PREMATURE AGING OF THE LUNGS OF THE OFFSPRING INDUCED BY MATERNAL NICOTINE EXPOSURE DURING GESTATION AND LACTATION: PROTECTIVE EFFECTS OF TOMATO JUICE

BY

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A thesis submitted in partial fulfillment of the requirements for the degree of Doctor Philosophiae, in the Department of Medical Biosciences, University of the Western Cape.

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KEYWORDS

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Tomato Juice

Lycopene

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Transgeneration

ABSTRACT

PREMATURE AGING OF THE LUNGS OF THE OFFSPRING INDUCED BY MATERNAL NICOTINE EXPOSURE DURING GESTATION AND LACTATION: PROTECTIVE EFFECTS OF TOMATO JUICE

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PhD thesis in the Department of Medical Biosciences, University of the Western Cape.

Tobacco smoking during pregnancy and lactation is a common habit and accounts for a significant percentage of fetal morbidity and mortality worldwide. The offspring is as a result exposed to nicotine through the blood and the milk of the mother. Nicotine is thus expected to interact with the developing fetus and the offspring of mothers who smoke or use NRT for smoking cessation, resulting in the interference with normal fetal and neonatal 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. This study aimed at investigating the effects of maternal nicotine exposure during gestation and lactation on lung development in the offspring; to establish whether tomato juice can have protective effects on the fetal lung development and function in the offspring; and to determine if nicotine cases premature aging of the lungs of the offspring. It was therefore shown that maternal exposure to nicotine during gestation and lactation

had no significant effect on the growth parameters of the offspring. Maternal nicotine exposure during gestation and lactation had no effect on the growth parameters of the offspring, but resulted in compromised lung structure and function. The morphometric results demonstrated decrease in alveolar number, increase in alveolar size, and decrease in lung parenchyma of the nicotine exposed animals showing a gradual deterioration of the lung parenchyma. Structural alterations include emphysematous lesions, where the latter was accompanied by an increase in alveolar size (Lm), and a decrease in the tissue volume of the lung parenchyma. Thickening of alveolar walls was also evident and serves as an indication of remodeling of the extracellular matrix, also a characteristic of emphysema. A consequence of the gradual deterioration of the lung parenchyma is a decrease in the alveolar surface area available for gas exchange. The present study showed that the emphysematous lesions were conceivably a result of a reduced rate of cell proliferation accompanied by the increase in senescent cells numbers in the alveolar walls of the exposed offspring. The data of this study suggests that maternal nicotine exposure during gestation and lactation induces premature aging of the lungs of the offspring rendering the lungs of the offspring more susceptible to disease later in life. Since these structural changes occurred later in the life of the offspring and long after nicotine withdrawal, it is suggested that it is programmed during gestation and lactation.

Smoking and NRT result in an increased load of oxidants in the mother and fetus. It also reduces the level of anti-oxidants and thereby compromising the ability of the mother to protect the fetus. It is hypothesized that this oxidant-antioxidant imbalance will program the lungs to age prematurely. The supplementation of the mother's diet with tomato juice, rich in lycopene, other anti-oxidants such as vitamin C, as well as phytonutrients protected the lungs of the offspring against the adverse effects of maternal nicotine exposure. This supports the hypothesis mentioned above.

The study further showed that the effects of grand-maternal nicotine exposure during gestation and lactation on the lungs of the F1 offspring is also transferred to the F2 offspring. This is most likely via the paternal and maternal germ line. Since tomato juice supplementation of the mother's diet with tomato juice prevented the adverse effects of maternal nicotine exposure on the lungs of the offspring, it is conceivable that it will prevent transfer of these changes to the F2 generation.

November 2012

DECLARATION

I declare that *Premature aging of the lungs of the offspring induced by maternal nicotine exposure during gestation and lactation: protective effects of tomato juice* 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 or quoted have been indicated and acknowledged as complete references.

Muyunda Mutemwa November 2012

Signed.....

DEDICATIONS

This thesis is dedicated to my supervisor Professor Gert S. Maritz for the influence he has had on my academic life and for the opportunity he afforded me of learning from him through his mentorship and supervision. It is also dedicated to my parents, Pastor Kwalela Mutemwa and Mrs. Priscah S Mutemwa whose never ceasing encouragement and support accompanied me throughout my studies.

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PUBLICATIONS ARISING FROM THIS THESIS

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2. Maritz GS**, Mutemwa M.** The effect of grand maternal nicotine exposure during gestation and lactation on the lung integrity of the F2 generation. *Pediatric Pulmonol.*

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LIST OF ABBREVIATIONS

α	Alpha
β	Beta
γ	Gamma
μ	Micro
π	Pi = 22/7
%	Percent
$\cdot O_2^-$	Oxygen free radical
° C	Degrees Celsius
3D	3-Dimensional
5-HT	5-hydroxytryptamine
ADH	Antidiuretic hormone
ALT	Alternative lengthening of telomeres
B	Alveolar shape constant
BMR	Basal Metabolic Rate
BW	Body Weight
CC	Chest circumference
CC/BW	Circumference to body weight ratio
CC/CRL	Crown rump length to body weight ratio
cm	Centimeter
CNS	Central nervous system
Con	Control

COPD	Chronic obstructive pulmonary disease
CRL	Crown rump length
Cst	Static lung compliance
DAPI	4',6-diamidino-2-phenylindole
DC	Dyskeratosis congenita
DMF	Dimethylformamide
DNA	2'-deoxy-5'-ribonucleic acid
EGF	Epidermal growth factor
ETS	Environmental tobacco smoke
F	Female
F0:	Mother exosed to nicotine during gestation and lactation
F1:	Offspring received nicotine via the placenta and mother's milk
F2:	Offspring from F1 generation.
FEV_1	Forced expiratory rate
FGF	Fibroblast growth factor
H &E	Haematoxylin and eosin
H_2O_2	Hydrogen peroxide
HNE	4-hydroxy-2-nonenal
HSPG	Heparan sulphate proteoglycans
IL	Interleukin
ISA	Internal surface area
IUGR	Intrauterine Growth Restriction

