are recognized as DNA breaks, consequently triggering a DNA damage response. Telomerase, an enzyme which is responsible for de novo synthesis of telomeric repeats and maintenance of telomeric length, can be expressed in germline, stem and cancer cells. However, it is undetectable in majority of normal somatic cells. An absence of telomerase is the major cause of replicative senescence (Kong et al., 2011).
1.8.2.2  Telomere-Independent Premature Senescence

Telomere-independent premature senescence is caused by the induction of many independent signals, such as aberrant activation of oncogenes, damage to chromatin structure, oxidative stress, DNA damage and inadequate culture conditions, as well as environmental stress associated with emphysema, such as cigarette smoke exposure and oxidants. Oxidative stress accelerates telomere shortening, possibly by inducing telomeric single strand breaks. These exposures induce alveolar cells to undergo premature senescence without telomere shortening (Tsuji et al., 2006, Kong et al., 2011). Since nicotine displays oxidant properties (Newman et al., 2002) and induce oxidant release (Maritz and Harding, 2011) it is conceivable that it may induce premature cell senescence.

1.8.2.3  Similarities between Replicative, and Stress-Induced Senescence

Replicative and stress-induced premature senescence share common changes in their cell cycle regulation and morphological properties. The expression of DNA damage checkpoints, inflammation, stress associated genes, genes encoding extracellular matrix proteins and the extracellular matrix are all altered. Senescence is now considered as a general stress response in normal cells to various types of cellular damage, however, senescent cells displays a unique pattern of gene expression and differs from proliferating or quiescent cells (Kong et al., 2011).
1.8.3 Mechanisms of Cellular Senescence

The molecular regulation of cellular senescence varies according to species and different cells types. Human fibroblasts may be taken as an example, where senescence is primarily caused by telomere shortening, whereas mouse fibroblasts are probably mediated by oxidative stress which is independent of telomere shortening. Contrary to this, diverse-inducing senescence stimuli are triggered through multiple genetic pathways, which usually seem to converge on p53 and pRb pathways. The inactivation of both p53 and pRb pathways are required to prevent the activation of senescence (Kong et al., 2011).

1.8.4 Senescence and Aging of the Lung

Biological aging is characterized by a progressive deterioration of physiological function in all tissues and organs over time, subsequent to developmental periods. It involves a variety of cellular, molecular and structural alterations based on several mechanisms. Although normally linked to chronological age, biological aging can occur earlier in life, being particularly independent from an individual’s chronological age. Premature aging is associated with increased susceptibility to major chronic diseases and ultimately, mortality (Balcombe and Sinclair, 2001, Kong et al., 2011). Aging and cellular senescence is closely related due to their shared ability to time and lifespan. It has been hypothesized that constant tissue regeneration leads to the accumulation of senescent cells in somatic tissues, which in turn limits tissue renewal and disturbs tissue homeostasis, consequently leading to aging. Many studies have shown that there is a relationship between accumulation of senescent cells and age-associated pathological conditions such
as osteoarthritis, artherosclerosis, dementia, liver cirrhosis, and respiratory disease. However, there is still uncertainty whether the accumulation of senescent cells are responsible for aging, or if it is accountable for age-related diseases (Kong et al., 2011).

The aging process is also controlled by telomere checkpoints. Aged human tissue is an example, which is at the sites of age-related pathological conditions, or which are associated with stress and obesity. Recent studies have observed that telomerase reactivation can reverse much of the premature aging phenotypes in telomerase-deficient mice, signifying that telomere attrition plays an important role in the aging process (Kong et al., 2011).

1.8.5 The Relationship between Aging and Chronic Diseases

Since aging is a multi-faceted process which involves many intimately woven factors such as: a) telomere attrition, b) cumulative DNA damage, c) impairment of DNA repair, d) epimutations in nuclear DNA, e) mutations in mitochondrial DNA, f) increased rigidity of cytoskeleton, g) increased cross-linking of extracellular matrix, h) protein damage, i) increased production of free radicals, j) and accumulation of waste products, it is necessary to seem prudent when interpreting findings, particularly with regards to animal data since it might not be evaluated from a single aspect. However, there is a parallel between the processes involving additional support for a relationship between aging and emphysema, as well as demonstrating differences between phenotypes of the disease, eg. homogeneous versus focal alterations, of which defects are viewed in vascular
maintenance in patients with emphysema and genetically modified rodents (Karrasch et al., 2008).

A variety of diseases already provides mechanistic proof which specifies a link to induced aging. COPD itself is a risk factor for other disorders such as cardiovascular disease, type II diabetes, or cognitive and functional deteriorations, all of which are age-related. Especially the association between arterial stiffness, osteoporosis and the severity of airflow obstruction has provided a supportive substantiation on premature aging in COPD (Vogelmeier and Bals, 2007). The structure and function of the human lung show a variety of alterations as part of the normal aging process which involves a) alveolar enlargement of air spaces; b) vascular remodelling; c) altered composition of extracellular matrix; d) reduced strength of respiratory muscles; e) impaired respiratory mechanisms; f) increased stiffness of chest wall; g) reduction of lung function reserves (volumes, flows) heterogeneity of ventilation; h) and impaired gas exchange capacity (Sprung et al., 2006).

1.8.6 The Relationship between Smoking and Cellular Senescence

Inhaled toxins, such as cigarette smoke, SO$_2$, O$_3$, and NO$_2$ injures alveolar epithelium. Under normal conditions, effective repair responses allows alveolar epithelial cells the ability to migrate, proliferate and differentiate to cover defects that results from injury. However, if alveolar epithelial cells are unsuccessful at restoring itself, then it may be presupposed that chronic lung disease would be the result thereof (Tsuji et al., 2004).
In diseases such as pulmonary emphysema and fibrosis, epithelial injury and regeneration are considered to prevail continually. These repeated cycles of epithelial cells damage proliferation and differentiation at the site of injury resulting in faster shortening of telomere length, in turn affecting replicative senescence. Moreover, stress-induced senescence may occur due to inhaled toxins regenerating oxidative stress and DNA damage in epithelial cells. However, once epithelial cells reach the senescence stage, they are no longer able to proliferate. Lung diseases may progress due to the cessation of repair responses in epithelial cells which affects the architectural and functional disruptions of alveolar epithelium (Tsuji et al., 2004).

Studies involving single exposure to cigarette smoke extract (CSE) pointed to cell growth inhibition at several stages of the cell cycle without killing the cells. In this study it was also indicated that, while there was a decrease in proliferation, there was a noted increase in p53 and p21 activity. When research was done on multiple exposures to CSE, it was found that cell growth decreased significantly, to the point that it nearly ceased to grow. The cells appeared to have a flat and enlarged morphology with an unregulated p16 and senescence associated β-galactosidase activity. These observations indicate that a single exposure to cigarette smoke will inhibit normal fibroblast proliferation, whereas multiple exposures seem to modify cells to a irreversible state of senescence (Tsuji et al., 2004). This will render the lungs more susceptible to stress, such as exposure to oxidants.
Studies also suggest that cigarettes smoke induced senescence of epithelial cells can be inhibited by an addition of an anti-oxidant called NAC, which indicates that oxidative stress is involved in the signalling pathways mediating the cigarette smoke induced senescence (Tsuji et al., 2004).

1.9 MOTIVATION FOR RESEARCH INVESTIGATION

Several studies conducted in the laboratories at the University of the Western Cape demonstrated an interference with the parenchymal lung tissue of the offspring when exposed to nicotine via the placenta and mother’s milk. This, in effect, decreases the amount of air sacs (alveolar number) resulting in a reduced surface area available for efficient gas exchange in the lung of the offspring. Although the offspring were only exposed to nicotine during gestation and lactation, emphysema-like lesions appear to develop after nicotine withdrawal in the offspring, which is after weaning on postnatal day 21. It has been proposed that exposure of the developing lung to nicotine changes the program that controls the maintenance of the structural and functional integrity of the lung as the animal age. It is further suggested that the lungs of these nicotine exposed offspring was programmed to age faster because of: 1) irreversible inhibition of glycolysis, 2) persistent high levels of AMP (adenosine monophosphate) during growth and development, 3) high levels of ROS (Maritz and Harding, 2011), and 4) genotoxic effects of nicotine (Demirhan et al., 2011). Previous studies have shown that supplementing the diet of mothers with strong anti-oxidants during gestation and lactation could possibly resist a change in the program which controls lung
development and integrity of the offspring in the long term (Maritz and Harding, 2011).

1.10 AIMS AND OBJECTIVES

It is known that lung development goes through several phases during which it develops into an efficient gas-exchanger. It has been shown that maternal nicotine exposure during gestation and lactation, and thus during all the phases of lung development, adversely affect the maintenance of lung integrity in the long-term. The aim of this project is to:

a) Establish whether maternal nicotine exposure during gestation, which is up to the saccular phase of lung development, will affect lung development in the short term and maintenance of structural integrity in the long term.

b) Determine whether maternal nicotine exposure during pregnancy will affect cell senescence in the alveolar walls.

c) Determine whether tomato juice supplementation will prevent the effects of maternal nicotine exposure on the lungs of the offspring.
CHAPTER TWO

Materials and Methods

2.1 ANIMALS AND ETHICAL CLEARANCE

White virgin female rats (Wistar descendents) were used in the present study. They were housed in the animal rooms at the Department of Medical Biosciences of the University of the Western Cape. Ethical clearance for this study was approved by the Ethical Committee of the University of the Western Cape in accordance to the guidelines of the Medical Research Council for Animal Care. Animals were fed a stock diet (Epol rat cubes and water as required) throughout the intended experiment. Room temperature was kept at 22±1°C and a day-night cycle of 12 hours (06:00 – 18:00) was maintained.

2.2 TREATMENT OF ANIMALS

Animals were mated overnight and afterwards randomly assigned to a control and three different experimental groups. At least 7 female rats were assigned to each group. Treatment of all the animals commenced on day 7 after mating. The pregnant experimental animals were divided into 4 groups (table 2.2): Group 1 received 1mg nicotine subcutaneously/ kg body weight/day. Group 2 received diluted tomato juice. Group 3 received both nicotine (1mg/ kg body weight/day) and diluted tomato juice. Group 4 was the control, receiving saline injections...
subcutaneously where the volume of saline was determined by the body weight of the mother.

### Table 2.2 Treatment of animals

<table>
<thead>
<tr>
<th>Group</th>
<th>1 (N)</th>
<th>2 (T)</th>
<th>3 (N+T)</th>
<th>4 (C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment (as from DAY 7 after mating)</td>
<td>Nicotine 1mg/BW/kg/day</td>
<td>Tomato Juice</td>
<td>Tomato Juice and Nicotine 1 mg/BW/kg/day</td>
<td>Control Saline</td>
</tr>
</tbody>
</table>

Table 2.2 Treatment of animals

The offspring received nicotine, lycopene and other nutrients in tomato juice only via the placenta and mother’s milk. Nicotine doses were not changed as the body weights of the animals increased during pregnancy; it remained standard according to the initial body weight of the female rats before mating. Nicotine doses were administered via subcutaneous injections with a 1ml tuberculin syringe.
F0 GENERATION: Mother exposed to nicotine during gestation.

F1 GENERATION: Babies received nicotine via placenta.

F1 GENERATION: Lungs of offspring were removed at postnatal days 14, 21, 42 and 84.

Figure 2.2 Rat model to illustrate that rats were exposed to nicotine and/or tomato juice in utero. F0 rats were mated to generate the F1 offspring. The lungs of the F1 offspring were exposed to nicotine and/or tomato juice via the placenta and mother’s breast milk (F0 generation).

The tomato juice (All Gold) containing 5.3mg lycopene/100ml was diluted 50/50 with distilled water on a daily basis due to its content being too viscous to move through the drinking bottles. It was freely available for the animals to drink from 200ml glass water bottles. The water and tomato juice intake was measured daily at a set time, and the average intake was recorded per week. The lycopene intake was calculated based on the tomato juice intake by the animals and recorded as the average weekly intake (mg/100g body weight/week). Apart from lycopene (5.3mg/100ml tomato juice), the tomato juice also contains protein (0.8g/100ml), carbohydrates (3.4g/100ml), fibre (0.55g/100ml), and sodium (200mg/100ml).

Lycopene in dietary sources mainly contains all-trans lycopene. However, research has shown that processed tomato products such as tomato paste and tomato puree contains a higher bioavailability than raw tomatoes (Boileau et al.,
2002). The pregnant rats within each of the experimental groups were weighed on a weekly basis to monitor any changes in their body weights and to determine if there were any abnormal changes in weights due to their different treatments. After birth the number of rat pups per litter was kept between 10 and 12 pups to ensure that the nutrient supply from the mother was adequate to support the normal growth and development of the offspring.

2.3 LUNG EXCISION PROCEDURE

Seven rats gave birth to seven litters within each of the four groups. At least 2 rat pups per litter were used for lung excision from each of the seven litters per group on postnatal days 14, 21, 42 and 84.

To remove lung tissue, the offspring were anaesthetized with an intraperitoneal injection of Sodium Pentobarbitone (90mg/kg/BW). Thereafter, the trachea was surgically cannulated and the diaphragm punctured. The fixative (10% buffered formalin, pH 7.2) was allowed to flow into the lungs via the trachea while a transpulmonary pressure gradient of 25cm fixative was maintained. After 30 minutes the lungs were ligated at the hilum of the lungs and removed via excision en bloc for morphologic and morphometric studies. The lungs were each separately labeled and stored in buffered formalin (pH 7.2).

Buffered formalin solution was made up of 4g Sodium Phosphate (Anhydrous) and 6g Sodium Phosphate (dehydrogenous) added to 900ml distilled water. Once
the chemicals were dissolved in the water, 100ml Formaldehyde solution was added to make up 1000ml buffered formalin solution.

2.4 DETERMINATION OF LUNG VOLUMES

Lung volumes were measured by the fluid displacement method (Scherle, 1970), whereby a 100ml beaker containing buffered formalin at a pH of 7.2 was weighed on a scale, and then zeroed. With the support of a surgical forceps, the extracted lung was then immersed in the beaker at a level of buoyancy, and the final lung volume was determined. Lung volumes were determined before and after the 24-hour fixation period to detect shrinkage. Due to minimal shrinkage (<2%), data was not corrected for shrinkage.

2.5 LUNG TISSUE PROCESSING

Subsequent to the determination of the lung volumes, the left inferior lobe of each pair of lungs were surgically removed with sterile blades and then placed in plastic embedding cassettes for further processing. Care was taken as not to damage the tissue during removal and processing. The lung-tissue cassettes were placed in a Histokinette™ tissue processor for the dehydration of lung tissue through a series of agents over a period of 18 hours through an automation process. Tissue was immersed in a series of solutions (Table 2.5), where it was dehydrated a gradual increase of alcohols, deparaffinized in xylene baths, then embedded in wax and left to dry on a cold plate.
Table 2.5  Illustration of lung tissue processing procedure (below).

<table>
<thead>
<tr>
<th>AGENT</th>
<th>TIME</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Dehydration Process</strong></td>
<td></td>
</tr>
<tr>
<td>1. 70% Ethanol</td>
<td>2 hours</td>
</tr>
<tr>
<td>2. 80% Ethanol</td>
<td>2 hours</td>
</tr>
<tr>
<td>3. 90% Ethanol</td>
<td>2 hours</td>
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<tr>
<td>4. Absolute Ethanol</td>
<td>2 hours</td>
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<tr>
<td>5. Absolute Ethanol</td>
<td>2 hours</td>
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<tr>
<td><strong>Deparaffinization Process</strong></td>
<td></td>
</tr>
<tr>
<td>6. Xylene Bath I</td>
<td>2 hours</td>
</tr>
<tr>
<td>7. Xylene Bath II</td>
<td>2 hours</td>
</tr>
<tr>
<td><strong>Tissue Embedding Preparation</strong></td>
<td></td>
</tr>
<tr>
<td>8. Wax Bath I</td>
<td>2 hours</td>
</tr>
<tr>
<td>9. Wax Bath II</td>
<td>2 hours</td>
</tr>
</tbody>
</table>

2.6  TISSUE EMBEDDING AND MICROTOMY

Subsequent to the automated tissue processing, tissues were manually removed from cassettes in order to begin the tissue embedding procedure. Lung tissue was carefully aligned and fixed into a mould block using warm wax to fill the mould. The mould hardened on an ice plate utilizing a tissue embedding machine. Tissue blocks were trimmed at right angles to enable accurate morphometric measurements. The paraffin tissue blocks were then cut into sections of 5µm thickness with a microtome for further microscopy. Every third section was used for morphology and morphometric evaluation. This prevented double counting of tissue structures. Once the sections were cut, they were floated on a warm water bath to remove wrinkles from the thinly cut paraffin lung tissue sections, which was then shifted onto a glass microscopic slide for further staining processes. Once slides were dried, it was placed in an oven at 80°C for ±30 minutes to allow fixation of lung tissue to the slide. All slides were initially examined to eliminate sections with evidence of inadequate preparation. A total of 32 slides for each postnatal group (day 14, 21, 42 and 84) were prepared and equally divided.
amongst each of their experimental groups (control, tomato juice, nicotine, and a combination of nicotine and tomato juice). The lung tissue slides were then ready for Haematoxylin and Eosin (H&E), Collagen and β-Galactosidase staining techniques.

