# MATERNAL NICOTINE EXPOSURE DURING GESTATION AND LACTATION INDUCE PREMATURE AGING OF THE LUNGS OF THE OFFSPRING

BY

MUYUNDA MUTEMWA

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Supervisor: Professor G S Maritz

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Muyunda Mutemwa

**KEYWORDS**

Tobacco Smoking

Nicotine Exposure

Lung Development

Maternal

Carotenoids

Tomato Juice

Lycopene

Alveoli

Emphysema

Premature Aging

# ABSTRACT


**MATERNAL NICOTINE EXPOSURE DURING GESTATION AND LACTATION INDUCE PREMATURE AGING OF THE LUNGS OF THE OFFSPRING**

M Mutemwa

MSc thesis, Department of Medical Biosciences, University of the Western Cape.

Tobacco smoking remains one of the leading causes of death worldwide. Despite all the efforts made by governments, researchers and communities to educate women about the dangerous effects of tobacco smoke and nicotine, smoking during pregnancy continues to be a common habit and accounts for a significant percentage of fetal morbidity and mortality. The offspring is, as a result, exposed to nicotine through the blood and the milk of the mother. Nicotine is therefore expected to interact with the developing fetus and the offspring of mothers who smoke or use Nicotine Replacement therapy for smoking cessation, resulting in the interference with normal fetal lung development. Maternal cigarette smoke or nicotine exposure produces adverse effects in the lungs of offspring, these include; intrauterine growth retardation, low birth weight, premature birth, reduced pulmonary function at birth, and a high occurrence of respiratory illnesses after birth. The main objectives of this study were to determine: 1) the effects of maternal nicotine exposure during gestation and lactation on lung

development in the offspring, 2) if there is evidence of premature aging of the lungs of the lungs of the nicotine exposed offspring, and 3) whether tomato juice can have protective effects on the fetal lung development and function in the offspring. From the study, it was established that maternal nicotine exposure had no significant effect on the growth parameters of the offspring. However, it results in the late onset of gradual parenchymal damage which resembles premature aging. The study also found that the consumption of tomato juice may have protective effects on the premature aging of the lungs of the offspring.

November 2009

# DECLARATION

I declare that "*Maternal Nicotine Exposure during Gestation and Lactation Induce Premature Aging of the Lungs of the Offspring"* is my own work, that it has not been submitted before for any degree or examination in any other university, and that all the sources I have used have been indicated and acknowledged as complete references.

Muyunda Mutemwa 13 November 2009




Signed: ……

# DEDICATIONS

This thesis is dedicated to my supervisor Professor Gert S. Maritz and to my parents Pastor Kwalela Mutemwa and Mrs. Priscah S. Mutemwa.

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| | |
|---|---|
| AMD | Age-related macular degeneration |
| AP-1 | Activator protein-1 |
| ARDS | Acute Respiratory Distress Syndrome |
| BW | body weight |
| C | Control |
| CC | Chest circumference |
| cm | centimeter |
| COPD | Chronic obstructive pulmonary disease |
| CR | Crown rump length |
| DAPI | 4'-6-Diamidino-2-phenylindole |
| DNA | Deoxyribonucleic acid |
| EGFR | Epidermal Growth Factor Receptor |
| ERK | extracellular signal–regulated kinases |
| g | gram |
| GSH | glutathione |
| H &E | Haematoxylin and Eosin |
| $H_2O_2$ | Hydrogen peroxide |
| IARC | International Agency for Research on Cancer |
| IL | Interleukin |
| JNK | c-jun N-terminal kinases |
| kg | kilogram |
| LDL | Low density lipoproteins |

| | |
|---|---|
| Lm | Linear Intercept |
| Lv | Lung Volume |
| MAP | mitogen-activated protein |
| MAPK | Mitogen-activated protein kinase |
| mg | milligram |
| ml | millilitre |
| mm | millimeter |
| MMP | Macrophage metallomatrix protein |
| N | Nicotine |
| N+T | Nicotine +Tomato |
| nAChR | Nicotinic acetylcholine receptors |
| NF-κB | Transcription factor |
| NO | nitric oxide |
| NRT | Nicotine Replacement Therapy |
| $O_2^-$ | oxygen free radical |
| ONOO- | peroxynitrite |
| PBS | Phosphate buffered saline |
| PTPs | Protein tyrosine phosphatases |
| RNS | reactive nitrogen species |
| ROS | reactive oxygen species |
| rTdT | Deoxy nucleotidyl Transferase, Recombinant |
| SA-gal | Senescence associated β-galactosidase |
| SSC | saline-sodium citrate |

| | |
|---|---|
| T | Tomato |
| TGF-β | transforming growth factor beta |
| Tsept | Alveolar wall thickness |
| Va | alveolar air volume density |
| $V_t$ | Volume Density |
| $\alpha_1$ | alpha-1 |
| β | beta |
| γ | gamma |
| μ | micro |
| % | percent |
| °C | degrees Celsius |
| 5-HT | 5-hydroxytryptamine |

# CHAPTER 1

## Literature Review

### 1.1 LUNG DEVELOPMENT

The adequate development and maturation of the lung, particularly during the prenatal stage of fetal development, is extremely important given that the survival of the infant at birth is dependent on this process (Copland and Post, 2004).

It has been reported that premature birth occurs in about 5 to 10% of all pregnancies. According to researchers, one of the main problems with premature birth appears to be the immaturity of the lung. The inadequate development of the lung causes approximately 75% of early mortality and long-term disability in prematurely born infants. The infants that have extremely low birth-weights, born between 24 and 28 weeks of gestation are the most susceptible to lung injury (Stevenson et al, 1998) because their lungs are fragile and have small volumes for gaseous exchange (Copland and Post, 2004).



Human lung development begins around day 26 of gestation, which is approximately the fourth week after conception. It includes the increase in the number of mature alveoli and continues postnatally until around the age of 7 (Boyden, 1977; Crapo et al, 1980; Jeffery, 1998). The main stages of lung development begin around week 8 and continue till week 40 of gestation ( which is about 32 weeks). The 32 weeks of development during the gestation period

are categorized into phases according to the visual appearance of lung tissue (Boyden, 1977). Normal lung development, which occurs as a series of complex, tightly regulated events, can be divided into five phases (Joshi and Kotecha, 2007). These phases include the embryonic phase, the pseudoglandular phase, the canalicular phase, the saccular phase, and the alveolar phase (Haddad, 2002).

### 1.1.1 Embryonic Stage

The embryonic phase of human lung development begins around the 4$^{th}$ week (day 26) of gestation in humans and continues until the 6$^{th}$ week (day 52) of gestation. This phase begins with the emergence of the lung from the base of the primordial foregut endoderm as the laryngo-tracheal groove (Ornitz et al, 1996; Cardoso, 2000). This forms the trachea and bronchial buds, which consecutively expands at the beginning of week 5 to form the main bronchi. The embryonic phase is mainly identified by the development of the lobular segments of the respiratory tree as tubes lined with columnar epithelium. The columnar epithelium is noticeable by the end of week 5 or 6 (Boyden, 1977).



### 1.1.2 Pseudoglandular Stage

The subsequent phase is the pseudoglandular phase which begins around week 5 or 6 (that is about day 52) and continues till week 16 or 17 of gestation. This phase is identified by the development of the fetal lungs as an exocrine gland

and the completion in the growth of the primal airways. During this phase, cartilage appears around the larger airways; smooth muscles also begin to evelop airways and blood vessels. At the end of this phase, acinar outlines first begin to appear as epithelial tubes and then continue to grow and branch. The columnar epithelial cells lining the tubular glandular structures that did not differentiate evolve into the many cell types that occupy the airways. These cells include serous, goblet, ciliated, clara and alveolar cells (Boyden, 1977; Mason and Williams, 1977; Crapo et al, 1980).

### 1.1.3 Canalicular Stage

The canalicular phase commences around week 16 or 17 of gestation and carries on until the 25$^{th}$ to the 27$^{th}$ week of gestation. This phase includes major developments of the fetal lung that are crucial to the extra-uterine life. These major developments include the enlargement of the lumina of the bronchi, and the terminal bronchioles, the development of capillaries at the site of the future air space where the alveoli would later form, and the appearance of surfactant. The subdivisions of the acini are also formed at this stage. Furthermore the lining of the epithilium begins to differentiate into alveolar type I and type II cells (Boyden, 1977; Mason and Williams, 1977) and the production of surfactant components by type II cells which are evident in the form of lamellar inclusion bodies by the 24$^{th}$ week of gestation (Kotecha, 2000).

### 1.1.4 Saccular Stage

The saccular or terminal sac phase begins around the 28th week of gestation and continues to about the 35th week of gestation. The phase is identified by the development of the terminal air sacs from alveolar ducts, refinement of the areas of gaseous exchange, a reduction in the thickness of the interstitial tissue, the thinning of the epithelium, the separation of the terminal air spaces as well as the differentiation of the terminal stages of alveolar type I and type II epithelial cells (Haddad, 2002).

### 1.1.5 Alveolar Stage

The ultimate phase is the alveolar phase which occurs during the last 5 weeks of fetal lung development. This phase begins at around the 36th week of gestation and is identified by the formation and maturation of the alveoli (Kotecha, 2000). During this phase, millions of alveoli are formed, with the interval surface area of the lung increased by thinning of the septal walls and reduction in the cuboidal epithelium. The phase further includes the separation of terminal subsaccules by loose connective tissue and the continuation of cellular maturation, more particularly alveolar type II epithelial cells which develop a greater density of lamellar bodies (Boyden, 1977; Mason and Williams, 1977). Further key determinants for lung development and maturation include maintenance of sufficient fetal lung fluid volume and fetal breathing movements, which appear to be crucial for the normal development of the lung (Kotecha, 2000).

## 1.2 FACTORS AFFECTING LUNG DEVELOPMENT

Despite efforts made by modern management, disorders of lung development and maturation together with the control of breathing, still remain central challenges that confront neonatologists today (Copland and Post, 2004). The normal development of the lung can be affected by various factors that are interrelated. These factors include factors that can be developmental, genetic, environmental or mechanical. Examples of these range from maternal and fetal nutrition, endocrine factors, fetal breath movements, normal fetal lung fluid production, sufficient amniotic fluid volume, adequate intrathoracic and extrathoracic space, positive transpulmonary pressure, to normal postnatal adaptation (Mesas-Burgos et al, 2009).


## 1.3 EMPHYSEMA

Emphysema is an important component of chronic obstructive pulmonary disease (COPD). It is a highly prevalent pulmonary disease (Snider et al, 1985) which is one of the major causes of morbidity and mortality worldwide (Harrison et al, 1997). Emphysema has been defined as a chronic and terminal disease in which the distal air spaces of the terminal bronchiole are abnormally and permanently enlarged. This abnormal enlargement of the air spaces is caused by the destruction of alveolar walls (Snider et al, 1985) which results in the restriction of airflow and the disturbance in blood oxygenation (Shapiro, 1995).

Although the pathogenesis of emphysema still remains unknown, a number of studies have proposed that genetic factors may have a role to play in determining individual susceptibility to the development of emphysema (Ito et al, 2005). The development of emphysema has been linked with smoking and atmospheric pollution by many researchers (Snider et al, 1985). Historically, emphysema has been linked to an excessive lung inflammation caused by the chronic inhalation of cigarette smoke and the resultant protease/anti-protease imbalance (Shapiro, 1995). It has been proposed that the destruction of tissue, which is a distinctive characteristic of emphysema, directly causes a decrease in lung tissue density. However, further loss of the tissue density comes from lung hyperinflation that is a result of enlarged lung compliance (Dirksen et al, 1997).



Studies have been done on lung changes that are characteristic of emphysema in both humans and animals through the use of different methodologies (Starcher and Williams, 1989; Janoff et al, 1983). A good number of these studies propose that connective tissue, particularly elastin, is the main target of the destruction observed in emphysema. It is generally believed that the structural rearrangement and loss of gaseous exchange surface brought about by elastin degradation in emphysema is permanent (Mercer and Crapo, 1992).

There is also increasing evidence in involvement of alveolar cell apoptosis in the pathogenesis of emphysema (Rangasamy et al, 2004). Since emphysema is characterized by a progressive impairment in alveolar gaseous exchange, which

is most likely a consequence of loss of alveolar capillaries, it has been suggested that there is a potential link between alveolar cell apoptosis and disruption of molecular and cellular signaling involved in alveolar structural maintenance and repair. This potential link has broader implications since the pathogenesis of alveolar damage induced by cigarette smoke may have common characteristics with alveolar enlargement due to aging (Tuder et al, 2006).

Pathologists have distinguished two major forms of emphysema which are closely related (Friedman, 2008). The first of these is centrilobular emphysema which is the most commonly observed form of emphysema. This form of emphysema is predominantly associated with the prolonged exposure to cigarette smoke (McCusker, 1992; Pryor et al, 1983). It is characterized by the breakdown of alveolar walls in the central portion of the acini, initially sparing the peripheral parts of the acinus and lobule. This form of emphysema is generally manifested in the upper regions of the lung. Panlobular emphysema on the other hand is recognized by the destruction involving all portions of the lobule out to the periphery, and is usually more severe in the lower lung regions (Friedman, 2008). It is further characterized by airspace enlargement throughout the acinus and is generally a consequence of a deficiency in the production of $\alpha_1$-protease inhibitor (McCusker, 1992).

## 1.4 THE PROTEINASE-ANTIPROTEINASE THEORY

Several studies have indicated that protease pathogenesis has an influence in a number of lung diseases, including asthma and COPD (Demedts et al, 2006); a disease characterized by the impairment of air flow (Eriksson, 1999). Generally, there appears to be a disturbance in the balance between proteolytic and anti-proteolytic molecules in lungs of patients with COPD (Demedts et al, 2006), resulting in an elevated proteolytic activity causing damage of healthy lung parenchyma, initiating the progression of emphysema. The increase in proteolytic activity may be a result of inflammation, which is characterized by the release of proteolytic enzymes by inflammatory cells such as macrophages and neutrophils, or could otherwise be a consequence of genetic factors such as deficiency of alpha-1 antitrypsin (Demedts et al, 2006).



The imbalance between proteases and anti-proteases results in the destruction of alveolar walls, thus developing into emphysema. Furthermore oxidative stress influences the proteinase-antiproteinase imbalance by stimulating proteases and inactivating antiproteinases. In addition to this, oxidants have an important role to play in the inflammatory reaction by stimulating the transcription factor (NF-κB), hence stimulating the transcription of pro-inflammatory genes (Demedts et al, 2006).

## 1.5 OXIDANT-ANTIOXIDANT STATUS

Oxidative stress is primarily caused by the imbalance between the production of reactive oxygen species (ROS) and the biologic scavenger system which detoxifies the reactive oxygen intermediates under normal physiologic conditions. Oxidative stress can also influence the molecular mechanisms that control lung inflammation (Guo and Ward, 2007).

The epithelium of the lung is frequently exposed to oxidants produced internally as part of normal metabolism, as well as oxidants in the ambient air, including ozone, nitrogen dioxide, diesel exhaust, and cigarette smoke (Rahman et al, 2006). A large amount of oxygen passes through the lung causing the lung to be highly susceptible to oxidative stress. Various levels of antioxidants are therefore required for protection against these vulnerabilities (Arab et al, 2002).



The main oxidants that are found in the lung are reactive oxygen species which include superoxide, hydrogen peroxide, and hydroxyl radicals; and the reactive nitrogen species (RNS) which include nitric oxide (NO) and its derivates peroxynitrite (ONOO-) and nitrogen dioxide. These oxidative and nitrosative species are the primary contributors to inflammatory injury (Lang et al, 2002). Molecules such as protein and lipid peroxide radicals can also contribute to oxidative stress (Musellim et al, 2006). The major oxidant sources in the lungs include neutrophils, eosinophils and alveolar macrophages, alveolar epithelial

cells, bronchial epithelial cells and endothelial cells. The formation of these oxidants is exacerbated by exposure to exogenous chemical and physical agents such as mineral dusts, ozone, nitrogen oxides, ultraviolet and ionizing radiation and tobacco smoke (De Paepe et al, 1999). Oxidative stress can lead to peroxidation of membrane lipids, reduction of nicotinamide nucleotides, rises in intracellular calcium ions, cytoskeleton disruption and deoxyribonucleic acid (DNA) damage (Foronjy and D'Armiento, 2006; Musellim et al, 2006). This subsequently results in an impaired cellular function, induces apoptosis, and stimulates dysfunctional matrix remodeling (Foronjy and D'Armiento, 2006).

According to what Harman (1956) suggested in the free radical theory of aging, oxidative stress is responsible for cellular senescence. Kondoh et al (2005) illustrated this by showing the link between senescence and the process of glycolysis. In their study, they demonstrated that an increase in glycolysis can bypass cellular senescence whereas, the inhibition of glycolysis results in premature senescence. By this they managed to show that indeed there is a link between oxidative stress and cellular senescence. This study was in agreement with studies done by Lee et al (1999) and Parrinello et al (2003) who proposed that the increase in ROS and oxidative stress have a part to play in the occurrence of senescence. Reports by Chen et al (1995; 2001) indicated that even mild oxidative stress such as low concentrations of hydrogen peroxide is enough to induce senescence. In summary, oxidative stress stimulates telomere-independent senescence. The prevention of oxidative stress is therefore

expected to give answers to the question of the immortality of cancer cells. It has actually been suggested that the protection from oxidative stress can significantly increase life span (Kondoh et al, 2007).

## 1.6 TOBACCO SMOKE AS SOURCE OF OXIDANTS

The exposure to tobacco smoke stimulates the airway and parenchymal inflammatory cells to produce proteinases mainly elastase from neutrophils and different metalloproteinase from alveolar macrophages. This leads to an overwhelming of the antiproteinase defense in the epithelial lining fluid. Lung tissue studies show that different biological processes are involved in the pathogenesis of emphysema, such processes include imbalances between oxidant stress and antioxidant defenses in the lungs (MacNee, 2000; Tuder et al, 2003) and between cellular apoptosis and regeneration (Aoshiba et al, 2003; Tuder et al, 2003).



ROS found in cigarette smoke such as peroxynitrite and hydrogen peroxide have been shown to promote the phosphorylation and stimulation of extracellular signal–regulated kinases (ERK), p38, and c-jun N-terminal kinases (JNK). These mitogen-activated protein (MAP) kinases are known to have a significant effect on immune responses in the lung. The activation of Mitogen-activated protein kinase (MAPK) by ROS depends on the stimulation of particular tyrosine kinases such as the epidermal growth factor receptor (EGFR), which when stimulated undergoes homodimerization or heterodimerization consequently leading to the

autophosphorylation and activation of tyrosine kinase. Oxidants produced during cigarette smoke can rapidly inhibit the critical protein tyrosine phosphatases (PTPs) that provide an inhibitory constriction upon the system, thereby altering the signaling response to exogenous stimuli (Foronjy and D'Armiento, 2006). Oxidative stress generated by tobacco smoke may initially deplete glutathione, followed by a rebound increase of glutathione in chronic smoke exposure as an adaptive response to oxidative stress, which occurs as a result of the upregulation of y-glutamylcysteine synthetase (Rahman and MacNee, 1999).

The most important defenses against oxidative stress are antioxidants; these include enzymatic and non-enzymatic antioxidants. The enzymatic antioxidants of the lung include glutamate, cysteine ligase, glutathione reductase, glutathione peroxidase, glucose-6-phosphate dehydrogenase, and in addition superoxide dismutases, catalase, heme oxygenase-1, peroxiredoxins, thioredoxins and glutaredoxins (Musellim et al, 2006; Chu et al, 2005). The non-enzymatic antioxidants are; glutathione, uric acid, alpha-tocopherol, bilirubin and lipoic acid, vitamins C and E, and beta-carotene (Rahman et al, 2004). These antioxidant defenses are found present in the lung epithelial lining fluids and within the lung cells (Arab et al, 2002) and are the first line of defense against the oxidants (Rahman et al, 2004).

Fluids in the lining of the airways and extracellular spaces of healthy lungs are maintained in an extremely reduced state through millimolar levels of reduced

glutathione (GSH) to protect the normal physiologic functions of the lungs. Consequently, the levels of antioxidants and oxidants in healthy lungs are balanced in favor of a reducing state. An elevation in oxidants or a decrease in antioxidants can disrupt this equilibrium and thus result in oxidative stress. The presence of an imbalance in the oxidant–antioxidant system is one of the first events that result in inflammatory reactions in the lung (Musellim et al, 2006).

The oxidant-antioxidant imbalance can result in the activation of cell surface receptors and inactivation of phosphatases, leading to the alteration of MAPK, NF-κB, and activator protein-1 (AP-1) signaling pathways, thus exacerbating inflammation (Foronjy and D'Armiento, 2006). Oxidative stress has been found to occur in many forms of lung disorders, such as pneumonia, acute respiratory distress syndrome (ARDS), idiopathic pulmonary disease, cystic fibrosis, bronchiectasis, ischemia–reperfusion injury, and lung cancer (Guo and Ward, 2007). An altered oxidant– antioxidant balance has also been implicated in age-related ocular diseases such as cataracts, age-related macular degeneration (AMD) and glaucoma (Yilmaz et al, 2005).



## 1.7 CAROTENOIDS

Carotenoids are common dietary constituents that are naturally contained in most fruits, including oranges or yellow and green vegetables. Carotenoids are known to exert antioxidant activities and prevent free radical-induced cellular damage (Bendich, 1993). Several researchers have investigated the potential role of

antioxidant nutrients in prevention of chronic diseases and aging processes in humans (Mayne, 2003). Evidence shows that the use of carotenoids is a prospective approach to protecting lung integrity and lung function. High concentrations of carotenoids, such as lycopene, together with other antioxidant vitamins including vitamin C and vitamin E in the lung epithelial lining and lining fluids in the lung may provide an additional level of protection against oxidative and ozone induced damage (Arab et al, 2002). Lycopene however is an effective antioxidant abundantly contained in tomato juice (Di Mascio et al, 1989). It has been utilized to test the hypothesis that oxidant-antioxidant imbalance could be important rather than inflammatory cell influx with increased burden of proteinases (Kasagi et al, 2006).



### 1.7.1 Lycopene

Lycopene, a bioactive carotenoid present in many fruits and vegetables is the major carotenoid in fresh tomatoes and tomato products (Takeoka et al, 2001; Kaplan et al, 1990). Lycopene has been shown to have many beneficial health effects (Giovannucci, 1999) and is considered to be the most effective biological carotenoid in quenching singlet oxygen (Di Mascio et al, 1989). Since tobacco smoke contains more than $10^{15}$ oxidant molecules (Church and Pryor, 1985), it has been suggested that dietary carotenoid intake may influence the development of tobacco smoke-induced emphysema (Kasagi et al, 2006). Previous literature has demonstrated that carotenoids have antioxidant properties and prevent cellular injury induced by free radicals (Bendich, 1993).

The prospective role of antioxidants in the prevention of chronic diseases and the aging process in humans has been investigated by researchers (Mayne, 2003).

Lycopene has antioxidant properties that have motivated interest in the tomato as a food with potential anticancer properties (Giovannucci, 1999). Although β-carotene had been effective against some chemically induced cancers, it was not effective against tumors in the respiratory tract (Obermueller-Jevic et al, 2002). Lycopene and β-carotene have been measured in lung tissue, which therefore adds to the possibility that carotenoids are a good defense mechanism against oxidative stress (Takeoka et al, 2001). An experiment done by Agarwal et al (2001) revealed that the intake of tomato products provided protection against oxidative damage to serum lipids, Low density lipoproteins (LDL) lipids, proteins and lymphocyte DNA. It was also noted from their study that lycopene supplementation causes a decrease in DNA damage which, as they suggested, might play a significant role in the lowering of cancer by decreasing the oxidation of proteins and DNA (Arab et al, 2002). It was also observed in their study that lycopene supplementation lowered serum LDL oxidation. This lowering of LDL oxidation might have an important role in the lowering of cardiovascular diseases as it is well known that oxidized LDL play a significant role in the formation of foam cell and arterial plague (Jialal and Devaraj, 1996; Parthasarathy, 1998). In addition to this, previous literature reveals that the increase in oxidized proteins has an important role to play in the development of chronic diseases and the process of aging. Because some proteins function as metabolic enzymes, their

oxidative damage may thus bring about a loss of this particular function (Hu, 1994; Stadtman, 1992).

## 1.8 TOBACCO SMOKING AND RESPIRATORY HEALTH

It has been estimated that cigarette smoking has caused 1 billion deaths in the twenty-first century (Ginzel, 2001). Studies have documented that cigarette smoke contains about 4800 compounds, 60 of which are identified as animal and/or human carcinogens. These compounds contained in tobacco include benzene, lead, and chlorinated dioxins and furans. Other components contained in tobacco smoke such as hydrogen cyanide and arsenic have been identified as harmful to the cardiovascular system. On the other hand, other components including acrolein, arsenic toluene, and phenol have been recognized to unfavorably affect the respiratory, reproductive and nervous systems (Fowles and Bates, 2000; International Agency for Research on Cancer, 2004).



## 1.9 MATERNAL SMOKING

Stein et al (1999) successfully demonstrated the association between smoking during pregnancy with an increase in the occurrence of respiratory tract diseases. Despite the evidence that maternal cigarette smoke during pregnancy is detrimental to the developing fetus (Schwartz et al, 1972), smoking still remains a widespread habit and accounts for a significant proportion of fetal morbidity and mortality through both a direct (fetal) and an indirect (placental) exposure (Salihu and Wilson, 2007). It is estimated that the exposure to maternal

tobacco or nicotine accounts for approximately 5 to 10% of all fetal and neonatal deaths (Proskocil et al, 2005). From previous work done on this subject, researchers have proposed that if smoking mothers were to give up the habit early during pregnancy, they should expect pregnancy results equal to those of nonsmoking mothers (Schwartz et al, 1972). However, it should be noted that smoking during early pregnancy can still cause unfavorable outcomes (Seller and Bnait, 1995).