KCl	Potassium chloride
kDa	Kilodalton
kg	Kilogram
KH_2PO_4	Potassium phosphate monohydride
L	Length of traverses
LDL	Low density lipoproteins
Lm	Mean linear intercept
LOX	Lysyl oxidase
Lv	Lung volume
Lv/BW	Lung Volume to Body Weight Ratio
m RNA	Messenger ribosenucleic acid
M	Male
m	Metre
M	Molar concentration
MAGP	Microfibril-associated glycoprotein
MAP	Mitogen activating protein
MDA	Malondialdehyde
mg	Milligram
ml	Millilitre
mm	Millimetre
MMP	Macrophage metallomatrix protein
N	Number of fields
Na	Number of alveoli per unit area

Na	Number of alveoli per unit area
$Na_2HPO_4.7H_2O$	Sodium phosphate heptahydrate
nAChR	Nicotinic acetylcholine receptor
NfCm	Nicotine exposed female mated with F1 control male
NF-κβ	Nuclear factor kappa beta
ng	Nanogram
Nic +TJ	Nicotine and tomato juice
Nic	Nicotine
NmCf	Nicotine exposed male mated with F1 control female
NmNf	Male exposed to nicotine mated female exposed to nicotine
SIDS	Sudden infant death syndrome
NNK	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone
NRT	Nicotine replacement therapy
NRT	Nicotine replacement therapy
PBS	Phosphate Buffered Saline
PCNA	Proliferating Cell Nuclear Antigen
PDGF	Platelet derived growth factor
pH	Measure of the acidity of a solution
ROS	Reactive oxygen species
rTdT	Terminal Deoxynucleotidyl Transferase, Recombinant, enzyme
Sa	Internal surface area
SA-β-Gal	Beta galactosidase
SP	Surfactant protein

SSC	Stock solution consists
TGF-β	Transforming growth factor beta
TJ	Tomato juice
TNF	Tumor necrosis factor
T*sept*	Interalveolar septal thickness
Va	Volume density of airspaces
VEGF	Vascular endothelial growth factor
Vt	Tissue volume
WHO	World Health Organization
X-Gal	5-bromo-4-chloro-3-indolyl P3-D-galactoside

CHAPTER ONE

Literature Review

1.1 Introduction

Tobacco continues to be the second major cause of death in the world and remains to be the leading global cause of unnecessary deaths (Villalbi et al., 2010). The 2011 World Health Reports revealed that tobacco has caused approximately 6 million deaths and is responsible for hundreds of billions of dollars of economic damage worldwide each year. The report indicated that judging from the current progress, by the year 2030; tobacco will be responsible for more than 8 million deaths worldwide each year. By the end of the 21st century, the use of tobacco might further cause a billion deaths (W.H.O., 2011). Diseases associated by tobacco smoking causes deleterious effects on the productivity of individuals exposed to smoke since it is harmful and destructive to the health of smokers and those that maybe exposed to it and increases hospital admissions.

Tobacco smoke contains large amounts of compounds potentially cytotoxic, such as nicotine, thiocyanate, carcinogens, carbon, and certain gases (Stellman and Djordjevic, 2009) that have harmful effects on various systems including; the cardiovascular, respiratory, reproductive and nervous systems (Fowles and Bates, 2000). Various studies have also shown that some of the diseases brought about and/or aggravated by tobacco smoke are induced by nicotine, the addictive

component in tobacco (Maritz et al., 2011b; Petre et al., 2011). A number of laboratory studies have demonstrated that nicotine alters the metabolism (Bruin et al., 2008c; Maritz et al., 2011b) and structure of organs (Holloway et al., 2005; Maritz et al., 2011b; Sekhon et al., 2001c) and consequently a down regulation of organ function together with an increase in susceptibility to disease (Maritz and Windvogel, 2003).

1.2 Fetal Lung Development

1.2.1 Phases of lung development

Lung development begins around day 26 of gestation in humans, 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 (about 32 weeks) (Whittaker et al.). 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).

The embryonic phase of human lung development begins around the 4th week (day 26) of gestation in humans and continues until the 6th 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 (Cardoso, 2000; Ornitz et al., 1996). 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). 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 envelop 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 line the airways. These cells include serous, goblet, ciliated, clara and alveolar cells (Boyden, 1977; Crapo et al., 1980; Mason and Williams, 1977).

The canalicular phase commences around week 16 or 17 of gestation and carries on until the 25th to the 27th 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 epithelium 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 24th week of gestation (Kotecha, 2000). 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).

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).

1.2.2 Stages of vulnerability during lung development

Since the embryonic phase of lung development is a phase during which the lungs undergo of cellular differentiation and branching morphogenesis, it thus is a crucial period when significant changes in lung structure thus affecting its purpose for gaseous exchange in later life. The subsequent phases of lung development coupled with sustained structural and functional growth and maturation at this stage, rapidly dividing cells are easily influenced by pollutants. This may result in progressive modification in lung development (Holloway et al., 2005; Harding and Maritz, 2012; Dyban and Dyban, 2006). During the early phases of development the lungs are highly sensitive to the destructive effects of xenobiotics and oxidants. This is due to the fact that most of the enzymes that provide defensive against xenobiotic compounds are not yet developed. Studies show that the development of such enzymes will be predisposed to these environmental toxicants (Li, 2002). The resultant effects of foreign substances are dependent upon the phase of development at the time of the impairment (Reik et al., 2001).

The response of the lung and the result of impairment on the developing lung is greatly dependent upon the phase of lung development at the time of exposure (Sasaki and Matsui, 2008). Recent studies suggest that the developing lung is less susceptible to substances such as tobacco smoke prior to the saccular phase of lung development. However, the increase in sensitivity to environmental changes seems to take place from the saccular phase onward. These changes in lung development are have been shown to have long term structural and functional consequences (Harding and Maritz, 2012).

1.3 Maternal Exposure to tobacco smoke

Tobacco smoking during pregnancy is an on-going challenge to society despite numerous unfavorable effects on maternal and fetal health. While research continues to show that maternal cigarette smoking during pregnancy is the primary cause of fetal morbidity and mortality and obstetric disease, many pregnant women carry on smoking (Jimenez-Ruiz et al., 2006). Maternal smoking during gestation and lactation has been linked with both short and long-term health risks extending from intrauterine growth restriction to psychological problems (Brion et al., 2010). Maternal smoking during pregnancy is closely associated with a high probability of low birth weight and perinatal complications, Sudden Infant Death Syndrome (SIDS), obstructive lung disease, altered neurodevelopment and childhood infections and cancers (Hofhuis et al., 2003) as well as an increased prevalence of lifestyle cardiovascular risk factors among offspring of smoking parents (Horta et al., 2011).