### 2.6.1 Haematoxylin and Eosin Staining

<table>
<thead>
<tr>
<th>CHEMICAL SOLUTION</th>
<th>TIME</th>
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<table>
<thead>
<tr>
<th>Deparaffination and Rehydration</th>
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<tbody>
<tr>
<td>1. Xylene bath I</td>
<td>5 mins</td>
</tr>
<tr>
<td>2. Xylene bath II</td>
<td>5 mins</td>
</tr>
<tr>
<td>3. Absolute ethanol I</td>
<td>5 mins</td>
</tr>
<tr>
<td>4. Absolute ethanol II</td>
<td>5 mins</td>
</tr>
<tr>
<td>5. 90% Ethanol</td>
<td>5 mins</td>
</tr>
<tr>
<td>6. 80% Ethanol</td>
<td>5 mins</td>
</tr>
<tr>
<td>7. 70% Ethanol</td>
<td>5 mins</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Haematoxylin Staining</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>8. Haematoxylin</td>
<td>15 mins</td>
</tr>
<tr>
<td>9. Rinse in tap water</td>
<td></td>
</tr>
<tr>
<td>10. Scott’s tap water</td>
<td>2 mins</td>
</tr>
<tr>
<td>11. 1% Acid alcohol – destaining agent</td>
<td>2 mins</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Eosin Staining and Dehydration</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>12. Eosin – counter stain</td>
<td>3 mins</td>
</tr>
<tr>
<td>13. Rinse in tap water</td>
<td></td>
</tr>
<tr>
<td>14. 80% Ethanol</td>
<td>2 mins</td>
</tr>
<tr>
<td>15. 90% Ethanol</td>
<td>2 mins</td>
</tr>
<tr>
<td>16. Absolute Ethanol</td>
<td>2 mins</td>
</tr>
<tr>
<td>17. Xylene bath I</td>
<td>2 mins</td>
</tr>
<tr>
<td>18. Xylene bath II</td>
<td>2 mins</td>
</tr>
<tr>
<td>19. Mount for observation using Xylene-based permount and coverslip slides, covering all the tissue.</td>
<td></td>
</tr>
</tbody>
</table>

Parenchymal tissue includes alveolar septa, alveolar ducts, respiratory bronchioles, and blood vessels with the diameter of <10µm. Samples were viewed at 100x magnification. Nuclei in cells stained blue and cytoplasm and red blood cells stained in various shades of red, pink and orange. An example of haematoxylin staining of lung tissue in the control group and nicotine group at postnatal day 84 is illustrated in Fig. 2.6.1.1.
2.6.2. Connective Tissue and Collagen Staining

The Elastica van Gieson staining kit for connective tissue was used to stain elastic fibers in tissue sections. It is a combination of the picrofuchsin method according to van Gieson and the nuclear staining technique according to Weigert's (Table 2.6.2).

Table 2.6.2 Connective and Collagen Staining Protocol (below):

<table>
<thead>
<tr>
<th>CHEMICAL SOLUTION</th>
<th>TIME</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Deparaffination and Rehydration</strong></td>
<td></td>
</tr>
<tr>
<td>1. Xylene bath I</td>
<td>5 mins</td>
</tr>
<tr>
<td>2. Xylene bath II</td>
<td>5 mins</td>
</tr>
<tr>
<td>3. Absolute ethanol I</td>
<td>5 mins</td>
</tr>
<tr>
<td>4. Absolute ethanol II</td>
<td>5 mins</td>
</tr>
<tr>
<td>5. 90% Ethanol</td>
<td>5 mins</td>
</tr>
<tr>
<td>6. 80% Ethanol</td>
<td>5 mins</td>
</tr>
<tr>
<td><strong>Connective and Collagen Stain</strong></td>
<td></td>
</tr>
<tr>
<td>7. Stain with Elastin according to Weigert</td>
<td>10 mins</td>
</tr>
<tr>
<td>8. Rinse under running tap water</td>
<td>1 min</td>
</tr>
<tr>
<td>9. Stain with Weiger's A&amp;B 1:1</td>
<td>5 mins</td>
</tr>
<tr>
<td>10. Rinse under running tap water</td>
<td>1 min</td>
</tr>
<tr>
<td>11. Stain with Picrofuchsin Solution</td>
<td>2 mins</td>
</tr>
<tr>
<td><strong>Dehydration</strong></td>
<td></td>
</tr>
<tr>
<td>12. 70% Ethanol</td>
<td>2 mins</td>
</tr>
<tr>
<td>13. 80% Ethanol</td>
<td>2 mins</td>
</tr>
<tr>
<td>14. 90% Ethanol</td>
<td>2 mins</td>
</tr>
<tr>
<td>15. Absolute Ethanol</td>
<td>2 mins</td>
</tr>
<tr>
<td>16. Xylene bath I</td>
<td>2 mins</td>
</tr>
<tr>
<td>17. Xylene bath II</td>
<td>2 mins</td>
</tr>
<tr>
<td>18. Mount for observation using Xylene-based permount and coverslip slides, covering all the tissue,</td>
<td></td>
</tr>
<tr>
<td>19. Once dried, observe under microscope at 100x total magnification.</td>
<td></td>
</tr>
</tbody>
</table>
Assessment of nuclei (black-brown pigment), elastic fibres (black pigment), collagen (red pigment) and muscle (yellow pigment) were observed. Fig. 2.6.2.1 illustrates examples of connective tissue staining in the lungs of the offspring, in the control and nicotine groups, at postnatal day 84.

![Fig.2.6.2.1 Connective Tissue Staining of Control Lung](image)

### 2.6.3. **β-Galactosidase Staining**

The purpose of this stain was to detect senescent cells in the alveolar walls of the lungs. Senescent cells are indicative of aging and represent an arrested state in which cells remain viable but unable to proliferate. Detection of cellular senescence histochemically, was done by means of staining for displays of senescence-associated expression of β-galactosidase (SA-β-Gal) activity in the cells of the alveolar walls. SA-β-Gal is only present in senescent cells, not in presenescent, quienscent or immortal cells.

#### 2.6.3.1 **Preparation for β-Galactosidase Staining**

Firstly, 10X PBS was prepared by weighing 80g NaCl, 2g KCl and 11.5g Na$_2$HPO$_4$, then solving it in 1000ml distilled water(dH$_2$O). 10ml of the prepared
10X PBS was then transferred into 900ml dH₂O in order to reach a 1 in 1000 dilution (1XPBS).

*Nuclear Fast Red*, a chemical solution which gives the cytoplasm of tissue its red pigment was prepared by adding 50g Potassium Aluminium Sulphate and 1g Nuclear Fast Red to 500ml dH₂O. The solution was then mixed until it was fully dissolved in the dH₂O, and then heated in a beaker for 30 minutes.

Subsequently, a *Fixative Solution* was prepared by adding 470µl staining solution and 25µl staining supplement to 125µl of 20mg/ml Xgal in DMF (Dimethylformaldehyde). When not used, it was stored in a fridge at -20°C in a dark glass container.

On completion of the preparatory solutions, β-Galactosidase staining was then conducted involving rehydration of tissue slides, tissue fixing, overnight slide incubation, cytoplasm staining, and dehydration processes (table 2.6.3).
Table 2.6.3. β-Galactosidase Staining Protocol

<table>
<thead>
<tr>
<th>CHEMICAL SOLUTION</th>
<th>TIME</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Rehydration</strong></td>
<td></td>
</tr>
<tr>
<td>1. Xylene Bath I</td>
<td>3 minutes</td>
</tr>
<tr>
<td>2. Xylene Bath II</td>
<td>3 minutes</td>
</tr>
<tr>
<td>3. Absolute Ethanol I</td>
<td>3 minutes</td>
</tr>
<tr>
<td>4. Absolute Ethanol II</td>
<td>3 minutes</td>
</tr>
<tr>
<td>5. 90% Ethanol</td>
<td>3 minutes</td>
</tr>
<tr>
<td>6. 80% Ethanol</td>
<td>3 minutes</td>
</tr>
<tr>
<td><strong>PBS Wash</strong></td>
<td></td>
</tr>
<tr>
<td>7. Immerse slides in the prepared 1XPBS bath</td>
<td>5 minutes</td>
</tr>
<tr>
<td><strong>Tissue Fixing</strong></td>
<td></td>
</tr>
<tr>
<td>8. In a foiled dark box, pipette droplets of the prepared Fixative Solution on each tissue slide, then cover to allow fixation.</td>
<td>15 minutes</td>
</tr>
<tr>
<td><strong>PBS Wash</strong></td>
<td></td>
</tr>
<tr>
<td>9. Immerse slides in a new 1XPBS bath</td>
<td>5 minutes</td>
</tr>
<tr>
<td><strong>Overnight Incubation</strong></td>
<td></td>
</tr>
<tr>
<td>10. Add the prepared Staining Solution Mix to each tissue slide. Incubate overnight in a dark box at 37°C.</td>
<td>24 hours</td>
</tr>
<tr>
<td><strong>PBS Wash</strong></td>
<td></td>
</tr>
<tr>
<td>11. After overnight incubation, immerse slides in a new 1XPBS bath</td>
<td>5 minutes</td>
</tr>
<tr>
<td><strong>Cytoplasm Staining</strong></td>
<td></td>
</tr>
<tr>
<td>12. Immerse slides in the prepared Nuclear Fast Red Solution</td>
<td>30 minutes to 60 minutes (preferably 60 minutes when staining lung tissue samples)</td>
</tr>
<tr>
<td><strong>Dehydration</strong></td>
<td></td>
</tr>
<tr>
<td>13. 80% Ethanol</td>
<td>2 minutes</td>
</tr>
<tr>
<td>14. 90% Ethanol</td>
<td>2 minutes</td>
</tr>
<tr>
<td>15. Absolute Ethanol Bath</td>
<td>2 minutes</td>
</tr>
<tr>
<td>16. Xylene Bath I</td>
<td>2 minutes</td>
</tr>
<tr>
<td>17. Xylene Bath II</td>
<td>2 minutes</td>
</tr>
<tr>
<td>18. Mount for observation using mounting media and coverslip slides, covering all the tissue.</td>
<td></td>
</tr>
</tbody>
</table>

Slides were then viewed under a microscope (Zeiss Axiostar Plus) for the development of a blue pigment along alveolar walls which is indicative of cellular senescence.
2.7 MORPHOMETRIC EVALUATION

In this study, the following morphometric techniques were used to quantify changes in the lungs of the offspring due to aging, nicotine exposure, and/or tomato juice supplementation during gestation.

2.7.1 Linear Intercept (Lm)

The Mean Linear Intercept (Lm) is a stereological method used to quantify the size of the alveoli in our animal models. It is the distance between alveolar walls and represents alveolar volume. Therefore, an increased Lm, is indicative of alveolar wall destruction, causing an enlargement of air spaces (alveoli), resulting in less surface area available for gas exchange in the lungs.

2.7.1.1 Determination of linear intercept (Lm)

- Equation: \( Lm = \frac{N \times L}{m} \)

- Where:
  - \( N \) = number of fields counted
  - \( L \) = length of crossline (2.02mm at 100x magnification)
  - \( m \) = sum of all intercepts

A micrometer containing a crossline, placed in the eye piece of a light microscope (100x magnification) was used to determine the number of alveolar intercepts. The Lm was calculated from the number of intercepts (m) where the crossline passes through the alveolar wall, and the number of times the line was placed on
the tissue sections. An alveolar wall that touches, but does not pass through a line is counted as 1 intercept, and an alveolar wall which passes through a line is counted as 2 intercepts.

- Example of Lm calculation:

<table>
<thead>
<tr>
<th>Field</th>
<th>Number of Intercepts</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>26</td>
</tr>
<tr>
<td>2</td>
<td>16</td>
</tr>
<tr>
<td>3</td>
<td>22</td>
</tr>
<tr>
<td>4</td>
<td>25</td>
</tr>
<tr>
<td>5</td>
<td>15</td>
</tr>
<tr>
<td>Total</td>
<td>104</td>
</tr>
</tbody>
</table>

Therefore:

\[
Lm = N \times \frac{L}{m} \\
= 2.02 \times \frac{5}{104} \\
= 0.097 \text{mm} \\
= 97 \mu\text{M}
\]

2.7.2 Point-Counting Method

The point-counting approach was used to measure \( V_t \) (tissue volume density) and \( V_a \) (alveolar air volume density) in the lungs of the control and experimental offspring (Bolender et al., 1993).
2.7.2.1  Determination of Vt

Vt was achieved by inserting a graticule, containing a point-counting grid in the eye piece of a light microscope (100x magnification).

The parenchymal fraction was calculated with the grid (122 points), where the points that fell over the lung tissue were counted, excluding points that fell over the blood vessels and bronchi with diameters larger than 2mm. At least 5 non-overlapping fields were randomly selected for point counting, for each lung tissue slide.

- Eg of Vt Calculation:

<table>
<thead>
<tr>
<th>Field</th>
<th>Number of Points Counted</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>49</td>
</tr>
<tr>
<td>2</td>
<td>29</td>
</tr>
<tr>
<td>3</td>
<td>36</td>
</tr>
<tr>
<td>4</td>
<td>48</td>
</tr>
<tr>
<td>5</td>
<td>41</td>
</tr>
</tbody>
</table>

Total: 203
Therefore:

\[
V_t = \frac{\text{total no. of points counted}}{\text{total no. of fields}}
\]

\[
= \frac{203}{5}
\]

\[
= \frac{40.6}{122 \text{ points (on grid)}}
\]

\[
= 33.28\%
\]

2.7.2.2 *Determination of Va:*

Va was achieved by subtracting the Vt by 100 in order to determine the percentage of the alveolar air space and volume available in the lungs after exposure to maternal nicotine, tomato juice and a combination of nicotine and tomato juice.

- Eg of Va Calculation:

  Day 14, control animals had a Vt of 33.69%

Therefore:

\[
V_a = 100 - V_t
\]

\[
= 100 - 33.69\%
\]

\[
= 66.31\%
\]

2.7.3 *Alveolar Number (Na)*

An estimate of the Na can give an indication as to the number of alveoli in the area available for gas exchange.
The Na was calculated in two parts, where the alveolar volume (Valv) was first determined from the Lm, and then the Na was determined from lung volumes (Lv), volume density of air (Va) and Valv (Massaro and Massaro, 2000).