Maternal cigarette smoke or nicotine exposure produces adverse effects in the lungs of offspring, these include; intrauterine growth retardation, low birth weight, premature birth, reduced pulmonary function at birth, and a high occurrence of respiratory illnesses after birth ( Proskocil et al, 2005; Egger and Aubert, 2005; Sekhon et al, 2004). In rats, the prenatal exposure to cigarette smoke decreases the number of pre-alveolar saccules and increases their size; this is expected to reduce the number of attachment points on small airways (Sekhon et al, 2004).



It is also well understood that cigarette smoke contains a high concentration of oxidant molecules that are thought to have an important role to play in the development of smoke-related lung diseases (Foronjy and D'Armiento, 2006). The presence of these free radicals results in DNA damage and increased inflammatory cells such as macrophages and neutrophils (De Paepe et al, 1999). These effects are likely to occur in human infants whose mothers smoke during pregnancy. More studies done on rat models revealed that cigarette smoke and

nicotine exposure during pregnancy and lactation can result in a reduced lung volume, number of saccules and septal crests, elastin fibers in fetal lungs, reduction in elastic tissue, increased number of lamellar bodies in the lungs of rat pups which result in emphysema-like changes (Sekhon et al, 2004).

In addition to these perinatal outcomes, some epidemiologic studies have suggested that a number of diseases observed in the offspring later on in life may also be related to maternal smoking during pregnancy. While some researchers have pointed out that maternal smoking during pregnancy increases the risk of certain forms of childhood cancers in the prenatally exposed offspring (Brooks et al, 2004; Filippini et al, 2000), other studies have linked maternal smoking with respiratory diseases such as asthma and atopy in the next generation (Raherison et al, 2007; Alati et al, 2006). In addition to this, more studies have linked prenatal smoke exposure with postnatal pathologies such as neurologic and behavioral disturbances (Lavezzi et al, 2005), obesity (Al Mamun et al, 2006), type 2 diabetes (Montgomery and Ekbom, 2002), and hypertension (Oken et al 2005; Ng and Zelikoff, 2007).



## 1.10 NICOTINE

Nicotine, a tertiary amine, (Buccafusco, 2004) is the main psychoactive component of tobacco smoke (Perkins et al, 1994) and is accountable for the habitual use of tobacco (Tutka et al, 2005). Nicotine functions biologically by binding to nicotinic acetylcholine receptors (nAChR) where it mimics the action of

acetylcholine. These receptors are members of the ligand-gated ion channels which are found in the brain. The nAChR are found to exist on neurons and on non-neuronal tissue such as muscles and immune cells (Gallowitsch-Puerta and Tracey, 2005). Normal lung cells and lung cancer cells have high affinity to nAChR (Pontieri et al, 1996; Maus et al, 1998). Literature suggests that the interaction of nicotine with nAChR on the surface of rodent epithelium found in the bronchioles promotes cell proliferation (Cattaneo et al, 1997).

### 1.10.1 Nicotine metabolism

Nicotine molecules have a low polarity and slight hydrophobicity which is caused by the existence of hydrophobic pyridine and pyrrolidine rings. This property is what makes it possible for nicotine to easily cross the blood–brain barrier and enter into the brain tissue (Oldendorf, 1974; Oldendorf et al, 1993; Spector and Goldberg, 1982) or, as shown in pregnant women, cross the placenta without restraint (Van Vunakis et al, 1974; Luck et al, 1982). The spread of nicotine in the organs is highly dependent upon its ability to bind to specific tissue. For example, the spleen, the liver, the lungs, and the brain have high affinity for nicotine, as opposed to adipose tissue which is shown to have a relatively low affinity to nicotine (Benowitz, 1986). About 5 to 10% of total elimination accounts for the renal excretion of unchanged nicotine. (Benowitz et al, 1983; Rosenberg et al, 1980). The rest is then metabolized mainly in the liver and in lungs although to a slight extent (Jacob et al, 1988; Benowitz et al, 1991).

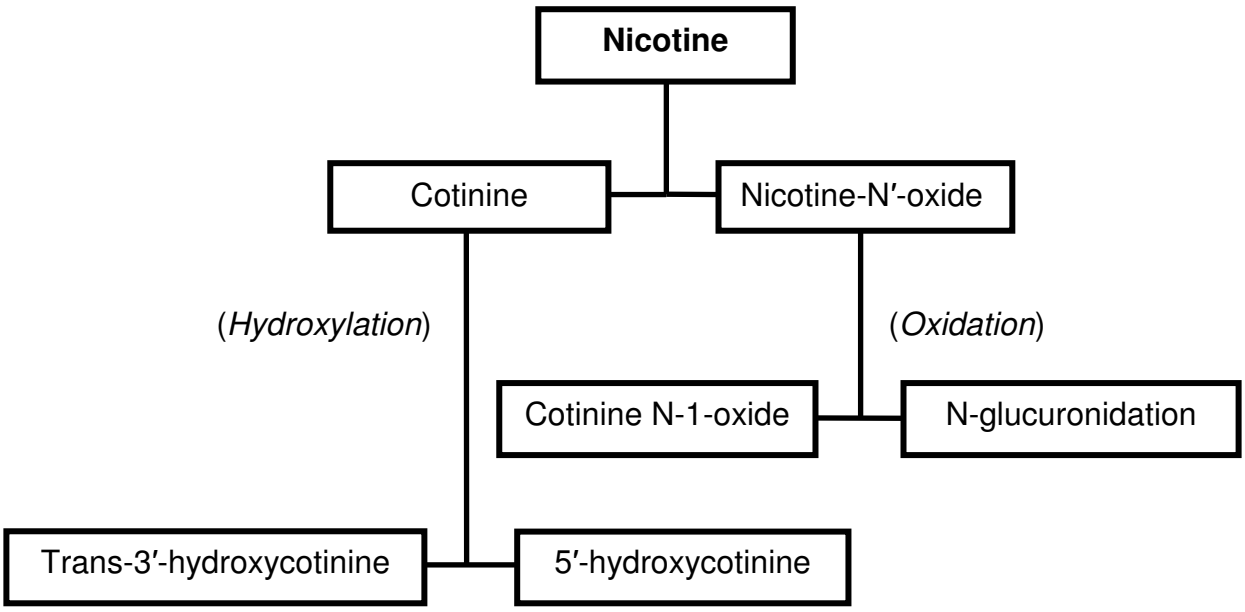



**Figure 1.10.1** The main metabolites of nicotine are cotinine and nicotine-N′-oxide. Cotinine is metabolized by hydroxylation to trans-3′-hydroxycotinine and 5′-hydroxycotinine, N-oxidation to cotinine N-1-oxide, and N-glucuronidation (Pogocki et al, 2007).



The major metabolites of nicotine are cotinine and nicotine-N′-oxide (figure 1.10.1). The formation of cotinine occurs in the liver. About 70 to 80% cotinine and its metabolites is responsible for nicotine metabolism in humans (Jacob et al, 1988; Benowitz et al, 1991; Hukkanen et al, 2005). The initial metabolism of cotinine includes oxidation of position 5′ of the pyrrolidine ring, a process which is mediated by a cytochrome P450 to an unstable intermediate 5′-hydroxynicotine (Murphy, 1973 ). The next step involves the metabolism of iminium cation to cotinine by a cytoplasmic aldehyde oxidase (Hibberd and Gorrod, 1983). Cotinine itself is also extensively metabolized, with less than 20% excreted unchanged in the urine (Benowitz et al, 1983). Nicotine is metabolized to nicotine-glucuronide (figure 1.10.1) and several other minor metabolites as well (Jacob et al, 1988; Benowitz et al, 1991; Hukkanen et al, 2005).

Nicotine has the ability to cross the placenta without restraint (Luck et al, 1985) and has been shown to occur in substantial amounts in the milk of smoking mothers (Luck and Nau, 1984). When maternally administered, nicotine is metabolized by the cell of the mother and n-oxide is taken to the developing fetus. It is therefore expected to interact with the developing fetus and the offspring of smoking mothers and/or mothers using nicotine replacement therapy (NRT) for smoking cessation (Maritz, 2008) resulting in the interference with normal fetal lung development (Maritz et al, 1993). The levels of nicotine that cross the placenta are sufficient to alter signaling by nicotinic receptors present in fetal lung (Conti-Fine et al, 2000). Some studies have suggested that nicotine and its metabolites can inhibit the apoptotic destruction of cells whose DNA has been damaged by genotoxic initiators of carcinogenesis. The continuous exposure to nicotine might lead to an altered phenotype of endothelial cells. This, therefore, brings forth a concern as to whether NRT might be a contributing factor to carcinogenesis, although it is advised that the resultant effects of persistent smoking are far worse than any short term exposure to nicotine (News and Views, 2007). Nicotine has been implicated as the potential factor that promotes the development of lung cancer (Chu et al, 2005).



As has previously been demonstrated by Maritz and Dennis (1998), exposure to maternal nicotine suppresses alveolarization of lungs of the offspring, causing a reduction in internal surface area for gas exchange. In rats, the prenatal

exposure to cigarette smoke decreases the number of pre-alveolar saccules and increases their size; this is therefore expected to reduce the number of attachment points on small airways (Collins et al, 1985). In another study done by Sekhon et al (2004), it was shown that the administration of nicotine in animal models caused lung hypoplasia and reduced surface complexity.

Considering the addictive nature of nicotine and that smoking is a problem that is not likely to go away in the near future, seeking ways to lessen the impact of smoking during pregnancy are important (Proskocil et al, 2005). Since some of the effects of smoking during pregnancy are mediated by nicotine which as previously mentioned crosses the placenta to interact with nicotinic receptors in the lung of the developing fetus (Luck et al, 1985).



### 1.11 NICOTINE REPLACEMENT THERAPY (NRT)

In view of the fact that nicotine is the addictive component of tobacco smoke (Ginzel et al, 2007); NRT has been documented as the most effective remedy for smoking cessation in non-pregnancy (Silagy et al, 2000). NRT is now advertised to a great extent as a therapy for smoking cessation (Ginzel et al, 2007**)** and has been presented by some researchers as a remedy even for pregnant mothers (Ruiz, 2006).

In support of this, these researchers argue that smoking during pregnancy already exposes the fetus to large quantities of the toxic substances present in

tobacco smoke, in addition to the high levels of nicotine. The use of NRT during pregnancy in contrast, exposes the fetus to lower levels of nicotine as opposed to the levels present in tobacco smoke. This will thus result in the elimination of the exposure to all other toxic substances present in tobacco smoke (Ruiz, 2006). They further argue that the cardiovascular effects of nicotine from NRT are less than those observed from smoking and that the regular use of NRT generates lower plasma nicotine concentrations (Dempsey and Benowitz, 2001).

In the presence of all these claims, we should not be ignorant of the adverse effects nicotine has on the developing fetus. Studies continue to demonstrate that nicotine causes damage to fetal lung, heart and nervous system (Kleinsasser et al, 2005; Argentin and Cicchetti, 2004). The unfavorable effects of nicotine on the lung have been shown by some researchers to persist even after the administration of nicotine has stopped (Ruiz, 2006). According to previous work done by Maritz and Windvogel (2005), the administration of nicotine during pregnancy and lactation has long term effects on the maintenance of lung integrity, the development of the respiratory system, susceptibility to lung diseases as well as a reduction in lung function (Maritz and Windvogel, 2005). Studies done on the lung tissue of the fetal and neonatal rats show that the maternal exposure to nicotine during gestation and lactation leads to a continuous inhibition of glycogenolysis and glycolysis (Maritz, 1987) which is a result of a reduction in the activity of phosphorylase in the developing fetal lung (Maritz, 1986).

It is suggested that, maternal exposure to nicotine during gestation and lactation results in an alteration to the 'program' that regulates the aging process of the developing lung. This will cause an alteration to the structure and function of the fetal lung making it to be more vulnerable to diseases (Maritz, 2008). As a consequence of the unfavorable effects nicotine has on the development of the fetus, some researchers have therefore proposed that the prescription of NRT is not advisable for pregnant or lactating mothers (Maritz, 2008; Ginzel et al, 2007).

## 1.12 SENESCENCE AND AGING OF THE LUNG

Senescence can be defined as a progressive deterioration of the structural and physiological function with age, leading to a decrease in productivity, increasing susceptibility to various diseases and death (Hasty and Vijg, 2004; Partridge and Mangel, 1999). This is a change that not only occurs at organ level but also at the tissue and cellular levels of the body. Additionally, some researchers have described aging to be a continuous process which is initiated at conception and continues till death (Balcombe and Sinclair, 2001). The word senescence is frequently used interchangeably with aging. Both senescence and aging are characterized by the progressive changes in the tissues or organs of the body. The increase in damage from exogenous and intrinsic mechanisms (Droge and Schipper, 2007) is a key factor in the association between aging and the development of chronic diseases of the brain and musculoskeletal or cardiovascular system (Karrasch et al, 2008).

### 1.12.1 Cellular senescence

Cellular senescence is defined as a complete and irreversible loss of replicative capacity occurring in primary somatic cells (Hayflick and Moorhead, 1961). It is characterized by the progressive morphological and functional change such as the decrease in proliferative activity in cells (Collado and Serrano, 2005); telomere shortening; a distinct, flat, and enlarged cell morphology (Pauwels et al, 2001); resistance to apoptosis (Murphy, 2000); altered production of inflammatory and growth mediators (Mannino et al, 2002); and an increase in senescence associated β-galactosidase (SA-gal) activity (Fishman, 2005).



Cellular senescence can be induced by telomere-dependent or telomere-independent mechanisms. Telomere-dependent mechanisms include the shortening of telomeres that, when it reaches a significant length, initiates DNA damage response and cell cycle arrest. Telomere shortening is the most important determinant of cellular aging (Hasty and Vijg, 2004). Telomere is defined as replicative DNA sequences that cap the ends of eukaryotic chromosomes (Blackburn, 1997). In order to prevent the activation of DNA damage signaling at the termini of the chromosome, telomere capping is required. It has been suggested that individuals are born with long telomeres and the decrease in telomere length results in aging (Nalapareddy et al, 2008), thus its main function is to cap chromosomes in order to prevent an activation of DNA

damage (Blackburn, 1997). Telomere shortening restricts the lifetime of primary human cells to a limited number of cell divisions (Hayflick, 1965).

### 1.12.2 Relationship between aging and chronic diseases

The relationship between aging, malnutrition and emphysema has also been well documented by researchers (Karrasch et al, 2008). Although normally linked with chronological aging which is characterized by the passing of time from birth and beyond, biological aging can occur prematurely and includes a diversity of cellular, molecular and structural changes based on various mechanisms at cellular, tissue and organ level (Karrasch et al, 2008). The increase in β-galactosidase activity is considered to arise from lysosomal β-galactosidase and reflect the increase in lysosomal biogenesis that naturally occurs during senescence (Campisi and d'Adda di Fagagna, 2007). The senescent cells can reach a permanent or irreversible quiescent state of G1 growth arrest in which they are resistant to mitogenic and various apoptotic stimuli (Swanson et al, 2009).



### 1.12.3 The mechanisms of cellular senescence

The major molecular and cellular mechanisms that have been linked with cellular aging include telomere attrition, cumulative DNA damage, the impairment of DNA repair, the epimutations in nuclear DNA, mutations in mitochondria, an increase in the rigidity of cytoskeleton, the increased cross-linking of the extracellular

matrix, protein damage, an increased production of free radicals, and the accumulation of waste products (Karrasch et al, 2008).

Researchers have proposed that the accumulation of DNA damage is a contributing factor to aging (Lieber and Karanjawala, 2004). Free radicals that target G-triplets can also directly damage telomeres (Midorikawa et al, 2002), thus inducing single strand breaks (Chen et al, 2001). Aging induced by external factors such as oxidative stress, can result in the reduction of telomere length. A study done by Müller et al (2006) demonstrated that cultured parenchyma lung fibroblasts from patients with emphysema did not show any alterations in telomere length although there were obvious signs of cellular senescence. This, therefore, indicates that telomere shortening could not have been the cause of senescence in this case, suggesting that alternative mechanisms could have been active in these cells (Müller et al, 2006).



Telomere-independent premature senescence on the other hand, is induced by mechanisms that initiate the development of ROS. The ROS can induce DNA damage, or alter chromatin structure. Senescence can also be induced by certain cell culture conditions, overexpression of oncogenes and the presence of anti-proliferative cytokines, in particular TGF-β (Campisi and d'Adda di Fagagna, 2007). There are various mechanisms that can stimulate DNA damage. These include replication errors, telomere shortening and the degeneration of ROS,

toxic metabolites, irradiation, ultraviolet radiations and exposure to environmental toxins (Vijg, 2008).

### 1.12.4 Relationship between smoking and senescence

Smokers show signs of premature aging (Kadunce et al, 1991; Tanaka at al, 2007; Yin et al, 2001). It has been proposed that the development of emphysema in COPD involves inflammation induced by cigarette smoke and leukocyte activation (fig 1.13), as well as the imbalances between oxidant-antioxidant and protease-antiprotease (Karrasch et al, 2008). Cigarette smoking is the most important risk factor for pulmonary emphysema and fibrosis. It is actually considered to be the cause of persistent epithelial injury and impaired repair. Some studies have demonstrated that cigarette smoke results in the death of alveolar epithelial cells (Lannan et al, 1994), and that it also hinders epithelial repair responses, such as chemotaxis, proliferation, and contraction of three-dimensional collagen gels (Wang et al, 2001).



The main physiological alterations that occur in the senile respiratory system may indicate mechanical or structural changes, most particularly reduced lung elasticity, an increase in stiffness of the chest wall, and reduced respiratory muscle strength (Dyer and Stockley, 2006).

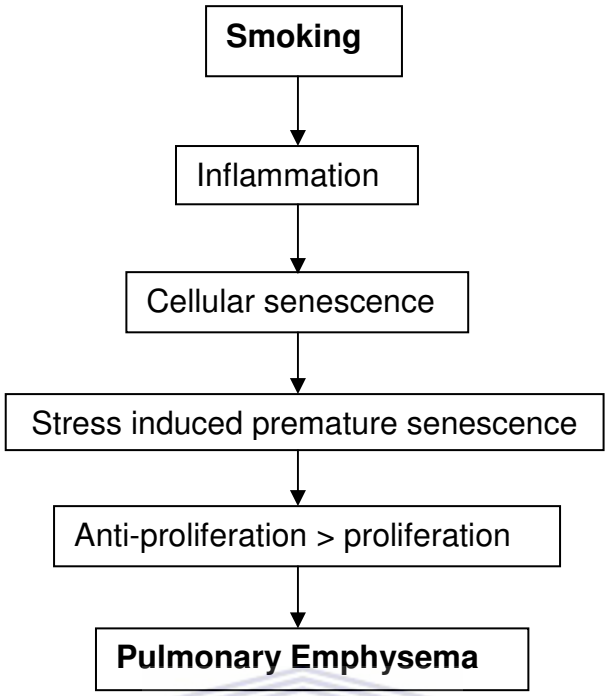



**Figure 1.13.4** Diagram illustrating a summary of the key aspects of aging and senescence that are involved in the development of lung emphysema (Karrasch et al, 2008).



Furthermore, there is a slight loss in the actual number of small airways (Thurlbeck, 1992); the distance between airspace walls is increased, whereas the internal surface area of the lung is decreased (Pierce et al, 1961). This is a result of larger pores that exist in the aged alveoli. There is also evidence of alveolar septal thickening with age and the alveoli becomes shallower (Verbeken et al, 1992).The human lung exhibits a number of functional and structural changes that are part of the normal aging process (Janssens et al, 1999; Sprung et al, 2003). These changes include the rarification of alveolar structures which is known to occur in older never-smokers (Pinkerton and Green, 2004). Although the structural alterations of the senile lung are considered to be nondestructive

(Janssens et al, 1999), and are rather homogeneous when compared with the more focal alterations in emphysema (Verbeken et al, 1992), the overall result appears to be similar with regard to the loss of tissue renewal and regenerative potential (Karrasch et al, 2008).

Evidence from previous studies indicates that smokers show signs of premature aging which is particularly evident in the skin. The link between aging and chronic disease is well-known (Karrasch et al, 2008). Oxidative stress is closely associated with the protease-antiprotease imbalance; it originates from compounds of cigarette smoke or inflammatory cells and has the capability to overcharge the antioxidative capacity of pulmonary tissue and further decrease the antiprotease defense (Wright and Churg, 2007).



The epithelium of the alveoli is frequently injured by a number of inhaled toxins, such as sulfur dioxide, ozone, nitrogen dioxide, and cigarette smoke, leading to the initiation of repair responses. These toxins also induce oxidative stress and DNA damage in epithelial cells consequently leading to stress-induced senescence. As soon as the epithelial cells reach the stage of senescence, they are no longer able to proliferate, leading to the cessation of the repair responses by alveolar epithelial cells. This cessation consequently, results in the disruption in the architectural integrity of the alveoli thus; the lung becomes more susceptible to diseases (Tsuji et al, 2004). The repair responses by alveolar epithelial cells require their integrated ability to migrate, proliferate, and

differentiate to cover defects that result from the injury (Rennard, 1999). It has been proposed that the inability of the epithelium to repair itself is an important cause of chronic lung diseases, such as pulmonary emphysema and fibrosis (Yokohori et al, 2004).

Several markers associated with telomere independent senescence have been identified, some of these include, thymidine labeling index, which monitors the number of replicating cells in the population under specific conditions which is related in a log/linear relationship to replicative life span and BrdU labeling and colony size distribution. Other markers such as lipofuscin deposition and lysosomal enzyme activity have also been proposed. SA-β-Gal measured at pH 6.0 selectively stains senescent cells. The pH 6.0 activity (also known as Senescence-associated β-galactosidase) is widely used to identify the presences of senescent cells in vitro. This method is not only easy, but also rapid and convenient to use (Cristofalo, 2005).



## 1.13 MOTIVATION OF THE STUDY

Several studies showed that maternal nicotine exposure results in the late onset of gradual parenchymal deterioration (Maritz, 1996; Maritz et al 1993; Müller et al, 2006; Verbeken et al, 1992). It is suggested that this is due to a change in the "program" that *controls lung aging* (Maritz, 2008). It is hypothesized that this change is brought about by an imbalance in the oxidant-antioxidant capacity of the lungs during fetal and neonatal development. Thus, the overall aim of this

study is to restore the oxidant-antioxidant capacity of the developing lungs by allowing the mothers to consume tomato juice and thus lycopene.

## 1.14 AIMS AND OBJECTIVES

The main objectives of this study are therefore:

- Firstly, to determine effects of maternal nicotine exposure during gestation and lactation on lung development in the offspring.
- Secondly, to establish whether tomato juice can have protective effects on the fetal lung development and function in the offspring.
- Thirdly, to determine if apoptosis is the cause of the thickening of the alveolar wall that is observed as the offspring increases in age or the development of emphysema.
- To determine whether tomato juice supplementation will prevent premature aging of the lungs of rats that was exposed to nicotine via the placenta and mother's milk.

# CHAPTER 2

# Materials and Methods

## 2.1 ANIMAL PREPARATION

White Wistar female rats were used in this study. The animals were mated for a period of seven days. During the seven days mating period, the rats all received water and the normal laboratory chow. The breeding program was maintained in the animal house of the Department of Medical Bio-Sciences at the University of the Western Cape. The room temperature was maintained at 22 ± 1℃, with day-night cycle of 12 hours. After the seven days mating was complete, the pregnant animals were weighed in order to determine the initial body weight. The animals were then randomly assigned to four different groups (with at least 6 animals per group) as follows;



- The first group was the control which received saline only.
- The second group received nicotine only.
- The third group received both nicotine and tomato juice.
- The fourth group received tomato juice.

The treatment of all four groups began as soon as the mating period was ended and was terminated at weaning when the offspring born to these mothers were 21 days old. It is important to note that the offspring were not treated with nicotine, saline or tomato juice. Since nicotine crosses the placenta and occurs in

the milk of smoking mothers (Luck and Nau, 1984), the offspring would therefore be exposed to nicotine only via the placenta and the mother's milk.

### 2.1.1 Ethical Clearance

The approval for the use of rats as experimental animals and the ethical clearance for the study were obtained from the Ethical Committee of the University of the Western Cape.

### 2.1.2 Nicotine Administration

The administration of nicotine to the mothers began as soon as the mating period was over. The nicotine was administered subcutaneously using sterile 1ml tuberculin syringes. The dose of nicotine and saline was 1mg/kg of the initial maternal body weight. This dose remained constant throughout the entire experiment. The nicotine solution was prepared by diluting 1mg nicotine with 100ml of distilled water. The nicotine solution was prepared weekly. All the animals were treated once a day between 08h30 and 09h30. Much care was taken when treating the animals in order to prevent any stress that could be caused during the process. The group that received tomato juice only was administered with neither nicotine nor saline. After the completion of the mating period, the administration of nicotine, saline and tomato juice to the experimental and control groups continued till weaning on postnatal day 21.