Maternal smoking is also strongly associated with a greater risk for pulmonary disorders and increased respiratory morbidity in the offspring of the smoking mother (Horta et al., 2007; Kramer, 1987). Epidemiological studies add by showing that a close link between abnormal pulmonary function in infants and children and the exposure to maternal cigarette smoke (Brion et al., 2010; Brion et al., 2008; Jaddoe et al., 2008).

As a consequence of this, the developing fetus or lactating child whose mothers smoke during pregnancy or lactation become susceptible to decreased lung function as well as the increased possibility of lung diseases as they get older (Ruiz, 2006). Although there is much supporting evidence that smoking during pregnancy is harmful to the unborn child (Schwartz et al., 1972), it has been estimated that about 15 to 20% of all women smoke during the entire duration of pregnancy (Andres and Day, 2000; Bergmann et al., 2003). Studies have estimated that the exposure to maternal tobacco or nicotine causes approximately 5 to 10% of all fetal and neonatal deaths (Proskocil et al., 2005).

Barker's hypothesis of 'fetal origins of adult disease' highlights the importance of early-life exposure (Barker and Clark, 1997). Worldwide longitudinal studies confirmed that poor fetal nutrition and low birth weight is associated with cardiovascular disease in adults. These studies also demonstrated that there is a link between low birth weight and increased incidences of poor respiratory health (Barker

and Martyn, 1984; Burri, 1984). Current studies lend support to evidence that exposure during early-life renders the lungs vulnerable to Chronic Obstructive Pulmonary Diseases (COPD) as well as other respiratory abnormalities (Bush et al., 2008; Canoy et al., 2007; Shi et al., 2007). The main principle for the origins of adult disease during early-life is that during early life, "programming" due to inadequate fetal nutrition leads to irreversible changes in organ structure, metabolism, and function (Gluckman et al., 2008; Gluckman et al., 2011).

It is estimated that the exposure to maternal tobacco or nicotine causes approximately 5 to 10% of all fetal and neonatal deaths (Proskocil et al., 2005). Previous studies suggest that if smoking mothers were to give up the habit early during pregnancy, they should expect pregnancy results equivalent to those of nonsmoking mothers (Schwartz et al., 1972). In spite of this, it should also be noted that smoking during early pregnancy can still cause unfavorable outcomes (Seller and Bnait, 1995). The adverse effects of maternal cigarette smoke or nicotine exposure include; intrauterine growth retardation (Hammoud et al., 2005; Nordentoft et al., 1996), low birth weight (Jaddoe et al., 2008; Bernstein et al., 2005), premature birth, reduced pulmonary function at birth, and a high occurrence of respiratory illnesses after birth (Egger and Aubert, 2005; Proskocil et al., 2005; Sekhon et al., 2004). Studies on rat models showed that 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).

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 in the lungs may result in an increase in oxygen in lungs 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).

Epidemiologic studies have also suggested that a number of diseases observed in the offspring later on in life may also be related to maternal smoking during pregnancy. 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). In addition to this, studies have associated 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 (Ng and Zelikoff, 2007; Oken et al., 2005). Maternal smoking is generally related with respiratory diseases such as asthma and atopy in the next generation (Alati et al., 2006; Raherison et al., 2007).

Prenatal exposure to maternal tobacco smoking leads to decreased lung function when evaluated shortly after birth, and a continuing decline through childhood to adult life. Reduced airway function in early life is accompanied by increased susceptibility to respiratory diseases although it may not predict persistent airflow limitation in adult life (Landau, 2008a). Evidence from previous studies suggests that prenatal exposure to maternal smoking has unfavorable effect the structural and functional development of small airways (Lødrup Carlsen et al., 1997; Stocks and Dezateux, 2003; Elliot et al., 2001). The modifications that occur in the developing airway structure and function as a result of maternal smoke are likely to persist even until childhood and may continue into adulthood. In infants, reduced lung function in occurs as a result of restricted airflow in small conducting airways (Henderson et al., 2010) after maternal smoke exposure. One of the subsequent changes observed in the developing conducting airways include airway hyperresponsiveness which may also carry on throughout childhood even till early adulthood, implying that effects of smoke exposure on the small airways are permanent (Goksor et al., 2007). As a result of reduced alveolarization, there will be a decrease in number of alveolar-bronchiolar attachments points, another potential mechanism for reduced lung function. It is conceivable that maternal smoking may have an effect on lung development by increasing oxidative stress in the lungs (Gilliland et al., 2000).

1.4 Maternal exposure to Nicotine

Nicotine is an alkaloid which comprises about 1.5% of commercial cigarette smoke. It includes about 95% of the total content of alkaloids. Nicotine is the primary psychoactive component of tobacco smoke and is accountable for the addictive nature as well as its high incidence of relapse of those who try to quite. The effects of nicotine are exerted on the Central Nervous System (CNS) and other organs such as the lungs through interacting with nicotinic acetylcholine receptors (nAChR) (Lavezzi et al., 2005).

1.4.1 Fetal and maternal absorption of Nicotine

It is commonly believed that the absorption of nicotine inhaled from cigarette smoke via the respiratory system is more rapid than via other routes such as the oral and transcutaneous routes. However, the lungs function as a reservoir for nicotine. The lungs slows down the entry of nicotine into the arterial circulation (Brewer et al., 2004) by 30 – 60 seconds or longer. As soon as nicotine is internalized it appears in the maternal circulation. Nicotine freely crosses the placenta and enters the fetal circulation (Matta et al., 2007b) and can enter the amniotic fluid where it may be absorbed via the skin of the fetus (Onuki et al., 2003). Nicotine concentrations in smokers generally range from 10 to 50 ng/ml (Hukkanen et al., 2005). It is expected that similar levels can be reached with nicotine administered through snuffing or nicotine replacement therapy (Teneggi et al., 2002). Nicotine bind with high affinity to organs such as the liver, kidney, spleen, brain and lung tissue whilst adipose tissue

has the lowest affinity for it (Henningfield et al., 1993; Hukkanen et al., 2005). Because of this, it is expected that nicotine accumulates in the lungs of the fetus (Henningfield et al., 1993; Hukkanen et al., 2005).