### 2.7.3.1 Determination of Na Calculation

- Where: \[\text{Valv} = \frac{\text{Lv} \times \pi}{3}\], and \[\text{Na} = \frac{\text{Lv} \times \text{Va}}{\text{Valv}}\]

- Example of Na Calculation
  - Control Day 14: \(\text{Lv} = 1.32\text{ml}, \text{Va} = 66.81\%\)

  - First Equation:
    \[\text{Valv} = \frac{\text{Lv} \times \pi}{3}\]
    \[= \frac{1.32\text{ml} \times 3.14}{3}\]
    \[= 1320\mu\text{l} \times 3.14/3\]
    \[= 1.3 \times 10^3\mu\text{l}\]

  - Second Equation:
    \[\text{Na} = \frac{\text{Lv} \times \text{Va}}{\text{Valv}}\]
    \[= \frac{1320\mu\text{l} \times 66.81}{1.3 \times 10^3}\]
    \[= 6.7 \times 10^4\text{alveoli}\]

### 2.7.4 Cellular Senescence

After the quantification of senescent cells, it was expressed as cells/100 \(\mu\text{m}\) of alveolar wall. The total number of senescent cells per length of alveolar wall was
determined by using the Image J software application. Pictures were taken at 400x magnification and used for determining cell numbers. A line was drawn along the alveolar wall, where the line started at the senescent cell and ended at a senescent cell. The B-galactosidase stained cell occurring between the start and the end points if the line was counted and the number of cells expressed as senescent cells/100 μm of alveolar wall. An example of B-galactosidase activity (dark stained spots along alveolar wall) was indicative that cellular senescence took place (see Fig. 2.7.4).

![Fig. 2.7.4 Digital image of senescent cells along alveolar wall](image)

This method was repeated 5-7 times for each group of animals (control, nicotine, tomato juice, nicotine and tomato juice exposed animals), for each age group. Calculations were done subsequent to the senescent cell counting method.
2.7.4.1 *Determination of Senescent Cell Count*

- Eg of Senescent Cell Calculation:

  - Equation:
    - $\frac{100}{\text{length of alveolar walls}} \times \text{no. of senescent cells along alveolar wall}$

  - Where:
    - $438.49\mu m = \text{length of alveolar wall}$
    - $7 = \text{number of cells along alveolar wall}$

    $= \frac{100}{438.49} \times 7$

    $= 1.60 \text{ cells/100µm alveolar wall}$

2.7.5 *Lung Compliance*

An estimate of the lung compliance is a measure of the ability of the lung to stretch when a volume of air is inhaled, and its ability to recoil when it is exhaled. Lung compliance was determined by determining the volume of the lungs of the control and experimental groups at different ages at a constant transpulmonary pressure of $25\text{cm H}_2\text{O}$. 
2.7.5.1 *Determination of Lung Compliance*

- Where:
  - \( Lv \) = Lung Volume of Animal
  - \( P = 25 \text{cm H}_2\text{O} \) (pressure constant)
  - \( BW \) = Body Weight of Animal

- Example of Lung Compliance calculation
  - Control Day 14;
  - \( Lv = 1.32 \text{ml} \);
  - \( BW = 25.17 \text{g} \)

  **Calculation:**
  \[
  \text{Lung Compliance} = \frac{Lv}{P} \div BW
  = \frac{1.32}{25} \div 25.17
  = 0.00209 \times 1000
  = 2.1 \text{ml/cm H}_2\text{O/kg}
  \]

The morphometric data at postnatal day 21 was pooled since no differences were observed between males and females.

2.8 **STATISTICAL ANALYSIS**

Statistical analysis of this study is expressed as means±SEM, where differences between experimental groups and differences between males and females within experimental groups were carried out by the use of the one way analysis of
variance (ANOVA) for unpaired data, followed by the Student-Newman-Kuels test for pairwise comparisons. A probability level of $P<0.05$ was designated as significant in this study.
CHAPTER THREE

Results

3.1 BODY WEIGHT (BW) AND TOMATO JUICE CONSUMPTION (TJC) OF PREGNANT RATS

3.1.1 Body Weights (BW) of Pregnant Rats

The BW of the pregnant rats increased throughout the gestational period (fig.3.1.1). A gradual increase from week 0 to 3 was maintained in all experimental groups (P>0.05). However, at gestational week 1, the animals that received both nicotine and tomato juice during gestation (256.54±6.47g) had BW of 15.6% (P<0.05) lower than the control group (303.9±3.4g), (Fig. 3.1.1(a)).

3.1.2 Tomato Juice Consumption (TJC) of Pregnant Rats

The TJC was measured throughout the gestational period (fig 3.1.2.). At gestational week 2, TJC was 38.1% (P<0.05) higher in the group of pregnant animals receiving tomato juice only (89.42±9.88ml/week), compared to animals receiving both nicotine and tomato juice (55.4±8.61ml/week). Both groups displayed a gradual increase in TJC throughout gestational week 1 and 2. However, at gestational week 3, the TJC of animals receiving tomato juice only decreased to a point where both groups showed a similar TJC (P>0.05).
3.1.3 Lycopene Consumption of Pregnant Rats

Tomato juice contains 5.3mg lycopene per 100ml. Thus it was possible to determine the lycopene consumption of animals who received tomato juice only, and a combination of nicotine and tomato juice throughout gestation (Fig. 3.1.2(a)). The consumption of lycopene follows the same trend as the consumption of tomato juice. The mothers that received tomato juice only had a higher lycopene (mg/ml/week) consumption compared to those mothers that received both nicotine and tomato juice throughout the gestational period. However, at gestational week 2, the pregnant mothers that received both nicotine and tomato juice consumed 4.44 mg/ml/week lycopene, whereas those mothers who received tomato juice only, consumed 4.24 lycopene mg/ml/week lycopene.

3.1.4 Litter Sizes at Birth of Offspring

The number of rat pups was counted per litter at the time of birth. This was to determine whether nicotine exposure via the placenta, or maternal tomato juice supplementation during gestation affected the litter size. The control group gave birth to litter sizes between 14 and 16 rat pups. Mothers who received both nicotine and tomato juice, or tomato juice only gave birth to litter sizes between 10 and 14 rat pups. Mothers who received nicotine during gestation gave birth to rat pups between 9 and 13 per litter (fig. 3.1.4).
Fig. 3.1.1 The effect of nicotine, tomato juice as well as nicotine and tomato juice supplementation on maternal body weight during gestation.

Fig. 3.1.1(a) The effect of nicotine, tomato juice as well as nicotine and tomato juice supplementation on maternal body weight at gestational week 2.

Table 3.1.1 The effect of nicotine, tomato juice as well as nicotine and tomato juice supplementation on maternal body weight during gestation (below).

<table>
<thead>
<tr>
<th>Gestation</th>
<th>C</th>
<th>C vs. T</th>
<th>T</th>
<th>T vs. N</th>
<th>N</th>
<th>C vs. NT</th>
<th>NT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Week 0</td>
<td>294.5±4.50</td>
<td>P&gt;0.05</td>
<td>266.2±6.96</td>
<td>P&gt;0.05</td>
<td>254.2±10.00</td>
<td>P&gt;0.05</td>
<td>249.7±14.44</td>
</tr>
<tr>
<td>Week 1</td>
<td>303.9±3.4</td>
<td>P&gt;0.05</td>
<td>280.8±6.8</td>
<td>P&gt;0.05</td>
<td>269.4±5.73</td>
<td>P&gt;0.05</td>
<td>256.5±6.47</td>
</tr>
<tr>
<td>Week 2</td>
<td>306.7±4.9</td>
<td>P&gt;0.05</td>
<td>315.5±4.26</td>
<td>P&gt;0.05</td>
<td>304.5±8.52</td>
<td>P&gt;0.05</td>
<td>289.6±6.72</td>
</tr>
<tr>
<td>Week 3</td>
<td>342.8±3.21</td>
<td>P&gt;0.05</td>
<td>354.5±6.78</td>
<td>P&gt;0.05</td>
<td>340.2±7.35</td>
<td>P&gt;0.05</td>
<td>339.1±2.33</td>
</tr>
</tbody>
</table>
Fig. 3.1.2  Tomato juice consumption (TJC) of pregnant rats throughout gestation

Fig. 3.1.3  Lycopene consumption of pregnant rats throughout gestation.

Table 3.1.2  Tomato juice and water consumption of pregnant rats throughout gestation.

Control (C) and Nicotine (N) groups received water. Tomato (T), as well as Nicotine and Tomato (NT) groups received diluted (50% water) tomato juice (ml/kg/week).

<table>
<thead>
<tr>
<th>Gestation</th>
<th><strong>C (water)</strong></th>
<th><strong>C vs. N</strong></th>
<th><strong>N (water)</strong></th>
<th><strong>T</strong></th>
<th><strong>T vs. NT</strong></th>
<th><strong>NT</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Week 0</td>
<td>62.5±9.24</td>
<td>P&lt;0.05</td>
<td>36.6±2.08</td>
<td>76.4±21.00</td>
<td>P&gt;0.05</td>
<td>40.4±8.37</td>
</tr>
<tr>
<td>Week 1</td>
<td>60±9.35</td>
<td>P&lt;0.05</td>
<td>57.8±3.11</td>
<td>89.2±49.88</td>
<td>P&lt;0.05</td>
<td>55.4±8.61</td>
</tr>
<tr>
<td>Week 2</td>
<td>47.5±1.99</td>
<td>P&lt;0.05</td>
<td>58.95±8.46</td>
<td>95.82±0.79</td>
<td>P&lt;0.05</td>
<td>83.69±8.13</td>
</tr>
<tr>
<td>Week 3</td>
<td>73.66±17.1</td>
<td>P&lt;0.05</td>
<td>40.2±3.2</td>
<td>87.85±5.75</td>
<td>P&lt;0.05</td>
<td>82.69±11.00</td>
</tr>
</tbody>
</table>

Table 3.1.3  Lycopene consumption of pregnant rats throughout gestation.

Tomato (T), as well as Nicotine and Tomato (NT) groups received diluted tomato juice (50% water) containing lycopene (mg/ml/week).

<table>
<thead>
<tr>
<th>Gestation</th>
<th><strong>T</strong></th>
<th><strong>NT</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Week 0</td>
<td>4.04</td>
<td>2.14</td>
</tr>
<tr>
<td>Week 1</td>
<td>4.74</td>
<td>2.94</td>
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<td>Week 2</td>
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<td>Week 3</td>
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<td>4.35</td>
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Fig. 3.1.4 Litter sizes of rats exposed to tomato juice, nicotine, and both nicotine and tomato juice at birth.
3.2 THE EFFECT OF MATERNAL NICOTINE EXPOSURE AND TOMATO JUICE INGESTION ON THE BW, LUNG VOLUME (Lv) AND BW/Lv RATIOS OF THE OFFSPRING.

3.2.1 Body Weights (BW) of the Offspring

BW changes in the offspring due to aging and the effect of maternal nicotine and tomato juice exposure during gestation is demonstrated in fig. 3.2.1. From birth up to postnatal day 42 there were no differences between male and female BW (P>0.05). However, from postnatal day 42 the BW of males increased faster compared to those of the female rats, so that at postnatal day 84, the BW of all male groups as shown in fig. 3.2.1(a) were significantly higher (P<0.05) than that of the females groups (fig 3.2.1(b)). Differences between experimental groups were shown mostly at postnatal day 84 (table 3.2.1).

3.2.2 Lung Volumes (Lv) of the Offspring

The effect of age, as well as maternal nicotine and tomato juice exposure during gestation on the lung volumes of the offspring are illustrated in Fig. 3.2.2. As expected, from postnatal day 14 up to postnatal day 84, there is a 6-7 fold increase (P<0.05) in Lv in both male and female control and experimental groups. From postnatal day 14 to postnatal day 42 the rate of increase of Lv was similar for all control and experimental groups. However, at postnatal day 84 the Lv of the male nicotine (11.66±1.05ml) group (fig.3.2.2 (a)) was 1.29-fold higher (P<0.05) than that of the control male (9.03±0.59ml) group. Also, at postnatal day 84, the Lv of the female offspring (fig.3.2.2 (b)) exposed to nicotine during gestation
(9.21±0.45ml) showed a 1.18-fold higher Lv than the control females (7.76±0.51ml). Furthermore, the Lv of males (11.64±0.95ml) and female offspring (10.1±0.67ml) that were exposed both nicotine and tomato juice during gestation, was at postnatal day 84 1.28-fold higher (P<0.02) compared to that of the control males (9.03±0.59ml) and females (7.76±0.51ml), respectively. This resembles the increases in BW of the rats born to mothers that received tomato juice during pregnancy.

Differences between male and female groups were observed at postnatal day 84. The increase in the Lv of the nicotine exposed male rats (11.66±1.05ml) was 1.26-fold more than the increase in Lv of (P<0.006) the nicotine exposed females (9.21±0.45ml). This implies that the response of the males and females to maternal nicotine exposure is different. Also, the Lv of the males exposed to both nicotine and tomato juice during gestation was 1.21-fold higher (P<0.02) than the female group exposed to both nicotine and tomato juice (table 3.2.2). This might be due to the faster increase in BW of the male rats during the same period of time.

### 3.2.3 Lung Volume/Body Weight Ratios (Lv/BW) of the Offspring

At postnatal day 14, animals exposed to nicotine showed a slightly higher (P<0.05) Lv/BW ratio compared to the rest of the experimental groups (table 3.2.3). Differences in Lv/BW ratios were displayed at postnatal day 42 in the female group exposed to both nicotine and tomato juice (P<0.05), showing a slightly higher Lv/BW compared to its control. At postnatal day 84, differences
were also observed between males (fig. 3.2.3(a)) and females (fig.3.2.3 (b)) of the control group (P<0.05). This was due to changes in the body composition that occurred once the offspring reached maturity, where males had a relatively higher BW and Lv than females.
Fig. 3.2.1  The effect of maternal nicotine and tomato juice exposure during gestation on the BW (g) of the male and female offspring.

Fig. 3.2.1(a) Bar graph illustration on BW of postnatal day 84 males exposed to maternal nicotine and tomato juice.

Fig. 3.2.1(b) Bar graph illustration on BW of postnatal day 84 females exposed to maternal nicotine and tomato juice.

Table 3.2.1 The effect of maternal nicotine, tomato juice, and a combination of nicotine and tomato juice during gestation on the body weight (g) of the male and female offspring at postnatal days 14, 21, 42 and 84 (below).

<table>
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<tr>
<th>Age (Days)</th>
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<th>C vs. T</th>
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<th>C vs. N</th>
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<td>F</td>
<td>25.17±0.80</td>
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<td>21</td>
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<td>36.71±2.15</td>
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<td>35.73±0.76</td>
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<td>37.43±1.68</td>
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Fig. 3.2.2. The effect of maternal nicotine and tomato juice exposure during gestation on the Lung Volumes (ml) of the male and female offspring.

Fig. 3.2.2(a) Bar graph illustration on Lung Volumes of postnatal day 84 males exposed to maternal nicotine and tomato juice.

Fig. 3.2.2(b) Bar graph illustration on Lung Volumes of postnatal day 84 females exposed to maternal nicotine and tomato juice.

Table 3.2.2 The effect of maternal nicotine, tomato juice, and a combination of nicotine and tomato juice during gestation on the lung volumes (ml) of the male and female offspring at postnatal days 14, 24, 42 and 84 (below).

<table>
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<tr>
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Fig. 3.2.3 The effect of maternal nicotine and tomato juice exposure during gestation on the Lv/BW ratio (ml/g) of the male and female offspring.

Fig. 3.2.3(a) Bar graph illustration on Lv/BW ratio (ml/g) of postnatal day 42 males exposed to maternal nicotine and tomato juice.

Fig. 3.2.3(b) Bar graph illustration on Lv/BW ratio (ml/g) of postnatal day 42 females exposed to maternal nicotine and tomato juice.

Table 3.2.3 The effect of maternal nicotine, tomato juice, and a combination of nicotine and tomato juice during gestation on the lung volume/body weight ratio (ml/g) of the male and female offspring at postnatal days 14, 24, 42 and 84 (below).

<table>
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<th>Age (Days)</th>
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<th>C vs. T</th>
<th>T</th>
<th>C vs. N</th>
<th>N</th>
<th>C vs. NT</th>
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3.3 THE EFFECT OF MATERNAL NICOTINE EXPOSURE AND TOMATO JUICE INGESTION DURING GESTATION ON THE CHEST CIRCUMFERENCE (CC) AND CROWN-RUMP LENGTH (CRL), CC/CRL RATIOS, AND CC/Lv RATIOS IN THE OFFSPRING.