### 2.1.3 Administration of Tomato Juice

As part of the experiment, tomato juice was freely available to drink by the animals that received a tomato juice supplementation. The tomato juice for these groups replaced their water intake. The brand of tomato juice that was used was the *All Gold Tomato juice* (containing 9mg of lycopene per 100ml). The tomato juice (lycopene) was prepared using a 50% dilution with tap water (to reduce its density) and given to the rats in water bottles. The control animals and the group treated with nicotine only received water. The total volume of liquid intake for the groups that received tomato juice and that of those that received water was measured per day for each rat. The liquid intake of the two groups was later compared to determine the total liquid intake for each group.

The mothers were all weighed weekly to determine if there were any changes in body weight within the different groups. The animals were all fed with the normal laboratory rat chow throughout the experiment.



## 2.2 LUNG EXTRACTION

The lungs were extracted from weaned pups at four different age groups namely postnatal days 21, 42, 63 and 84. Lungs from least 3 pups from each litter were used and at least 4 litters from each of the four age groups. The rat lungs were fixed in 10% buffered formaldehyde solution after the removal from the thorax.

The following reagents were used to make up 10% buffered formaldehyde solution:

- Formaldehyde 100ml
- Distilled water 900ml
- Sodium phosphate (anhydrous) 4g
- Sodium phosphate (dehydrogenous) 6g

Before the rats were sacrificed, they were weighed and the weight was recorded. The chest circumference and the crown-rump length were subsequently measured and recorded. The pups were sacrifices by injecting them with an overdose of approximately 0.5ml per kg BW of sodium pentobarbitone solution. When the rat was completely unconscious, the thoracic cavity was carefully opened. Special care was taken to prevent damage to the lungs. Lungs that were damaged during the dissection were discarded. The fixative was allowed to flow freely into the lungs at a transpulmonary pressure of 25cm water. When the flow stopped, the lungs were kept in the thorax at this pressure for 10 minutes before it was removed. The trachea was closed before removal by tube by binding it with a string that was then tightly placed around the trachea to prevent any leakage of liquid from the opening made in the trachea. 10% neutral buffered formaldehyde was used to inflate the lungs whilst still in the ribcage. The ribcage acts as a protective mechanism from over expansion of the lung that could lead to breaking of the lung tissue. Inflating the lungs in the ribcage assists in keeping the natural environment of the thoracic cavity to ensure that the inflation is proportional to the maximum air volume the lung can naturally hold. After the lungs were fully inflated, the intra-tracheal installation tube was removed and the

string was then tied tightly around the trachea to ensure that the liquid does not leak out. Following that, the lungs were rapidly and delicately extracted from the ribcage, the heart and trachea were removed.

The lung volume was then determined using the fluid displacement technique of Scherle (1970). The lungs were afterward placed in 10% buffered formalin where they kept for a maximum time of a week before further processing.

## 2.3 PROCESSING AND EMBEDDING OF THE LUNG TISSUE

An automatic tissue processor was used. The lungs were delicately handled at the trachea to avoid any damage that could be caused to the lung tissue and placed on a clean glass tile. For each lung, the left lobe was cut off gently using a scalpel, and perfectly placed in a properly labeled cassette. When all the cassettes containing the lung tissue were ready, they were placed into the tissue processing rack of the automatic tissue processor. The processor was programmed so that the lungs to be processed followed an 18 hour cycle as follows;



- Firstly, in ascending graded ethanol solutions (70%, 80% and 90%) for two hours in each solution.
- Then, for 2 hours in 100% ethanol (repeated once for a total of 4 hours in 100% ethanol solution).
- Followed by 2 hours in 100% xylene (repeated once for a total of 4 hours in 100% xylene solution).

- Lastly, for 2 hours in wax (repeated once for a total of 4 hours in wax).

After the 18 hour cycle was complete, the cassettes were removed from the tissue processing rack. A small amount of wax was left to run into a mould to ensure that it covered the base of the mould smoothly. The lobe was then removed from the cassette and placed onto the mould with the lateral surface facing downwards. More wax was then let to run onto the mould ensuring that the tissue sample was fully covered with wax. The mould was afterward placed on a laboratory freezer for about 10 minutes so as to solidify the wax to facilitate in the sectioning.



## 2.4 MICROSCOPY AND SLIDE PREPARATION

When the wax had solidified, the cassettes were removed from the mould. The access wax was then removed from the cassettes using a dissecting knife. A microtome was used to cut the tissue into sections. The bevel angle used was $6^{o}$ and a 5µm sections were obtained for the study.

The cut sections were carefully placed in a water bath (heated to 45 $^{o}$ C) in order to flatten them. The sections were then picked up using clean microscope slides and left over night to be fixed on to the slide. Following this, the slides were placed on a staining rack and then put in a hot air oven at 80 $^{o}$ C for approximately 5 minutes to further fix the tissue and to melt the wax.

## 2.5 STAINING

### 2.5.1 H and E staining

- The tissue sections (attached to microscope slides) were immersed in fresh xylene for 5 minutes and repeated once for a total of two xylene washes (that is 10 minutes).
- Slides containing the tissue sections were then dehydrated using absolute alcohol for 5 minutes in 100% ethanol solution and repeated once for a total of 10 minutes in 100% ethanol.
- This process was continued by repeating the procedure described above in 90% and then 80% ethanol for 5 minutes each. The slides were rinsed well in running tap water.
- Staining was subsequently done in Haematoxylin for about 15 minutes and then rinsed in running tap water.
- Next, the slides were blued in Scott's tap water for 2 minutes then rinsed under tap water and differentiated in 1% acid alcohol, and rinsed under tap water again.
- Following that, a counter stain in Eosin was done for 3 minute done, after which the slides were rinsed again in running tap water to rinse excess stain.
- The slides were then placed in 80% and 90% ethanol solutions for 2 minutes each and then placed in xylene for another 2 minutes and then left to dry for about 30 minutes

- The slides were mounted then allowed to dry for morphometric and morphologic analysis.

### 2.5.2 Staining for Apoptosis

#### *2.5.2.1 Principle*

In the present study, the DeadEnd™ Fluorometric TUNEL kit which evaluates apoptotic cell death in systems such as cultured cells or paraffin-embedded tissue sections was used for detection of apoptosis. This System may be used for the determination of nuclear DNA fragmentation, which is an important biochemical characteristic of apoptosis in many cell types. It determines fragmented DNA of apoptotic cells by catalytically incorporating fluorescein-12-dUTP at 3´-OH DNA ends using the Terminal Deoxynucleotidyl Transferase, Recombinant, enzyme (rTdT). rTdT forms a polymeric tail using the principle of the TUNEL. The fluorescein-12-dUTPlabeled DNA may be observed by using fluorescence microscopy. Samples are analyzed under a fluorescence microscope a standard fluorescein filter set can be used to observed green fluorescence of fluorescein at 520 ± 20nm.



#### *2.5.2.2 Method*

- Tissue sections were immersed in fresh xylene for 5 minutes at room temperature. This was repeated once for a total of two xylene washes.
- The samples were then washed by immersing the slides in 100% ethanol for 5 minutes at room temperature.

- Next, the samples were rehydrated by sequentially immersing the slides through graded ethanol washes (100%, 95%, 85%, 70%, and 50%) for 3 minutes each at room temperature.
- Samples were then washed by immersing the slides in 0.85% NaCl for 5 minutes and then immersed in PBS for 5 minutes at room temperature.
- The samples were subsequently fixed by immersing the slides in 4% methanol-free formaldehyde solution in PBS for 15 minutes at room temperature and afterwards washed using in PBS for 5 minutes at room temperature. This step was then repeated once for a total of two PBS washes.
- The excess liquid was removed from the tissue using a paper towel and the slides were placed on a flat surface and 100µl of 20µg/ml Proteinase K was added to each slide to cover the tissue section. Slides were afterward incubated for 8–10 minutes at room temperature.



- Samples were again washed by immersing the slides in PBS for 5 minutes at room temperature and fixed in 4% methanol-free formaldehyde solution in PBS for 5 minutes at room temperature.
- A final wash was then done by immersing the slides in PBS for 5 minutes at room temperature.
- The excess liquid was removed by tapping the slides, the tissue samples were then covered with 100µl of equilibration buffer. The slides were left to equilibrate at room temperature for about 5–10 minutes.

- While the tissue samples were equilibrating, the Nucleotide Mix was thawed on ice and a sufficient rTdT incubation buffer for all samples was prepared.
- The area around the equilibrated surface was then blotted with tissue paper to remove most of the 100µl of Equilibration Buffer and 50µl of rTdT incubation buffer was subsequently added to the tissue samples on a 5 $cm^2$ area and exposure of slides. Light was avoided from this step onward.
- Tissue samples were covered with Plastic cover slips to ensure the even distribution of the reagent. Paper towels soaked with water then placed at the bottom of a humidified chamber. The slides were incubated for 60 minutes at 37℃ inside the humidified chamber to permit the tailing reaction to occur. The chamber was covered with aluminum foil to protect it from direct light.
- A 20X SSC 1:10 dilution was made with distilled water and a sufficient amount of the resulting 2X SSC. The plastic cover slips were removed at this stage.
- The reaction was then stopped the by immersing the slides in the 2X SSC in a Coplin jar for 15 minutes at room temperature.
- The samples were washed again by immersing the slides in fresh PBS for 5 minutes at room temperature.
- The samples were then stained using DAPI solution freshly diluted to 1ml: 10ml in PBS for 5 minutes at room temperature in the dark.

- Following this, a final wash was done in PBS for 5 minutes. This was repeated two times for a total of 3 washes in PBS.
- The excess water was subsequently drained off from the slides and the area surrounding the tissue samples was wiped with tissue paper. Samples were analyzed immediately under a fluorescence microscope to view the green fluorescence of fluorescein.

## 2.6 MORPHOMETRIC TECHNIQUES

The morphometric techniques used in this study included:

- Volume Density (Va and Vt)
- Mean Linear intercept (Lm)
- Alveolar wall thickness (Tsept)
- Lung Volume (Lv)



### 2.6.1 Volume Density (Va and Vt)

#### *2.6.1.1 Principal*

Alveolar volume gives an indication of the size of the alveolus and thus the volume of air that occupies it. It is expected that in alveoli with larger volumes, the total surface area for gaseous exchange in the lung would be reduced. This also would indicate that there would be a possibility of less alveolar surface area for gaseous exchange and less alveoli. According to Blanco et al (1991), the average size of an individual alveolus increases with age.

### *2.6.1.2 Method*

The alveolar air volume density (Va) and alveolar tissue volume density (Vt) were determined as illustrated by Bolender et al (1993). A 122-point eyepiece graticule was used at 100 x magnifications for the point counting technique which helped in determining the alveolar air volume density Va and Vt.

A 10x eyepiece and a 10x objective were used to obtain a total magnification of 100x. Two blocks were taken from the upper lung lobe, 1 from the middle lobe and two from the lower lobe. Non-parenchyma tissue included bronchus and blood vessels which had a diameter of >1.1mm. The alveoli that were found within the graticule and those that touched the lower and right borders of the graticule were included. The alveoli were excluded from the count including those that were outside the square on the upper and the left side of the graticule. Furthermore, the fields surround by non-parenchymatous tissue were excluded from the counts. At least 5 randomly selected non-overlapping fields were analysed for each slide.



The alveoli that contributed to the count included:

- Those that were found within the graticule, and
- Those that touched the right lower borders of the graticule.

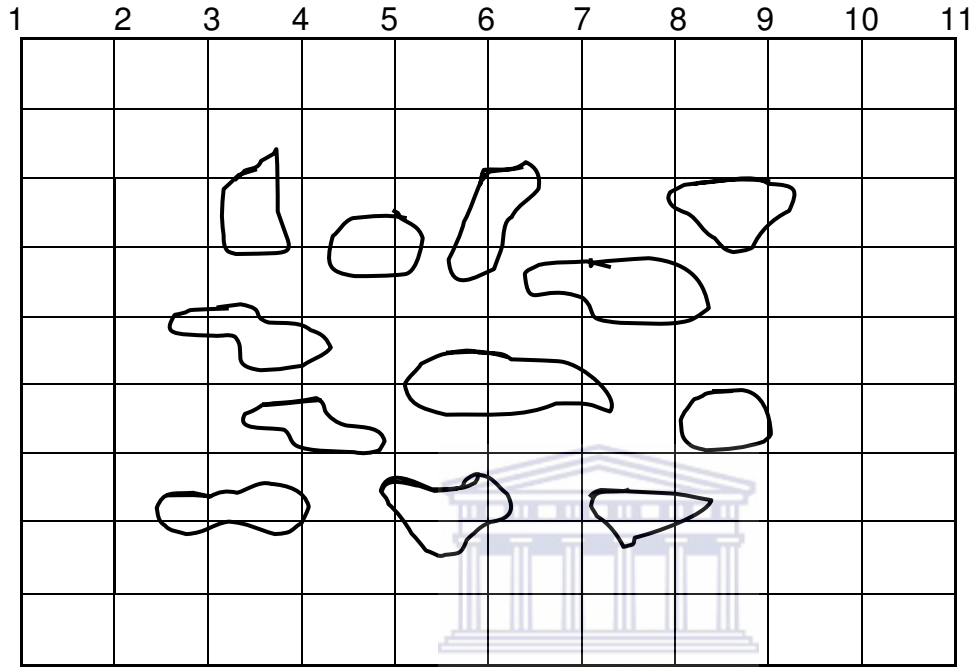
The alveoli that did not contribute in the count included:

- Those found outside the square on the upper left side of the graticule.

- Areas that contained non-parenchymatous tissue were excluded from the counts. At least 5 randomly selected non-overlapping fields per slide were analysed.

The following method was used to calculate the number count (Na):

Example:






5 points on the lines

Total lines/points = 110

Therefore, 5/110 X 100 = 4.55%

### 2.6.2 Determination of Mean Linear Intercept (Lm)

#### *2.6.2.1 Principle*

The Mean linear intercept (Lm), is the average distance found between alveolar walls and indicates the diameter of the alveolus (Dunnill, 1962). During normal lung maturation, the Lm increases with decreasing air-tissue interface. In

microscopic emphysema however, the Lm has been observed to increase, indicating alveolar wall destruction and an increase in alveolar volume. Increase in Lm is indicative of a decrease in surface area available for gaseous exchange.

#### *2.6.2.2 Method*

The linear intercept was calculated as follows;

**Lm = L x L/m**

Where

**N** = number of fields counted

**L** = length of cross line (0.02 mm at 100x magnification)

**M** = sum of all intercepts


An intercept is where the cross line passes through the alveolar wall. Crossing an alveolar wall = 2 intercepts. The line just touches an alveolar wall = 1 intercept. The number of alveolar intercepts (m) was determined using an eyepiece micrometer at 100x magnification. For each slide 5 fields were used to determine the mean linear intercept.

Example:

| Field | # of intercepts |
|---|---|
| 1 | 26 |
| 2 | 16 |
| 3 | 22 |
| 4 | 25 |
| 5 | 15 |
| | Total: 104 |

Lm = 2.02 x 5/104

= 10.1/104

= 0.097mm

= 97µM



The following alveolar walls contributed to the intercept count:

- Those that touched without crossing the left side of the vertical line,
- Those that touched without crossing the upper end of the horizontal line, and
- Those that intercepted the cross hairs.

Cut blood vessels were each counted as half an intercept.

Structures and Alveolar walls that did not contribute to the mean linear intercept included:

- Those that touched but not cross the right border of the vertical arm, and
- Those that touched but not cross the lower border of the horizontal arm.

### 2.6.3 Alveolar wall thickness (Tsept)

#### *2.6.3.1 Principal*

The Alveolar wall thickness (Tsept) is the distance between alveoli that are adjacent to each other or the thickness of the wall of the alveoli between adjacent alveoli. Tsept is determined by utilizing the point counting and linear intercept method as described by Weibel (1963). The Weibel no. 1 graticule at 100x magnification was used to determine the number of points on the alveolar septum and number of alveolar intercepts. At least 5 non-overlapping fields that were randomly selected were analysed for each slide.



#### *2.6.3.2 Method*

The calculation of the Tsept was done using the following equation:

**T*sept* = z x Pse/2x Ise**,

Where:

**z** = lengths of lines on graticule (um)

**Pse** = points on alveolar walls

**Ise** = number of intercepts of alveolar walls

### 2.6.4 Statistical Analysis (Morphometry)

The results were analysed on the Microsoft Excel data analysis programme using standard error bars and a one-way analysis of variance test (ANOVA) for unpaired data and the Student t-test for pair-wise comparisons. A probability level of $P<0.05$ was chosen as significant to the study and the values were recorded as means ± standard error of means.

# CHAPTER 3

# Results

## 3.1 THE EFFECTS OF MATERNAL NICOTINE AND TOMATO JUICE ON THE BODY WEIGHT (BW) OF THE OFFSPRING.

From the data summarized in table 3.1 and illustrated in figure 3.1, it is clear that up to postnatal day 42 the body weight (BW) of the males and females were the same ($P > 0.05$). However, from postnatal day 42 the BW of the males increased faster ($P < 0.05$) than that of the females so that on postnatal days 63 and 84, the BW of the males was higher ($P < 0.05$) than that of the females.

The exposure of the mothers during gestation and lactation to tomato juice, nicotine, or to both nicotine and tomato juice had no effect on the BW the males or females ($P > 0.05$).



## 3.2 THE EFFECTS OF MATERNAL NICOTINE AND TOMATO JUICE ON CC/BW AND CC/CR OF THE OFFSPRING.

From postnatal day 21 to postnatal day 84, the chest circumference (CC) of the control and experimental male animals increased between 1.65 and 1.94-fold; the CC of the female control animals increased between 1.42 and 1.71-fold. By postnatal day 84, the CC of the males within all the groups was higher ($P<0.01$) than that of the females. There was no difference ($P>0.05$) between the CC of the control animals and that of the experimental groups at postnatal day 84.

From table 3.2 and figures 3.2 it is clearly indicated that no differences ($P > 0.2$) in the CC/BW ratios were observed between the control and the nicotine group on postnatal day 21. However, the CC/BW ratios of the control animals were lower than that of the animals that were exposed to tomato juice and those exposed to both nicotine and tomato juice. At postnatal day 84, there were no differences observed ($P>0.05$) in the CC/BW ratios of the control male animals and the male experimental animals. Similarly, there were no differences between the CC/BW ratios of the control female animals and that of the female experimental groups of the same age. At postnatal days 63 and 84, the CC/BW ratios of the female animals of each group was significantly higher ($P<0.01$) than that of the males. Within the tomato group, the CC/BW ratios of the male animals were 32% higher ($P>0.001$) than that of the female animals. On postnatal day 84, no differences ($P>0.05$) were observed between the control and the experimental animals.

**Table 3.1** The effects of maternal exposure to nicotine, tomato juice, or both nicotine and tomato juice on the body weight (g) of the male and the females offspring at postnatal days 21, 42, 63, and 84. P-value: No differences within experimental groups up to day 42 ($P > 0.05$). Postnatal day 63 Males vs. Females ($P < 0.05$). Postnatal day 84 Males vs. Females ($P < 0.05$).

| Age (days) | Sex | Control | C vs. N | Tomato | C vs. T | Nicotine | C vs. N+T | N+T |
|---|---|---|---|---|---|---|---|---|
| **21** | Males | 40.36±0.88 | P>0.05 | 36.84±1.22 | P>0.05 | 44.77±1.27 | P>0.05 | 36.43±1.56 |
| | Females | 40.36±0.88 | P>0.05 | 36.84±1.22 | P>0.05 | 44.77±1.27 | P>0.05 | 36.43±1.56 |
| **M vs. F** | | P>0.05 | | P>0.05 | | P>0.05 | | P>0.05 |
| **42** | Males | 140.21±7.07 | P>0.3 | 121.98±3.67 | P<0.04 | 131.30±5.59 | P>0.9 | 141.00±5.58 |
| | Females | 130.14±2.79 | P>0.4 | 111.85±3.88 | P<0.001 | 135.43±7.11 | P>0.1 | 122.70±3.57 |
| **M vs. F** | | P>0.1 | | P>0.8 | | P>0.6 | | P>0.1 |
| **63** | Males | 269.33±5.59 | P>0.5 | 245.61±6.97 | P<0.02 | 262.51±11.35 | P<0.04 | 248.94±4.49 |
| | Females | 210.14±9.61 | P>0.6 | 184.77±5.05 | P<0.03 | 216.41±7.46 | P>0.06 | 186.61±5.70 |
| **M vs. F** | | P<0.001 | | P<0.001 | | P<0.002 | | P<0.001 |
| **84** | Males | 330.43±2.40 | P>0.6 | 339.49±7.66 | P>0.2 | 338.25±15.16 | P>0.6 | 325.03±12.26 |
| | Females | 237.26±6.30 | P>0.9 | 229.98±6.92 | P>0.4 | 231.19±10.70 | P>0.9 | 230.07±4.31 |
| **M vs. F** | | P<0.002 | | P<0.001 | | P<0.001 | | P<0.001 |

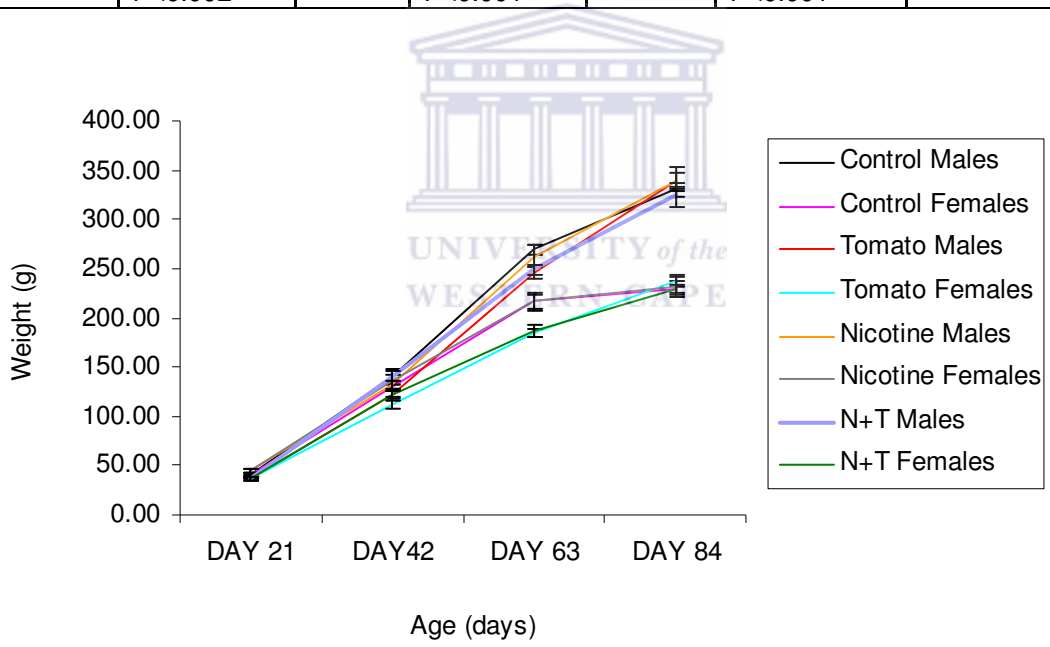



**Figure 3.1** The effects of maternal exposure to nicotine, tomato juice, and both nicotine and tomato juice on the body weight of the male and female offspring at postnatal days 21, 42, 63, and 84.

Table 3.3 and figure 3.3 indicate that no significant differences ($P > 0.05$) in the CC/CR ratios were seen when the control was compared to the experimental animals on postnatal day 21. On postnatal day 84, the CC/CR ratios of the female control animals was slightly but significantly lower ($P<0.04$) than that of the female N+T group. However, there were no differences observed when the CC/CR ratios of the control male and female animals were compared to that of the males and females that were exposed to tomato juice and those that were exposed to both tomato juice and nicotine on postnatal day 84. However, the CC/CR ratios of male nicotine group was significantly higher ($P<0.009$) than that of the female nicotine groups at postnatal day 84.

Although the data in tables 3.2 and 3.3 indicate that the CC/BW ratios of male animals increased faster than that of the female animals, the CC/BW and the CC/CR ratios were not affected by the maternal exposure to tomato juice, nicotine, or to both nicotine and tomato juice ($P>0.05$) during gestation and lactation. It also means that growth was proportional for the control as well as the experimental animals.