During maternal smoking, nicotine is introduced into the circulation of the fetus through the placenta. Even though there is no evidence that nicotine is metabolized by the placenta, there is substantial evidence that indicate that it freely crosses the placenta (Luck et al., 1984). For that reason it is conceivable that the blood concentrations of nicotine reached in the fetus are similar to those in the mother. However, there is currently no evidence to support this belief. A study by Luck et al. (1985) Subsequent to the administration of nicotine, the highest nicotine levels in the pregnant mother's blood is reached after 15-30 minutes (Suzuki et al., 1974). A large amount of the nicotine that enters the fetus returns to the mother's circulation for elimination, the rest enters the amniotic fluid via the fetal urine. As a result of this distribution, nicotine and cotinine accumulate in the amniotic fluid of the pregnant smoker as the nicotine eliminated by the fetus is combined with the nicotine originating from the blood vessels of the amniochorionic membrane (Luck et al., 1985a). As a result of this, it is feasible that the fetus is exposed to nicotine long after concentrations in maternal blood have decreased.

1.4.2 Nicotine Metabolism

There is an increased blood flow to the liver as well as rapid breakdown of nicotine and cotinine in the mother. The metabolism of nicotine in the fetal liver is slow because the enzymatic protection mechanisms of the fetus are not properly matured. Due to that reason, a longer half-life of nicotine in the fetus is therefore to be expected (Frank and Sosenko, 1987; Walther et al., 1991). This accounts for the more rapid metabolism of nicotine and its metabolite cotinine during pregnancy (Dempsey and Benowitz, 2001). This argument is substantiated by the high concentrations of nicotine in fetal tissue in contrast to maternal blood levels (Lambers and Clark, 1996; Luck et al., 1985a). The developing fetal lung as well as other organs are as a result exposed to high concentrations of nicotine for longer periods of time and thus its potential destructive effects on cell structure and integrity (Kleinsasser et al., 2005a). Previous studies done in rat models showed that both higher concentrations and longer exposure-time may stimulate the production of oxidants (Bruin et al., 2008c). In a study by Bruin et al. (2008 it was shown that the exposure of βeta cell mitochondria to ROS contributed to the loss of respiratory enzyme function and mitochondrial structure. It is important to note that proliferating cells at this stage of fetal development are highly susceptible to the influences of foreign substances like nicotine (Rehan et al., 2007), it is therefore expected that nicotine exposure during gestation and early postnatal life via maternal milk may also interfere with the growth and development of the fetus and neonate. Previous research has demonstrated that, nicotine in mother's milk is 2 to 3 times higher than in the mother's plasma

(Dahlstrom et al., 1990b; Luck et al., 1985b). During gestation and lactation, nicotine can have direct effect on cells by decreasing the supply nutrients to the developing offspring. Studies continue to show that long-term nicotine exposure instigates an inclination for genetic vulnerabilities (Guo et al., 2005; Hartwell and Kastan, 1994; Sastry et al., 1998). This thereby leads to alterations in the genetic "program" that regulates and maintains lung development, structure and ultimately aging of lung tissue thus rendering the lungs more vulnerable to respiratory diseases.

1.4.3 Fetal and neonatal oxidant/antioxidant status

From previous studies it is undeniable that the maternal exposure to nicotine results in oxidative stress in fetal, neonatal and later life (Husain et al., 2001; Orhon et al., 2009; Maritz et al., 2011b). The electron transport chain enzyme complexes in the inner membrane of the mitochondria are highly vulnerable to ROS inactivation (Wallace, 2005). Because of this, reactive oxygen species (ROS) directly influences the mitochondria, and mitochondrial DNA which is extremely susceptible to the harmful effects of ROS (Droge, 2002). Nicotine not only stimulates an overproduction of oxidants, but also causes a decrease in the activity of SOD and catalyse and produces in reduced levels of low molecular weight antioxidants such as vitamins C and E (Zaken et al., 2001). The reduced antioxidant capacity of the body results in the increased concentration of malondialdehyde (Sandberg et al.), which signifies oxidant damage to the cells (Halima et al., 2010; Özokutan et al., 2005). This results in a disruption and imbalance in the oxidant/antioxidant system which

may be prolonged even after nicotine withdrawal (Özokutan et al., 2005) and persist as the offspring gets older (Bruin et al., 2008c).

Fetal and neonatal ROS is an end result of maternal smoking or NRT and may bring about several undesirable consequences including damage to mitochondrial DNA and nuclear DNA. Maternal nicotine and ROS may therefore alter the capability of the mitochondria to provide energy and meet energy requirements of the body as well as to fulfill its homeostatic functions. By altering the "program" that controls growth, tissue maintenance, cellular aging and metabolism, the long term exposure to nicotine renders the offspring of the smoking mother susceptible to

30 premature aging coupled with the increased susceptibility to disease.

1.4.4 Effects of Maternal Nicotine on Lung Development

Seeing that tobacco contains a large number of compounds that presents high risk not only to the smoking expecting mother but also to the unborn child, as the addictive component of tobacco smoke (Ginzel et al., 2007); several researchers have recommended the use Nicotine Replacement Therapy (NRT) for smoking cessation. NRT comes in various forms such as; nicotine-containing gums, lozenges, sprays and patches as a remedy for smoking cessation (Ginzel et al., 2007; Silagy et al., 2000). NRT has been promoted to a great extent and has been presented by some researchers as a remedy even for pregnant mothers (Ruiz, 2006). However, it has been made clear from ongoing studies that nicotine on its own is harmful to the fetal lung, heart and nervous system (Argentin and Cicchetti, 2004; Kleinsasser et al., 2005a). Nicotine

easily crosses the placenta (Luck et al., 1985b) and occurs in significant amounts in the milk of smoking mothers (Luck and Nau, 1984). As a result it interacts with the developing fetus and the offspring of smoking mothers or mothers using nicotine replacement therapy (NRT) for smoking cessation (Maritz, 2008). This interaction results in the interference of the normal fetal lung development (Maritz et al., 1993). The levels of nicotine that cross the placenta are sufficient to cause an alteration to the signaling by nicotinic receptors present in fetal lung (Conti-Fine et al., 2000). Nicotine and its metabolites have the ability to obstruct the apoptotic destruction of cells whose DNA has been damaged by genotoxic initiators of carcinogenesis. Continuous exposure to nicotine results in an altered phenotype of endothelial cells. The fact that NRT might be a contributing factor to carcinogenesis despite the fact that it is recommended as a remedy for smoking cessation thus have to be reviewed (N.R.C, 2007).