3.3.1 The Chest Circumference (CC) of the Offspring

The data in Table 3.3.1 illustrates the effect of aging, as well as maternal nicotine exposure, and tomato juice ingestion during gestation, on the CC of the offspring. At postnatal day 14, the CC of all the experimental groups was smaller than that of the controls (P<0.05). At postnatal day 21, animals exposed to nicotine, and to both nicotine and tomato juice, had a CC of between 1.11-fold to 1.20-fold (P<0.05) smaller than the control group, respectively (fig. 3.3.1). No differences in CC were observed in the female group at postnatal day 42. However, at postnatal day 42, the CC of the males (93±3.20mm) born to mothers that ingested tomato juice during gestation had a CC of 1.12-fold lower (P<0.05) than that of the control group (104±2.09mm).

Female differences in CC between experimental groups (fig. 3.3.1 (b)) were observed at postnatal day 84 (P<0.05). Females that received tomato juice via the mother during gestation (107.76±4.25mm) had a CC lower than the control group (113.56±2.94mm) at postnatal day 84. Whereas females exposed to nicotine (120.76±4.18mm) via the placenta had a CC that is higher than that of the control group of the same age; and females exposed to both maternal nicotine and tomato
juice (128.32±4.38mm) during gestation had a CC of 1.12-fold higher than the control group (113.56±2.94mm), at postnatal day 84.

At postnatal day 84, the response of male and female offspring to maternal nicotine exposure, or supplementing the mother’s diet with tomato juice or exposure of the mother to both nicotine and tomato juice was different. The CC of the nicotine exposed males (134.15±7.86mm) was higher (P<0.05) than the nicotine exposed females (120.76±4.18mm). Similarly, the males (139.21±5.58mm) exposed to both nicotine and tomato juice during gestation, had a CC higher (P<0.05) compared to the females (128.32±4.38 mm) of the same group, (fig. 3.3.1 (a) and fig. 3.3.1 (b)).

3.3.2 The Crown-Rump Length (CRL) of the Offspring

The CRL (fig. 3.3.2) in the offspring at postnatal day 14 differed between groups (P<0.05). However, at postnatal day 21, only the group exposed to tomato juice, and to both nicotine and tomato juice displayed higher CRL measurements compared to the controls of the same age (P<0.05). At postnatal day 84, males exposed to both nicotine and tomato juice (196.5±4.71mm) was higher (P<0.05) than that of the control (184.29±3.67mm). In the 84-day-old female group, differences in CRL was shown in animals exposed to nicotine via the placenta, as well as those females exposed to both nicotine and tomato juice (P<0.05). The CRL of the females exposed to nicotine (179.95±1.86mm) via the placenta had a higher CRL than the controls (173.31±2.48mm). The data also shows that the females exposed to both tomato juice and nicotine (182.89±3.74mm) via the
placenta had a significantly higher CRL than the control group (173.31±2.48mm) of the same age.

### 3.3.3 The Chest Circumference/Crown-Rump Length Ratios (CC/CRL) of the Offspring

The effect of maternal nicotine exposure during gestation or supplementing the mother’s diet with tomato juice on the CC/CRL ratios in the offspring showed (fig. 3.3.3) that at postnatal day 14, no differences were apparent within experimental groups (P>0.05). However, at postnatal day 21, only the offspring exposed to nicotine via the placenta expressed a lower (P<0.05) CC/CRL ratio when compared to its control. At postnatal day 42, males who were born to females that received tomato juice only during pregnancy appeared to have a slightly lower (P<0.05) CC/CRL ratio in comparison to the rest of the experimental groups (P>0.05). The female group that received a combination of nicotine and tomato juice, was the only group who had a lower (P<0.05) CC/CRL ratio compared to the rest of the experimental groups at postnatal day 84. Table 3.3.3(a) and table 3.3.3(b) illustrates that no significant differences were established between male and female groups at postnatal day 42 and 84 (P>0.05).

### 3.3.4 The Chest Circumference/Lung Volume Ratios (CC/Lv) of the Offspring

CC/Lv ratios between males and females of all experimental groups were similar (P>0.05) at postnatal day 14 (fig. 3.3.4 and table 3.3.4). However, differences did occur within experimental groups at postnatal day 21, when compared to its
control group (P<0.05) as seen in fig. 3.3.4(a). Animals exposed to tomato juice during gestation had a CC/Lv of (27.62±1.27mm/ml) that was 1.19-fold lower (P<0.05) than that of the controls (33.03±1.15mm/ml). Also, the CC/Lv ratio of animals exposed to nicotine (26.50±1.32mm/ml) via the placenta was 1.24-fold lower than the control (33.03±1.15mm/ml). Also, the CC/Lv ratio of the offspring that was exposed to both maternal tomato juice and nicotine (29.09±0.86mm/ml) via the placenta was lower than that of the 21-day-old controls (33.03±1.15mm/ml).

Table 3.3.4 shows that at postnatal day 42 and 84 no differences in CC/Lv ratios between experimental groups were apparent (P>0.05).
Fig. 3.3.1 The effect of maternal nicotine and tomato juice exposure during gestation on the CC (mm) of the male and female offspring.

Fig. 3.3.1(a) Bar graph illustration on CC (mm) of postnatal day 84 males exposed to maternal nicotine and tomato juice.

Fig. 3.3.1(b) Bar graph illustration on CC (mm) of postnatal day 84 females exposed to maternal nicotine and tomato juice.

Table 3.3.1 The effect of maternal nicotine, tomato juice, and a combination of nicotine and tomato juice during gestation on the chest circumference (mm) of the male and female offspring at postnatal days 14, 24, 42 and 84 (below).

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<td>M vs. F</td>
<td></td>
<td>P&lt;0.05</td>
<td>P&lt;0.05</td>
<td>P&lt;0.05</td>
<td>P&lt;0.05</td>
<td>P&lt;0.05</td>
<td>P&lt;0.05</td>
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</tr>
<tr>
<td>21</td>
<td>M</td>
<td>73.27±1.47</td>
<td>P&lt;0.05</td>
<td>73.14±2.26</td>
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<td>65.89±2.32</td>
<td>P&lt;0.05</td>
<td>60.78±1.59</td>
</tr>
<tr>
<td>21</td>
<td>F</td>
<td>73.27±1.47</td>
<td>P&lt;0.05</td>
<td>73.14±2.26</td>
<td>P&lt;0.05</td>
<td>65.89±2.32</td>
<td>P&lt;0.05</td>
<td>60.78±1.59</td>
</tr>
<tr>
<td>M vs. F</td>
<td></td>
<td>P&lt;0.05</td>
<td>P&lt;0.05</td>
<td>P&lt;0.05</td>
<td>P&lt;0.05</td>
<td>P&lt;0.05</td>
<td>P&lt;0.05</td>
<td></td>
</tr>
<tr>
<td>42</td>
<td>M</td>
<td>104.7±2.09</td>
<td>P&lt;0.05</td>
<td>93.8±3.20</td>
<td>P&lt;0.05</td>
<td>100.5±2.74</td>
<td>P&lt;0.05</td>
<td>111.2±4.54</td>
</tr>
<tr>
<td>42</td>
<td>F</td>
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<td>P&lt;0.05</td>
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<td>P&lt;0.05</td>
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<tr>
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<td>P&lt;0.05</td>
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<td>P&lt;0.05</td>
<td>P&lt;0.05</td>
<td>P&lt;0.05</td>
<td>P&lt;0.05</td>
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</tr>
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Fig. 3.3.2  The effect of maternal nicotine and tomato juice exposure during gestation on the CRL (mm) of the male and female offspring.

Fig. 3.3.2(a)  Bar graph illustration on CRL (mm) of postnatal day 84 males exposed to maternal nicotine and tomato juice.

Fig. 3.3.2(b)  Bar graph illustration on CRL (mm) of postnatal day 84 females exposed to maternal nicotine and tomato juice.

Table 3.3.2  The effect of maternal nicotine, tomato juice, and a combination of nicotine and tomato juice during gestation on the crown-rump length (mm) of the male and female offspring at postnatal days 14, 24, 42 and 84 (below).

<table>
<thead>
<tr>
<th>Age (Days)</th>
<th>Sex</th>
<th>C</th>
<th>C vs. T</th>
<th>T</th>
<th>C vs. N</th>
<th>N</th>
<th>C vs. NT</th>
<th>NT</th>
</tr>
</thead>
<tbody>
<tr>
<td>14</td>
<td>M</td>
<td>69.14±1.27</td>
<td>P&lt;0.05</td>
<td>73.67±1.57</td>
<td>P&lt;0.05*</td>
<td>78.94±1.11</td>
<td>P&lt;0.05</td>
<td>76.48±1.59</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>69.14±1.27</td>
<td>P&lt;0.05</td>
<td>73.67±1.57</td>
<td>P&lt;0.05*</td>
<td>78.94±1.11</td>
<td>P&lt;0.05</td>
<td>76.48±1.59</td>
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<td>M vs. F</td>
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<td>P&lt;0.05</td>
<td></td>
<td>P&lt;0.05</td>
<td></td>
<td>P&lt;0.05</td>
<td></td>
<td>P&lt;0.05</td>
</tr>
<tr>
<td>21</td>
<td>M</td>
<td>93.17±0.77</td>
<td>P&lt;0.05</td>
<td>104.57±4.09</td>
<td>P&gt;0.05</td>
<td>94.06±2.43</td>
<td>P&lt;0.05</td>
<td>103.81±2.27</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>93.17±0.77</td>
<td>P&lt;0.05</td>
<td>104.57±4.09</td>
<td>P&gt;0.05</td>
<td>94.06±2.43</td>
<td>P&lt;0.05</td>
<td>103.81±2.27</td>
</tr>
<tr>
<td>M vs. F</td>
<td></td>
<td>P&gt;0.05</td>
<td></td>
<td>P&gt;0.05</td>
<td></td>
<td>P&gt;0.05</td>
<td></td>
<td>P&gt;0.05</td>
</tr>
<tr>
<td>42</td>
<td>M</td>
<td>146.3±2.13</td>
<td>P&lt;0.05</td>
<td>141.4±3.41</td>
<td>P&gt;0.05</td>
<td>142.2±3.10</td>
<td>P&gt;0.05</td>
<td>156.6±3.32</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>142.8±2.59</td>
<td>P&lt;0.05</td>
<td>141.7±5.09</td>
<td>P&gt;0.05</td>
<td>139.4±3.41</td>
<td>P&gt;0.05</td>
<td>151.6±2.74</td>
</tr>
<tr>
<td>M vs. F</td>
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<td>P&lt;0.05</td>
<td></td>
<td>P&lt;0.05</td>
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<td>P&lt;0.05</td>
<td></td>
<td>P&lt;0.05</td>
</tr>
<tr>
<td>84</td>
<td>M</td>
<td>184.3±3.67</td>
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<td>191.8±6.65</td>
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<td>192.4±5.0</td>
<td>P&gt;0.05</td>
<td>190.5±4.71</td>
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<tr>
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<td>F</td>
<td>173.3±2.48</td>
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<td>176.8±2.24</td>
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<td>182.9±2.74</td>
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<td>M vs. F</td>
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<td>P&lt;0.05</td>
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<td>P&lt;0.05</td>
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</table>
Fig. 3.3.3 The effect of maternal nicotine and tomato juice exposure during gestation on the CC/CRL (mm/mm) of the male and female offspring.

Fig. 3.3.3(a) Bar graph illustration on CC/CRL ratio (mm/mm) of postnatal day 84 males exposed to nicotine and tomato juice.

Fig. 3.3.3(b) Bar graph illustration on CC/CRL ratio (mm/mm) of postnatal day 84 females exposed to nicotine and tomato juice.

Table 3.3.3 The effect of maternal nicotine, tomato juice, and a combination of nicotine and tomato juice during gestation on the chest circumference/crown-rump length (mm/mm) of the male and female offspring at postnatal days 14, 24, 42 and 84 (below).

<table>
<thead>
<tr>
<th>Age (Days)</th>
<th>Sex</th>
<th>C</th>
<th>C vs. T</th>
<th>T</th>
<th>C vs. N</th>
<th>N</th>
<th>C vs. NT</th>
<th>NT</th>
</tr>
</thead>
<tbody>
<tr>
<td>14</td>
<td>M</td>
<td>0.89±0.03</td>
<td>P&gt;0.05</td>
<td>0.83±0.02</td>
<td>P&gt;0.05</td>
<td>0.86±0.02</td>
<td>P&gt;0.05</td>
<td>0.84±0.01</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>0.89±0.03</td>
<td>P&gt;0.05</td>
<td>0.83±0.02</td>
<td>P&gt;0.05</td>
<td>0.86±0.02</td>
<td>P&gt;0.05</td>
<td>0.84±0.01</td>
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<tr>
<td>21</td>
<td>M</td>
<td>0.79±0.02</td>
<td>P&gt;0.05</td>
<td>0.72±0.03</td>
<td>P&lt;0.05</td>
<td>0.70±0.02</td>
<td>P&gt;0.05</td>
<td>0.75±0.01</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>0.79±0.02</td>
<td>P&gt;0.05</td>
<td>0.72±0.03</td>
<td>P&lt;0.05</td>
<td>0.70±0.02</td>
<td>P&gt;0.05</td>
<td>0.75±0.01</td>
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<tr>
<td>42</td>
<td>M</td>
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<td>0.66±0.02</td>
<td>P&lt;0.05</td>
<td>0.71±0.01</td>
<td>P&gt;0.05</td>
<td>0.74±0.01</td>
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<tr>
<td></td>
<td>F</td>
<td>0.72±0.02</td>
<td>P&lt;0.05</td>
<td>0.66±0.02</td>
<td>P&lt;0.05</td>
<td>0.71±0.01</td>
<td>P&gt;0.05</td>
<td>0.74±0.01</td>
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<tr>
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<td>P&gt;0.05</td>
<td>0.71±0.03</td>
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<td>0.67±0.02</td>
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<td>0.70±0.02</td>
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<td>P&gt;0.05</td>
<td>P&gt;0.05</td>
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Fig. 3.3.4 The effect of maternal nicotine and tomato juice exposure during gestation on the CC/Lv ratio (mm/ml) of the male and female offspring.

Fig. 3.3.4(a) Bar graph illustration on CC/Lv ratio (mm/ml) of postnatal day 21 animals exposed to maternal nicotine and tomato juice.

Table 3.3.4 The effect of maternal nicotine, tomato juice, and a combination of nicotine and tomato juice during gestation on the chest circumference/lung volume ratio (mm/ml) of the male and female offspring at postnatal days 14, 24, 42 and 84 (below).

<table>
<thead>
<tr>
<th>Age (Days)</th>
<th>Sex</th>
<th>C</th>
<th>C vs. T</th>
<th>T</th>
<th>C vs. N</th>
<th>N</th>
<th>C vs. NT</th>
<th>NT</th>
</tr>
</thead>
<tbody>
<tr>
<td>14</td>
<td>M</td>
<td>43.94±0.87</td>
<td>P&gt;0.05</td>
<td>41.25±2.76</td>
<td>P&gt;0.05</td>
<td>45.4±1.02</td>
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</tr>
<tr>
<td>14</td>
<td>F</td>
<td>43.94±0.87</td>
<td>P&gt;0.05</td>
<td>41.25±2.76</td>
<td>P&gt;0.05</td>
<td>45.4±1.02</td>
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<td></td>
</tr>
<tr>
<td>M vs. F</td>
<td></td>
<td>P&gt;0.05</td>
<td></td>
<td>P&gt;0.05</td>
<td></td>
<td>P&gt;0.05</td>
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<td></td>
</tr>
<tr>
<td>21</td>
<td>M</td>
<td>33.03±1.15</td>
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<td>26.50±1.32</td>
<td>P&gt;0.05</td>
<td>29.09±0.86</td>
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<td>F</td>
<td>33.03±1.15</td>
<td>P&gt;0.05</td>
<td>26.50±1.32</td>
<td>P&gt;0.05</td>
<td>29.09±0.86</td>
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<tr>
<td>M vs. F</td>
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<td>P&gt;0.05</td>
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<td>P&gt;0.05</td>
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<td>42</td>
<td>M</td>
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<td>P&gt;0.05</td>
<td>23.01±1.00</td>
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<tr>
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<td>F</td>
<td>23.18±1.52</td>
<td>P&gt;0.05</td>
<td>21.82±1.36</td>
<td>P&gt;0.05</td>
<td>23.01±1.00</td>
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<tr>
<td>M vs. F</td>
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<td>P&gt;0.05</td>
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<td>P&gt;0.05</td>
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<td>P&gt;0.05</td>
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</tr>
<tr>
<td>84</td>
<td>M</td>
<td>13.71±0.87</td>
<td>P&gt;0.05</td>
<td>12.69±0.79</td>
<td>P&gt;0.05</td>
<td>12.16±0.92</td>
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<td>F</td>
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<td>12.91±0.97</td>
<td>P&gt;0.05</td>
<td>15.19±0.93</td>
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<tr>
<td>M vs. F</td>
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<td>P&gt;0.05</td>
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<td>P&gt;0.05</td>
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</table>
3.4 THE EFFECT OF MATERNAL NICOTINE EXPOSURE AND TOMATO JUICE INTAKE DURING GESTATION ON THE ALVEOLAR AIR (Va) AND TISSUE VOLUME (Vt) IN THE LUNGS OF THE OFFSPRING

3.4.1 The Alveolar Air (Va) in the Lungs of the Offspring

The Va in the lungs of the offspring rapidly increased between postnatal days 14 and 21, then stabilized until postnatal day 84 (table 3.4.1). There were no differences in Va between experimental groups from postnatal day 14 to day 42 (P>0.05). However, at postnatal day 84 (fig. 3.4.1), animals exposed to nicotine via the placenta had a Va (82.44±0.81%) that was higher (P<0.05) than the control (71.21±2.22%). Animals exposed both to nicotine and tomato juice via the placenta showed a Va similar to those animals exposed to tomato juice only (P>0.05). This means that maternal administration of tomato juice, together with nicotine during gestation, prevented the lungs of the offspring from the harmful effects of nicotine on the developing lung.