**Table 3.2** The effect of maternal exposure to nicotine, tomato juice, and both nicotine and tomato juice on the CC/BW ratio of the male and female offspring at postnatal days 21, 42, 63, and 84. P-value: No significant difference within experimental groups and within age groups from day 21 to day 84 (P>0.05).

| Age (days) | Sex | Control | C vs. N | Tomato | C vs. T | Nicotine | C vs. N+T | N+T |
|---|---|---|---|---|---|---|---|---|
| | Males | 20.43±0.43 | P>0.2 | 22.62±0.72 | P<0.01 | 19.64±0.48 | P<0.003 | 23.00±0.76 |
| **21** | Females | 20.43±0.43 | P>0.2 | 22.62±0.72 | P<0.01 | 19.64±0.48 | P<0.003 | 23.00±0.76 |
| **M vs. F** | | P>0.05 | | P>0.05 | | P>0.05 | | P>0.05 |
| | Males | 8.62±0.33 | P>0.2 | 9.53±0.29 | P>0.05 | 9.19±0.31 | P>0.5 | 8.30±0.34 |
| **42** | Females | 8.76±0.22 | P>0.2 | 9.73±0.17 | P<0.01 | 9.23±0.40 | P>0.06 | 9.38±0.21 |
| **M vs. F** | | P>0.7 | | P>0.6 | | P>0.9 | | P<0.01 |
| | Males | 5.57±0.10 | P>0.8 | 5.85±0.18 | P>0.2 | 5.50±0.30 | P>0.2 | 5.79±0.16 |
| **63** | Females | 6.30±0.23 | P>0.9 | 7.09±0.21 | P<0.01 | 6.26±0.24 | P<0.03 | 7.04±0.21 |
| **M vs. F** | | P<0.01 | | P<0.001 | | P>0.06 | | P<0.003 |
| | Males | 4.80±0.09 | P>0.6 | 4.64±0.12 | P>0.3 | 4.90±0.24 | P>0.8 | 4.78±0.12 |
| **84** | Females | 6.10±0.18 | P>0.8 | 6.12±0.16 | P>0.9 | 6.14±0.20 | P>0.5 | 6.22±0.13 |
| **M vs. F** | | P<0.001 | | P<0.001 | | P<0.003 | | P<0.001 |

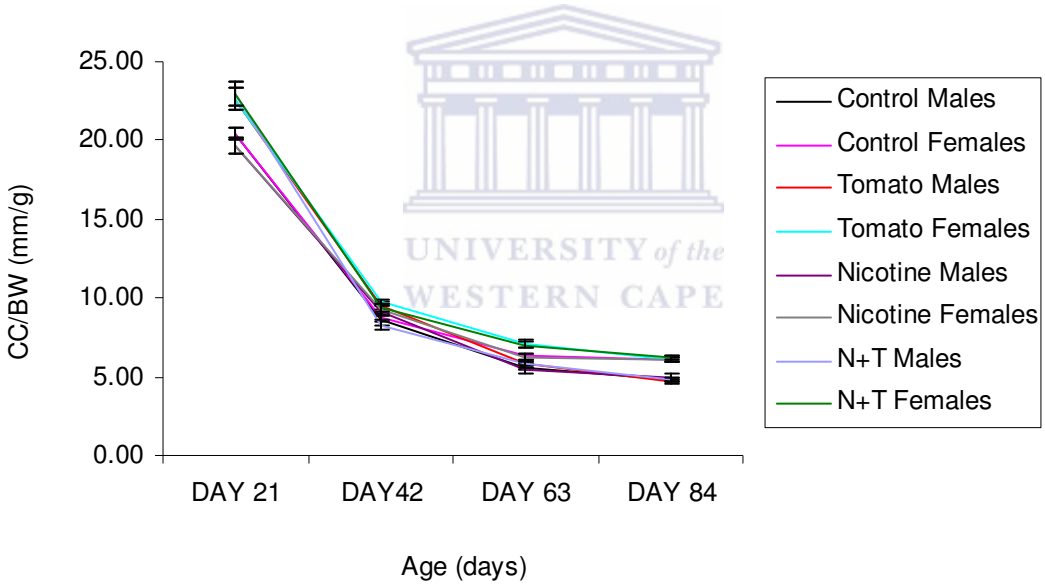





**Figure 3.2** The effect of maternal exposure to nicotine, tomato juice, and both nicotine and tomato juice during gestation and lactation on the CC/BW ratio at postnatal days 21, 42, 63, and 84.

**Table 3.3** The effects of maternal exposure to nicotine, tomato juice and the combination of both nicotine and tomato juice on CC/CR ratio at postnatal days 21, 42, 63, and 84. P-value: No significant difference within experimental groups and within age groups from day 21 to day 84 (P>0.05).

| Age (days) | Sex | Control | C vs. N | Tomato | C vs. T | Nicotine | C vs. N+T | N+T |
|---|---|---|---|---|---|---|---|---|
| 21 | Males | 8.93±0.07 | P>0.2 | 9.03±0.11 | P>0.4 | 8.78±0.12 | P>0.9 | 8.93±0.09 |
| | Females | 8.93±0.07 | P>0.2 | 9.03±0.11 | P>0.4 | 8.78±0.12 | P>0.9 | 8.93±0.09 |
| **M vs. F** | | P>0.05 | | P>0.05 | | P>0.05 | | P>0.05 |
| 42 | Males | 8.19±0.13 | P>0.8 | 8.10±0.13 | P>0.6 | 8.24±0.19 | P>0.08 | 7.88±0.11 |
| | Females | 7.84±0.11 | P<0.01 | 7.88±0.11 | P>0.8 | 8.41±0.19 | P>0.05 | 8.22±0.14 |
| **M vs. F** | | P>0.05 | | P>0.2 | | P>0.5 | | P>0.06 |
| 63 | Males | 7.90±0.13 | P>0.05 | 7.52±0.10 | P<0.03 | 7.53±0.11 | P>0.3 | 7.70±0.20 |
| | Females | 7.42±0.13 | P>0.7 | 7.42±0.11 | P>0.9 | 7.47±0.12 | P>0.5 | 7.52±0.08 |
| **M vs. F** | | P<0.01 | | P>0.5 | | P>0.7 | | P>0.3 |
| 84 | Males | 7.84±0.17 | P>0.2 | 7.74±0.15 | P>0.7 | 8.09±0.11 | P>0.5 | 7.69±0.17 |
| | Females | 7.54±0.17 | P>0.9 | 7.84±0.13 | P>0.2 | 7.53±0.14 | P<0.04 | 8.01±0.11 |
| **M vs. F** | | P>0.2 | | P>0.6 | | P<0.009 | | P>0.1 |

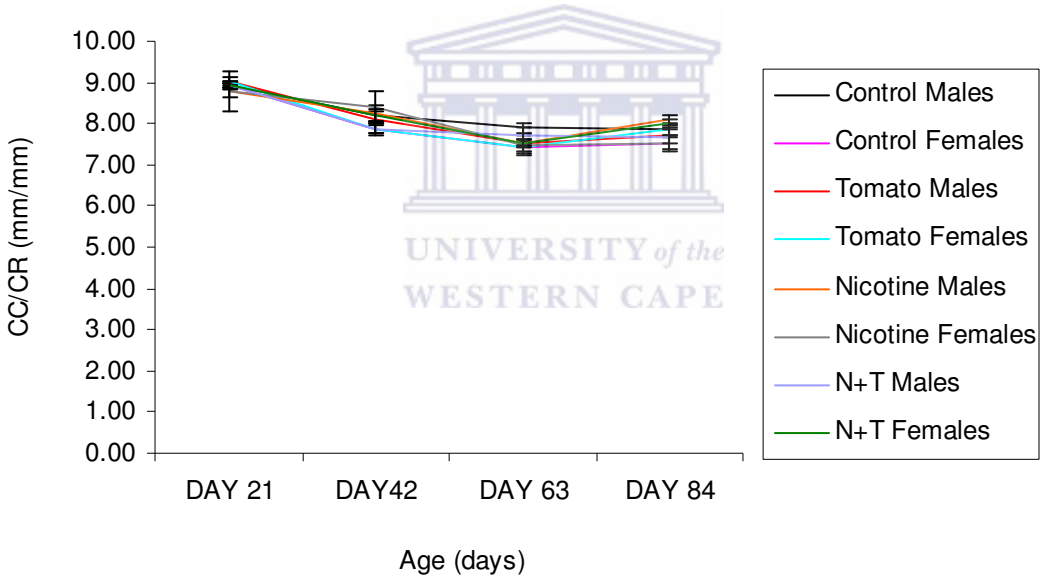





**Figure 3.3** The effect of maternal exposure to nicotine, tomato juice, and both nicotine and tomato juice during gestation and lactation on the CC/CR ratio at postnatal days 21, 42, 63, and 84.

## 3.3 THE EFFECTS OF MATERNAL NICOTINE EXPOSURE AND TOMATO JUICE ON THE LUNG VOLUME (LV).

Table 3.4 and figure 3.4 shows that the Lv of all the male and female animals; both the control and the experimental groups increased by 4 to 5-fold ($P<0.001$) between postnatal days 21 and 84.

Up to postnatal day 42, the Lv of the control animals and the experimental groups, both male and female, increased at the same rate. The increase was such that at postnatal day 42, there was no difference in Lv between males and females as well as between the control and the experimental groups. However, after postnatal day 42, the Lv of the male nicotine group increased at a faster rate so that at postnatal day 84 it was 26.38% more ($P<0.001$) than that of the male control animals. There was no difference ($P>0.2$) observed between the Lv of the female control animals and that of the of the nicotine female group from postnatal day 21 to postnatal day 42. However, at postnatal day 63, the Lv of the control female animals was lower ($P<0.003$) than that of the female nicotine group. Interesting enough, at postnatal day 84, there was no difference ($P>0.2$) between the Lv of the female control animals and that of the female nicotine group. This indicates that from postnatal day 42, the Lv of the female nicotine group increased faster than the female control Lv. Thereafter, the increase slowed down so that on postnatal day 84, the Lv of the nicotine exposed animals was slightly but not significantly lower ($P>0.05$) than that of the control animals.

**Table 3.4** The effects of maternal exposure to nicotine, tomato juice, and both nicotine and tomato juice on the Lung Volumes (Lv) (ml) of the males and female animals at postnatal days 21, 42, 63, and 84. P-value: No differences within experimental groups up to day 42 (P > 0.05). From day 63 to day 84, C vs. N male is significant (P<0.05). On day C vs. N females is significant but not significant on day 84.

| **Age (days)** | Sex | **Control** | **C vs. N** | **Tomato** | **C vs. T** | **Nicotine** | **C vs. N+T** | **N+T** |
|---|---|---|---|---|---|---|---|---|
| **21** | Males | 2.45±0.12 | P>0.05 | 2.48±0.13 | P>0.7 | 2.84±0.10 | P>0.6 | 2.54±0.19 |
| | Females | 2.45±0.12 | P>0.05 | 2.48±0.13 | P>0.05 | 2.84±0.10 | P>0.05 | 2.54±0.19 |
| **M vs. F** | | P>0.05 | | P>0.05 | | P>0.05 | | P>0.05 |
| **42** | Males | 5.95±0.40 | P>0.1 | 5.37±0.29 | P>0.2 | 5.05±0.33 | P>0.3 | 5.43±0.40 |
| | Females | 5.33±0.23 | P>0.8 | 5.09±0.37 | P>0.5 | 5.28±0.27 | P>0.9 | 5.31±0.24 |
| **M vs. F** | | P>0.05 | | P>0.05 | | P>0.05 | | P>0.05 |
| **63** | Males | 9.58±0.37 | P<0.02 | 9.68±0.62 | P>0.8 | 11.00±0.46 | P>0.3 | 8.87±0.81 |
| | Females | 8.08±0.33 | P<0.003 | 7.91±0.66 | P>0.8 | 9.59±0.31 | P>0.9 | 8.13±0.27 |
| **M vs. F** | | P<0.008 | | P>0.07 | | P<0.001 | | P>0.2 |
| **84** | Males | 11.22±0.29 | P<0.001 | 11.86±0.37 | P>0.2 | 14.18±0.64 | P>0.09 | 11.18±0.33 |
| | Females | 10.42±0.40 | P>0.2 | 9.66±0.71 | P>0.3 | 11.14±0.55 | P>0.1 | 9.45±0.36 |
| **M vs. F** | | P>0.1 | | P<0.02 | | P<0.001 | | P<0.005 |

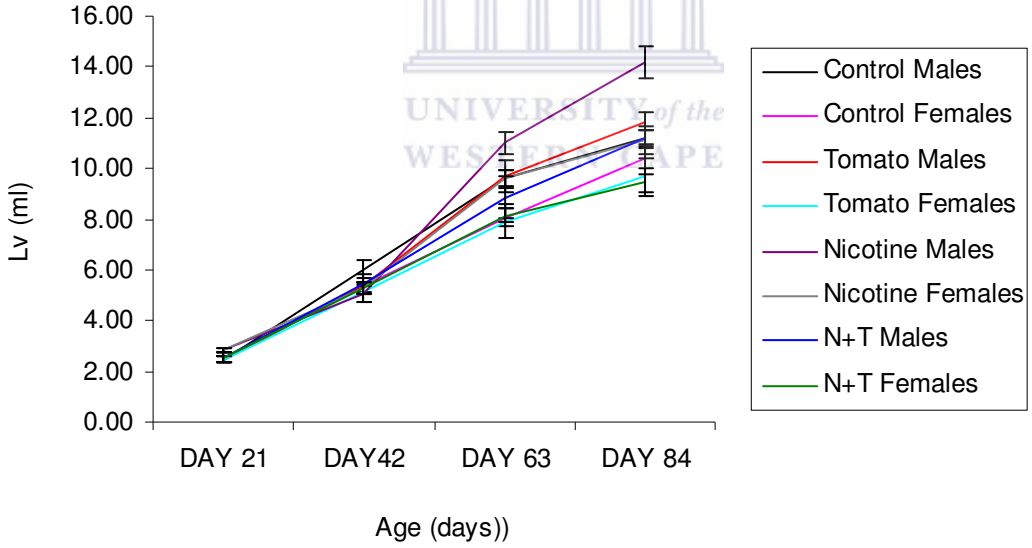



**Figure 3.4** The effects of maternal exposure to nicotine, tomato juice, and both nicotine and tomato juice on the Lung Volumes (Lv) of the males and female animals at postnatal days 21, 42, 63, and 84.

**Table 3.5** The effects of maternal exposure to nicotine, tomato juice, and both nicotine and tomato juice on the Lv/BW (ml/g) ratio at postnatal days 21, 42, 63, and 84. P-value: No significant difference within experimental groups and within age groups from day 21 to day 84 (P>0.05).

| Age (days) | Sex | Control | C vs. N | Tomato | C vs. T | Nicotine | C vs. N+T | N+T |
|---|---|---|---|---|---|---|---|---|
| | Males | 6.05±0.26 | P>0.3 | 6.81±0.27 | P>0.05 | 6.37±0.19 | P>0.05 | 6.86±0.33 |
| **21** | Females | 6.05±0.26 | P>0.3 | 6.81±0.27 | P>0.05 | 6.37±0.19 | P>0.05 | 6.86±0.33 |
| **M vs. F** | | P>0.05 | | P>0.05 | | P>0.05 | | P>0.05 |
| | Males | 4.26±0.21 | P>0.5 | 4.40±0.18 | P>0.6 | 3.96±0.40 | P>0.1 | 3.83±0.20 |
| **42** | Females | 4.09±0.15 | P>0.7 | 4.56±0.32 | P>0.1 | 4.00±0.36 | P>0.2 | 4.32±0.14 |
| **M vs. F** | | P>0.5 | | P>0.6 | | P>0.9 | | P>0.07 |
| | Males | 3.57±0.16 | P<0.001 | 3.98±0.33 | P>0.5 | 4.22±0.21 | P>0.7 | 3.58±0.37 |
| **63** | Females | 3.90±0.21 | P>0.3 | 4.31±0.37 | P>0.1 | 4.45±0.12 | P>0.1 | 4.40±0.22 |
| **M vs. F** | | P>0.2 | | P>0.5 | | P>0.3 | | P>0.06 |
| | Males | 3.40±0.10 | P<0.02 | 3.51±0.15 | P>0.3 | 4.22±0.14 | P>0.9 | 3.45±0.09 |
| **84** | Females | 4.54±0.20 | P<0.03 | 4.06±0.26 | P>0.3 | 4.84±0.20 | P>0.1 | 4.11±0.16 |
| **M vs. F** | | P<0.001 | | P>0.1 | | P<0.03 | | P<0.004 |

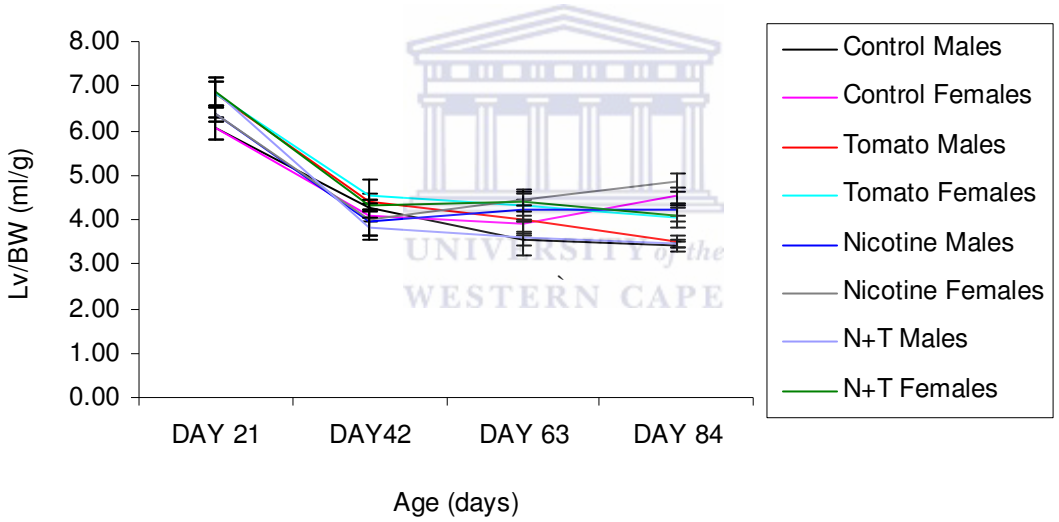



**Figure 3.5** The effects of maternal exposure to nicotine, tomato juice, and both nicotine and tomato juice on the Lv/BW ratio at postnatal days 21, 42, 63, and 84.

**Table 3.6** The effects of maternal exposure to nicotine, tomato juice, and both nicotine and tomato juice on the CC/Lv (mm/ml) ratio at postnatal days 21, 42, 63, and 84. P-value: No significant difference within experimental groups and within age groups from day 21 to day 84 (P>0.05).

| Age (days) | Sex | Control | C vs. N | Tomato | C vs. T | Nicotine | C vs. N+T | N+T |
|---|---|---|---|---|---|---|---|---|
| | Males | 35.28±1.64 | P>0.07 | 34.86±2.06 | P>0.8 | 31.16±0.99 | P>0.9 | 35.34±2.15 |
| **21** | Females | 35.28±1.64 | P>0.07 | 34.86±2.06 | P>0.8 | 31.16±0.99 | P>0.9 | 35.34±2.15 |
| **M vs. F** | | P>0.05 | | P>0.05 | | P>0.05 | | P>0.05 |
| | Males | 20.68±1.22 | P>0.09 | 21.98±1.14 | P>0.4 | 24.45±1.81 | P>0.4 | 22.36±0.90 |
| **42** | Females | 21.78±1.05 | P>0.2 | 21.80±1.37 | P>0.9 | 23.82±1.52 | P>0.9 | 21.90±0.90 |
| **M vs. F** | | P>0.4 | | P>0.9 | | P>0.7 | | P<0.9 |
| | Males | 15.85±0.81 | P<0.02 | 15.40±1.52 | P<0.04 | 13.03±0.39 | P>0.8 | 16.67±1.59 |
| **63** | Females | 16.41±0.79 | P<0.01 | 17.68±1.66 | P>0.7 | 14.11±0.54 | P>0. 6 | 16.21±0.64 |
| **M vs. F** | | P<0.6 | | P>0.3 | | P>0.1 | | P>0.7 |
| | Males | 14.16±0.29 | P<0.001 | 13.30±0.36 | P>0.09 | 11.61±0.34 | P>0.6 | 13.89±0.47 |
| **84** | Females | 13.52±0.61 | P>0.2 | 15.49±1.25 | P>0.2 | 12.72±0.37 | P>0.09 | 15.25±0.69 |
| **M vs. F** | | P>0.3 | | P>0.1 | | P>0.05 | | P>0.1 |

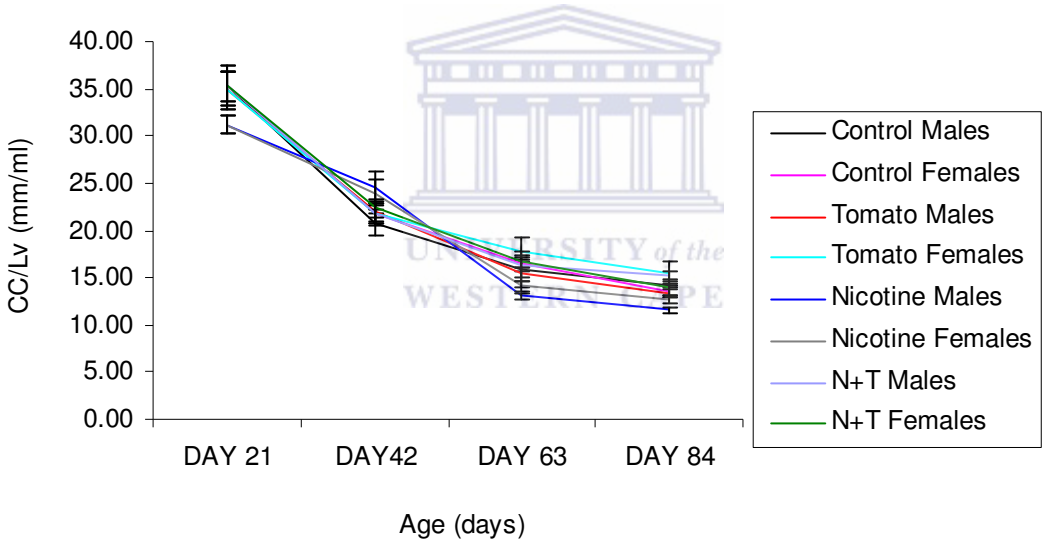



**Figure 3.6** The effects of maternal exposure to nicotine, tomato juice, and both nicotine and tomato juice on the CC/Lv ratio at postnatal days 21, 42, 63, and 84.

Table 3.5 and figure 3.5 show that between postnatal day 21 and 42, there was no difference ($P>0.05$) between the Lv/BW ratios of the control animals and the experimental groups. On postnatal day 63, the Lv/BW ratios of the male control group was significantly lower ($P<0.001$) than that of the nicotine males, whilst the ratios between the control females and the nicotine females showed no difference ($P>0.3$). At postnatal day 84, however, both the male and female Lv/BW ratios of the control animals were significantly lower ($P<0.05$) than those of the male and female nicotine groups.

Table 3.6 illustrates that at postnatal day 84, the CC/Lv ratios of the male control animals were higher than ($P<0.001$) than that of the nicotine male animals whilst the comparison between the control female animals and the nicotine female group showed no difference ($P>0.2$). In addition to that, there was no difference observed ($P>0.05$) between the CC/Lv ratios of the control animals and the CC/Lv ratios of the experimental animals.



From the results obtained in tables 3.4, 3.5 and 3.6, it can be concluded that the maternal exposure to nicotine during gestation and lactation increases the lung volume of the male offspring born to these mothers. We can also deduce that the tomato juice has protective towards the adverse effects nicotine exerts on the lungs of the offspring when maternally administered together with nicotine.

## 3.4 THE EFFECTS OF MATERNAL NICOTINE AND TOMATO JUICE ON THE VOLUME DENSITY ($V_T$) OF THE LUNG OF THE OFFSPRING.

The data presented in table 3.7 and figure 3.7 demonstrates the volume density of the lung tissue ($V_t$) from postnatal day 21 up until postnatal day 84. The $V_t$ of the of the control animals was higher ($P<0.05$) than that of the nicotine animals. The $V_t$ of the control as well as that of the experimental animals exposed to tomato juice and to both nicotine and tomato juice increased with age so that the $V_t$ at postnatal day 84 it was higher ($P<0.001$) than at postnatal day 21. From the data it was also evident that while the $V_t$ of the control and the experimental groups increased with age, the $V_t$ of the nicotine groups (both males and females) decreased ($P<0.001$) with age. From postnatal day 21 to postnatal day 84, there were no differences ($P<0.05$) observed between the males and female animals within each group.



The much lower values obtained from the day 63 and 84 nicotine treated group show the obvious destructive effects of nicotine on the lung parenchyma. In addition to this, it can be noted that the maternal intake of tomato juice combined with nicotine during gestation and lactation prevented the lungs of offspring of from the harmful effects of nicotine exerts on the developing rat lung.