The harmful effects of nicotine on the fetal lung persist even after the administration of nicotine has stopped (Ruiz, 2006). The administration of nicotine during gestation and lactation have long term effects on the development of the respiratory system, the maintenance of lung integrity, susceptibility to lung diseases and a reduction in lung function (Maritz and Windvogel, 2005). Previous studies show that the maternal exposure to nicotine during gestation and lactation results in the persistent inhibition of glycogenolysis and glycolysis (Maritz, 1987) a consequence of the reduced activity of phosphorylase in the developing fetal lung (Maritz, 1986). Fetal nicotine exposure

also suppresses alveolarization in the lungs of the offspring, resulting in a reduced internal surface area for gaseous exchange (Maritz and Dennis, 1998). In rat models, it was shown that the prenatal exposure to cigarette smoke resulted in a reduced number of pre-alveolar saccules and increased size of saccules; consequently this is expected to decrease the number of attachment points on small airways (Collins et al., 1985). The administration of nicotine in animal models has also been shown to cause lung hypoplasia and reduced surface complexity. Prenatal nicotine exposure affects the development of fetal pulmonary vasculature which results in pulmonary hypertension due to nicotine to nicotinic acetylcholine receptor interaction in pulmonary vessels (Sekhon et al., 2004). The long term consequences of fetal and neonatal nicotine on the developing nervous system have been reported (Pauly and Slotkin, 2008; Winzer-Serhan, 2008). Smoking during pregnancy causes intrauterine growth restriction (Robinson et al., 2000; Widerøe et al., 2003) and low birth weight which is a risk factor for obesity, hypertension s well as type II diabetes. Ongoing studies continue to show the relationship between maternal cigarette smoking and the increased risk for cancers such as brain tumors and childhood leukemia of childhood (Sasco and Vainio, 1999).

Once in the fetal circulation, nicotine interacts with nicotinic acetylcholine receptors (nAChRs) in the fetal lung altering lung structure and function in the offspring. It is understood that nicotine increases collagen accumulation, up-regulates surfactant protein gene expression, and stimulates neuro-endocrine cell hyperplasia in fetal

lungs. These alterations together changes pulmonary function (Sekhon et al., 2001c). In addition to this, it is expected that fetal and neonatal nicotine exposure alter the activities of this autocrine cholinergic loop and by this altering lung development in the offspring. Prenatal exposure to nicotine periods can increase the risk for developing diseases later in life (Morgan and Martinez, 1998). Maternal and grand-maternal smoking during pregnancy has been linked to greater risk of asthma in childhood, implying that the effects of prenatal and postnatal smoking are not only permanent but heritable as well. This is verified by the observation that long-term exposure to nicotine leads to in an inclination to the initiation of genetic instability. The exposure to foreign substances increases the risk for poor health later in life by altering the epigenome (Heijmans et al., 2009; Li et al., 2005).

The developmental period of fetal development as well as the early postnatal period are most vulnerable to extenal substances (Bateson, 2007; Heindel, 2006). Since several processes are occurring during these developmental stages, if permanently transformed, subsequent organ growth and function will be altered as well. The exposure to environmental toxicants, such as maternal smoking during gestation and lactation, can alter the epigenome to increase the vulnerability to adult onset of disease (Bollati and Baccarelli, 2010). Exposure to nicotine has various undesirable outcomes such as; DNA damage (Ginzkey et al., 2009) and increase in ROS production which also induce DNA damage (Wiseman and Halliwell, 1996).

Maternal nicotine exposure during prenatal and postnatal development may cause structural alteration that may become progressively worse over time, due to programming changes during organ development during the prenatal and postnatal period. It is therefore expected that maternal smoking or NRT administered during pregnancy and lactation may trigger epigenetic alterations in the lungs of the offspring transferrable to subsequent generations and result in inclinations to adult respiratory disease (unpublished data).

1.5 Cellular Senescence

Cellular senescence has been defined as a state of permanent G1 arrest during which primary somatic cells have a complete irreversible loss of their replicative capacity (Hayflick, 1965). According to Hayflick and Moorhead (1961; 1965), normal human fibroblasts proliferation are restricted to a limited number of times in vitro (Hayflick, 1965; Hayflick and Moorhead, 1961). The maximum number of times the cells can divide is called the 'Hayflick limit' and which maybe about 50 to 70 division times in tissue culture. Once the 'Hayflick limit' is reached, the cells will stop dividing and enters into a type of cell arrest called replicative senescence (Serrano and Blasco, 2001). The cell will undergo a series of morphological biochemical and functional changes which are suggestive of ageing, for that reason, this process is known as senescence (Dimri et al., 1995).

The concept of the ‘Heyflick limit’ proposes that normal somatic cells possess a cell division cycle counting mechanism or ‘clock’ (Hayflick and Moorhead, 1961). According to the telomere hypothesis of senescence, the counting mechanism is progressive with telomere shortening which takes place during cell division (Olovnikov, 1973). Telomere forms a protective cap at the end of the chromosome. This cap prevents it from being recognized as double-stranded breaks and also prevents the DNA ends from degradation and recombination (Chen et al., 2001b; Lange et al., 2005). Since the chromosome does not have the ability to duplicate the ends of linear molecules, telomeres become progressively shorter every time the cell enters cell division (Blasco, 2005). Ultimately, telomeres reach a significantly short length, appearing as double-stranded DNA breaks that stimulate the p53 tumor inhibitor protein leading to telomere-initiated senescence or apoptosis (de Lange, 2005; Zglinicki and Martin-Ruiz, 2005). The telomere is elongated by telomerase which is a ribonucleoprotein with DNA polymerase activity (Greider and Blackburn, 1985). In most adult tissue, the level of activity is inadequate to prevent progressive telomere destruction which occurs with aging balanced (Collins and Mitchell, 2002). By using a generation of telomerase-deficient mice Blasco et al. (1997) showed that telomerase is the most important cellular activity which accounts for the maintenance of telomere length.