3.4.2 The Tissue Volume (Vt) in the Lungs of the Offspring

Fig. 3.4.2 illustrates the effect of aging, as well as of nicotine, tomato juice only, and nicotine and tomato juice, on the Vt in the lungs of the offspring. As expected, no differences in the experimental groups were observed at postnatal days 14, 21 and 42 (P>0.05). However, at postnatal day 84 (table 3.4.2), Vt of the control group (28.79±2.22%) was 1.64-fold higher (P<0.05) than that of the nicotine group (17.56±0.81%). Interestingly, the animals exposed to both nicotine
and tomato juice via the placenta of the mother showed a Vt similar to those animals exposed to tomato juice only during gestation (P>0.05). This indicates that supplementing the mother’s diet with tomato juice during gestation, while exposing the unborn offspring to nicotine, helped prevent the adverse effects that nicotine has when administered alone.
Fig. 3.4.1  The effect of maternal nicotine and tomato juice exposure during gestation on the Va in the lungs of the offspring.

Table 3.4.1 The effect of maternal nicotine, tomato juice, and a combination of nicotine and tomato juice during gestation on the alveolar air volume in the lungs of the offspring at postnatal days 14, 24, 42 and 84 (below).

<table>
<thead>
<tr>
<th>Age (Days)</th>
<th>C</th>
<th>C vs. T</th>
<th>T</th>
<th>C vs. N</th>
<th>N</th>
<th>C vs. NT</th>
<th>NT</th>
</tr>
</thead>
<tbody>
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<td>73.11±1.89</td>
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<td>68.09±0.73</td>
<td>P&gt;0.05</td>
<td>70.02±1.81</td>
</tr>
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<td>21</td>
<td>72.65±1.29</td>
<td>P&gt;0.05</td>
<td>73.47±0.41</td>
<td>P&gt;0.05</td>
<td>77.47±2.21</td>
<td>P&gt;0.05</td>
<td>76.09±0.21</td>
</tr>
<tr>
<td>42</td>
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<td>P&gt;0.05</td>
<td>71.33±0.40</td>
<td>P&gt;0.05</td>
<td>77.1±2.04</td>
<td>P&gt;0.05</td>
<td>74.71±0.31</td>
</tr>
<tr>
<td>84</td>
<td>71.21±2.32</td>
<td>P&gt;0.05</td>
<td>71.53±4.04</td>
<td>P&gt;0.05</td>
<td>82.44±0.81</td>
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<td>75.09±0.33</td>
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Fig. 3.4.2  The effect of maternal nicotine and tomato juice exposure during gestation on the Va in the lungs of the offspring.

Table 3.4.2 The effect of maternal nicotine, tomato juice, and a combination of nicotine and tomato juice during gestation on the alveolar air volume in the lungs of the offspring at postnatal days 14, 24, 42 and 84 (below).

<table>
<thead>
<tr>
<th>Age (Days)</th>
<th>C</th>
<th>C vs. T</th>
<th>T</th>
<th>C vs. N</th>
<th>N</th>
<th>C vs. NT</th>
<th>NT</th>
</tr>
</thead>
<tbody>
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<td>P&gt;0.05</td>
<td>26.90±1.89</td>
<td>P&gt;0.05</td>
<td>31.71±0.73</td>
<td>P&gt;0.05</td>
<td>29.97±1.81</td>
</tr>
<tr>
<td>21</td>
<td>27.35±1.29</td>
<td>P&gt;0.05</td>
<td>26.53±0.40</td>
<td>P&gt;0.05</td>
<td>22.53±2.21</td>
<td>P&gt;0.05</td>
<td>23.91±0.21</td>
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<tr>
<td>42</td>
<td>27.83±1.29</td>
<td>P&gt;0.05</td>
<td>28.47±0.40</td>
<td>P&gt;0.05</td>
<td>22.9±2.04</td>
<td>P&gt;0.05</td>
<td>25.29±0.21</td>
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<tr>
<td>84</td>
<td>28.79±2.22</td>
<td>P&gt;0.05</td>
<td>24.1±1.28</td>
<td>P&lt;0.05</td>
<td>17.56±0.81</td>
<td>P&gt;0.05</td>
<td>24.92±0.33</td>
</tr>
</tbody>
</table>
### 3.5 The Effect of Maternal Nicotine and Tomato Juice on the Linear Intercept (Lm) in the Lungs of the Offspring

#### 3.5.1 The Linear Intercept (Lm) in the Lungs of the Offspring

Changes in the Lm of the lungs of the offspring as a function of age, as well as a result of exposure to nicotine, or to tomato juice, or to both nicotine and tomato juice during gestation is shown in fig. 3.5.1. The Lm measurement indicates the size of the alveoli in the lungs. As the animals age, it is expected for the size of the alveoli to gradually increase, thereby increasing the Lm. This means that the surface area available for gas exchange would slowly decrease. According to the data in fig 3.5.1, no significant differences were found in Lm between all the experimental and the control group at postnatal day 14 (P>0.05). However, at postnatal day 21, the offspring exposed to nicotine (48.32±0.76µm) via the placenta had an Lm that was 1.33-fold higher (P<0.05) than the control (36.06±0.64µm). At postnatal day 42, no differences were observed between the experimental groups except for those animals exposed to nicotine during gestation (48.32±0.76µm), which was 1.45-fold higher (P<0.05) than in the control group (37.19±2.18µm). This was also observed at postnatal day 84, where the Lm of the nicotine exposed animals (61.84±2.81µm) was higher than that of the animals whose mothers received a tomato juice supplementation as well as those that were exposed to nicotine and was receiving a tomato juice supplementation and the control (38.18±2.94µm) (fig.3.5.1 (a)). The Lm those animals exposed to both
nicotine and tomato juice during gestation was the same as for the control animals (P>0.05).

The results achieved in this study and those reflecting changes in Vt and Va as illustrated in fig. 3.4.1 and fig. 3.4.2, shows that maternal nicotine exposure during gestation results in a gradual degradation of lung parenchymal tissue in the lungs of the nicotine exposed offspring as the animals age. Interestingly, supplementing the mother’s diet with tomato juice, together with nicotine administration during gestation only prevented the harmful effects of nicotine on the lung tissue of the offspring.
Fig. 3.5.1 The effect of maternal nicotine and tomato juice exposure during gestation on the Lm (µm) of the offspring.

Fig. 3.5.1(a) Bar graph illustration on Lm (µm) of postnatal day 84 animals exposed to maternal nicotine and tomato juice.

Table 3.5.1 The effect of maternal nicotine, tomato juice, and a combination of nicotine and tomato juice during gestation on the linear intercept (µm) of the offspring at postnatal days 14, 24, 42 and 84 (below).

<table>
<thead>
<tr>
<th>Age (Days)</th>
<th>C</th>
<th>C vs. T</th>
<th>T</th>
<th>C vs. N</th>
<th>N</th>
<th>C vs. NT</th>
<th>NT</th>
</tr>
</thead>
<tbody>
<tr>
<td>14</td>
<td>31.19±3.06</td>
<td>P&gt;0.05</td>
<td>26.89±1.88</td>
<td>P&gt;0.05</td>
<td>31.91±0.75</td>
<td>P&gt;0.05</td>
<td>29.90±1.25</td>
</tr>
<tr>
<td>21</td>
<td>36.84±0.64</td>
<td>P&gt;0.05</td>
<td>37.72±5.32</td>
<td>P&gt;0.05</td>
<td>48.32±0.76</td>
<td>P&gt;0.05</td>
<td>36.54±0.94</td>
</tr>
<tr>
<td>42</td>
<td>37.19±2.18</td>
<td>P&gt;0.05</td>
<td>32.14±1.91</td>
<td>P&gt;0.05</td>
<td>54.29±1.55</td>
<td>P&gt;0.05</td>
<td>39.12±3.53</td>
</tr>
<tr>
<td>84</td>
<td>38.18±2.94</td>
<td>P&gt;0.05</td>
<td>36.03±1.61</td>
<td>P&gt;0.05</td>
<td>61.84±2.81</td>
<td>P&gt;0.05</td>
<td>38.63±1.44</td>
</tr>
</tbody>
</table>
3.6 THE EFFECT OF MATERNAL NICOTINE EXPOSURE AND TOMATO JUICE INTAKE DURING GESTATION ON THE ALVEOLAR NUMBER (Na) IN THE LUNGS OF THE OFFSPRING

3.6.1 The Alveolar Number (Na) in the Lungs of the Offspring

Fig. 3.6.1 provides an estimate of the Na in the lungs of the offspring exposed to nicotine via the placenta and those offspring that received tomato juice during gestation. The data also showed the effect of exposure to both nicotine and tomato juice during pregnancy on the Na of the offspring as they grew older. As a function of age, the Na tends to increase with time as the body weight of the offspring increased. At postnatal days 14 and 21, the Na in the lungs of the control offspring was similar to that of all the experimental groups (P>0.05). However, as the animals approached postnatal day 42, the Na of the nicotine exposed group increased much slower than that of the control animals and in the other experimental groups. To illustrate; at postnatal day 42, nicotine exposed animals had a Na of 0.16±0.03 million which was 4.93-fold less than the control (0.79±0.38 million). At postnatal day 84, nicotine exposed animals had an Na of 0.43±0.1 million which was 5.27-fold less than the control group (2.27±0.25 million). This indicates that those offspring exposed to maternal nicotine during gestation, continued to suffer the effects of nicotine relatively late in life. Those animals exposed to both maternal nicotine and tomato juice however, had the same Na as the control rats (P>0.05), suggesting that tomato juice prevented the breakdown of the alveolar walls, thus keeping the Na within normal range as the animals age.
Fig. 3.6.1 The effect of maternal nicotine and tomato juice exposure during gestation on the Na in the lungs of the offspring.

Fig. 3.6.1(a) Bar graph illustration on Na of postnatal day 84 offspring exposed to maternal nicotine and tomato juice.

Table 3.6.1 The effect of maternal nicotine, tomato juice, and a combination of nicotine and tomato juice during gestation on the alveolar number (x 10^6) in the lungs of the offspring at postnatal days 14, 24, 42 and 84 (below).

<table>
<thead>
<tr>
<th>Age (Days)</th>
<th>C (10^6)</th>
<th>C vs. T</th>
<th>T (10^6)</th>
<th>C vs. N</th>
<th>N (10^6)</th>
<th>C vs. NT</th>
<th>NT (10^6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>14</td>
<td>0.21±0.03</td>
<td>P&gt;0.05</td>
<td>0.35±0.04</td>
<td>P&gt;0.05</td>
<td>0.3±0.02</td>
<td>P&gt;0.05</td>
<td>0.22±0.06</td>
</tr>
<tr>
<td>21</td>
<td>0.21±0.01</td>
<td>P&gt;0.05</td>
<td>0.18±0.02</td>
<td>P&gt;0.05</td>
<td>0.18±0.02</td>
<td>P&gt;0.05</td>
<td>0.19±0.05</td>
</tr>
<tr>
<td>42</td>
<td>0.79±0.38</td>
<td>P&gt;0.05</td>
<td>0.64±0.09</td>
<td>P&gt;0.05</td>
<td>0.16±0.03</td>
<td>P&gt;0.05</td>
<td>0.95±0.18</td>
</tr>
<tr>
<td>84</td>
<td>2.27±0.23</td>
<td>P&gt;0.05</td>
<td>2.33±0.21</td>
<td>P&gt;0.05</td>
<td>0.43±0.1</td>
<td>P&gt;0.05</td>
<td>2.01±0.23</td>
</tr>
</tbody>
</table>
3.7 THE EFFECT OF MATERNAL NICOTINE EXPOSURE AND TOMATO JUICE INTAKE DURING GESTATION ON THE STATIC COMPLIANCE (Cst) OF THE LUNGS OF THE OFFSPRING

3.7.1 The Static Compliance (Cst) of the Lungs of the Offspring

Fig. 3.7.1 illustrates the effect of aging, as well as nicotine, supplementing the pregnant mothers diet tomato juice only, and giving her both nicotine and tomato juice during gestation, on the static compliance of the lungs of the offspring. No differences between males and females were initially observed (P>0.05).

No differences (P>0.05) were observed between experimental groups at postnatal days 14 and 42. However, at postnatal day 84, males exposed to nicotine (0.466±0.040 ml/ml H₂O/kg) via the placenta during gestation, had a higher (P<0.05) Cst compared to its control (0.361±0.020 ml/ml H₂O/kg) (table 3.7.1(a)). Also, at postnatal day 84, females exposed to nicotine (0.339±0.010 ml/ml H₂O/kg) via the placental during gestation, had a higher (P<0.05) Cst than the control group (0.310±0.020 ml/ml H₂O/kg) (table 3.7.1(b)).
Fig. 3.7.1 The effect of maternal nicotine and tomato juice exposure during gestation on the static compliance (ml/ml H_2O/kg) of the lungs of the male and female offspring.

Fig. 3.7.1 (a) The effect of maternal nicotine and tomato juice exposure during gestation on the static compliance (ml/ml H_2O/kg) of the lungs of the male offspring at postnatal day 84.

Fig. 3.7.1 (b) The effect of maternal nicotine and tomato juice exposure during gestation on the static compliance (ml/ml H_2O/kg) of the lungs of the female offspring at postnatal day 84.

Table 3.7.1 The effect of maternal nicotine, tomato juice, and a combination of nicotine and tomato juice during gestation on the lung compliance (ml/ml H_2O/kg) of the lungs of the male and female offspring at postnatal days 14, 24, 42 and 84 (below).