**Table 3.7** The effects of maternal exposure to nicotine, tomato juice, and both nicotine and tomato juice on the volume density % ($V_t$) of the parenchymal tissue of the male and female offspring at postnatal days 21, 42, 63, and 84. P-value: significant difference between C and N is evident from as early as day 21until day 84. P-value: No significant difference between C vs. T and C vs. N+T from day 21 to day 84 (P>0.05).

| Age (days) | Sex | Control | C vs. N | Tomato | C vs. T | Nicotine | C vs. N+T | N+T |
|---|---|---|---|---|---|---|---|---|
| | Males | 20.19± 0.29 | P<0.02 | 22.76± 1.13 | P<0.01 | 18.77± 0.47 | P>0.05 | 22.73± 0.75 |
| **21** | Females | 20.19± 0.29 | P<0.02 | 22.76± 1.13 | P<0.01 | 18.77± 0.47 | P>0.05 | 22.73± 0.75 |
| **M vs. F** | | P>0.05 | | P>0.05 | | P>0.05 | | P>0.05 |
| | Males | 22.73±0.49 | P<0.01 | 23.50±0.30 | P>0.2 | 17.81±1.11 | P>0.3 | 23.28±0.25 |
| **42** | Females | 24.64±0.58 | P<0.001 | 23.17±0.70 | P>0.1 | 17.27±0.66 | P>0.2 | 22.84±1.33 |
| **M vs. F** | | P>0.06 | | P>0.6 | | P>0.6 | | P>0.7 |
| | Males | 26.17±1.13 | P<0.002 | 27.65±0.30 | P>0.9 | 14.70±1.18 | P>0.8 | 25.79±1.13 |
| **63** | Females | 25.08±0.33 | P<0.003 | 27.54±0.43 | P<0.01 | 15.79±1.49 | P<0.02 | 23.77±0.09 |
| **M vs. F** | | P>0.4 | | P>0.8 | | P>0.1 | | P>0.5 |
| | Males | 28.69±1.52 | P<0.001 | 28.52±1.00 | P>0.9 | 15.85±0.27 | P>0.3 | 26.78±1.19 |
| **84** | Females | 26.45±0.57 | P<0.001 | 27.81±0.91 | P>0.2 | 15.25±0.09 | P>0.9 | 26.34±1.37 |
| **M vs. F** | | P>0.2 | | P>0.6 | | P>0.8 | | P>0.1 |

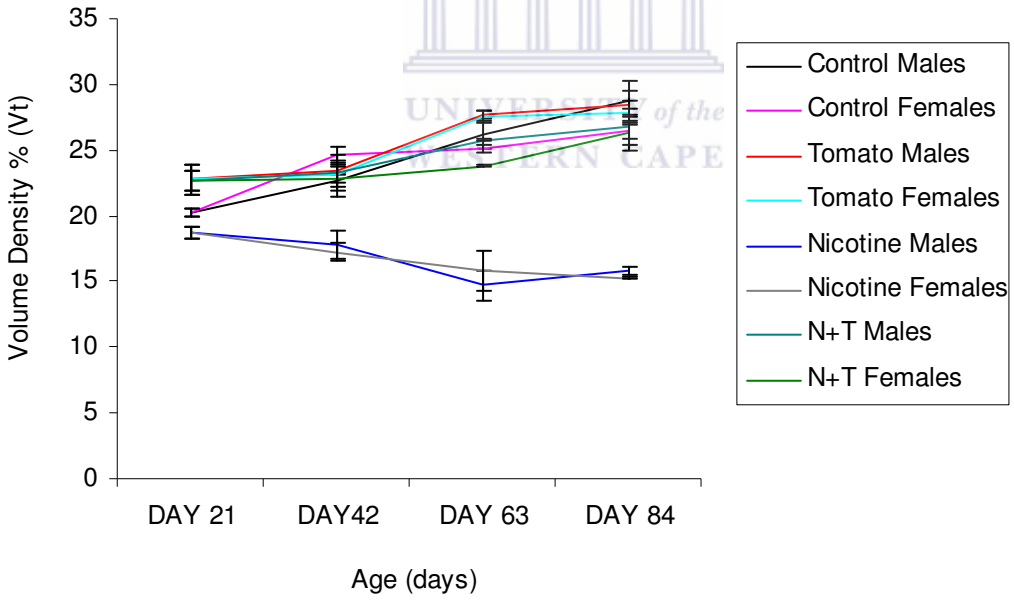



**Figure 3.7** The effects of maternal exposure to nicotine, tomato juice, and both nicotine and tomato juice on the volume density the parenchymal tissue % ($V_t$) of the male and female offspring at postnatal days 21, 42, 63, and 84.

## 3.5 THE EFFECTS OF MATERNAL NICOTINE AND TOMATO JUICE ON ALVEOLAR LINEAR INTERCEPT (LM).

The Lm measurement is an indication of the size of the alveoli. According to the data in table 3.8 and figure 3.8, the Lm of the 21 day old control animals was slightly but significantly higher ($P<0.05$) than that of all the experimental groups. However, from postnatal day 21 up to postnatal day 84, the Lm of the control animals gradually decreased so that on postnatal day 84, the Lm of the control animals were much lower ($P<0.001$) than that of the nicotine group. This is in contrast with the gradual increase in Lm of the nicotine exposed rats during the same period of time.



The results obtained in table 3.8 and figure 3.8 are in agreement with the results obtained in table 3.7 and figure 3.7, showing that, the maternal exposure to nicotine during gestation and lactation will result in the gradual degradation of the lung tissue of the offspring. The maternal administration of tomato juice, together with nicotine during gestation and lactation, prevented the lungs of offspring of from the harmful effects of nicotine exerts on the developing rat lung.

**Table 3.8** The effects of maternal exposure to nicotine, tomato juice, and both nicotine and tomato juice on the linear intercept (Lm) (µm) of the male and the female offspring at postnatal days 21, 42, 63, and 84. P-value: significant difference between C and N is evident from day 21 until day 84. P-value: No significant difference when C vs. T and C vs. N+T where compared from day 21 to day 84 (P>0.05).

| **Age (days)** | Sex | **Control** | **C vs. N** | **Tomato** | **C vs. T** | **Nicotine** | **C vs. N+T** | **N+T** |
|---|---|---|---|---|---|---|---|---|
| | Males | 43.77±1.57 | P<0.01 | 35.23±1.63 | P>0.05 | 36.87±.075 | P>0.05 | 38.90±1.47 |
| **21** | Females | 43.77±1.57 | P<0.01 | 35.23±1.63 | P>0.05 | 36.87±0.75 | P>0.05 | 38.90±1.47 |
| **M vs. F** | | P>0.05 | | P>0.05 | | P>0.05 | | P>0.05 |
| | Males | 32.27±1.47 | P<0.04 | 29.97±1.32 | P>0.2 | 49.55±7.07 | P>0.8 | 35.19±1.56 |
| **42** | Females | 32.20±1.34 | P<0.001 | 33.39±0.82 | P>0.2 | 40.97±0.35 | P>0.4 | 32.67±2.19 |
| **M vs. F** | | P>0.9 | | P>0.05 | | P>0.2 | | P>0.3 |
| | Males | 32.97±1.76 | P<0.004 | 36.12±1.41 | P>0.3 | 44.73±2.79 | P>1.00 | 33.09±1.76 |
| **63** | Females | 33.00±1.79 | P<0.01 | 35.10±1.08 | P>0.9 | 46.07±3.09 | P>1.00 | 30.95±1.79 |
| **M vs. F** | | P>0.9 | | P>0.2 | | P>0.7 | | P>0.7 |
| | Males | 32.97±0.99 | P<0.001 | 32.56±0.83 | P>0.4 | 46.77±2.20 | P>0.6 | 33.79±0.74 |
| **84** | Females | 33.65±1.80 | P<0.001 | 32.13±2.16 | P>0.7 | 47.50±2.17 | P>0.6 | 34.18±0.56 |
| **M vs. F** | | P>0.7 | | P>0.8 | | P>0.8 | | P>0.6 |

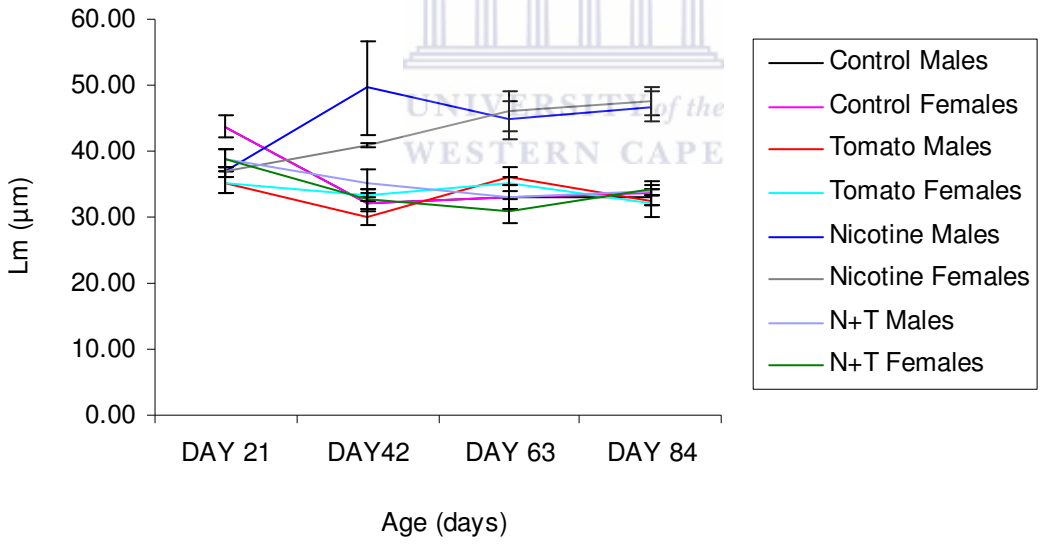



**Figure 3.8** The effects of maternal exposure to nicotine, tomato juice, and both nicotine and tomato juice on the linear intercept (Lm) of the male and the female offspring at postnatal days 21, 42, 63, and 84.

## 3.6 THE EFFECTS OF MATERNAL NICOTINE AND TOMATO JUICE ON INTERALVEOLAR SEPTAL THICKNESS (TSEPT) OF THE LUNGS OF THE OFFSPRING.

The results of the Interalveolar septal thickness (Tsept) as shown in table 3.9 and illustrated in figure 3.9 shows that the Tsept of the nicotine group increased at a faster rate than that of the other groups so that on postnatal day 84, the Tsept of the nicotine groups was 71% higher ($P<0.04$) in the control males and 44% higher ($P<0.04$) in the control females. There was no difference ($P>0.05$) between the Tsept of the control and that of the tomato juice group and the group that was exposed to both nicotine and tomato juice at postnatal days 21 and 84. There was also no difference ($P>0.05$) in the Tsept of the male and female rats in the nicotine groups.

**Table 3.9** The effects of maternal exposure to nicotine, tomato juice, and both nicotine and tomato juice on the Interalveolar septal thickness (Tsept) (µm) of the male and the female offspring at postnatal days 21, 42, 63, and 84. P-value: No difference within experimental groups and within age groups from day 21 to day 63 (P>0.05). A significant difference between C and N is can be noted on day (P<0.05).

| Age (days) | Sex | Control | C vs. N | Tomato | C vs. T | Nicotine | C vs. N+T | N+T |
|---|---|---|---|---|---|---|---|---|
| 21 | Males | 9.27±1.20 | P>0.4 | 7.22±0.70 | P>0.1 | 8.08±0.90 | P>0.1 | 6.91±0.95 |
| | Females | 9.27±1.20 | P>0.4 | 7.22±0.70 | P>0.1 | 8.08±0.90 | P>0.1 | 6.91±0.95 |
| 42 | Males | 6.53±0.45 | P>0.1 | 7.40±0.77 | P>0.3 | 7.61±0.37 | P>0.1 | 8.23±0.92 |
| | Females | 6.62±0.34 | P>0.09 | 6.60±0.50 | P>0.9 | 9.05±1.13 | P>0.2 | 7.66±0.73 |
| 63 | Males | 7.36±0.83 | P>0.2 | 8.50±0.96 | P>0.4 | 9.77±1.45 | P>0.6 | 7.36±0.83 |
| | Females | 8.96±1.50 | P>0.5 | 9.90±1.80 | P>0.7 | 10.33±1.28 | P>0.8 | 9.55±2.02 |
| 84 | Males | 9.60±0.06 | P<0.04 | 10.12±0.84 | P>0.6 | 16.42±3.15 | P>0.7 | 9.84±0.70 |
| | Females | 9.44±0.72 | P<0.04 | 8.50±1.06 | P>0.5 | 13.59±1.26 | P>0.9 | 9.14±1.60 |

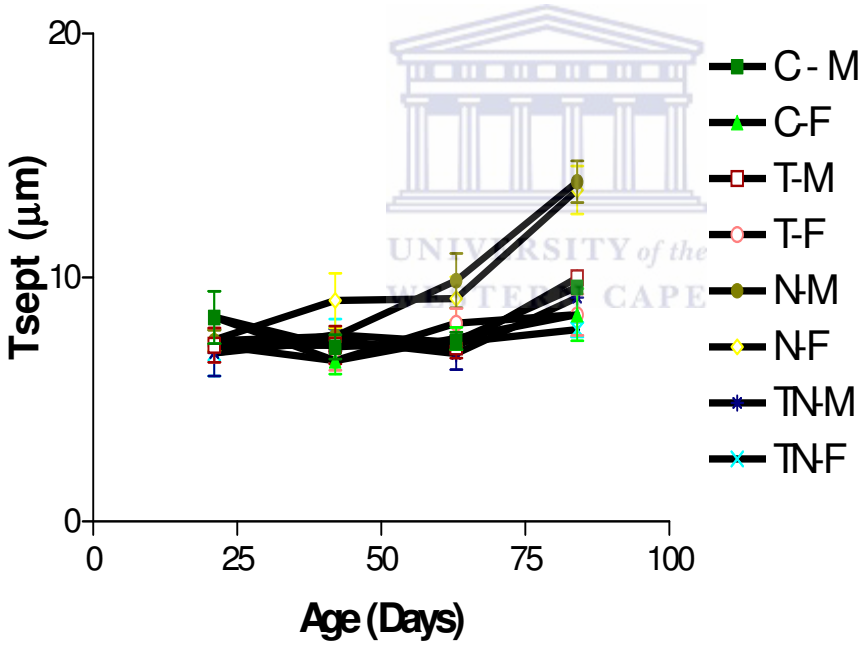



**Figure 3.9** The effects of maternal exposure to nicotine, tomato juice, and both nicotine and tomato juice on the Interalveolar septal thickness (Tsept) of the male and the female offspring at postnatal days 21, 42, 63, and 84.

## 3.7 THE EFFECTS OF MATERNAL NICOTINE AND TOMATO JUICE EXPOSURE ON THE MORPHOLOGY OF THE LUNGS OF THE OFFSPRING.

A view of the lungs of the 84-day-old animals as shown in figure 3.10 indicates that the control animals (A), the tomato group (B), and the N+T group (C), show the same architecture. These results are congruent to the results obtained from the morphometric results. The comparison of the control animals with the nicotine groups (D) demonstrates that on postnatal day 84, the nicotine group showed larger alveolar spaces compared to the control. The similarity between the control and the N+T group supports that the administration of tomato juice together with nicotine does have protective effects on the lungs of offspring that were maternally exposed to nicotine during gestation and lactation.



## 3.8 THE EFFECT OF MATERNAL EXPOSURE TO NICOTINE AND TOMATO JUICE ON APOPTOSIS IN THE LUNGS OF THE 84-DAY-OLD OFFSPRING.

From fig 3.11 it appears as if the number of apoptotic cells were the same for the lung tissue of the 84-day-old rats of all the groups. However, when the volume densities of the apoptotic cells were determined (fig. 3.12) it was noted that the volume density of the apoptotic cells of the lungs of the 84-day-old control rats (27.90 ± 1.94 %) was lower ($P<0.05$) than that of the animals that were exposed to tomato juice (36.12 ± 1.98 %) or to a both nicotine and tomato juice (36.33 ±

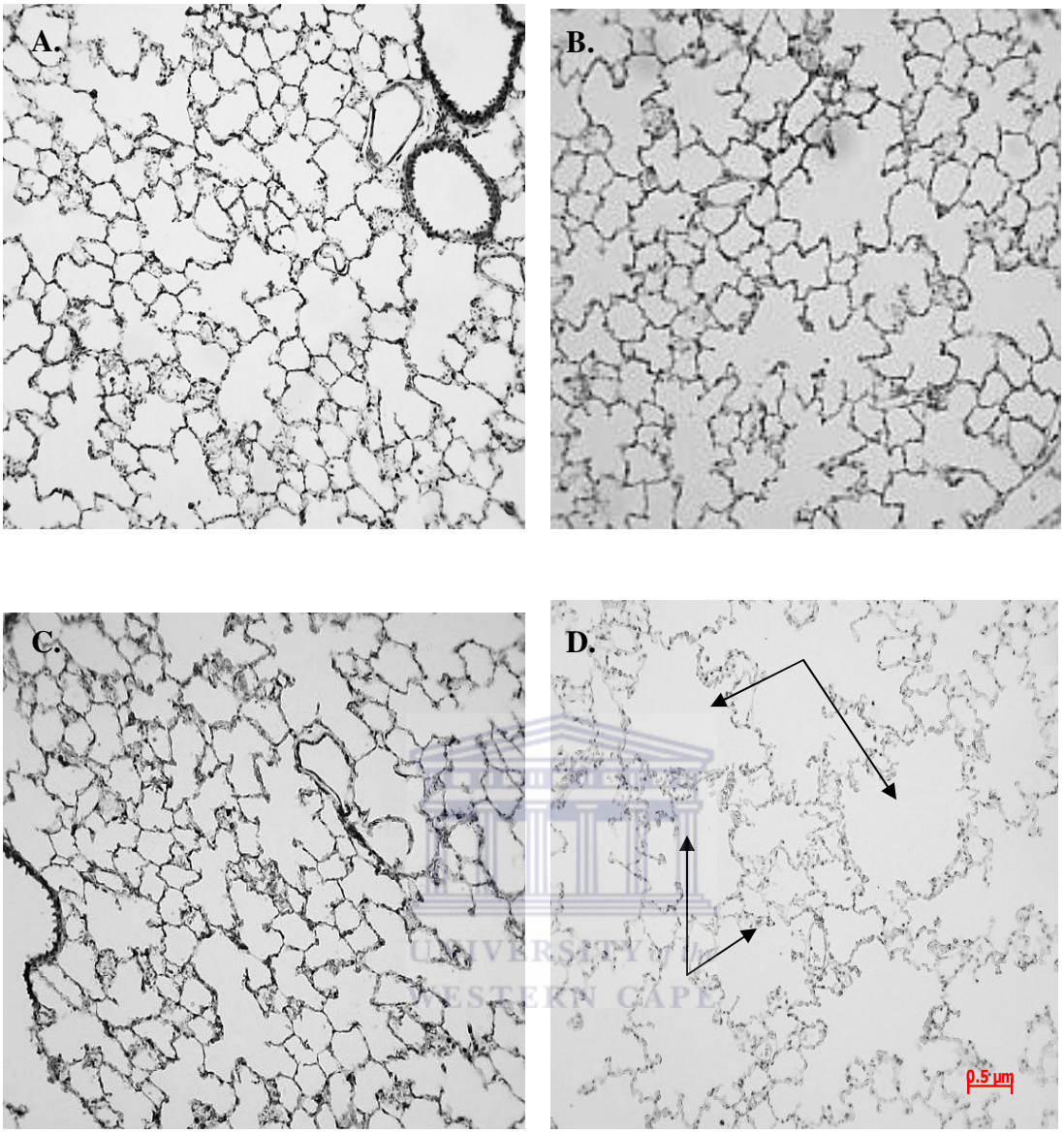



**Figure 3.10** The influence of maternal exposure to nicotine, tomato juice and a combination of nicotine and tomato juice on lung parenchyma of the 84-day-old offspring. A. = Control; B. = Tomato juice; C. = Nicotine + tomato juice; D. = Nicotine.
Arrows = Emphysema. (Bar = 0.5µm).

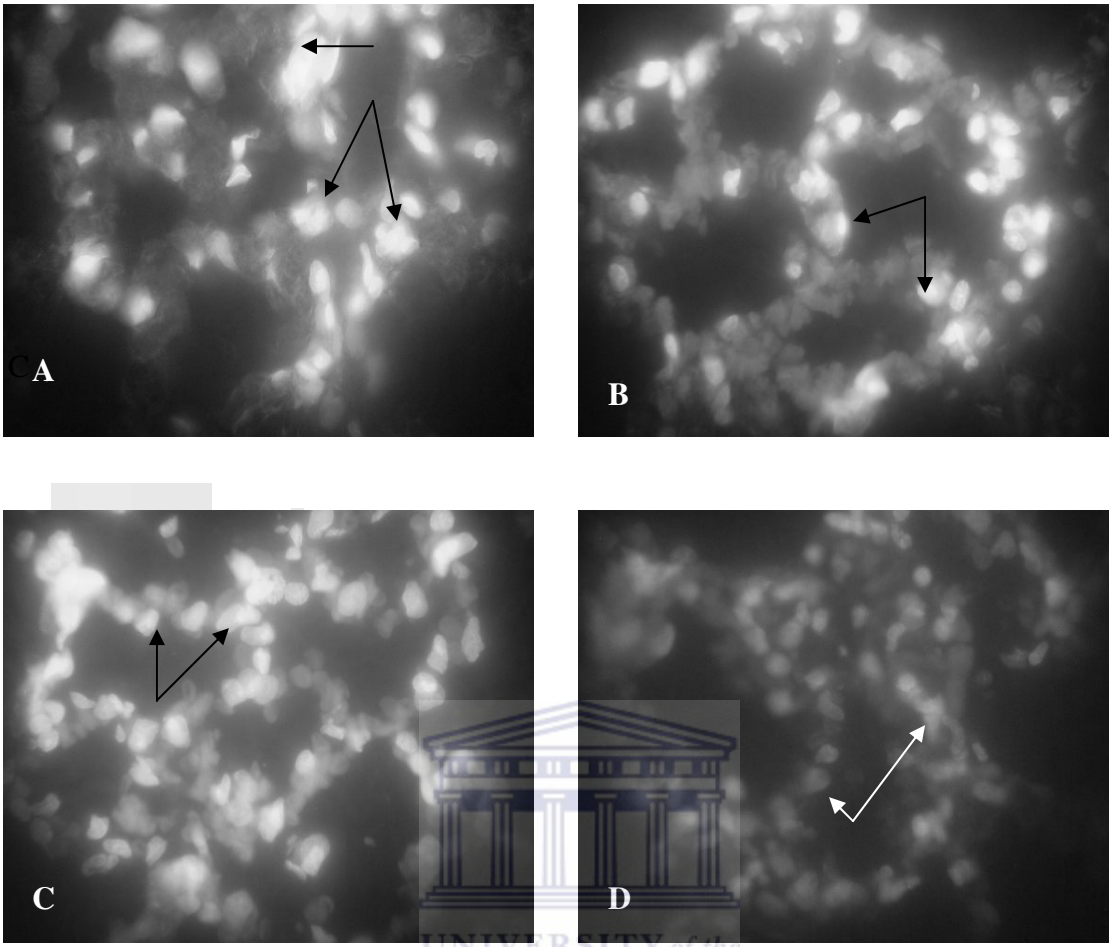



**Figure 3. 11** The effect of maternal exposure during gestation and lactation to, A) nicotine, B) tomato juice only , C) control and tomato juice, or D) to nicotine only, on apoptosis in the lungs of 84-day-old offspring. Arrows pointing some apoptotic cells.

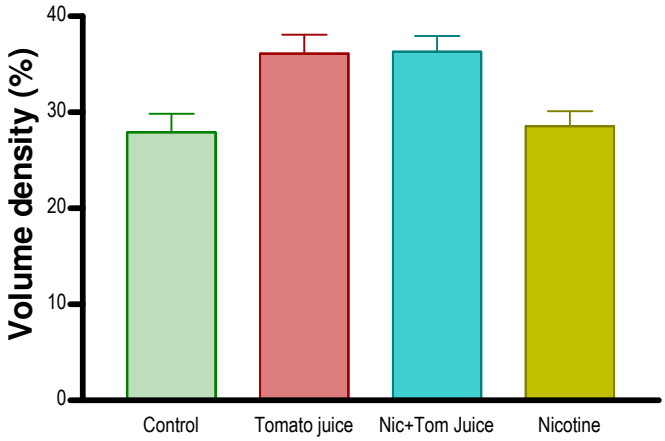



**Figure. 3. 12** The effect of maternal exposure to nicotine, tomato juice, or both nicotine and tomato juice on the volume density of the apoptotic cells of the lungs of the 84-day-old offspring.

1.62 %). No difference was observed between the control and the animals that were exposed to nicotine only (28.54 ± 1.57 %). The $V_t$ of the apoptotic cells of the nicotine group was lower than those that were exposed to tomato juice or to both nicotine and tomato juice.

# CHAPTER 4

## Discussion of Results

### 4.1 INTRODUCTION

Epidemiologic studies indicate that cigarette smoking is a major cause of COPD such as chronic bronchitis and emphysema (Morbidity and mortality weekly report, 1989). Cigarette smoke also contains abundant free radicals, including nitric oxide (Stedman, 1968), and may therefore induce some of its harmful effects by the free radical mechanism (Pryor et al, 1983). Reports indicate that approximately 20 to 30% of all newborns are exposed to components of tobacco smoke either before or after birth, increasing their susceptibility to respiratory diseases (Hofhuis et al, 2003). Several studies have indicated that reduced lung function caused by maternal exposure to cigarette smoke is predominant in the offspring of smoking mothers. The effects of maternal smoking during pregnancy on the offspring *in utero* and during postnatal life may persist even until the later stages of life (Cunningham et al, 1994).



Some of the effects of maternal smoking include an increase the distance between alveolar attachment points onto small alveolar airways causing a decrease in the connection of the airways with the lung parenchyma (Elliot et al, 2001). The adverse impact of maternal smoking on the alveolar airway-parenchymal tethering may increase lung compliance and increase the

susceptibility of the respiratory system to COPD. Similarly, from studies done in rats, it was observed that the prenatal exposure to cigarette smoke not only decreased the number of pre-alveolar saccules but also increased their size, thus decreasing the number of attachment points on the small airways (Collins et al, 1985). It is clear from the literature that, maternal smoking during prenatal development has major consequences on lung structure and function in early childhood as well as in the long-term (Maritz et al, 2005). Maternal smoking during gestation has been further demonstrated to accelerate deterioration of the forced expiratory volume ($FEV_1$) which is suggestive of premature aging of the lung (Green and Pinkerton, 2004). These changes may compromise respiratory health in the long term.



Cigarette smoke contains a range of radicals and oxidants which have the potential to increase the rate of oxidative damage. It is, therefore, conceivable that when the antioxidant defenses become overwhelmed by the oxidants, the resultant injury can lead to cell death. Some of the alterations of biological molecules brought about by cigarette smoke include oxidation of nucleic acids, cell proteins, and membrane lipids (Saha-Otterbein et al, 1998). These changes may results in accelerated cellular aging (Morla et al, 2006).