Senescent cells assume distinct, flat and enlarged cell morphology; this appearance can be used to distinguish senescence cells from non-senescent cells. In addition,

senescent cells have been observed to have an increase in the number of vacuoles and lysosomal bodies that contain UV-fluorescent pigments. The philological change leads to a reduced response to growth factors as well as other mitotic stimuli. Consequently, the sensitivity to toxins, drugs, irradiation, stress and other environmental changes is elevated. This leads to a general decrease in respiration and energy metabolism (Rattan, 1995). Senescent cells also exhibit a senescence associated β-galactosidase activity (Dimri et al., 1995) which may be indicative of an increased lysosomal-mass (Lee et al., 2006). Furthermore, since there is an increase in the rate of protein synthesis and degradation, senescent cells have an increased number of altered and inactivated proteins (Rattan, 1995; Serrano and Blasco, 2001).

1.5.1. The role of senescence in apoptosis

Senescence and apoptosis occur throughout the normal growth and development of cells and organisms. It has been proposed that senescence is an important mechanism in the preservation and maintenance of a physiological balance within a system (Hengartner, 1995). Senescence is also necessary in protecting the differentiation and growth of potential tumor cells (Sager, 1991). It is understood that these potential tumor cells possess the ability to replicate an unlimited number of times, a vital step in the malignant transformation of normal cells (Reddel, 2000). This means that the progressive shortening of telomere that occurs with every cycle of cell division not only restricts the replication of normal noncancerous cells, but also the proliferation cells that are in the process of neoplastic transformation. The exception to this

includes immortal mammalian cell lines and tumors which preserve their telomeres as they are deficient in telomerase and by means of alternative lengthening of telomeres (ALT). This is a mechanism that is comprised of homologous recombination between telomeres (Muntoni and Reddel, 2005).

This then confirms the theory that telomerase is a tumorigenic factor that facilitates the development of tumors. There are two exceptions to this general trend and it is if there is telomerase deficiency coupled with a p53deficiency (Chin et al., 1999) otherwise the overexpression of the telomere-binding protein TRF2, which recruits the nuclease XPF to telomeres and degrades them (Blanco et al., 2007). This leads to cells proliferation in the presence of rampant chromosomal aberrations, thus stimulating the development of cancer (Artandi et al., 2000).

Zglinicki and Martin-Ruiz (2005) showed that oxidative damage may accelerate the rate of telomere shortening and thus aging is associated with the increase of oxidative damage. This implies therefore that telomere shortening could reflect the proliferative history of a cell, as well as the accumulation of oxidative damage. The decrease in telomere length is associated with age in several tissues as well as diseases associated with aging (Canela et al., 2004; Cawthon et al., 2003; Ogami et al., 2004; Panossian et al., 2003). Furthermore, a number of human premature aging syndromes, including dyskeratosis congenita (DC) and aplastic anemia, have also been linked to mutations in telomerase or in proteins that directly affect telomerase activity and have been

considered to have a faster rate of telomere degradation progressive with age (Mason and Bessler, 2004).

Previous studies have demonstrated that mice deficient in telomerase activity have short telomeres and age prematurely (Blasco et al., 1997; Lee et al., 1998). Garcia-Cao et al. (2006) further showed that even the first generation of telomerase-deficient mice will have a shortened lifespan, which interestingly enough becomes shorter and shorter in successive generations. In addition, mice that that show an overexpression of telomerase are prone to develop tumors (Canela et al., 2004; González-Suárez et al., 2001).

1.5.2. 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).

1.5.3 The mechanisms of cellular senescence

The main molecular and cellular mechanisms that are associated with cellular aging include telomere attrition; cumulative DNA damage; an impairment of DNA repair; the epimutations in nuclear DNA; mutations in mitochondria; an increase in the rigidity of cytoskeleton; an increased cross-linking of the extracellular matrix, protein damage; an increased production of free radicals; and an 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., 2001b). 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).

1.5.4 Smoking and senescence

Smokers show signs of premature aging (Kadunce et al., 1991; Tanaka et 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, 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).

Furthermore, there is a slight loss in the actual number of small airways (Thurlbeck, 1992); the distance between airspace walls is increased, and the internal surface area of the lung is decreased (Pierce et al., 1961). This results from larger pores that exist in the aging 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 that 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 that 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).

1.5.5 The role of proteases and oxidative stress in the aging process

The epithelium of the alveoli is frequently injured by a number of inhaled toxins that include; 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. Once 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 results in the disruption in the architectural integrity of the alveoli thus, the lung becomes more susceptible to diseases (Tsuji et al., 2004b). 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., 2004b).

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).

1.5.6 The Effect of Carotenoids on the Oxidant/Antioxidant Balance

As indicated by Harman, 1956 in the free radical theory of aging, oxidative stress is responsible for cellular senescence. In agreement with this, Kondoh et al (2005) demonstrated that there is a close relationship between senescence and the process of glycolysis. In this study, it was shown that an increase in glycolysis can bypass cellular senescence while the inhibition of glycolysis may lead to premature senescence. Results of other studies done by Lee et al (1999) and Parrinello et al (2003) found that the increase in ROS and oxidative stress is associated with the occurrence of senescence (Lee et al., 1999; Parrinello et al., 2003). Chen et al (1995) pointed out that even mild oxidative stress such as low concentrations of hydrogen peroxide is enough to provoke senescence (Chen et al., 2001a).

Since oxidative stress is capable of inducing telomere-independent senescence, it therefore expected that the prevention of oxidative stress may thus give answers to the question to the immortality of cancer cell. It has in fact been suggested that the protection from oxidative stress can significantly increase life span (Kondoh et al.,

2007). In addition to this, it has been clearly documented that nicotine induces oxidant damage in different organs. This is achieved by increasing the products of lipid peroxidation and reducing the endogenous antioxidant activity (Şener et al., 2005). In pregnant mothers using nicotine, it is expected that the fetus is exposed to high levels of nicotine which may as consequently lead to an oxidant/ antioxidant imbalance. This will therefore adversely affect the development and survival of the organs of the unborn child.