<table>
<thead>
<tr>
<th>Age (Days)</th>
<th>Sex</th>
<th>C</th>
<th>C vs. T</th>
<th>T</th>
<th>C vs. N</th>
<th>N</th>
<th>C vs. NT</th>
<th>NT</th>
</tr>
</thead>
<tbody>
<tr>
<td>14</td>
<td>M</td>
<td>C</td>
<td>P&lt;0.05</td>
<td>C</td>
<td>P&lt;0.05</td>
<td>N</td>
<td>P&lt;0.05</td>
<td>N</td>
</tr>
<tr>
<td>14</td>
<td>F</td>
<td>0.21±0.01</td>
<td>P&lt;0.05</td>
<td>0.28±0.05</td>
<td>P&lt;0.05</td>
<td>0.25±0.02</td>
<td>P&lt;0.05</td>
<td>0.24±0.01</td>
</tr>
<tr>
<td>M vs. F</td>
<td></td>
<td>P&lt;0.05</td>
<td>P&lt;0.05</td>
<td>P&lt;0.05</td>
<td>P&lt;0.05</td>
<td>P&lt;0.05</td>
<td>P&lt;0.05</td>
<td>P&lt;0.05</td>
</tr>
<tr>
<td>21</td>
<td>M</td>
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<td>0.11±0.008</td>
<td>P&lt;0.05</td>
<td>0.10±0.003</td>
<td>P&lt;0.05</td>
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</tr>
<tr>
<td>21</td>
<td>F</td>
<td>0.21±0.01</td>
<td>P&lt;0.05</td>
<td>0.31±0.01</td>
<td>P&lt;0.05</td>
<td>0.28±0.01</td>
<td>P&lt;0.05</td>
<td>0.25±0.02</td>
</tr>
<tr>
<td>M vs. F</td>
<td></td>
<td>P&lt;0.05</td>
<td>P&lt;0.05</td>
<td>P&lt;0.05</td>
<td>P&lt;0.05</td>
<td>P&lt;0.05</td>
<td>P&lt;0.05</td>
<td>P&lt;0.05</td>
</tr>
<tr>
<td>42</td>
<td>M</td>
<td>0.19±0.010</td>
<td>P&lt;0.05</td>
<td>0.19±0.010</td>
<td>P&lt;0.05</td>
<td>0.20±0.020</td>
<td>P&lt;0.05</td>
<td>0.22±0.020</td>
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<tr>
<td>42</td>
<td>F</td>
<td>0.19±0.012</td>
<td>P&lt;0.05</td>
<td>0.21±0.020</td>
<td>P&lt;0.05</td>
<td>0.20±0.020</td>
<td>P&lt;0.05</td>
<td>0.21±0.020</td>
</tr>
<tr>
<td>M vs. F</td>
<td></td>
<td>P&lt;0.05</td>
<td>P&lt;0.05</td>
<td>P&lt;0.05</td>
<td>P&lt;0.05</td>
<td>P&lt;0.05</td>
<td>P&lt;0.05</td>
<td>P&lt;0.05</td>
</tr>
<tr>
<td>84</td>
<td>M</td>
<td>0.36±0.020</td>
<td>P&lt;0.05</td>
<td>0.42±0.020</td>
<td>P&lt;0.05</td>
<td>0.46±0.040</td>
<td>P&lt;0.05</td>
<td>0.46±0.030</td>
</tr>
<tr>
<td>84</td>
<td>F</td>
<td>0.31±0.020</td>
<td>P&lt;0.05</td>
<td>0.35±0.010</td>
<td>P&lt;0.05</td>
<td>0.33±0.010</td>
<td>P&lt;0.05</td>
<td>0.40±0.020</td>
</tr>
<tr>
<td>M vs. F</td>
<td></td>
<td>P&lt;0.05</td>
<td>P&lt;0.05</td>
<td>P&lt;0.05</td>
<td>P&lt;0.05</td>
<td>P&lt;0.05</td>
<td>P&lt;0.05</td>
<td>P&lt;0.05</td>
</tr>
</tbody>
</table>
3.8 THE EFFECT OF MATERNAL NICOTINE EXPOSURE AND TOMATO JUICE INTAKE DURING GESTATION ON CELLULAR SENESCENCE IN THE LUNGS OF THE OFFSPRING

3.8.1 Cellular Senescence in the Lungs of the Offspring

Fig. 3.8.1 illustrates the effect of aging, gender, as well as nicotine, tomato juice, and exposure to both nicotine and tomato juice on senescent cell count/100μm length of alveolar wall. At postnatal day 14, the number of senescent cells/100μm was the same as for the control and experimental groups (P>0.05). At postnatal days 42 and 84, no differences were observed between the control, and the experimental animals exposed to tomato juice only, or to both nicotine and tomato juice (P>0.05). On the other hand, at postnatal days 42, the nicotine exposed offspring had a senescent cell count of 45% (P<0.001) higher at 0.42±0.04 cells/100μm alveolar walls than that of the control at 0.19±0.02 cells/100μm group. At postnatal day 84, the nicotine exposed offspring (0.46±0.03 cells/100μm) also displayed a significant 47.8% higher (P<0.01) senescent cell count when compared with the control group at 0.22±0.09 cells/100μm alveolar wall (table 3.8.1) of the same age.

In only the nicotine group, the cellular senescence increased between postnatal days 14 and 84 (P<0.05). The number of senescent cells per 100μm of alveolar wall of the other experimental groups between postnatal days 14, 42 and 84, displayed no differences (P>0.05). Between postnatal day 14 (0.32±0.03 cells/100μm) and 42 (0.42±0.04 cells/100μm) of the nicotine exposed offspring,
the senescent cell count has increased by 76.1% (P<0.05), and between postnatal day 14 (0.32±0.03 cells/100µm) and postnatal day 84 (0.46±0.03 cells/100µm), the senescent cell count has increased by 69.6% (P<0.05) in the nicotine exposed offspring as well.

Although the number of senescent cells/100µm alveolar wall in the nicotine exposed offspring increased from 0.32±0.03 cells/100µm at postnatal day 14 to 0.42±0.04 cells/100µm on postnatal day 42 (P>0.05), it reached a plateau between postnatal days 42 and 84.

Between postnatal days 14 and 42 of the control animals, the number of senescent cells decreases from 0.23±0.0 cells/100µm to 0.19±0.02 cells/100µm on postnatal day 42. Like for the nicotine group the number of senescent cells per length of alveolar wall remains unaltered between postnatal days 42 and 84.
Fig. 3.8.1 The effect of maternal nicotine and tomato juice exposure during gestation on the senescent cell count (cell/100µm) of the offspring.

Table 3.8.1 The effect of maternal nicotine, tomato juice, and a combination of nicotine and tomato juice during gestation on the senescent cell count (cell/100 µm) of the offspring at postnatal days 14, 42 and 84 (below).

<table>
<thead>
<tr>
<th>Age (Days)</th>
<th>C vs. T</th>
<th>T vs. N</th>
<th>C vs. NT</th>
<th>NT</th>
</tr>
</thead>
<tbody>
<tr>
<td>14</td>
<td>0.23±0.04</td>
<td>P&gt;0.05</td>
<td>0.23±0.02</td>
<td>P&gt;0.05</td>
</tr>
<tr>
<td>D14 vs. D42</td>
<td>P&gt;0.05</td>
<td>P&gt;0.05</td>
<td>P&gt;0.05</td>
<td>P&gt;0.05</td>
</tr>
<tr>
<td>42</td>
<td>0.19±0.02</td>
<td>P&gt;0.05</td>
<td>0.17±0.01</td>
<td>P&lt;0.05</td>
</tr>
<tr>
<td>D42 vs. D84</td>
<td>P&gt;0.05</td>
<td>P&gt;0.05</td>
<td>P&gt;0.05</td>
<td>P&gt;0.05</td>
</tr>
<tr>
<td>84</td>
<td>0.22±0.09</td>
<td>P&gt;0.05</td>
<td>0.2±0.03</td>
<td>P&gt;0.05</td>
</tr>
<tr>
<td>D84 vs. D14</td>
<td>P&gt;0.05</td>
<td>P&gt;0.05</td>
<td>P&gt;0.05</td>
<td>P&gt;0.05</td>
</tr>
</tbody>
</table>
3.9 THE EFFECT OF MATERNAL NICOTINE EXPOSURE AND TOMATO JUICE INTAKE DURING GESTATION ON THE CONNECTIVE TISSUE IN THE LUNGS OF THE OFFSPRING

The connective tissue framework is essential for the structural integrity of the lungs. Fig. 3.9(a) is the control group of day-84-old offspring, and fig. 3.9(b) are those animals that were exposed the intake of tomato juice during gestation via the mother, at the same age. The connective tissue framework seems intact, showing healthy lungs as the rats mature. In fig. 3.9(c), however, there are breakages in the connective tissue framework of the lungs of the 84-day-old rats caused by nicotine exposure via the placenta during gestation. Interestingly, those animals exposed to both maternal nicotine and tomato juice intake (fig. 3.9(d)) by the mother during gestation did not show destruction of the connective tissue framework in the lungs, suggesting that tomato juice prevented the harmful effects of nicotine.

Also, the Cst was higher at postnatal day 84 for both males and females exposed to nicotine via the placenta during gestation. The breaks in the connective tissue (fig. 3.9(c)) resulted in an increased Cst in the nicotine exposed offspring.
Fig. 3.9. Connective tissue stain of lung tissue of the 84-day-old offspring: a) Control, b) Tomato Juice Exposed, c) Nicotine Exposed, d) Exposed to both Tomato Juice and Nicotine during Gestation. Arrows indicate breaks in connective tissue in alveolar walls.
Breaks in the connective tissue are evident in the alveolar walls of the nicotine exposed offspring. No such breaks were evident in the lungs of the controls and the other experimental groups (fig. 3.9).

Also, the results in this project points to the fact that the offspring that was exposed to nicotine via the placenta during gestation, results in changes in the lung parenchyma which resembles emphysema-like lesions in their adult life (fig. 3.10 (D)). And that the offspring whose mother’s diet was supplemented with tomato juice, and who received nicotine via the placenta, showed a preventative effect of tomato juice against the adverse consequences of nicotine exposure during gestation (fig. 3.10 (C)). This indicates that tomato juice is an effective supplement that can assist in maintaining the integrity of the lungs of the offspring.
Fig. 3.10. The influence of maternal exposure to nicotine, tomato juice, and a combination of nicotine and tomato juice on lung parenchyma of the 84-day-old offspring. (A) Control. (B) Tomato juice. (C) Nicotine and tomato juice. (D) Nicotine. Where arrows indicate emphysema-like lesions (Bar = 0.5 mm.)
CHAPTER FOUR

Discussion

4.1 INTRODUCTION

4.1.1 Smoking Prevalence
The WHO estimates that there are approximately 1.1 billion smokers worldwide. About 800 million of these smokers are in developing countries. An estimate of about 50% of men and 8% of women in developing countries are smokers (WHO 2002).

In South Africa however, prevalence rates for daily adult smoking over the past decade have continuously decreased. Adult (15 years smoking history) daily smoking rates illustrated a decrease that fell 30.2% in 1995 to 24.1% in 2004. As a result, approximately 2.5 million smokers stopped their smoking habits during this period (Steyn et al., 2002, van Walbeek, 2002).

4.1.2 Smoking during Pregnancy
In South Africa, it has been estimated that about 20% of female smokers are smoking during pregnancy, of which coloured women had the highest rates (Steyn et al., 1997). At Tygerberg hospital in the Western Cape, smoking during pregnancy significantly increased the risk of the two leading causes of perinatal
death, namely preterm labour and abruption placentae (Odendaal et al., 2001). Yet only 12% of pregnant smokers presenting at the hospital were aware of these risks. Unfortunately, only few doctors in the public sector, antenatal services, in Cape Town advise their patients about the risks of smoking or quitting the habit, whereas the rest do not regard smoking as a priority issue, especially during pregnancy (Viljoen and Odendaal, 2005).

### 4.1.3 Mortality

In the year 2000, an estimated 4.84 million premature deaths were attributed to cigarette smoking, worldwide. Of this 4.84 million, 2.41 million were in lower-income countries, and 2.43 million were industrialised countries. This measured up to 12% of the total global adult (30+) mortality (Ezzati and Lopez, 2003). In South Africa about 8% of adult deaths were attributable to smoking in 1998 which amounts to approximately 21500 deaths per year during that period. As the smoking epidemic is still increasing, it is expected that the proportion of deaths from tobacco continues to grow in the near future (Sitas et al., 2004).

In high-income countries, cardiovascular diseases and lung cancer are the main causes of death from smoking whereas in South Africa, the leading causes of death from smoking are chronic obstructive pulmonary disease (COPD), tuberculosis (TB), as well as lung cancer and ischaemic heart disease (IHD) (table 4.1.3) (Sitas et al., 2004). If people stopped smoking, it is estimated that approximately 58% of lung cancer deaths, 37% of COPD, 20% of TB deaths, and
23% of vascular deaths could be avoided. Smoking evidently has an impact on health in South Africa, and also adversely affects the productivity of individuals due to a higher likelihood of hospitalisation of smokers with associated costs to the economy (Sitas et al., 2004).

### Table 4.1.3 Smoking related deaths in 2000

<table>
<thead>
<tr>
<th>Disease caused by smoking in 2000</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chronic Obstructive Pulmonary Disease (COPD)</td>
<td>28</td>
</tr>
<tr>
<td>Tuberculosis (TB)</td>
<td>19</td>
</tr>
<tr>
<td>Lung Cancer</td>
<td>13</td>
</tr>
<tr>
<td>Ischaemic Heart Disease (IHD)</td>
<td>12</td>
</tr>
<tr>
<td>Cancer of the Lip, Mouth, Pharynx and Oesophagus</td>
<td>10</td>
</tr>
<tr>
<td>Strokes and Vascular Diseases</td>
<td>9</td>
</tr>
<tr>
<td>Other Conditions</td>
<td>9</td>
</tr>
</tbody>
</table>

An estimated five million people in the world die prematurely each year as a result of tobacco use and tobacco smoke exposure. Epidemiological studies have shown that cigarette smoke kills more Americans than alcohol, car accidents, suicide, AIDS, homicide and illegal drugs combined (Li et al., 2011). Although no data is available, it is plausible that the same is true for South Africa. Deaths of infants due to parental smoking are not taken into account in these studies.

### 4.1.4 Smoking and its Effect on Growth and Development of the Offspring

Epidemiological studies have shown that an estimated 20% of women persist in their smoking habits throughout their pregnancy, even though there are well documented information emphasizing the dangers of smoking on the unborn child (Steyn et al., 1997). The smoking mother inhales approximate 4000 chemicals in tobacco, some of which readily crosses the placenta and entering the fetal
circulation together with the nutrients. Maternal smoking induces low birth weight, and is the principal cause of fetal growth restriction especially in well developed countries. Studies have shown that the chemicals inhaled during maternal cigarette smoking inhibits fetal growth through direct effects on fetal and placental cells, or via altered expressions of hormones and growth factors known to be important for fetal growth and development. It has also been shown that a risk to low fetal birth weight is directly correlated with nicotine concentrations in maternal blood (Gruslin et al., 2009).

The effects of maternal smoking during gestation can be due to multiple factors, such as poor nutritional status since a suppressed appetite can occur as an effect of tobacco smoke, as well as the anorexigenic effect of nicotine itself (Wickstrom, 2007). After absorption of nicotine, distribution of it in the body is rapid and it has a particularly high affinity for the brain, heart and lungs (Wickstrom, 2007).

The effect of tobacco smoke during pregnancy allows the compounds contained in the cigarette to accumulate in the utero-placental environment, thus affecting the developing fetus. Nicotine, the addictive component of tobacco smoke accumulates in the developing fetal lung; it not only suppresses energy metabolism in the neonatal lung, but also interferes with neonatal lung cellular integrity (Maritz and van Wyk, 1997). The consequent inhibition of glucose metabolism in the neonatal lung is not due to the inadequate transport thereof, but rather due to the suppression of glycolysis which may cause an interference with lung development since glycolysis plays an important role in maintaining the
integrity of the lung, as well as the supply of energy which is essential for cell
proliferation to occur in the developing lung (Iba et al., 1999). Glucose, vitamin A
and vitamin C are essential nutrients for lung development and optimum function
thereof after birth and into old age (Elsayed, 1987, Nakamura et al., 2012).

Also, studies have shown that glucose is the main source of α-glycerophosphate
for pulmonary surfactant synthesis. It is, therefore, essential for normal lung
development, structure, and function in newborns and adults (Maritz and Harding,
2011). Type II alveolar epithelial cells are the main source of surfactant. In the
fetal lung, surfactant is produced in increasing quantities towards the end of
gestation and after birth, from glycogen resulting in a lecithin/sphingomyelin
increase. This means that the glucose supply during gestation is essential to build
up the glycogen stores of the lung for surfactant synthesis to prepare the lungs for
birth. Inadequate glucose supply during fetal lung development may result in
compromised surfactant synthesis.Studies have indeed shown that cigarette
exposure of rats led to lower production of surfactant in the lung tissue (Rooney,
1984). This might be attributed to nicotine by virtue of its inhibitory effect of
glycolysis and glycogenolysis (Maritz and Harding, 2011).