Nicotine is a well recognized component of cigarette smoke (Perkins et al, 1994). It easily crosses the placenta to interact with nicotinic receptors in the lungs of the developing fetus (Sekhon et al, 1999). Nicotine has also been found in the

milk of smoking mothers (Luck and Nau, 1984; Page-Sharp et al. 2003). Furthermore, Rylander et al, (1995) found nicotine and cotinine in the urine of 10-month-old non-suckling infants, indicating uptake of nicotine by passive smoking. Since the offspring in my study received nicotine only via the placenta and mother's milk, the uptake of nicotine by the offspring *in utero* and during lactation was also passive.

In a study by Benowitz et al (1982) and Murrin et al (1987) the plasma nicotine concentration was determined in female rats 2 days after birth of the offspring. These rats received 1.5 mg nicotine/kg body weight/day.They illustrated that the plasma nicotine content of these females rats were the same as that of human smokers. It is therefore, possible that in my study the increase in nicotine content of the mother's milk after a single injection was initially of the same magnitude. It is known that pregnancy has variable and unpredictable effects on drug metabolism. It is, therefore possible that the plasma nicotine concentrations measured after birth are not likely to reflect the actual concentrations during the various stages of pregnancy, especially during critical windows of organogenesis. However, the immaturity of the antioxidant capacity of the fetal rat lung makes it vulnerable to the effects of nicotine from the mother's blood and radical-mediated cell injury (Yam et al, 1978; Tanswell and Freeman, 1984; Gerdin et al, 1985; Hayashibe et al, 1990). Due to the slow metabolism of nicotine in the fetus, the half-life of nicotine will be much longer in the lungs of the offspring (Luck et al,

1985). This implies that the impact of nicotine on all those metabolic processes that are affected by nicotine, will last much longer than in the lungs of the adult.

## 4.2 THE EFFECTS OF GENDER AND MATERNAL EXPOSURE TO NICOTINE, TOMATO JUICE AND TO BOTH NICOTINE AND TOMATO JUICE ON GROWTH AND DEVELOPMENT OF THE OFFSPRING.

### 4.1.1 Gender differences

It has been established from previous research that young male rats show a higher and more rapid increase in BW than do females from the same litter (Kim et al, 1952). The observation from the current study shows that from birth to postnatal day 42, there were no differences in BW between the males and the female animals within all the different groups. This means that growth up to postnatal day 42 was not affected by gender differences. On the other hand, the increase in body weight of male animals was faster between postnatal days 42 and 84 than for the female animals. Kim et al (1952) suggested that the difference in the growth rate and ultimately weight of male and female animals may be due to a difference in the amount of food consumed or to the difference in the absorption and utilization of the food after it has been consumed.



However, there is very little information available on whether the rapid increase in weight that is observed in the males is merely due to an elevated consumption of increase in male BW after postnatal day 42 can be attributed only to the intake of food compared to the females (Kim et al, 1952). It is unlikely that the faster

increase in male BW after postnatal day 42 can be attributed only to the intake of more food than the females because it is not a function of food intake only but of a variety of factors. The faster increase in BW in males is rather due to a physiological difference between males and females that comes into play at this stage of growth, development and maturation. It is plausible that the male rats continue to grow at the same rate but that the growth rate of the females slowed down as part of the physiologic maturation process, which includes hormonal changes related to gender differences. This is illustrated by the almost linear increase in BW of the male animals throughout the time of the study, while the female BW increase slowed down from after postnatal day 42. This suggestion is supported by studies of other researchers that proposed that one of the reasons for the difference in body weight is the release of gonadal steroids, which have been shown to have important effects on the regulation of energy balance. It is well known that androgenic hormones contribute significantly to the composition and control of BW (Gentry and Wade, 1976). The faster growth of the males is, therefore, most likely due to hormonal and metabolic changes that occur as part of their overall maturation. Due to this difference, males and females appear to have significant variations in the systems that regulate energy balance and body weight (Shi and Clegg, 2009). Woods and colleagues (1998) suggested that the regulation of body weight occurs through negative feedback mechanisms which characterize most systems. The catabolic signal to the brain is provided by the hormone leptin which is secreted from adipose tissue. Leptin controls body weight through the activation of the sympathetic nervous system (Campfield et al,

1995). It has been suggested that females have more subcutaneous fat than males (Dua, 1996). Since studies have shown that leptin levels are more associated with subcutaneous fat than visceral fat, it is conceivable that the adiposity message conveyed to the brain in males differs from that in females (Clegg et al, 2003 and 2006). According to Demerath et al (1999) females show higher leptin levels than males which will contribute to a lower food intake by the females compared to males. This is evident even before puberty. In addition, several studies have proposed that, estradiol which is one of the natural forms of estrogen, may also influence food intake and body weight regulation. Estradiol is involved in several physiologic functions that include development, growth and energy homeostasis (Houpt et al, 1979; Friend, 1971; Eckel et al, 2000; Czaja and Goy, 1975; Gong et al, 1989).



This study is, among existing studies the first to show that the increase in BW of males and females is the same for up to 6 weeks after birth, and that the slower increase in the BW of the females only occurred around that time. It is interesting to note that male and female rats reach sexual maturity from 7 weeks after birth (Quin, 2005) and is conceivably the reason for the later onset of difference in the increase in BW between males and females.

### 4.2.2 Effect of nicotine.

Several studies have shown that fetal and neonatal nicotine exposure results in reduced body weight in rats during early postnatal life (Lawrence et al, 2008;

Cliver et al, 1995; Kramer, 1987). Some studies suggested that nicotine results in the constriction of placental blood vessels which consequently leads to a decreased placental blood flow. The nutritional supply to the fetus will, as a result, decrease during the period of vasoconstriction. This means that the exposure of the mother to nicotine during pregnancy could result in restricted fetal growth, resulting in lower birth weight of the offspring (Phillip et al, 1984; Birnbaum et al 1994). This is contrary to the findings of the present study, where no difference in BW was observed between the control and nicotine exposed offspring. It is possible that the doses of nicotine of 6 and 3 mg nicotine/kg body weight/day used by Phillip and co-workers (1984) and Birnbaum et al (1994) was too high. This is supported by the observations of Murrin et al, (1987) and Benowitz et al, (1982) that showed that when pregnant rats receive 1.5 mg nicotine per day, the maternal plasma nicotine concentration is similar to the values observed for human smokers. They showed that birth weight of the offspring and postnatal weight gain was not affected. It is, therefore, conceivable that the nicotine levels in my study were within the range of human smokers and conceivable that it will not affect the BW of the offspring. Also, since nicotine was given only once per day, it is possible that the time that placental vasoconstriction lasted, and thus the time of lower nutrient supply, was too short to have a significant impact on the growth of the offspring.



The present study also demonstrated that neither maternal nicotine exposure nor intake of tomato juice during gestation and lactation had any effect on the chest circumference (CC) or the crown-rump length (CR) or on the CC/CR ratios. This

means that nicotine, as well as tomato juice had no effect on the proportional growth of the offspring. This means that all the changes seen in the lung structure of the offspring that was exposed to nicotine via the placenta and mother's milk, was not due to an inadequate nutrient supply to the developing fetus and neonate. It is, therefore, conceivable that these changes can be attributed to the effects of nicotine or products of nicotine metabolism.

## 4.3 THE EFFECT OF MATERNAL NICOTINE EXPOSURE ON LUNG DEVELOPMENT IN THE OFFSPRING.

Apart from the BW, the results from this study further illustrated that from birth to postnatal day 42, there was no difference between the Lv of the male and female groups. The results furthermore showed that between postnatal day 42 and postnatal day 84, the lung volumes (Lv) of the male nicotine group increased more rapidly compared to that of the females. The reason for the faster increase in the Lv of the male rats can be attributed to the bigger body weights and thus greater oxygen demand of the male rats due to faster growth from around postnatal day 42. This is supported by a study done by Gehr et al (1981) which showed that bigger animals require larger internal surface area in the lungs to meet the demands of the bigger body. Supporting studies indeed show that adult males have larger Lv than females (Becklake and Kauffman, 1999; Hyde et al 2007; Thurlbeck, 1982), and that the increase in Lv is proportional to the increase in BW (Tenney and Remmers, 1963). These observations are supported by the data generated in this project, which shows that the Lv/BW ratios are the same

for male and female rats, except for the 84-day-old control male rats and those that were exposed to both nicotine and tomato juice, which was higher than that of the control female rats of the same age. This can be attributed to a faster increase in BW of the male rats between postnatal days 42 and 84.

While maternal nicotine exposure had no effect on the lung volumes of the male rats up to postnatal day 42, the Lv of these rats increased faster after postnatal day 42 than that of the control male rats. Consequently, the Lv of these males was, at postnatal day 84, 26.4% higher than that of the controls. Contrary to this, the Lv of the control female and nicotine exposed rats was not affected by maternal nicotine exposure. This explains why the:

1. Lv/BW ratio of the 84-day-old nicotine exposed male rats was 23.2 % higher than that of the control rats of the same age, and,
2. CC/Lv ratio of the control rats of the same age was higher than that of the nicotine exposed rats.



The reason for the different response of the male and female rats to maternal nicotine exposure is not known. It is possible that, the lungs of the males are more sensitive for the effect of nicotine. The bigger lung volume of the male nicotine exposed rats is conceivable due to higher lung compliance and not due to a larger chest circumference since the latter was not affected by maternal nicotine exposure. If this is so, it implies that the connective tissue framework of

the lungs of the male nicotine exposed rats was compromised. Studies to investigate this are currently planned.

This above observation further implies that the lungs of the male nicotine exposed offspring will have a higher propensity for respiratory disease than the female rats that were exposed to nicotine via the placenta and mother's milk. This observation is contrary to the findings of researchers who showed that female smokers have a higher risk of developing respiratory disease than males (Prescott et al, 1997; Patel et al, 2009). On the other hand, researchers in Australia and Japan showed that female smokers have lower incidences of COPD than male smokers (Sato et al, 2001; NSW Dept of Health, 2006) and supports the suggestion that males that were exposed to nicotine *in utero* and during lactation may have a higher propensity for respiratory disease than females The reason for the conflicting results is not known. The question that needs to be answered is whether or not *in utero* exposure to nicotine indeed elicits a different response in males and females in the long term.



The Morphometric data shows that the alveolar diameter (Lm), and the alveolar volume of the lungs of the control rats, as well as the rats that were born from mothers that received tomato juice during gestation and lactation, decrease as the lungs mature. However, the alveolar volume of those rats that were exposed to nicotine via the placenta and mother's milk increased as the animals age. This means that alveolar formation occurred in the control animals as opposed to the

gradual breakdown of alveolar walls in the lungs of the nicotine exposed offspring. The gradual breakdown of the alveolar walls resulted in the decrease in the tissue volume ($V_t$) of the lungs of the nicotine exposed rats as a function of age. This is in contrast to the increase in the $V_t$ of the lungs of the control rats as well as those rats that were born to mothers that received tomato juice supplements, as well as those that received nicotine as well as tomato juice supplements. This slow increase in the $V_t$ is a normal response of the lung to aging (Kovar et al, 2002).

The decrease in Vt of the lung parenchyma of the nicotine exposed animals is to be expected because of the gradual deterioration of the lung parenchyma in these rats. The gradual deterioration of the lung parenchyma and the increase in alveolar volume, and consequently the reduced alveolar number, explains the decrease in the alveolar surface area available for gas exchange in the lungs of these rats. These observations were made for both the gender groups.



In the light of the decrease in lung tissue volume of the nicotine exposed offspring, the increase in alveolar wall thickness (Tsept) of the lungs of these rats was unexpected. The fact that the tissue volume of these rats decreased despite the increase in alveolar wall thickness, was in all likelihood due to a faster deterioration of the lung parenchyma and the late onset of alveolar wall thickening. Consequently, the increase in alveolar wall thickness was masked by

this. The reason for the increase in alveolar wall thickness is not clear, but it can be due to:

1. ***an increased production of extracellular matrix.***

The production of the extracellular matrix is tightly controlled. Since the connective tissue components, such as collagen and elastin, are important in maintaining the 3D structure of the lung, it is essential to ensure that a balance between synthesis and degradation is maintained. Epithelial-mesenchymal interaction is necessary to maintain this balance and thus alveolar integrity (Rehan et al, 2007). It has been shown that nicotine exposure *in utero* induces lung injury by disrupting the epithelial-mesenchymal control of extracellular matrix formation (Rehan et al, 2007). This may result in an imbalance in the homeostatic mechanisms that control the extracellular matrix content and composition of the lungs of these animals. In a study by Maritz and Thomas (1994) it was also shown that maternal nicotine exposure result in type I cell damage followed by type II cell proliferation and differentiation. They also showed that, in addition to the above, swelling of the mitochondria of these cells. This was not reflected in a slower oxygen utilization of the lungs which implies that it is unlikely that these cells were metabolically compromised (Maritz and Thomas, 1994). This means that the energy metabolism of the type II cells of the lungs of the nicotine exposed offspring was normal. The cell-cell interaction between type II cells and the fibroblasts will not be affected due to inadequate

energy metabolism, but rather if interaction between these two cell types is compromised.

The composition of the extracellular matrix of the aging lung also changes. Studies by Huang et al (2007 a and b) showed that elastin fiber content decrease as the animals age while the collagen content increase. This will compromise the lung compliance as the animals age. It is plausible that the premature thickening of the alveolar walls of the lungs of the nicotine-exposed rats was due to a premature aging of the lungs, since aging of the lungs goes with a decline in homeostatic mechanisms involved in controlling extracellular metabolism and thus composition (MacNee, 2009).



**2. *an increase in the cellularity of the alveolar walls*.**

An increase in the cellularity of the alveolar walls can be due to a) cell proliferation, or b) inhibition of apoptosis, or, c) both proliferation and suppression of apoptosis.

*a) cell proliferation*

Type II epithelial cell growth and differentiation is essential for the maintenance of lung alveolar integrity (Rehan et al, 2007). Mesenchymal-epithelial cell interactions are critically important for normal lung development as well as for repair of injury Shannon and Hyatt, 2004). It was suggested that due to the irreversible inhibition of glycolysis, the main energy source of the type I cells, it is

conceivable that it will result in death of the type I epithelial cells, followed by type II cell proliferation and differentiation to maintain alveolar integrity and thus internal surface area available for gas exchange (Maritz, 1987). They indeed illustrated an increase in the type II cell numbers of rats that were exposed to nicotine via the placenta and mother's milk. These experiments were performed while the rat pups were still exposed to nicotine. Whether type II cell proliferation will still be enhanced after nicotine withdrawal is not known. If it is indeed enhanced, it is conceivable that the increase in alveolar wall thickness is due to an increased cellularity. No direct evidence is available yet to support such a suggestion. Whether that is true at this late phase of lung maturation is not known and warrant further study.

*b. a slower apoptotic process.*

Apoptosis plays an important role in lung development. According to Bruce et al (1999), the initial thinning of the alveolar walls during lung maturation, when the thick walled alveolar saccules are changed into thin walled alveoli, is due to apoptosis of the interstitial fibroblasts. Although epithelial type II cell proliferation occur when type I cell are damaged (Evans et al, 1975; Adamson and Bowden 1974), it has been shown that there is no correlation between cell proliferation and apoptosis (Imai et al, 2005). My results showed that maternal nicotine exposure during gestation and lactation result in microscopic emphysema in the adult rat. Cell-based mechanisms associated with emphysematous parenchymal damage include increased apoptosis and

cell proliferation. Apoptosis correlate with parenchymal deterioration and enlargement of airspaces in the aging lung (Imai et al, 2005). Although it is possible that apoptosis was affected by nicotine while the offspring was exposed to nicotine via the placenta and mothers milk, it had no effect on apoptosis in the lungs of the 84-day-old offspring. It is, therefore, unlikely that thickening of the alveolar walls of the 84-day-old rats as they age was due to a slower apoptosis or even increased cell proliferation. It can in all likelihood be attributed to an increase in the extracellular matrix. Further studies to investigate this are currently undertaken.

It is important to note that some of the changes in the integrity of the lungs of the nicotine exposed offspring only become visible from around postnatal day 42. The increase in alveolar wall thickness only became apparent around postnatal day 84. This means that these changes developed after all the nicotine was removed from the lungs of the offspring. It is therefore plausible that maternal nicotine exposure during lung development *in utero* and during early postnatal life, when cell proliferation and differentiation was rapid, induced changes to the “program” that controls lung development, maintenance of structural integrity and aging. Increased alveolar volume, thickening of alveolar walls and development of emphysema-like lesions in the lung parenchyma are all signs of lung aging. Since these structural changes occur in the lungs of the nicotine exposed offspring, it is a sign of premature aging of the lungs of these animals.

In summary, it noticeable from this study and from previous literature that the exposure to nicotine during gestation and lactation may result in the failure to maintain lung structure and functional integrity, as the animal gets older. The acceleration in the aging of the lung, however, implies that nicotine does alter the 'programming' of the normal aging of the lung (Maritz et al, 2005). Premature aging of the lungs due to maternal nicotine exposure during gestation and lactation may render the fetal lungs more vulnerable to lung disease (Plummer et al, 2005). This premature aging is associated with a compromised antioxidant capacity of the mother and fetus. It is suggested that supplementing the mother's diet with tomato juice rich in lycopene will prevent the change in the "program" and thus allow for a normal aging of the lungs of the offspring.



## 4.4 THE ROLE OF TOMATO JUICE

Maritz et al (2005) suggested that exposure to nicotine compromise the antioxidant capacity of the mother and that of the developing fetus and neonate. If this is so, strategies to normalize the antioxidant capacity of the mother and the fetus will prevent the adverse effects of maternal nicotine exposure on the "program" that controls lung maintenance and aging in the long term. One such strategy is to supplement the mother's diet with tomato juice where tomato juice is rich in lycopene, a carotenoid, and other phytonutrients with strong antioxidant capacity (Rao and Argarwal, 1999).

Carotenoids have been documented to have antioxidant and preventative properties against free radical-induced cellular damage (Bendich, 1993). Several researchers have explored the potential function of antioxidant nutrients in the prevention of chronic diseases and the aging processes in humans (Mayne, 2003). Lycopene is recognized as the main carotenoid in tomatoes (Kaplan, 1990) and is further expected to be the most effective biological carotenoid in reducing or eradicating singlet oxygen (Di Mascio, 1989). The intake of dietary carotenoid may have the potential to influence the progress of tobacco smoke-induced emphysema (Kasagi et al, 2006); given that tobacco smoke contains more than $10^{15}$ oxidant molecules/puff (Church, 1985). This is supported by the work of Kasagi et al (2006) who demonstrated that the combined use of tomato juice and chronic tobacco smoke exposure completely protected senescence-prone mouse strain from smoke-induced emphysema.



Tomatoes are the main dietary source of lycopene (Gerster, 1997). It has been indicated by previous researchers that at least 85% of our dietary lycopene comes from tomatoes and tomato based products such as juice, pastes, sauce and many more (Bramley, 2000). Markovic and colleagues (2006) found in their study that tomato extracts rich in lycopene was effective against oxidative stress. This means therefore, that tomato extract or lycopene may be effective against oxidative stress induced by chemicals or drugs to some organs.

Since nicotine induce oxidant activity in cells (Dasgupta et al, 2006), and since lycopene, a strong antioxidant (Rao and Argarwal, 1999), is present in tomato juice (Takeoka et al, 2001; Kaplan et al, 1990), we decided to establish whether tomato juice will prevent the adverse effects of maternal nicotine exposure during gestation and lactation on lung development in the offspring.

Supplementing the mother's diet with tomato juice had no effect on the growth and development of the offspring. It also had no effect on the structural development of the lung. Tomato juice supplementation also prevented all the adverse effects of maternal nicotine exposure on lung development and aging in the offspring. It is likely that it will be effective in the prevention of the harmful effects of maternal smoking to the offspring of smoking pregnant women. This also suggests that tomato juice intake during gestation and lactation by smoking mothers, or mothers using nicotine replacement therapy to quit the habit, may prevent changes of the 'program' that controls lung maintenance and premature aging in the offspring. This will ensure an improved respiratory health in the offspring of smoking mothers.



It is interesting to note that although maternal intake of tomato juice had no effect on lung growth and development or on the aging of the lungs of the offspring, it enhances apoptosis in the lungs of the 84-day old offspring. Whether apoptosis was faster during the earlier phases of lung development, while the offspring received lycopene and other phytonutrients via the placenta and mother's blood,

is not known. Since apoptosis is still enhanced at day 84 after birth, that is 9 weeks after tomato juice supplementation was terminated, implies that it has a long term effect on the offspring. The consequences of the enhanced apoptosis are not known. Since tomato juice supplementation during gestation and lactation had no effect on lung development and aging in the offspring at this late stage of lung maturation, it is not likely to have a detrimental impact on lung integrity and respiratory health.

## 4.5 CONCLUSION AND FUTURE PROSPECTIVES

Studies show that despite all the efforts made by governments, researchers and communities to educate and inform women about the harmful effects of tobacco smoke and nicotine, smoking during gestation and lactation still remains a common habit and accounts for a significant percentage of fetal morbidity and mortality (Salihu and Wilson, 2007; Proskocil et al, 2005). The offspring is as a result exposed to nicotine through the blood and the milk of the mother (Luck and Nau, 1984). The results in this project support the findings in previous studies that showed that maternal exposure to nicotine during gestation and lactation alters the "program" that controls the maintenance on the lung structure in the long term (Maritz and Windvogel, 2003).

The present study further suggests that maternal nicotine exposure during gestation and lactation result in premature aging of the lungs of the offspring. This will increase the risk of respiratory disease in the adult. Furthermore, the

data from the present study showed that maternal supplementation with tomato juice prevents all the adverse effects of maternal nicotine exposure. Further studies are essential to further investigate the mechanism of action of nicotine and to refine develop strategies to prevent the adverse effects of smoking and nicotine on lung development of the offspring. These include:

1. Assessing cell senescence in the lungs of the offspring
2. determine the reason for alveolar wall thickening
3. establish why tomato juice enhance apoptosis and the long term outcome of it.

In conclusion, it is apparent from this study that the nicotine intake during pregnancy and lactation presents harmful effects on the health and development of the fetal lung. The present study determined the effects of maternal nicotine exposure during gestation and lactation on lung development in the offspring. The study also established that the consumption of tomato juice may have protective effects on the premature aging of the lungs of the offspring.

# REFERENCES

Adamson, I.Y.R. & Bowden, D.H. 1974, "The type 2 cell as progenitor of alveolar epithelial regeneration: A cytodynamic study in mice after exposure to oxygen", *Laboratory Investigation,* vol. 30, no. 1, pp. 35-42.

Agarwal, A., Shen, H., Agarwal, S. & Rao, A.V. 2001, "Lycopene content of tomato products: Its stability, bioavailability and in vivo antioxidant properties", Journal of Medicinal Food, vol. 4, no. 1, pp. 9-15.

Al Mamun, A., Lawlor, D.A., Alati, R., O'Callaghan, M.J., Williams, G.M. & Najman, J.M. 2006, "Does maternal smoking during pregnancy have a direct effect on future offspring obesity? Evidence from a prospective birth cohort study", *American Journal of Epidemiology,* vol. 164, no. 4, pp. 317-325.

Alati, R., Al Mamun, A., O'Callaghan, M., Najman, J.M. & Williams, G.M. 2006, "In utero and postnatal maternal smoking and asthma in adolescence", *Epidemiology,* vol. 17, no. 2, pp. 138-144.

Aoshiba, K., Yokohori, N. & Nagai, A. 2003, "Alveolar wall apoptosis causes lung destruction and emphysematous changes", *American Journal of Respiratory Cell and Molecular Biology,* vol. 28, no. 5, pp. 555-562.

Arab, L., Steck-Scott, S. & Fleishauer, A.T. 2002, "Lycopene and the lung", *Experimental biology and medicine,* vol. 227, no. 10, pp. 894-899.

Argentin, G. & Cicchetti, R. 2004, "Genotoxic and antiapoptotic effect of nicotine on human gingival fibroblasts", *Toxicological Sciences,* vol. 79, no. 1, pp. 75-81.

Balcombe, N.R. & Sinclair, A. 2001, "Ageing: Definitions, mechanisms and the magnitude of the problem", *Bailliere's Best Practice and Research in Clinical Gastroenterology,* vol. 15, no. 6, pp. 835-849.

Becklake, M.R. & Kauffmann, F. 1999, "Gender differences in airway behaviour over the human life span", *Thorax,* vol. 54, no. 12, pp. 1119-1138.

Bendich, A. 1993, "Biological functions of dietary carotenoids", *Annals of the New York Academy of Sciences,* vol. 691, pp. 61-67.

Benowitz, N.L. 1986, "Clinical pharmacology of nicotine.", *Annual Review of Medicine,* vol. 37, pp. 21-32.



Benowitz, N.L., Hall, S.M. & Herning, R.I. 1983, "Smokers of low-yield cigarettes do not consume less nicotine", *New England Journal of Medicine,* vol. 309, no. 3, pp. 139-142.

Benowitz, N.L., Jacob, P., Kyerematen, G.A., Morgan, M.L., Chattopadhyay, B., DeBethizy, J.D. & Vesell, E.S. 1991, "Nicotine metabolism in humans [1]", *Clinical pharmacology and therapeutics,* vol. 50, no. 4, pp. 462-464.

Benowitz, N.L., Kuyt, F. & Jacob, P. 1982, "Circadian blood nicotine concentrations during cigarette smoking", *Clinical pharmacology and therapeutics,* vol. 32, no. 6, pp. 758-764.