It is therefore important to seek ways to restore the antioxidant status of the mother and the unborn child even though the mother continues to smoke or use NRT. Several researchers have shown the prospective role of antioxidant nutrients in prevention of chronic diseases and aging processes in humans (Mayne, 2003). The supplementation of antioxidants in relatively high concentrations has been recommended for pregnant mothers who smoke or use NRT. Carotenoids are also known to exert antioxidant activities and prevent free radical-induced cellular damage (Bendich, 1993). 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). Kashara and co-workers (2001) described lung epithelial

and endothelial alveolar septal death resulting from a decrease in endothelial cell maintenance factors such as VEGF, VEGF-R2 protein and mRNA expression in human subjects with smoke induced emphysema. In this study, it was shown that vascular factors play an important role in the pathogenesis of emphysema, with endothelial cells undergoing apoptosis to a greater extent than lung cells when exposed to cigarette smoke.

1.6 Relationship between apoptosis and Emphysema

Apoptosis has been implicated in the loss of alveolar wall cells in patients with emphysema (Kasahara et al., 2001). The increased incidence of apoptosis in alveolar wall cells of patients with emphysema has been linked with a decrease in the expression of endothelial survival factors such as vascular endothelial growth factor and its receptor, kinase insert domain-containing receptor. Segura-Valdes et al. (2000) demonstrated that an elevated level of apoptosis in alveolar wall cells and the enhanced expression of metalloproteinases in the lungs of patients with emphysema. Aoshiba et al. (2003) also showed that the stimulation of apoptosis in alveolar epithelial cells leads the loss of alveolar wall structures and emphysematous changes in mice. This indicates that apoptosis in alveolar wall cells is may lead to the development of emphysema. This suggests that alveolar wall cells are lost, at least in part, as a result of apoptosis in emphysematous lungs. Since apoptosis leads to the loss of alveolar wall cell, the remaining cells must proliferate in order to maintain the pulmonary architecture (Aoshiba et al., 2003; Segura-Valdez et al., 2000). In a human

study done by Tao et al. (1998), it was shown that the level of proliferation in vascular wall cells is was elevated in the lungs of patients with emphysema. Sekhon et al. (1994) showed that exposure to cigarette smoke stimulated cell proliferation in small airways and arteries in rat models (Tao et al., 1998; Sekhon et al., 1994).

1.7 Tomato juice and Lycopene

The use of lycopene, a bioactive carotenoid present in many fruits and vegetables is the major carotenoid in fresh tomatoes and tomato products (Kaplan et al., 1990; Takeoka et al., 2001). 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 1015 oxidant molecules (Church and Pryor, 1985a), 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 (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 (Agarwal et al., 2001). 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 et al., 1998). In addition to this, previous literature reveals that the increase in oxidized proteins have 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 AIMS AND OBJECTIVES

The key objectives of this study are to determine:

1. The effects of maternal nicotine exposure during gestation and lactation on lung development in the offspring.
2. 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.
3. If maternal nicotine exposure during gestation and lactation induces premature cellular senescence in the lungs of the offspring
4. 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.
5. Whether the effects of grand-maternal nicotine exposure is transferred to the F2 generation.

CHAPTER TWO

Materials and Methods

2.1 Ethical Clearance

The approval for the use of the animals as well as the experimental procedures followed in this study was obtained from the ethical committee of the research committee of the University of the Western Cape.

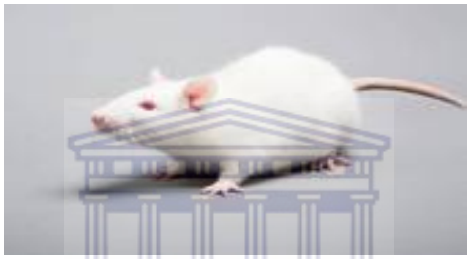
2.2 Animal preparation

2.2.1 F0 Generation

2.2.1.1 Nicotine Administration

White virgin female Wistar rats were used in the study. The animals were fed a stock diet of Epol rat cubes during the course of the experiment. The animals received food and water as required. The room temperature was maintained at 22°± 1°C and a day-night cycle of 12 hours (06:00 – 18:00) throughout the entire experiment. The F0 generation of animals were mated overnight and afterwards randomly allocated to control and experimental groups.

Generation F0: Mother exosed to nicotine during gestation and lactation



Generation F1: Offspring received nicotine via the placenta and mother's milk

Generation F2: Offspring from F1 generation. No nicotine exposure of the F1 generation after weaning from the F0 generation. F1 males and females were mated to generate the F2 generation

Figure 2.2: Rat model to illustrate the generation of the F1 and F2 generations. Only the F0 mothers were directly exposed to nicotine. The F1 offspringonly received nicotine via the placenta and mother's milk.

At least 6 female rats were assigned to each group. The experimental animals were divided into 3 groups; group one received 1 mg nicotine/ kg body weight/ day. Group 2 received only tomato juice, and a third group received both nicotine (1mg/ kg body weight/ day) and tomato juice. The administration of both nicotine and tomato juice to all the mothers in the treatment groups was from the onset of pregnancy up to weaning on postnatal day 21, this means that the treatment covered the entire period of gestation and lactation. The offspring received nicotine and lycopene in tomato juice only via the placenta and mother's milk. The control animals received saline. The nicotine dose was not changed as the body weights of the animals increased during pregnancy. The nicotine was administered subcutaneously.

After birth, the number of rats per litter was kept between 8 to 10 pups to ensure that the nutrient supply from the mother was adequate to support normal growth and development.

2.2.1.2 Administration of Tomato Juice

The brand of tomato juice that was used was the All Gold Tomato juice. In order to reduce its density, the tomato juice was diluted 50/50 with distilled water. It was made freely available for the animals to drink from water bottles. The water and tomato juice intake was measured on a daily basis and the average intake recorded per week. The lycopene intake was calculated based on the tomato juice intake by the animals as well as the lycopene content of the tomato juice and recorded as the

average intake (mg/100g body weight/week). Apart from lycopene (5.3 mg/100ml tomato juice), the tomato juice also contains protein (0.8g/100 ml), carbohydrates (3.4 g/100 ml), fibre (0.55 g/100 ml), and sodium (200mg/100ml).