It has also been shown that maternal nicotine exposure during gestation causes
alterations in lung structure and lung surfactant which may be important in the
pathogenesis of impaired pulmonary function in infants and children exposed to
tobacco smoke due to parental smoking or nicotine replacement therapy (NRT)
(Chen et al., 2004).
From the above it is clear that strategies to assist smokers to quit are important so that the general health of a population can be improved. Some health professionals prescribe nicotine replacement therapy (NRT) as a strategy to assist smokers, including pregnant women, to quit. However, studies implicated nicotine in tobacco smoke, as the cause of lower birth weight and compromised lung function of the offspring of smokers. It has been shown that maternal nicotine exposure during alveolar formation adversely affects lung structure in the longer term (Windvogel, 2007), which may affect lung structural integrity and function in the longer term. The alveolar phase of lung development is preceded by other phases of the lung starting with the embryonic phase, followed by the pseudoglandular phase and saccular phases (Jeffrey, 1998). The latter phase is followed by the alveolar phase. The aim of this study was therefore to establish whether maternal nicotine exposure up to the saccular phase will interfere with lung development, and if so, whether it will return to normal as the lung matures. A further objective was to develop a strategy to prevent the adverse effects of maternal nicotine exposure during pregnancy on the lungs of the offspring.

4.2 MOTIVATION FOR THE USE OF 1mg NICOTINE/kg BODY WEIGHT/DAY

The daily nicotine intake of human males and females who smoke tobacco varies between 10.5 and 78.6 mg (Kurtoglu et al., 2007). Based on the assumption that 90% of nicotine is absorbed by inhalation (Duan et al., 1991), the nicotine intake of a 60kg female will be between 0.16 and 1.18mg nicotine/kg body weight/day. The dose used in this study (1mg nicotine/kg pre-pregnancy weight/day) was
therefore within the range of intake of habitual smokers. Because nicotine readily crosses the placenta and occurs in the milk of the human and rat mother (Maritz and Harding, 2011), the fetal rats, like in humans, will be exposed to nicotine via the placenta. Studies have shown that the breast milk from human smoking mothers contains a mean of 33.1 ng/ml (Stepans and Wilkerson, 1993). It has also been shown that the milk/plasma nicotine ratio after smoking is 2.9. In humans the amount of nicotine transferred to the infant via the mother’s milk increases from 0.09 to 1.03 μg/kg infant body weight when mothers smoke before breast feeding and the daily dose of nicotine via the mother’s milk was 6 μg/kg body weight (Stepans and Wilkerson, 1993, Maritz and Harding, 2011).

Since nicotine readily crosses the placenta and occurs in the milk or rats, and since the dose of nicotine used in this study is within the range of intake by human smokers, it is conceivable that the nicotine content of the female rats was the same as that for humans. This means that the perinatal and postnatal exposure to nicotine of the rat pups in all likelihood resembled that of human offspring. The data generated in this study can therefore be assumed to be the same as the effects of nicotine, inhaled by smokers, on the offspring.
4.3 THE EFFECT OF TOMATO JUICE ONLY, NICOTINE ONLY, OR BOTH NICOTINE AND TOMATO JUICE ON GROWTH AND DEVELOPMENT

4.3.1 The Pregnant Female

The nutritional status of the mother is used as a nutritional barometer by the fetus to prepare for the world outside the womb. Any interference with the mother’s nutritional status or her health may thus impact on the development of the fetus. Reports have indeed shown an association between disturbances in the offspring’s cardiovascular health, such as hypertension, to poor maternal health. Various studies have, for example, also shown that maternal obesity during pregnancy is related to adverse perinatal outcomes which may persist for several generations (Thangaratinam et al., 2012). Therefore, it is conceivable that any changes in body weight or body composition of the pregnant mother due to nicotine exposure will also have an effect on the development of the offspring and consequently the health thereof (Thangaratinam et al., 2012).

In this study, no significant differences were observed between mothers receiving tomato juice only, or both nicotine and tomato juice during gestation. However, the body weights of the mothers receiving nicotine were slightly lower than the rest of the experimental groups throughout gestation. This could possibly be due to the anorexic effects of nicotine slightly suppressing appetite, thereby decreasing body weight. As a result, nutrient intake may also be lowered, which
may affect the developing fetus. Supporting studies have indeed shown that nicotine decreases food intake (Mineur et al., 2011).

Interestingly, pregnant mothers receiving both nicotine and tomato juice, had no effect on body weight of the offspring. This suggests that tomato juice containing anti-oxidants such as lycopene and other phytonutrients, assisted in preventing appetite and weight loss, thereby ensuring that sufficient quantities of nutrients are transported to the developing fetus via the placenta.

### 4.3.2 The Offspring

#### 4.3.2.1 Body Weight (BW)

The BW of the offspring increased gradually after birth. Only from postnatal day 42, gender differences were observed. This suggests that the growth and development of the males and females were the same up to postnatal day 42, which means all metabolic and growth control mechanisms, was the same. After postnatal day 42 however, the increase in the body weights of the females slowed down. It is interesting to note that at postnatal day 84, the body weights of the male and female rats that were exposed to nicotine via the placenta was higher than that of the control animals of the same age.

Smokers generally gain weight once they quit the smoking habit. This may be due to an increase in appetite as well as metabolic rate after nicotine has been eliminated from the body (Audrain-McGovern and Benowitz, 2011). It has been shown that early life chemical exposures may induce obesity and type II diabetes.
in the offspring (Fernandez-Twinn and Ozanne, 2006). These exposures to foreign chemicals that change the *in utero* environment within which the fetus develops may have a significant impact on the metabolism and health of the individual in the long term. An example is that maternal smoking during pregnancy produces metabolic programming that leads to an increased risk of obesity in the offspring (Al Mamun et al., 2006). In a study by Ferrara et al (2001) it was found that smoking cessation for 4 weeks was accompanied by an increase in BW and the activity of adipose tissue lipoprotein lipase (AT-LPL). The increase in AT-LPL activity suggests an increase in the efficiency of the body to store energy in the fat stores of the body and of weight gain. This increase in the efficiency to store fat is not accompanied by an increase in lipolytic activity which further increases the efficiency of fat storage. These are plausible explanations why the offspring in this study that were exposed to nicotine via the placenta, had increased BW at postnatal day 84. Interestingly, at postnatal day 42, the BW of the nicotine exposed offspring had BW similar to that of the control. Yet, upon reaching postnatal day 84, their BW significant increased when compared to the rest of the experimental groups of the same age. This suggests that weight gain is indeed followed by nicotine withdrawal (Fornari et al., 2007), even though these animals were only exposed to nicotine via the placenta during gestation. Importantly, the late increase in BW of the nicotine exposed offspring suggests that the offspring were programmed during pregnancy to be more susceptible to obesity later in life. It is interesting to note that this effect of maternal nicotine exposure on the BW gain of the offspring later in life was prevented by supplementing the diets of the pregnant mothers with tomato juice. This implies that by maintaining the anti-
oxidant capacity of the mother and fetus during pregnancy prevented the programming of the fetus to become obese later in life.

The BW of human smokers gradually increases after quitting, gaining between 3.2 and 8.8kg within 8 years of smoking (Audrain-McGovern and Benowitz, 2011) and it is possible that nicotine in tobacco smoke is the causative factor.

It is plausible that nicotine induce a metabolic adjustment which favours an increase in BW after quitting. This adjustment is prevented by tomato juice. The question is though why nicotine exposure during pregnancy only resulted in an increased BW of the male and female offspring as opposed to those who were exposed to nicotine during gestation and lactation or lactation only where BW was not affected (Maritz and Windvogel, 2003).

4.3.2.2 Lung Volume and Chest Circumference

From birth up to postnatal day 42, lung volumes of the offspring increased in a similar trend as in the body weights. No male or female differences were observed. Since the increase in body weight allows for an increase in the oxygen demand of the body, lung volumes will increase and a concomitant increase in alveolar formation and a consequent equivalent increase in the surface area for gas exchange in the lungs (Forrest, 1970). Although the body weights of the female rats were lower than that of the males at the same age, the lung volume/body weight ratio was the same for control male and female rats because of the possible lower oxygen requirements/demands of the females at postnatal day 84.
Studies have shown that chest dimensions and pulmonary function are positively correlated in healthy individuals (Borkan et al., 1981). On the other hand, older smokers were characterised by larger chests than non-smokers or former smokers. Younger smokers, however, showed no differences in chest dimensions among men (Borkan et al., 1981). This might be due to the shorter time that they were smoking, was too short for nicotine to induce a gradual increase in the chest circumference of these smokers. In this study, however, it has been demonstrated that the chest circumference of young rats born from mothers that were exposed to nicotine during pregnancy, was initially smaller than that of the control animals of the same age up to postnatal day 21. Contrary to this the chest circumference of the mature nicotine exposed offspring (postnatal day 84) was larger than that of the control animals. This is difficult to explain since after weaning on postnatal day 21, the animals were not exposed to nicotine. However, if chest size is a reflection of lung volume, it can be expected that it is due to the bigger volumes of the lungs of the 84-day-old nicotine-exposed offspring. This is supported by the observation that the chest circumference/lung volume ratio of the control male and female rats at postnatal day 84 was the same as for those rats exposed to nicotine via the placenta. This means that the increase in chest circumference of these nicotine exposed rats was proportional to the increase in lung volume. The impact of maternal nicotine exposure during pregnancy on the chest circumference of the offspring was, like for the increase in the lung volume, not prevented by supplementing the mother’s diet with tomato juice.
The crown rump length (CRL) of the offspring was also not affected by maternal nicotine exposure or the use of tomato juice as a supplement during pregnancy. This was in contrast to the increased body weight of the 84-day-old nicotine exposed rats. What this observation implies is that the increase in body weight was not due to an increase in the overall size of the body of the nicotine exposed offspring. There are two possible explanations for the increase in BW despite the fact that the CRL was not affected by maternal nicotine exposure during gestation, namely: 1) it is likely that it is due to an increased capacity to store fat, and 2) it has been shown that nicotine have anti-diuretic properties (Burn et al., 1945) which implies that the increased body weight of the older animals can partly be attributed to water retention. More research into this effect of maternal nicotine exposure on BW changes in the offspring is required because of possible health consequences for the offspring in the longer term of smoking mothers or mothers using NRT.

4.4 THE EFFECT OF MATERNAL NICOTINE EXPOSURE DURING PREGNANCY ON LUNG DEVELOPMENT IN THE OFFSPRING

4.4.1 Lung Structure

The lungs of rats are still in the saccular phase of lung development at the time of birth (Joshi and Kotecha, 2007). The walls of the saccules are thick due to a double capillary layer. At postnatal day 4, rapid alveolar formation commences in rats and ends at postnatal day 13 (Schittny et al., 2008). Following postnatal day 13, alveolar formation continues to take place as the animal grows, but at a much
slower rate. The double capillary layer converges into a single layer during alveolarisation and maturation, allowing the alveolar walls to become thinner. This offers explanation for the decrease in tissue volume as the lungs of the offspring mature. While the tissue volume of the control lungs reached a plateau after postnatal day 21, the tissue volume of the animals that were exposed to nicotine via the placenta decreased further after postnatal day 42. This was followed by an increase in the volume of the air component of the lungs of the nicotine exposed animals. The decrease in the tissue volume of the nicotine exposed rats was due to a slow but gradual breakdown of the lung parenchyma as illustrated by the appearance of microscopic emphysema in the lungs of the offspring at around postnatal day 42. The maintenance of the constant tissue volume from postnatal day 21 in the control animals is due to a balance between formation of new parenchymal tissue and degradation thereof. On the other hand, the gradual decrease in the volume of the lung parenchyma of the nicotine exposed lungs can be ascribed to faster tissue degradation than tissue formation. It is important to note that the offspring of the nicotine exposed mothers received nicotine only up to birth. After birth the offspring never received any nicotine. Nicotine only has a half-life of 90 minutes (Fontaine, 2005), therefore, it is highly unlikely that any nicotine was still present in the lungs of the offspring at 9 weeks after nicotine exposure was stopped. This implies that maternal nicotine exposure during pregnancy programmed the lungs of the offspring to degrade faster than that of the control animals. This means that impaired tissue homeostasis gradually developed in these animals followed over time with an imbalance between tissue regeneration and tissue degeneration. This becomes apparent at around postnatal
day 42 when emphysema-like lesions appear in the lungs of the mature nicotine exposed offspring.

Although most research in the past has solely referred to alveolar tissue, it is important to note that lung parenchyma also consists of bronchioles, bronchi, blood vessels, as well as interstitium. However, the largest component consists of the alveolar component. Alveoli, which are large in number, are thin-walled, allowing for an enormous surface area for proper gas exchange to be maintained. This implies that if degradation of the lung parenchyma exceeds the replacement of parenchymal tissue, alveolar size and numbers will be adversely affected with a consequent decrease in the surface area available for gas exchange. In this study it was indeed demonstrated that the alveolar size, as indicated by the mean linear intercept (Lm), of the control animals decreased as the animals’ age due to formation of new alveoli. Contrary to this, the alveolar size of the rats that were exposed to nicotine via the placenta increased over time. This is an indication of alveolar wall destruction with a consequent increase in the alveolar volume and a decrease in alveolar numbers in the lungs of the nicotine exposed offspring. Consequently while the alveolar number of the control and tomato juice groups increased over time, that of the nicotine exposed animals was considerably lower than that of the control animals. These effects of maternal nicotine exposure during gestation were observed after weaning on postnatal day 21 and gradually worsened as the animals aged. Since no nicotine is present in the body, these observations suggest that nicotine exposure during gestation interfered with the genetically determined processes that are responsible for maintenance of lung
structure and function. This interference can be at DNA level, or epigenetic level or both since nicotine is genotoxic (Maritz and Harding, 2011). It also increases the oxidative stress in the offspring (Bruin et al., 2008). Nicotine also displays antioxidant activity (Newman et al., 2002).

Therefore, since nicotine and the oxidants are able to induce DNA damage such as strand breaks (Jorgensen et al., 2010), it may result in transcriptional mutagenesis. Nicotine also impair DNA repair (Sellappa et al., 2009). It also induces epigenetic changes (Maritz and Harding, 2011). Taken together these effects of nicotine may have important implications for biologic systems such as the respiratory system in that it may result in programming an imbalance between tissue degradation and synthesis.

It has already been determined that DNA damage induced by reactive oxygen species is involved in aging and various human diseases (Kregel and Zhang, 2007). If this is so, it explains why tomato juice rich in lycopene and the phytonutrients associated with its anti-oxidant function, protects the lung against the adverse effects of nicotine on the lungs of the offspring of mothers that were exposed to nicotine during pregnancy. It is therefore conceivable that nicotine, and thus NRT, may induce DNA damage that may render the respiratory system more prone to premature aging and thus increased susceptibility to respiratory disease.
The connective tissue framework of the lung is important for maintenance of lung structure and function. The extracellular matrix is an important component of the lung parenchyma, which has regulatory effects on cellular physiology, leading to reorganization and remodelling of the extracellular matrix, in turn, having an effect on lung function (Faffe and Zin, 2009). The major components of connective tissue are collagen, elastin, proteoglycans, and glycosaminoglycans (Mercer and Crapo, 1990). Collagen, especially collagen type IV is an important protein on the basement membrane of the extracellular matrix (Jalali et al., 2010). In my study, the offspring exposed to nicotine via the placenta during gestation showed a low connective tissue content in the lungs at postnatal day 84. Supporting studies have shown that as nicotine crosses the placenta barrier and occurs in the mother's milk during lung development of the offspring, it causes adverse effects on the connective tissue of the lung (Jalali et al., 2010). This implies that not only maternal smoking, but NRT as well can damage the connective tissue framework of the lungs of the offspring, consequently affecting lung structure maintenance, and function in the long term.