Birnbaum, S.C., Kien, N., Martucci, R.W., Gelzleichter, T.R., Witschi, H., Hendrickx, A.G. & Last, J.A. 1994, "Nicotine- or epinephrine-induced uteroplacental vasoconstriction and fetal growth in the rat", *Toxicology,* vol. 94, no. 1-3, pp. 69-80.

Blackburn, E.H. 1997, "The telomere and telomerase: Nucleic acid-protein complexes acting in a telomere homeostasis system. A review", *Biochemistry (Moscow),* vol. 62, no. 11, pp. 1196-1201.

Blanco, L.N., Massaro, D. & Massaro, G.D. 1991, "Alveolar size, number, and surface area: Developmentally dependent response to 13% O2", *American Journal of Physiology - Lung Cellular and Molecular Physiology,* vol. 261, no. 6 5-3, pp. L370-L377.

Bolender, R.P., Hyde, D.M. & Dehoff, R.T. 1993, "Lung morphometry: A new generation of tools and experiments for organ, tissue, cell, and molecular biology", American Journal of Physiology - Lung Cellular and Molecular Physiology, vol. 265, no. 6 9-6, pp. L521-L548.

Boyden, E.A. 1977, "Development and growth of the airways", *Lung biology in health and disease: Development of the lung,* pp. 4-35.

Bramley, P.M. 2000, "Is lycopene beneficial to human health?", *Phytochemistry,* vol. 54, no. 3, pp. 233-236.

Brooks, D.R., Mucci, L.A., Hatch, E.E. & Cnattingius, S. 2004, "Maternal smoking during pregnancy and risk of brain tumors in the offspring. A prospective study of 1.4 million Swedish births", *Cancer Causes and Control,* vol. 15, no. 10, pp. 997-1005.

Bruce, M.C., Honaker, C.E. & Cross, R.J. 1999, "Lung fibroblasts undergo apoptosis following alveolarization", *American Journal of Respiratory Cell and Molecular Biology,* vol. 20, no. 2, pp. 228-236.

Buccafusco, J.J. 2004, "Neuronal nicotinic receptor subtypes: Defining therapeutic targets", *Molecular Interventions,* vol. 4, no. 5, pp. 285-295.

Campfield, L.A., Smith, F.J., Guisez, Y., Devos, R. & Burn, P. 1995, "Recombinant mouse OB protein: Evidence for a peripheral signal linking adiposity and central neural networks", *Science,* vol. 269, no. 5223, pp. 546-549.



Campisi, J. & D'Adda Di Fagagna, F. 2007, "Cellular senescence: When bad things happen to good cells", *Nature Reviews Molecular Cell Biology,* vol. 8, no. 9, pp. 729-740.

Cardoso, W.V. 2000, "Lung morphogenesis revisited: Old facts, current ideas", *Developmental Dynamics,* vol. 219, no. 2, pp. 121-130.

Cattaneo, M.G., D'Atri, F. & Vicentini, L.M. 1997, "Mechanisms of mitogen-activated protein kinase activation by nicotine in small-cell lung carcinoma cells", *Biochemical Journal,* vol. 328, no. 2, pp. 499-503.

Chen, Q., Fischer, A., Reagan, J.D., Yan, L.-. & Ames, B.N. 1995, "Oxidative DNA damage and senescence of human diploid fibroblast cells", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 92, no. 10, pp. 4337-4341.

Chen, Q.M., Prowse, K.R., Tu, V.C., Purdom, S. & Linskens, M.H.K. 2001, "Uncoupling the senescent phenotype from telomere shortening in hydrogen peroxide-treated fibroblasts", *Experimental cell research,* vol. 265, no. 2, pp. 294-303.

Chu, M., Guo, J. & Chen, C. 2005, "Long-term exposure to nicotine, via Ras pathway, induces cyclin D1 to stimulate G1 cell cycle transition", *Journal of Biological Chemistry,* vol. 280, no. 8, pp. 6369-6379.

Church, D.F. & Pryor, W.A. 1985, "Free-radical chemistry of cigarette smoke and its toxicological implications", *Environmental health perspectives,* vol. VOL. 64, pp. 111-126.

Clegg, D.J., Brown, L.M., Woods, S.C. & Benoit, S.C. 2006, "Gonadal hormones determine sensitivity to central leptin and insulin", *Diabetes,* vol. 55, no. 4, pp. 978-987.

Clegg, D.J., Riedy, C.A., Smith, K.A.B., Benoit, S.C. & Woods, S.C. 2003, "Differential sensitivity to central leptin and insulin in male and female rats", *Diabetes,* vol. 52, no. 3, pp. 682-687.

Cliver, S.P., Goldenberg, R.L., Cutter, G.R., Hoffman, H.J., Davis, R.O. & Nelson, K.G. 1995, "The effect of cigarette smoking on neonatal anthropometric measurements", *Obstetrics and gynecology,* vol. 85, no. 4, pp. 625-630.

Collado, M. & Serrano, M. 2005, "The senescent side of tumor suppression", *Cell Cycle,* vol. 4, no. 12, pp. 1722-1724.

Collins, M.H., Moessinger, A.C. & Kleinerman, J. 1985, "Fetal lung hypoplasia associated with maternal smoking: A morphometric analysis", *Pediatric research,* vol. 19, no. 4, pp. 408-412.

Conti-Fine, B.M., Navaneetham, D., Lei, S. & Maus, A.D.J. 2000, "Neuronal nicotinic receptors in non-neuronal cells: New mediators of tobacco toxicity?", *European journal of pharmacology,* vol. 393, no. 1-3, pp. 279-294.

Copland, I. & Post, M. 2004, "Lung development and fetal lung growth", *Paediatric Respiratory Reviews,* vol. 5, no. Supplement 1, pp. S259-S264.

Crapo, J.D., Barry, B.E., Foscue, H.A. & Shelburne, J. 1980, "Structural and biochemical changes in rat lungs occurring during exposures to lethal and adaptive doses of oxygen", *American Review of Respiratory Disease,* vol. 122, no. 1, pp. 123-143.

Cristofalo, V.J. 2005, "SA β Gal staining: Biomarker or delusion", *Experimental gerontology,* vol. 40, no. 10, pp. 836-838.

Cunningham, J., Dockery, D.W. & Speizer, F.E. 1994, "Maternal smoking during pregnancy as a predictor of lung function in children", *American Journal of Epidemiology,* vol. 139, no. 12, pp. 1139-1152.

Czaja, J.A. & Goy, R.W. 1975, "Ovarian hormones and food intake in female guinea pigs and rhesus monkeys", *Hormones and behavior,* vol. 6, no. 4, pp. 329-349.

Dasgupta, P., Rastogi, S., Pillai, S., Ordonez-Ercan, D., Morris, M., Haura, E. & Chellappan, S. 2006, "Nicotine induces cell proliferation by β-arrestin-mediated activation of Src and Rb-Raf-1 pathways", *Journal of Clinical Investigation,* vol. 116, no. 8, pp. 2208-2217.

De Paepe, M.E., Sardesai, M.P., Johnson, B.D., Lesieur-Brooks, A.M., Papadakis, K. & Luks, F.I. 1999, "The role of apoptosis in normal and accelerated lung development in fetal rabbits", *Journal of pediatric surgery,* vol. 34, no. 5, pp. 863-871.

Demedts, I.K., Demoor, T., Bracke, K.R., Joos, G.F. & Brusselle, G.G. 2006, "Role of apoptosis in the pathogenesis of COPD and pulmonary emphysema", *Respiratory Research,* vol. 7.

Demerath, E.W., Towne, B., Wisemandle, W., Blangero, J., Chumlea, W.C. & Siervogel, R.M. 1999, "Serum leptin concentration, body composition, and gonadal hormones during puberty", *International journal of obesity,* vol. 23, no. 7, pp. 678-685.

Dempsey, D.A. & Benowitz, N.L. 2001, "Risks and benefits of nicotine to aid smoking cessation in pregnancy", *Drug Safety,* vol. 24, no. 4, pp. 277-322.

Di Mascio, P., Kaiser, S. & Sies, H. 1989, "Lycopene as the most efficient biological carotenoid singlet oxygen quencher", *Archives of Biochemistry and Biophysics,* vol. 274, no. 2, pp. 532-538.

Dirksen, A., Friis, M., Olesen, K.P., Skovgaard, L.T. & Sørensen, K. 1997, "Progress of emphysema in severe α1-antitrypsin deficiency as assessed by annual CT", *Acta radiologica,* vol. 38, no. 5, pp. 826-832.

Droge, W. & Schipper, H.M. 2007, "Oxidative stress and aberrant signaling in aging and cognitive decline", *Aging Cell,* vol. 6, no. 3, pp. 361-370.

Dua, A., Hennes, M.I., Hoffmann, R.G., Maas, D.L., Krakower, G.R., Sonnenberg, G.E. & Kissebah, A.H. 1996, "Leptin: A significant indicator of total body fat but not of visceral fat and insulin insensitivity in African-American women", *Diabetes,* vol. 45, no. 11, pp. 1635-1637.

Dunnill, M.S. 1962, "Quantitative methods in the study of pulmonary pathology", *Thorax,* vol. 17, pp. 320-328.

Dyer, C.A.E. & Stockley, R.A. 2006, "The aging lung", *Reviews in Clinical Gerontology,* vol. 16, no. 2, pp. 99-111.

Eckel, L.A., Houpt, T.A. & Geary, N. 2000, "Spontaneous meal patterns in female rats with and without access to running wheels", *Physiology and Behavior,* vol. 70, no. 3-4, pp. 397-405.

Egger, B. & Aubert, J. 2005, "Pulmonary emphyzema: Mechanisms and therapeutic perspectives", *Revue Medicale Suisse,* vol. 1, no. 41, pp. 2664-2672.

Elliot, J., Carroll, N., Bosco, M., McCrohan, M. & Robinson, P. 2001, "Increased airway responsiveness and decreased alveolar attachment points following in utero smoke exposure in the guinea pig", *American Journal of Respiratory and* Critical Care Medicine, vol. 163, no. 1, pp. 140-144.

Eriksson, S. 1999, "Alphax-antitrypsin deficiency", *Journal of Hepatology, Supplement,* vol. 30, no. 1, pp. 34-39.

Evans, M.J., Cabral, L.J., Stephens, R.J. & Freeman, G. 1975, "Transformation of alveolar type 2 cells to type 1 cells following exposure to NO2", *Experimental and molecular pathology,* vol. 22, no. 1, pp. 142-150.

Filippini, G., Farinotti, M. & Ferrarini, M. 2000, "Active and passive smoking during pregnancy and risk of central nervous system tumours in children", *Paediatric and perinatal epidemiology,* vol. 14, no. 1, pp. 78-84.

Fishman, A.P. 2005, "One hundred years of chronic obstructive pulmonary disease", *American Journal of Respiratory and Critical Care Medicine,* vol. 171, no. 9, pp. 941-948.

Foronjy, R. & D'Armiento, J. 2006, "The effect of cigarette smoke–derived oxidants on the inflammatory response of the lung", *Clinical and Applied Immunology Reviews,* vol. 6, no. 1, pp. 53-72.

Fowles, J. & Bates, M. 2000, *The Toxic Constituents of Tobacco and Tobacco Smoke: Priorities for Harm Reduction,* .

Friedman, P.J. 2008, "Imaging studies in emphysema", *Proceedings of the American Thoracic Society,* vol. 5, no. 4, pp. 494-500.

Friend, D.W. 1971, "Self-selection of feeds and water by swine during pregnancy and lactation.", *Journal of animal science,* vol. 32, no. 4, pp. 658-666.

Gallowitsch-Puerta, M. & Tracey, K.J. 2005, "Immunologic role of the cholinergic anti-inflammatory pathway and the nicotinic acetylcholine alpha 7 receptor.", *Annals of the New York Academy of Sciences,* vol. 1062, pp. 209-219.



Gehr, P., Mwangi, D.K. & Ammann, A. 1981, "Design of the mammalian respiratory system. V. Scaling morphometric pulmonary diffusing capacity to body mass: Wild and domestic mammals", *Respiration physiology,* vol. 44, no. 1, pp. 61-86.

Gentry, R.T. & Wade, G.N. 1976, "Androgenic control of food intake and body weight in male rats", *Journal of comparative and physiological psychology,* vol. 90, no. 1, pp. 18-25.

Gerdin, E., Tyden, O. & Eriksson, U.J. 1985, "The development of antioxidant enzymatic defense in the perinatal rat lung: Activities of superoxide dismutase, glutathione peroxidase, and catalase", *Pediatric research,* vol. 19, no. 7, pp. 687-691.

Gerster, H. 1997, "The potential role of lycopene for human health", *Journal of the American College of Nutrition,* vol. 16, no. 2, pp. 109-126.

Ginzel, K. 2001, "After some 100 million deaths - What's next? American Council of Science and Health", *Priorities for Health,* vol. 13, pp. 24-39.

Ginzel, K.H., Maritz, G.S., Marks, D.F., Neuberger, M., Pauly, J.R., Polito, J.R., Schulte-Hermann, R. & Slotkin, T.A. 2007, "Critical review: Nicotine for the fetus, the infant and the adolescent?", *Journal of Health Psychology,* vol. 12, no. 2, pp. 215-224.



Giovannucci, E. 1999, "Tomatoes, tomato-based products, lycopene, and cancer: Review of the epidemiologic literature", Journal of the National Cancer Institute, vol. 91, no. 4, pp. 317-331.

Gong, E.J., Garrel, D. & Calloway, D.H. 1989, "Menstrual cycle and voluntary food intake", *American Journal of Clinical Nutrition,* vol. 49, no. 2, pp. 252-258.

Green, F.H.Y. & Pinkerton, K.E. 2004, "Environmental determinants of lung aging", *The Lung: Development, Aging and the Environment,* , pp. 377-395.

Guo, R.-. & Ward, P.A. 2007, "Role of oxidants in lung injury during sepsis", *Antioxidants and Redox Signaling,* vol. 9, no. 11, pp. 1991-2002.

Haddad, J.J. 2002, "Oxygen-sensing mechanisms and the regulation of redox-responsive transcription factors in development and pathophysiology", *Respiratory Research,* vol. 3.

Harman, D. 1956, "Aging: A theory based on free radical and radiation chemistry", *J.Gerontol.,* vol. 11, pp. 298-300.

Harrison, P.T.C., Hoskins, J.A., Brown, R.C., Hext, P.M. & Pigott, G.H. 1997, "Pulmonary hyperplastic and neoplastic changes in rats treated concurrently with chrysotile asbestos and N-nitrosoheptamethyleneimine (NHMI)", *The Annals of Occupational Hygiene,* vol. 41, no. Supplement 1, pp. 293-297.

Hasty, P. & Vijg, J. 2004, "Accelerating aging by mouse reverse genetics: A rational approach to understanding longevity", *Aging Cell,* vol. 3, no. 2, pp. 55-65.

Hayashibe, H., Asayama, K., Dobashi, K. & Kato, K. 1990, "Prenatal development of antioxidant enzymes in rat lung, kidney, and heart: marked increase in immunoreactive superoxide dismutases, glutathione peroxidase, and catalase in the kidney", *Pediatric research,* vol. 27, no. 5, pp. 472-475.

Hayflick, L. & Moorhead, P.S. 1961, "The serial cultivation of human diploid cell strains", *Experimental cell research,* vol. 25, no. 3, pp. 585-621.

Hayflick, L. 1965, "The limited in vitro lifetime of human diploid cell strains", *Experimental cell research,* vol. 37, no. 3, pp. 614-636.

Hibberd, A.R. & Gorrod, J.W. 1983, "Enzymology of the metabolic pathway from nicotine to cotinine, in vitro", *European journal of drug metabolism and pharmacokinetics,* vol. 8, no. 2, pp. 151-162.

Hofhuis, W., De Jongste, J.C. & Merkus, P.J.F.M. 2003, "Adverse health effects of prenatal and postnatal tobacco smoke exposure on children", *Archives of Disease in Childhood,* vol. 88, no. 12, pp. 1086-1090.

Houpt, K.A., Coren, B., Hintz, H.F. & Hilderbrant, J.E. 1979, "Effect of sex and reproductive status on sucrose preference, food intake, and body weight of dogs", *Journal of the American Veterinary Medical Association,* vol. 174, no. 10, pp. 1083-1085.



Hu, M. 1994, "Measurement of protein thiol groups and glutathione in plasma", *Methods in enzymology,* vol. 233, pp. 380-385.

Huang, K., Mitzner, W., Rabold, R., Schofield, B., Lee, H., Biswal, S. & Tankersley, C.G. 2007 (a), "Variation in senescent-dependent lung changes in inbred mouse strains", *Journal of applied physiology,* vol. 102, no. 4, pp. 1632-1639.

Huang, K., Rabold, R., Schofield, B., Mitzner, W. & Tankersley, C.G. 2007 (b), "Age-dependent changes of airway and lung parenchyma in C57BL/6J mice", *Journal of applied physiology,* vol. 102, no. 1, pp. 200-206.

Hukkanen, J., Jacob, P. & Benowitz, N.L. 2005, "Metabolism and disposition kinetics of nicotine", *Pharmacological reviews,* vol. 57, no. 1, pp. 79-115.

Hyde, D.M., Blozis, S.A., Avdalovic, M.V., Putney, L.F., Dettorre, R., Quesenberry, N.J., Singh, P. & Tyler, N.K. 2007, "Alveoli increase in number but not size from birth to adulthood in rhesus monkeys", *American Journal of Physiology - Lung Cellular and Molecular Physiology,* vol. 293, no. 3.

Imai, K., Mercer, B.A., Schulman, L.L., Sonett, J.R. & D'Armiento, J.M. 2005, "Correlation of lung surface area to apoptosis and proliferation in human emphysema", *European Respiratory Journal,* vol. 25, no. 2, pp. 250-258.



International Agency for Research on Cancer *(IARC).* 2000, "Monographs programme on the evaluation of carcinogenic risks to humans".

Ito, I., Nagai, S., Handa, T., Muro, S., Hirai, T., Tsukino, M. & Mishima, M. 2005, "Matrix metalloproteinase-9 promoter polymorphism associated with upper lung dominant emphysema", *American Journal of Respiratory and Critical Care Medicine,* vol. 172, no. 11, pp. 1378-1382.

Jacob, P.J., Benowitz, N.L. & Shulgin, A.T. 1988, "Recent studies of nicotine metabolism in humans", *Pharmacology Biochemistry and Behavior,* vol. 30, no. 1, pp. 249-253.

Janoff, A., Chanana, A.D. & Joel, D.D. 1983, "Evaluation of the urinary desmosine radioimmunoassay as a monitor of lung injury after endobronchial elastase instillation in sheep", *American Review of Respiratory Disease,* vol. 128, no. 3, pp. 545-551.

Janssens, J.P., Pache, J.C. & Nicod, L.P. 1999, "Physiological changes in respiratory function associated with ageing", *European Respiratory Journal,* vol. 13, no. 1, pp. 197-205.

Jeffery, P.K. 1998, "The development of large and small airways", American Journal of Respiratory and Critical Care Medicine, vol. 157, no. 5 II SUPPL., pp. S174-S180.



Jialal, I. & Devaraj, S. 1996, "Low-density lipoprotein oxidation, antioxidants, and atherosclerosis: A clinical biochemistry perspective", *Clinical chemistry,* vol. 42, no. 4, pp. 498-506.

Joshi, S. & Kotecha, S. 2007, "Lung growth and development", *Early human development,* vol. 83, no. 12, pp. 789-794.

Kadunce, D.P., Burr, R., Gress, R., Kanner, R., Lyon, J.L. & Zone, J.J. 1991, "Cigarette smoking: Risk factor for premature facial wrinkling", Annals of Internal Medicine, vol. 114, no. 10, pp. 840-844.

Kaplan, L.A., Lau, J.M. & Stein, E.A. 1990, "Carotenoid composition, concentrations, and relationships in various human organs", *Clinical physiology and biochemistry,* vol. 8, no. 1, pp. 1-10.

Karrasch, S., Holz, O. & Jörres, R.A. 2008, "Aging and induced senescence as factors in the pathogenesis of lung emphysema", *Respiratory medicine,* vol. 102, no. 9, pp. 1215-1230.

Kasagi, S., Seyama, K., Mori, H., Souma, S., Sato, T., Akiyoshi, T., Suganuma, H. & Fukuchi, Y. 2006, "Tomato juice prevents senescence-accelerated mouse P1 strain from developing emphysema induced by chronic exposure to tobacco smoke", *American Journal of Physiology - Lung Cellular and Molecular Physiology,* vol. 290, no. 2, pp. L396-L404.

Kim, K.S. & Ivy, A.C. 1952, "Factors influencing cholesterol absorption", *Am J Physiol,* vol. 171, pp. 302-318.

Kleinsasser, N.H., Sassen, A.W., Semmler, M.P., Harréus, U.A., Licht, A.-. & Richter, E. 2005, "The tobacco alkaloid nicotine demonstrates genotoxicity in human tonsillar tissue and lymphocytes", *Toxicological Sciences,* vol. 86, no. 2, pp. 309-317.

Kondoh, H., Lleonart, M.E., Bernard, D. & Gil, J. 2007, "Protection from oxidative stress by enhanced glycolysis; a possible mechanism of cellular immortalization", *Histology and histopathology,* vol. 22, no. 1-3, pp. 85-90.

Kondoh, H., Lleonart, M.E., Gil, J., Beach, D. & Peters, G. 2005, "Glycolysis and cellular immortalization", *Drug Discovery Today: Disease Mechanisms,* vol. 2, no. 2, pp. 263-267.

Kotecha, S. 2000, "Lung growth: Implications for the newborn infant", *Archives of Disease in Childhood: Fetal and Neonatal Edition,* vol. 82, no. 1, pp. F69-F74.

Kovar, J., Sly, P.D. & Willet, K.E. 2002, "Postnatal alveolar development of the rabbit", Journal of applied physiology, vol. 93, no. 2, pp. 629-635.

Kramer, M.S. 1987, "Intrauterine growth and gestational duration determinants", *Pediatrics,* vol. 80, no. 4, pp. 502-511.



Lang, J.D., McArdle, P.J., O'Reilly, P.J. & Matalon, S. 2002, "Oxidant-antioxidant balance in acute lung injury", *Chest,* vol. 122, no. 6 SUPPL., pp. 314S-320S.

Lannan, S., Donaldson, K., Brown, D. & MacNee, W. 1994, "Effect of cigarette smoke and its condensates on alveolar epithelial cell injury in vitro", *American Journal of Physiology - Lung Cellular and Molecular Physiology,* vol. 266, no. 1 10-1, pp. L92-L100.

Lavezzi, A.M., Ottaviani, G. & Matturri, L. 2005, "Adverse effects of prenatal tobacco smoke exposure on biological parameters of the developing brainstem", *Neurobiology of disease,* vol. 20, no. 2, pp. 601-607.

Lawrence, J., Xiao, D., Xue, Q., Rejali, M., Yang, S. & Zhang, L. 2008, "Prenatal nicotine exposure increases heart susceptibility to ischemia/reperfusion injury in adult offspring", *Journal of Pharmacology and Experimental Therapeutics,* vol. 324, no. 1, pp. 331-341.

Lee, A.C., Fenster, B.E., Ito, H., Takeda, K., Bae, N.S., Hirai, T., Yu, Z.-., Ferrans, V.J., Howard, B.H. & Finkel, T. 1999, "Ras proteins induce senescence by altering the intracellular levels of reactive oxygen species", *Journal of Biological Chemistry,* vol. 274, no. 12, pp. 7936-7940.

Lieber, M.R. & Karanjawala, Z.E. 2004, "Ageing, repetitive genomes and DNA damage", *Nature Reviews Molecular Cell Biology,* vol. 5, no. 1, pp. 69-75.

Luck, W. & Nau, H. 1984, "Nicotine and cotinine concentrations in serum and milk of nursing smokers", *British journal of clinical pharmacology,* vol. 18, no. 1, pp. 9-15.

Luck, W., Hansen, R., Steldinger, R. & Nau, H. 1982, "Nicotine and Cotinine - two pharmacologically active substances as parameters for the strain on fetuses and babies of mothers who smoke", *Journal of perinatal medicine,* vol. 10, no. Suppl. 2, pp. 107-108.

Luck, W., Nau, H., Hansen, R. & Steldinger, R. 1985, "Extent of nicotine and cotinine transfer to the human fetus, placenta and amniotic fluid of smoking mothers", *Developmental pharmacology and therapeutics,* vol. 8, no. 6, pp. 384-395.

MacNee, W. 2000, "Oxidants/antioxidants and COPD", *Chest,* vol. 117, no. 5 SUPPL. 1, pp. 303S-317S.

MacNee, W. 2009, "Accelerated lung aging: A novel pathogenic mechanism of chronic obstructive pulmonary disease (COPD)", *Biochemical Society transactions,* vol. 37, no. 4, pp. 819-823.

Mannino, D.M., Homa, D.M., Akinbami, L.J., Ford, E.S. & Redd, S.C. 2002, "Chronic obstructive pulmonary disease surveillance--United States, 1971-2000.", *Respiratory care,* vol. 47, no. 10, pp. 1184-1199.



Maritz, G. 1986, "Pre- and postnatal carbohydrate metabolism of rat lung tissue. The effect of maternal nicotine exposure", *Archives of Toxicology,* vol. 59, no. 2, pp. 89-93.

Maritz, G.S. & Dennis, H. 1998, "Maternal nicotine exposure during gestation and lactation interferes with alveolar development in the neonatal lung", *Reproduction, Fertility and Development,* vol. 10, no. 3, pp. 255-261.