4.4.2 Cellular Senescence

From the saccular phase to the alveolar phase of lung development, interstitial fibroblasts play a critical role. Type II cell proliferation and the formation of Type I cells also occur as part of the process whereby new alveoli are formed. During this transition from the one phase to the other, the number of interstitial fibroblasts in the neonatal lung of the rat increased 4-fold (Jauniaux et al., 1999). Therefore, any interference with fibroblast proliferation and function in the developing lung
will not only impact on lung growth and development, but also its role in the maintenance of lung structural and functional integrity. Studies have shown that perturbations such as hypoxia, barotrauma, and steroid therapy interferes with alveolar formation in the rat (Luck et al., 1985), baboon (Murdzoska et al., 2010), and human infants (Maritz et al., 2011). These perturbations which are significant, often permanent, decreases the number of alveoli and thus the surface available for gas exchange. The smaller the surface area available for gas exchange, the lower the lung capacity of the individual would be, and consequently it will not meet the increasing demand for oxygen by the body during exercise.

An important function of fibroblasts is the formation of mature alveoli, as well as supplying the 3D connective tissue scaffold to support the lung and thereby maintain structural stability that is important for optimal lung function. A significant component of the 3D scaffold is the elastic fibres in the lung, which plays an important role in alveolarisation. Fibroblast-Type II cell communication is also essential for alveolar formation and maintenance (Fehrenbach, 2001) Studies have shown that the inhibition of elastic fibre assembly has been linked to impaired septation (Raunio et al., 1999). Damage to the elastic tissue compartment or impaired formation of this compartment due to impaired fibroblast function and communication with Type II alveolar epithelial cells will thus impair alveolar formation and maintenance. This will result in larger, but fewer alveoli in the lungs, as well as a decrease in the surface available for gas exchange. In neonatal lung fibroblasts of the rat, elastin expression is the highest during the phase of rapid alveolarisation, and declines rapidly thereafter.
(Gamieldien and Maritz, 2004). This suggests that interference with lung fibroblast integrity may result in impaired lung development.

Fibroblast proliferation and migration is inhibited by exposure to tobacco smoke, by increasing the cell cycle transit time, thus reducing the rate of alveolarisation. Consequently a smaller internal surface area available for gas exchange can be expected in the lungs of neonates born to parents that smoke. Tobacco smoke exposure also compromises fibroblast-induced repair responses, which may be a factor that contributes to the development of smoke induced diseases (Carmella et al., 1997) such as emphysema and asthma. Furthermore, nicotine also accumulates in the lung fibroblasts which cause glycolysis to be suppressed, in turn further compromising the role of fibroblasts to maintain alveolarisation and lung structure maintenance in the long term. This will eventually result in a compromised lung function.

Research has shown that studies done in vitro displayed no effects on the fibroblasts of human fetal lungs when exposed to nicotine (Carmella et al., 1997). On the other hand, in vivo studies also shown that nicotine had no long-term effect of metabolism in the lungs of adult animals as opposed to permanent suppression of energy metabolism of animals that were exposed to nicotine during lung development (Maritz and Burger, 1992). These in vitro studies were performed on cells that were not metabolically permanently compromised as oppose to the fibroblasts of lung cells of neonatal rats that had been exposed to nicotine during gestation and lactation.
Since studies have confirmed that maternal nicotine exposure during gestation and lactation interferes with glucose metabolism and apoptosis in the fetal and neonatal lung (Kordom et al., 2003), and that it may cause disruption of the interaction between lung fibroblast glucose metabolism and fibroblast function (Maritz and Harding, 2011), it is plausible that it will also adversely affect the long-term maintenance of lung structure.

Interestingly, it has also been shown that patients with emphysema, showed a reduced proliferation rate of their lung fibroblasts (Holz et al., 2004) as well as premature aging (Muller et al., 2006). Therefore, it is conceivable that the gradual deterioration of the connective tissue framework of the lungs of those rat pups that were exposed to nicotine via the placenta was partially due to compromised fibroblast proliferation and premature aging. Premature aging of the cells in the alveolar wall may also reduce the number of, for example fibroblasts, which are capable of proliferation and thereby reduce the capacity of the alveolar wall to maintain itself. A consequence of this is a slow degradation of the lung parenchyma.

Glycolysis plays an important role in the maintenance of lung cells (Lunt and Vander Heiden, 2011). Studies have shown that glycolysis is suppressed in animals exposed to maternal nicotine during gestation and lactation, with a consequent reduction of glucose flux through the glycolytic pathway (Maritz, 1987). Additionally, AMP (adenosine monophosphate) also accumulated in the lungs of the nicotine exposed rat pups. Subsequently, the AMP content of the
lungs of the nicotine exposed offspring increased even after the withdrawal of nicotine (Maritz and Burger, 1992). Studies have shown that the onset of premature cell senescence is associated with persistent reduced glycolytic activity as well as high levels of AMP (Kondoh et al., 2005a, Zwerschke et al., 2003). Supporting studies shows that enhancement of glycolysis bypasses cellular senescence (Kondoh et al., 2007). Therefore, it is conceivable that maternal nicotine exposure during gestation induce premature aging of the lungs of the offspring by irreversible suppression of glycolysis and the persistent high levels of AMP in the lungs of the offspring (fig. 4.4.2).

The structural integrity of the lung needs to be maintained by replacing the alveolar cells lost by apoptosis, with cell proliferation. Studies have shown that emphysema is associated with increased levels of alveolar cell proliferation as well as apoptosis (Newman et al., 2002). Upon reaching the senescence stage of the cell cycle, it causes cell proliferation to stop, thereby not compensating for the loss of alveolar cells by apoptosis to be replaced. According to this study, animals exposed to nicotine via the placenta during gestation showed signs of cellular senescence from as early as postnatal day 14, and the number of senescent cells/100µm of alveolar wall had a faster, yet gradual increase up to postnatal day 84, compared to the rest of the experimental groups that received both tomato juice and nicotine via the placenta, tomato juice only, and the control. This implies that alveolar cell loss contributes to pulmonary destruction and reduced lung surface area in the lungs of the offspring, which are characteristics of emphysema-like changes (Newman et al., 2002). Supporting studies have shown
that cellular senescence may play a role in changes that occur in the lungs during the pathogenesis of emphysema (Tsuji et al., 2006).

Therefore, premature aging and the gradual deterioration of the lung parenchyma will compromise lung function. This means that smoking and NRT will program the lungs of the offspring to become structurally and functionally compromised due to premature aging of the cells of the alveolar wall.

![Diagram illustrating metabolic changes induced by nicotine](image)

Fig.4.4.2. Diagram to illustrate the metabolic changes that induce premature aging in the lung parenchyma of rats exposed to nicotine via the placenta and mother's milk.

4.5 LUNG FUNCTION

Lung function and lung structure are in close relation to each other (Berend et al., 1981). This suggests that changes in structure of the lungs may have adverse effects on the function of the lungs. As described in the results of this study, a decrease in alveolar number and the consequent decrease in internal surface area available for gas exchange imply that the diffusion capacity for oxygen of the lung of the nicotine exposed offspring will be compromised in the long term. Diffusion capacity is a measure of how effectively oxygen and carbon dioxide are transferred across the blood-air barrier between the lungs and the blood. During
exercise, the body has an increased demand for oxygen. However, if there is a
decrease in the alveolar number due to factors such as cigarette smoke, the lungs
will thus have a smaller internal surface area available for gas exchange, which
may adversely impact the ability of such individuals to execute strenuous
exercises.

In this study the static compliance of the lungs of the control male and female and
those born to mothers that received tomato juice supplementation during gestation
was the same. Compliance was also not affected for those rats that were exposed
to nicotine via the placenta up to postnatal day 42. However at postnatal day 84
the compliance of the male rats was markedly higher than that of the control
animals of the same age. The compliance of the female rats was not affected
though. The reason for this gender difference in response to maternal nicotine
exposure is not known.

From the data it appears that the increase in the compliance of the nicotine
exposed offspring is only observed later in the life of the offspring, and at a stage
when the structural integrity was compromised most. It is plausible that as the
animals age, compliance will further increase due to the gradual deterioration of
the lung parenchyma, due to elastic tissue damage. Although no direct evidence is
available, it is conceivable that premature senescence of fibroblasts and other cells
in the alveolar walls will result in a gradual deterioration of the alveolar walls
with the development of emphysema-like lesions. Also, emphysematous lesions,
as shown in this study, are associated with degradation of the elastic tissue which will adversely affect lung recoil.

4.6 EFFECTS OF TOMATO JUICE

Many females resort to nicotine replacement therapy (NRT) to quit their smoking habits, even during pregnancy. Studies have shown that nicotine readily crosses the placenta and is present in the fetal circulation and amniotic fluid at higher levels than in the maternal circulation (Sastry et al., 1998). This suggests that the mere intake of nicotine, whether it is via smoking or NRT, changes the *in vitro* environment within which the fetus develops by reducing the anti-oxidant capacity of the mother as well as in the developing fetus or neonate. It also increases the oxidant levels in the *in utero* environment and the fetus (Aycicek et al., 2005). Therefore if the oxidant-antioxidant balance of the mother, fetus, and offspring can be restored by supplementing the diets with antioxidants, it will be possible to prevent the adverse effects of nicotine on lung development.

There is a large body of epidemiological evidence suggesting that tomatoes contain several antioxidants that may reduce the risk for oxidant associated diseases. These antioxidants include ascorbic acid, vitamin E, carotenoids, flavonoids, as well as phenolic acids. The disease-preventing potential of a food, such as tomatoes, is a consequence of several such constituents which may show some synergistic interactions (Erdman et al., 2009). This implies that one micronutrient, together with other micronutrients in the correct ratios may be much more effective as opposed to supplementing an individual’s diet with a
single micronutrient only. Research studies has in fact shown that pure lycopene is not as effective as an antioxidant when used on its own, compared to when it is in the presence of the phytonutrients such as tomatoes (Bjelakovic et al., 2008). Experimental research has indeed shown that supplementing the diet of adult rats with antioxidants and phytonutrients, prevented nicotine induced damage to the lung parenchyma of rats (Kasagi et al., 2006). Therefore, one of the aims in this study was to establish whether the oxidative effect on the lungs caused by nicotine can be prevented by the anti-oxidant properties in tomato juice in the lungs of the offspring. The diets of the mothers in this study was supplemented with tomato juice containing trans-lycopene, a potent anti-oxidant, as well as other phytonutrients and vitamins to work effectively against the harmful effects of nicotine administered during gestation.

As a result, the supplementation of tomato juice in the mothers’ diet during gestation had no effect on the growth and development of the offspring. Tomato juice also showed no effect on the structural development of the lungs in the offspring, thereby preventing the adverse effects of nicotine exposure via the placenta as well maintaining the average rate at which the animals’ age. This suggests than not only maternal smoking, but also NRT should not be an option for pregnant mothers, and that supplementing the diet with tomato juice throughout pregnancy for those who are trying to quit the smoking habit could prevent severe damage to the lungs and general health in the postnatal life of the offspring.
It is evident that in this study, stress-induced cellular senescence was caused by the harmful effects of nicotine in the lungs of the offspring via the placenta, allowing DNA damage and oxidative stress to worsen as the animals’ age. However, tomato juice supplementation with nicotine administration via the placenta showed no increase in the senescent cell count/100µm of alveolar wall in the lungs of the offspring from postnatal day 14 up to postnatal day 84. This implies that tomato juice, containing lycopene and other phytonutrients, prevented alveolar cells from reaching a state of cell senescence, which means that apoptosis and proliferation continued to function normally even though the rats was exposed to nicotine via the placenta during gestation.

In view of the fact that tomato juice supplementation in the mother’s diet had no effect on the lung development and aging in the offspring up to postnatal day 84, it implies that upon reaching lung maturation, further damage is less likely to occur. This also suggests that those animals exposed to nicotine via the placenta during gestation only, surely experienced the adverse effects thereof, such as increased body weights, increased lung volumes, increased cellular senescence and decreased lung surface area and decreased alveolar numbers] in their adult life. This confirms that a change in the ‘program’ of lung development was altered in utero. And it also confirms that tomato juice is able to prevent such detrimental effects on the respiratory health of the offspring.
4.7 NICOTINE REPLACEMENT THERAPY (NRT)

As a result of the dangers of smoking, NRT is now widely promoted by health practitioners as a safe way to quit smoking. However, many studies illustrate that nicotine indeed interferes with the development of the fetal lungs, heart, as well as the central nervous system. Nicotine is genotoxic (Argentin and Cicchetti, 2004, Kleinsasser et al., 2005) and studies have shown that its toxic effects persists in the fetus even after administration thereof has stopped (Maritz, 2002). In monkeys, research has shown that the damaging effects of nicotine during pregnancy increases the development of α7 nicotinic receptors in cells implicated in lung development (Sekhon et al., 1999). This implies that more nicotine molecules can enter these cells to interfere with metabolism and organelle integrity and function.

Studies have also shown that lysyl oxidase activity is suppressed by maternal nicotine exposure, consequently contributing to the gradual deterioration of the lung parenchyma of the offspring. Nicotine also induces peroxidation of membrane lipids (Kalpana and Menon, 2004) which changes the oxidant/antioxidant status of the lungs of the offspring. This is supported by the decrease in the vitamin C and E content of the lungs of the offspring (Maritz and Rayise, 2011). Studies also show a clear decrease in the levels of enzymes that catalyze the removal of antioxidants from the lung. Research done in our laboratories have shown that the level of superoxide dismutase in the lungs of rats that were exposed to nicotine via the mother’s blood remains significantly lower than that of rats not exposed to nicotine (unpublished data). This implies that apart
from its immediate effect in the lungs of those who use NRT, nicotine intake during pregnancy and lactation will have a long-term effect on the maintenance of lung integrity and respiratory health of the offspring (Bruin et al., 2008).

### 4.8 IMPACTS ON THE HEALTH OF THE OFFSPRING IN THE LONG TERM

Even though more research regarding the long-term effects of NRT in humans are required, there is a substantial amount of evidence in animal studies which signifies that fetal and neonatal exposure leads to widespread postnatal health consequences. In animal experimental research, it has been shown that maternal nicotine exposure during gestation and lactation had an effect of the pulmonary, cardiovascular, reproductive, metabolism as well as hypoxia-sensing outcomes in the offspring (Bruin et al., 2010).

Interestingly, the adverse effects of maternal nicotine are not limited to the first generation offspring (F1). Studies have shown that maternal nicotine exposure also appears to affect the second generation of offspring (F2), where the mothers of the F2 generation had elevated blood pressure, increased fasting serum insulin, as well as an enhanced insulin response to an oral glucose challenge (Holloway et al., 2007).

It is therefore important not to only consider the effect of smoking or NRT on the lungs of the respiratory health of the first generation progeny, but also on the subsequent generations. The long term implications should therefore be
considered by health authorities when making policy decisions regarding the safety if NRT especially during pregnancy.

4.9 CONCLUSION

In conclusion, the outcomes of my study showed that maternal nicotine exposure during gestation resulted in a change in the program that controls aging of the lungs of the offspring. Consequently, this renders the lungs more susceptible to disease and reduces lung function. Based on findings of other studies done in our laboratories, the effect of nicotine on lung development is also transferable between generations. This is conceivable due to a combination of placental changes as well as the direct effect of nicotine on cell DNA. It is therefore not advisable to prescribe NRT to pregnant and lactating women. However, a more relative approach would be to ensure that the antioxidant capacity of pregnant and lactating women are maintained, as it will protect the fetus and neonate against the adverse effects of oxidants in the atmospheric air and especially in tobacco smoke.

More data generated in this particular study will provide a better understanding and interpretation of results concerning the sites of mechanism of action of nicotine intake by the mother and on the lungs of the offspring as well as the long term consequences regarding the susceptibility of these lungs to damage, as well as the irreversible growth arrest of cellular senescence. It will also further confer
evidence as to why NRT is not an option to stop the smoking habit, especially in pregnant and lactating females.
REFERENCES


FONTAINE, B. 2005. [Smoking and breastfeeding: how can we help mothers stop smoking?].
Tabagisme et allaitement: quelles techniques d'aide a l'arret du tabac proposer aux meres? *Journal de gynecologie, obstetrique et biologie de la reproduction*, 34 Spec No 1, 3S209-12.


MINEUR, Y. S., ABIZAID, A., RAO, Y., SALAS, R., DILEONE, R. J., GUNDISCH, D., DIANO, S., DE BIASI, M., HORVATH, T. L., GAO,


SELLAPPA, S., PRATHYUMNAN, S., JOSEPH, S., KEYAN, K. S., BALAKRISHNAN, M. & SASIKALA, K. 2009. XRCC1399 and