Maritz, G.S. & Thomas, R. 1994, "The influence of maternal nicotine exposure on the interalveolar septal status of neonatal rat lung", *Cell biology international,* vol. 18, no. 7, pp. 747-757.

Maritz, G.S. & Windvogel, S. 2003, "Chronic maternal nicotine exposure during gestation and lactation and the development of the lung parenchyma in the offspring: Response to nicotine withdrawal", *Pathophysiology,* vol. 10, no. 1, pp. 69-75.

Maritz, G.S. & Windvogel, S. 2005, "Does maternal nicotine exposure during different phases of lung development influence the program that regulates the maintenance of lung integrity in the offspring? A comparative morphologic and morphometric study", *Trends Comp Biochem Physiol,* vol. 11, pp. 63-73.

Maritz, G.S. 1987, "Maternal nicotine exposure and carbohydrate metabolism of fetal and neonatal lung tissue: Response to nicotine withdrawal", *Respiration,* vol. 51, no. 3, pp. 232-240.

Maritz, G.S. 1996, "Ascorbic acid. Protection of lung tissue against damage.", *Sub-cellular biochemistry,* vol. 25, pp. 265-291.

Maritz, G.S. 2008, "Maternal nicotine exposure: Long-term effect on lung integrity in the offspring", *Current Women's Health Reviews,* vol. 4, no. 3, pp. 153-161.

Maritz, G.S., Morley, C.J. & Harding, R. 2005, "Early developmental origins of impaired lung structure and function", *Early human development,* vol. 81, no. 9, pp. 763-771.

Maritz, G.S., Woolward, K.M. & Du Toit, G. 1993, "Maternal nicotine exposure during pregnancy and development of emphysema-like damage in the offspring", *South African Medical Journal,* vol. 83, no. 3, pp. 195-198.

Markovic, K., Hruškar, M. & Vahčić, N. 2006, "Lycopene content of tomato products and their contribution to the lycopene intake of Croatians", Nutrition Research, vol. 26, no. 11, pp. 556-560.

Mason, R.J. & Williams, M.C. 1977, "Type II alveolar cell. Defender of the alveolus", *American Review of Respiratory Disease,* vol. 115, no. 6 II, pp. 81-91.

Maus, A.D.J., Pereira, E.F.R., Karachunski, P.I., Horton, R.M., Navaneetham, D., Macklin, K., Cortes, W.S., Albuquerque, E.X. & Conti-Fine, B.M. 1998, "Human and rodent bronchial epithelial cells express functional nicotinic acetylcholine receptors", *Molecular pharmacology,* vol. 54, no. 5, pp. 779-788.

Mayne, S.T. 2003, "Antioxidant nutrients and chronic disease: Use of biomarkers of exposure and oxidative stress status in epidemiologic research", *Journal of Nutrition,* vol. 133, no. 3 SUPPL., pp. 933S-940S.

McCusker, K. 1992, "Mechanisms of respiratory tissue injury from cigarette smoking", *American Journal of Medicine,* vol. 93, no. 1 SUPPL.1, pp. 1A-18S-1A-21S.

Mercer, R.R. & Crapo, J.D. 1992, "Structural changes in elastic fibers after pancreatic elastase administration in hamsters", *Journal of applied physiology,* vol. 72, no. 4, pp. 1473-1479.

Mesas-Burgos, C., Nord, M., Didon, L., Eklof, A.-. & Frenckner, B. 2009, "Gene expression analysis after prenatal tracheal ligation in fetal rat as a model of stimulated lung growth", *Journal of pediatric surgery,* vol. 44, no. 4, pp. 720-728.

Midorikawa, K., Hirakawa, K. & Kawanishi, S. 2002, "Hydroxylation of deoxyguanosine at 5′ site of GG and GGG sequences in double-stranded DNA induced by carbamoyl radicals", *Free radical research,* vol. 36, no. 6, pp. 667-675.



Montgomery, S.M. & Ekbom, A. 2002, "Smoking during pregnancy and diabetes mellitus in a British longitudinal birth cohort", *British medical journal,* vol. 324, no. 7328, pp. 26-27.

Morbidity and mortality weekly report, 1989. "The Surgeon General's Report on Reducing the Health Consequences of Smoking: 25 Years of Progress.", 1989, *MMWR.,* vol. 38 Suppl 2, pp. 1-32.

Morla, M., Busquets, X., Pons, J., Sauleda, J., MacNee, W. & Agustí, A.G.N. 2006, "Telomere shortening in smokers with and without COPD", *European Respiratory Journal,* vol. 27, no. 3, pp. 525-528.

Müller, K.-., Welker, L., Paasch, K., Feindt, B., Erpenbeck, V.J., Hohlfeld, J.M., Krug, N., Nakashima, M., Branscheid, D., Magnussen, H., Jörres, R.A. & Holz, O. 2006, "Lung fibroblasts from patients with emphysema showmarkers of senescence in vitro", *Respiratory Research,* vol. 7.

Murphy, P.J. 1973, "Enzymatic oxidation of nicotine to nicotine 1'(5') iminium ion. A newly discovered intermediate in the metabolism of nicotine.", *Journal of Biological Chemistry,* vol. 248, no. 8, pp. 2796-2800.

Murphy, S.L. 2000, "Deaths: final data for 1998.", National vital statistics reports : from the Centers for Disease Control and Prevention, National Center for Health Statistics, National Vital Statistics System, vol. 48, no. 11, pp. 1-105.

Murrin, L.C., Ferrer, J.R., Wanyun, Z. & Haley, N.J. 1987, "Nicotine administration to rats: Methodological considerations", *Life Sciences,* vol. 40, no. 17, pp. 1699-1708.

Musellim, B., Ikitimur, H., Uzun, H. & Ongen, G. 2006, "The oxidant-antioxidant balance in systemic sclerosis cases with interstitial lung involvement", *Rheumatology international,* vol. 27, no. 2, pp. 163-167.

Nalapareddy, K., Jiang, H., Gutierrez, L.M.G. & Rudolph, K.L. 2008, "Determining the influence of telomere dysfunction and DNA damage on stem and progenitor cell aging - what markers can we use?", *Experimental gerontology,* vol. 43, no. 11, pp. 998-1004.

News and Views, August 2007. "Nuclear medicine communications", vol. 28, no. 8, pp. 673.

Ng, S.P. & Zelikoff, J.T. 2007, "Smoking during pregnancy: Subsequent effects on offspring immune competence and disease vulnerability in later life", *Reproductive Toxicology,* vol. 23, no. 3, pp. 428-437.

NSW Health 2006 Report of the NSW Chief Health Officer, Sydney: NSW Department of Health, 2006. Available at http://www.health.nsw.gov.au/public-health/chorep/. Accessed 11 November 2009.



Obermueller-Jevic, U.C., Espiritu, I., Corbacho, A.M., Cross, C.E. & Witschi, H. 2002, "Lung tumor development in mice exposed to tobacco smoke and fed β-carotene diets", *Toxicological Sciences,* vol. 69, no. 1, pp. 23-29.

Oken, E., Huh, S.Y., Taveras, E.M., Rich-Edwards, J.W. & Gillman, M.W. 2005, "Associations of maternal prenatal smoking with child adiposity and blood pressure", *Obesity research,* vol. 13, no. 11, pp. 2021-2028.

Oldendorf, W.H. 1974, "Lipid solubility and drug penetration of the blood brain barrier", *Proceedings of the Society for Experimental Biology and Medicine,* vol. 147, no. 3, pp. 813-816.

Oldendorf, W.H., Stoller, B.E. & Harris, F.L. 1993, "Blood-brain barrier penetration abolished by N-methyl quaternization of nicotine", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 90, no. 1, pp. 307-311.

Ornitz, D.M., Xu, J., Colvin, J.S., McEwen, D.G., MacArthur, C.A., Coulier, F., Gao, G. & Goldfarb, M. 1996, "Receptor specificity of the fibroblast growth factor family", Journal of Biological Chemistry, vol. 271, no. 25, pp. 15292-15297.

Otterbein, L.E., Chin, B.Y., Mantell, L.L., Stansberry, L., Horowitz, S. & Choi, A.M.K. 1998, "Pulmonary apoptosis in aged and oxygen-tolerant rats exposed to hyperoxia", American Journal of Physiology - Lung Cellular and Molecular Physiology, vol. 275, no. 1 19-1, pp. L14-L20.

Page-Sharp, M., Hale, T.W., Hackett, L.P., Kristensen, J.H. & Ilett, K.F. 2003, "Measurement of nicotine and cotinine in human milk by high-performance liquid chromatography with ultraviolet absorbance detection", *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences,* vol. 796, no. 1, pp. 173-180.

Parrinello, S., Samper, E., Krtolica, A., Goldstein, J., Melov, S. & Campisi, J. 2003, "Oxygen sensitivity severely limits the replicative lifespan of murine fibroblasts", *Nature cell biology,* vol. 5, no. 8, pp. 741-747.

Parthasarathy, S., Santanam, N. & Augé, N. 1998, "Oxidized low-density lipoprotein, a two-faced janus in coronary artery disease?", *Biochemical pharmacology,* vol. 56, no. 3, pp. 279-284.

Partridge, L. & Mangel, M. 1999, "Messages from mortality: The evolution of death rates in the old", *Trends in Ecology and Evolution,* vol. 14, no. 11, pp. 438-442.

Patel, S.M., Ratcliffe, S.J., Reilly, M.P., Weinstein, R., Bhasin, S., Blackman, M.R., Cauley, J.A., Sutton-Tyrrell, K., Robbins, J., Fried, L.P. & Cappola, A.R. 2009, "Higher Serum Testosterone Concentration in Older Women is Associated with Insulin Resistance, Metabolic Syndrome, and Cardiovascular Disease", *Journal of Clinical Endocrinology Metabolism,* .

Pauwels, R.A., Buist, A.S., Calverley, P.M.A., Jenkins, C.R. & Hurd, S.S. 2001, "Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease: NHLBI/WHO Global Initiative for Chronic Obstructive Lung Disease (GOLD) workshop summary", *American Journal of Respiratory and Critical Care Medicine,* vol. 163, no. 5, pp. 1256-1276.

Perkins, K.A., Sexton, J.E., Reynolds, W.A., Grobe, J.E., Fonte, C. & Stiller, R.L. 1994, "Comparison of acute subjective and heart rate effects of nicotine intake

via tobacco smoking versus nasal spray", *Pharmacology Biochemistry and Behavior,* vol. 47, no. 2, pp. 295-299.

Pierce, J.A., Hocott, J.B. & Hefley, B.F. 1961, "Elastic properties and the geometry of the lungs", *J Clin Invest,* vol. 40, pp. 1516-1524.

Pinkerton, K.E. & Green, F.H.Y. 2004, "Normal aging of the lung", *The Lung: development, aging and the environment,* .

Plummer, H.K., Dhar, M. & Schuller, H.M. 2005, "Expression of the α7 nicotinic acetylcholine receptor in human lung cells", *Respiratory Research,* vol. 6.

Pogocki, D., Ruman, T., Danilczuk, M., Danilczuk, M., Celuch, M. & Wałajtys-Rode, E. 2007, "Application of nicotine enantiomers, derivatives and analogues in therapy of neurodegenerative disorders", European journal of pharmacology, vol. 563, no. 1-3, pp. 18-39.



Pontieri, F.E., Tanda, G., Orzi, F. & Di Chiara, G. 1996, "Effects of nicotine on the nucleus accumbens and similarity to those of addictive drugs", *Nature,* vol. 382, no. 6588, pp. 255-257.

Prescott, J. & Blackburn, E.H. 1997, "Functionally interacting telomerase RNAs in the yeast telomerase complex", *Genes and Development,* vol. 11, no. 21, pp. 2790-2800.

Proskocil, B.J., Sekhon, H.S., Clark, J.A., Lupo, S.L., Jia, Y., Hull, W.M., Whitsett, J.A., Starcher, B.C. & Spindel, E.R. 2005, "Vitamin C prevents the

effects of prenatal nicotine on pulmonary function in newborn monkeys", *American Journal of Respiratory and Critical Care Medicine,* vol. 171, no. 9, pp. 1032-1039.

Pryor, W.A., Hales, B.J., Premovic, P.I. & Church, D.F. 1983, "The radicals in cigarette tar: Their nature and suggested physiological implications", Science, vol. 220, no. 4595, pp. 425-427.

Quinn, R. 2005, "Comparing rat's to human's age: How old is my rat in people years?", Nutrition, vol. 21, no. 6, pp. 775-777.

Raherison, C., Pénard-Morand, C., Moreau, D., Caillaud, D., Charpin, D., Kopfersmitt, C., Lavaud, F., Taytard, A. & Annesi-maesano, I. 2007, "In utero and childhood exposure to parental tobacco smoke, and allergies in schoolchildren", *Respiratory medicine,* vol. 101, no. 1, pp. 107-117.



Rahman, I. & MacNee, W. 1999, "Lung glutathione and oxidative stress: Implications in cigarette smoke-induced airway disease", *American Journal of Physiology - Lung Cellular and Molecular Physiology,* vol. 277, no. 6 21-6, pp. L1067-L1088.

Rahman, I., Biswas, S.K. & Kode, A. 2006, "Oxidant and antioxidant balance in the airways and airway diseases", *European journal of pharmacology,* vol. 533, no. 1-3, pp. 222-239.

Rahman, I., Marwick, J. & Kirkham, P. 2004, "Redox modulation of chromatin remodeling: Impact on histone acetylation and deacetylation, NF-κB and pro-inflammatory gene expression", *Biochemical pharmacology,* vol. 68, no. 6, pp. 1255-1267.

Rangasamy, T., Cho, C.Y., Thimmulappa, R.K., Zhen, L., Srisuma, S.S., Kensler, T.W., Yamamoto, M., Petrache, I., Tuder, R.M. & Biswal, S. 2004, "Genetic ablation of Nrf2 enhances susceptibility to cigarette smoke-induced emphysema in mice", *Journal of Clinical Investigation,* vol. 114, no. 9, pp. 1248-1259.

Rao, A.V. & Agarwal, S. 1999, "Role of lycopene as antioxidant carotenoid in the prevention of chronic diseases: A review", *Nutrition Research,* vol. 19, no. 2, pp. 305-323.

Rehan, V.K., Wang, Y., Sugano, S., Santos, J., Patel, S., Sakurai, R., Boros, L.W., Lee, W. & Torday, J.S. 2007, "In utero nicotine exposure alters fetal rat lung alveolar type II cell proliferation, differentiation, and metabolism", *American Journal of Physiology - Lung Cellular and Molecular Physiology,* vol. 292, no. 1, pp. L323-L333.

Rennard, S.I. 1999, "Inflammation and repair processes in chronic obstructive pulmonary disease", *American Journal of Respiratory and Critical Care Medicine,* vol. 160, no. 5 II, pp. S12-S16.

Rosenberg, J., Benowitz, N.L., Jacob, P. & Wilson, K.M. 1980, "Disposition kinetics and effects of intravenous nicotine", *Clinical pharmacology and therapeutics,* vol. 28, no. 4, pp. 517-522.

Ruiz, C.A.J. 2006, "Nicotine Replacement Therapy During Pregnancy", *Archivos de Bronconeumologia,* vol. 42, no. 8, pp. 404-409.

Rylander, E., Pershagen, G., Eriksson, M. & Bermann, G. 1995, "Parental smoking, urinary cotinine, and wheezing bronchitis in children", *Epidemiology,* vol. 6, no. 3, pp. 289-293.

Salihu, H.M. & Wilson, R.E. 2007, "Epidemiology of prenatal smoking and perinatal outcomes", *Early human development,* vol. 83, no. 11, pp. 713-720.

Sato, S., Kaneko, T., Nakamura, Y., Asamizu, E., Kato, T. & Tabata, S. 2001, "Structural analysis of a Lotus japonicus genome. I. Sequence features and mapping of fifty-six TAC clones which cover the 5.4 Mb regions of the genome", *DNA Research,* vol. 8, no. 6, pp. 311-318.

Scherle, W. 1970, "A simple method for volumetry of organs in quantitative stereology.", *Mikroskopie,* vol. 26, no. 1, pp. 57-60.

Schwartz, D., Goujard, J., Kaminski, M. & Rumeau-Rouquette, C. 1972, "Smoking and pregnancy. Results of a prospective study of 6,989 women.", *Revue europeenne d''etudes cliniques et biologiques.European journal of clinical and biological research,* vol. 17, no. 9, pp. 867-879.

Sekhon, H.S., Proskocil, B.J., Clark, J.A. & Spindel, E.R. 2004, "Prenatal nicotine exposure increases connective tissue expression in foetal monkey pulmonary vessels", *European Respiratory Journal,* vol. 23, no. 6, pp. 906-915.

Sekhon, H.S., Yibing, J., Raab, R., Kuryatov, A., Pankow, J.F., Whitsett, J.A., Lindstrom, J. & Spindel, E.R. 1999, "Prenatal nicotine increases pulmonary α7 nicotinic receptor expression and alters fetal lung development in monkeys", *Journal of Clinical Investigation,* vol. 103, no. 5, pp. 637-647.

Seller, M.J. & Bnait, K.S. 1995, "Effects of tobacco smoke inhalation on the developing mouse embryo and fetus", *Reproductive Toxicology,* vol. 9, no. 5, pp. 449-459.

Shannon, J.M. & Hyatt, B.A. 2004, "Epithelial-mesenchymal interactions in the developing lung".



Shapiro, S.D. 1995, "The pathogenesis of emphysema: the elastase:antielastase hypothesis 30 years later.", *Proceedings of the Association of American Physicians,* vol. 107, no. 3, pp. 346-352.

Shi, H. & Clegg, D.J. 2009, "Sex differences in the regulation of body weight", *Physiology and Behavior,* vol. 97, no. 2, pp. 199-204.

Silagy, C., Mant, D., Fowler, G. & Lancaster, T. 2000, "Nicotine replacement therapy for smoking cessation.", *Cochrane database of systematic reviews (Online : Update Software),* , no. 2.

Snider, G.L., Kleinerman, J., Thurlbeck, W.M. & Bengali, Z.K. 1985, "The definition of emphysema: Report of a national heart, lung and blood institute, division of lung diseases, workshop", *Am.Rev.Respir.Dis,* vol. 182-185.

Spector, R. & Goldberg, M.J. 1982, "Active transport of nicotine by the isolated choroid plexus in vitro", *Journal of neurochemistry,* vol. 38, no. 2, pp. 594-596.

Sprung, J., Whalley, D.G., Falcone, T., Wilks, W., Navratil, J.E. & Bourke, D.L. 2003, "The effects of tidal volume and respiratory rate on oxygenation and respiratory mechanics during laparoscopy in morbidly obese patients", Anesthesia and Analgesia, vol. 97, no. 1, pp. 268-274.

Stadtman, E.R. 1992, "Protein oxidation and aging", *Science,* vol. 257, no. 5074, pp. 1220-1224.

Starcher, B. & Williams, I. 1989, "The beige mouse: Role of neutrophil elastase in the development of pulmonary emphysema", *Experimental lung research,* vol. 15, no. 5, pp. 785-800.

Stedman, R.L. 1968, "The chemical composition of tobacco and tobacco smoke", *Chemical reviews,* vol. 68, no. 2, pp. 153-207.

Stein, R.T., Holberg, C.J., Sherrill, D., Wright, A.L., Morgan, W.J., Taussig, L. & Martinez, F.D. 1999, "Influence of parental smoking on respiratory symptoms during the first decade of life: The Tucson Children's Respiratory Study", *American Journal of Epidemiology,* vol. 149, no. 11, pp. 1030-1037.

Stevenson, D.K., Wright, L.L., Lemons, J.A., Oh, W., Korones, S.B., Papile, L., Bauer, C.R., Stoll, B.J., Tyson, J.E., Shankaran, S., Fanaroff, A.A., Donovan, E.F., Ehrenkranz, R.A. & Verter, J. 1998, "Very low birth weight outcomes of the National Institute of Child Health and Human Development Neonatal Research Network, January 1993 through December 1994," *American Journal of Obstetrics and Gynecology,* vol. 179, no. 6, pp. 1632-1639.

Swanson, K.S., Vester, B.M., Apanavicius, C.J., Kirby, N.A. & Schook, L.B. 2009, "Implications of age and diet on canine cerebral cortex transcription", Neurobiology of aging, vol. 30, no. 8, pp. 1314-1326.

Takeoka, G.R., Dao, L., Flessa, S., Gillespie, D.M., Jewell, W.T., Huebner, B., Bertow, D. & Ebeler, S.E. 2001, "Processing effects on lycopene content and antioxidant activity of tomatoes", *Journal of Agricultural and Food Chemistry,* vol. 49, no. 8, pp. 3713-3717.



Tanaka, H., Ono, Y., Nakata, S., Shintani, Y., Sakakibara, N. & Morita, A. 2007, "Tobacco smoke extract induces premature skin aging in mouse", Journal of dermatological science, vol. 46, no. 1, pp. 69-71.

Tanswell, A.K. & Freeman, B.A. 1984, "Pulmonary antioxidant enzyme maturation in the fetal and neonatal rat. I. Developmental profiles", *Pediatric research,* vol. 18, no. 7, pp. 584-587.

Tenney, S.M. & Remmers, J.E. 1963, "Comparative quantitative morphology of the mammalian lung: Diffusing area", *Nature,* vol. 197, no. 4862, pp. 54-56.

Thurlbeck, W.M. 1982, "Postnatal human lung growth", *Thorax,* vol. 37, no. 8, pp. 564-571.

Thurlbeck, W.M. 1992, "Prematurity and the developing lung", *Clinics in perinatology,* vol. 19, no. 3, pp. 497-519.

Tsuji, T., Aoshiba, K. & Nagai, A. 2004, "Cigarette smoke induces senescence in alveolar epithelial cells", *American Journal of Respiratory Cell and Molecular Biology,* vol. 31, no. 6, pp. 643-649.

Tuder, R.M., Yoshida, T., Arap, W., Pasqualini, R. & Petrache, I. 2006, "Cellular and molecular mechanisms of alveolar destruction in emphysema: An evolutionary perspective", *Proceedings of the American Thoracic Society,* vol. 3, no. 6, pp. 503-510.

Tuder, R.M., Zhen, L., Cho, C.Y., Taraseviciene-Stewart, L., Kasahara, Y., Salvemini, D., Voelkel, N.F. & Flores, S.C. 2003, "Oxidative stress and apoptosis interact and cause emphysema due to vascular endothelial growth factor receptor blockade", *American Journal of Respiratory Cell and Molecular Biology,* vol. 29, no. 1, pp. 88-97.

Tutka, P., Mosiewicz, J. & Wielosz, M. 2005, "Pharmacokinetics and metabolism of nicotine", *Pharmacological Reports,* vol. 57, no. 2, pp. 143-153.

Van Vunakis, H., Langone, J.J. & Milunsky, A. 1974, "Nicotine and cotinine in the amniotic fluid of smokers in the second trimester of pregnancy", *American Journal of Obstetrics and Gynecology,* vol. 120, no. 1, pp. 64-66.

Verbeken, E.K., Cauberghs, M., Mertens, I., Clement, J., Lauweryns, J.M. & Van de Woestijne, K.P. 1992, "The senile lung; Comparison with normal and emphysematous lungs. 1. Structural aspects", *Chest,* vol. 101, no. 3, pp. 793-799.

Vijg, J. 2008, "The role of DNA damage and repair in aging: New approaches to an old problem", *Mechanisms of ageing and development,* vol. 129, no. 7-8, pp. 498-502.

Wang, H., Liu, X., Umino, T., Sköld, C.M., Zhu, Y., Kohyama, T., Spurzem, J.R., Romberger, D.J. & Rennard, S.I. 2001, "Cigarette smoke inhibits human bronchial epithelial cell repair processes", *American Journal of Respiratory Cell and Molecular Biology,* vol. 25, no. 6, pp. 772-779.

Weibel, E.R. 1963, "Principles and methods for the morphometric study of the lung and other organs", *Lab Invest,* vol. 12, pp. 131-155.

Woods, S.C., Seeley, R.J., Porte Jr., D. & Schwartz, M.W. 1998, "Signals that regulate food intake and energy homeostasis", *Science,* vol. 280, no. 5368, pp. 1378-1383.

Wright, J.L. & Churg, A. 2007, "Current concepts in mechanisms of emphysema", *Toxicologic pathology,* vol. 35, no. 1, pp. 111-115.

Yam, M.H., Egan, P.O., Frieze, W.E. & Hughes, V.W. 1978, "Positronium fine-structure interval Δv in oxide powders", *Physical Review A,* vol. 18, no. 2, pp. 350-353.

Yilmaz, A., Adigüzel, U., Tamer, L., Yildirim, O., Oz, O., Vatansever, H., Ercan, B., Değirmenci, U. & Atik, U. 2005, "Serum oxidant/antioxidant balance in exfoliation syndrome", *Clinical and Experimental Ophthalmology,* vol. 33, no. 1, pp. 63-66.

Yin, L., Morita, A. & Tsuji, T. 2001, "Skin aging induced by ultraviolet exposure and tobacco smoking: Evidence from epidemiological and molecular studies", Photodermatology Photoimmunology and Photomedicine, vol. 17, no. 4, pp. 178-183.



Yokohori, N., Aoshiba, K., Nagai, A. & Kuriyama, T. 2004, "Increased Levels of Cell Death and Proliferation in Alveolar Wall Cells in Patients with Pulmonary Emphysema", *Chest,* vol. 125, no. 2, pp. 626-632.