2.2.2 F1 Generation

Once the animals gave birth the number of rat pups per litter was kept at between 8 and 10 pups to ensure that the nutrient supply from the mother was adequate to support normal growth and development. Since the daily nicotine intake of human males and females who smoke tobacco varies between 10.5 and 78.6 mg (Benowitz and Jacob, 1984), assuming that 90% of the nicotine is absorbed on inhalation (Gleason, 1963), the nicotine intake of a 60 kg female will be between 0.16 and 1.18 mg nicotine/kg body weight/day. The dose used in this study was therefore within the range of intake of habitual smokers. Because nicotine readily crosses the placenta and occurs in the milk of the mother (Luck and Nau, 1984), the fetal and neonatal rats will be exposed to nicotine via the placenta and mother's milk. The breast milk from smoking mothers contains a mean of 33.1 ng/ml (Stepans and Wilkerson, 1993). It was shown that the milk/plasma nicotine ratio after smoking is 2.9. The amount of nicotine transferred to the infant via the mother's milk increased from 0.09 to 1.03 μg/kg infant body weight when mothers smoke before breast feeding. The dose of nicotine given to the pregnant rats was 1 mg/kg maternal body weight subcutaneously from the onset of gestation. The dose of nicotine remained constant for the period of

the study. This implies that the offspring only received nicotine via the mother's blood and milk (Dahlstrom et al., 1990a).

The F1 nicotine exposed males and females were exposed to nicotine via the placenta and mother's milk (F0 generation) only. Although the F1 rats received nicotine only via the placenta and mother's milk, they never received nicotine after weaning. The control animals were never exposed to nicotine. For the F1 generation of animals, lung tissue samples were collected at ages 14, 21, 42 and 84. The morphometric data at postnatal day 21 of the F1 progeny was pooled since no differences were observed between males and females.

2.2.3 F2 Generation

Males and females of the F1 generation were mated in order to determine the whether the effect of grand maternal nicotine exposure during gestation and lactation is transferrable to the F2 generation. While the F1 generation was exposed to nicotine via the placenta and mother's milk, the F2 generation was never exposed to any nicotine. The F2 progeny were divided into groups as follows:

1. Control (F1 Control male mated with F1 control female)
2. NmCf (F1 nicotine exposed male mated with F1 control female),
3. NfCm (F1 nicotine exposed female mated with F1 control male),
4. NmNf (F1 male exposed to nicotine mated with F1 female also exposed to nicotine).

2.3 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 (CC) and the crown-rump length (CRL) were subsequently measured and recorded as shown in figure 2.3. The pups were then injected with a 6% 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.

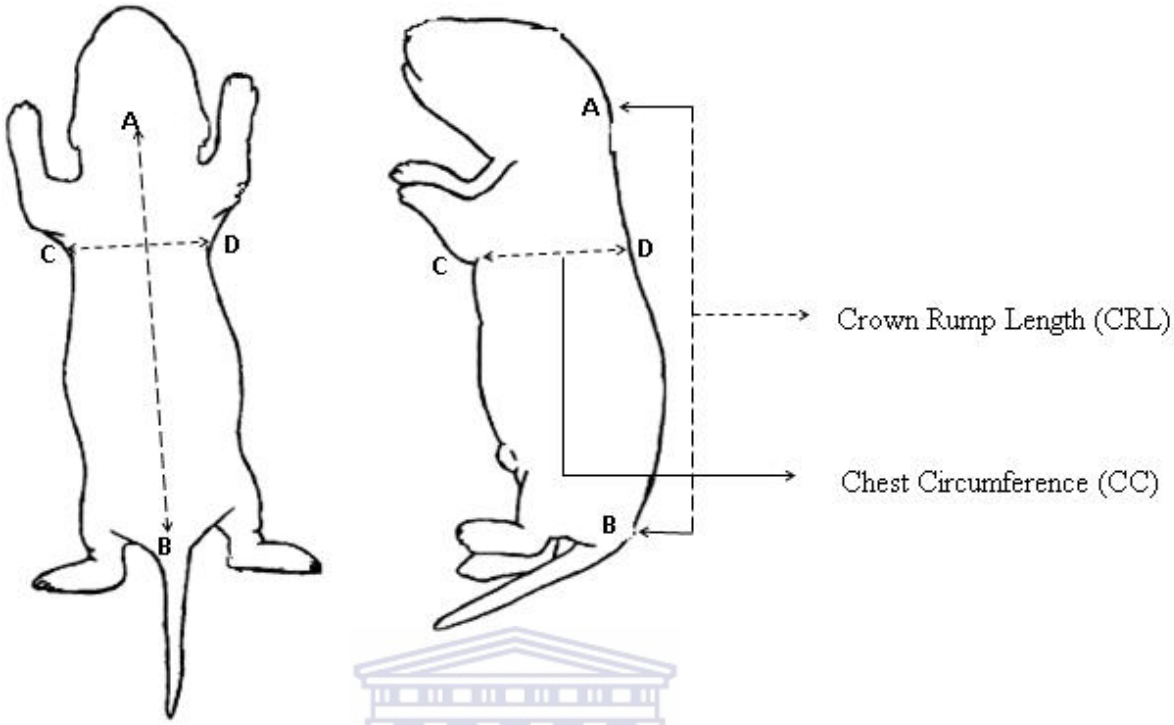


Figure 2.3.1: Rat model to illustrate the measurement of the Crown rump length (CRL)-A to B and the Chest curcumference measurement (CC) C to D. Only the F1 offspring and the F2 generation were used.

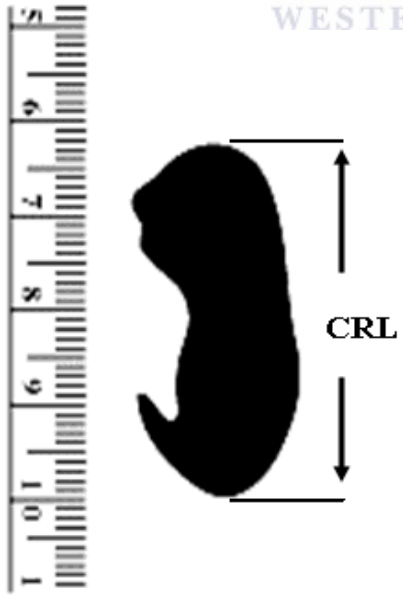


Figure 2.3.2: Rat model to illustrate the measurement of the Crown rump length (CRL)-. Only the F1 offspring and the F2 generation were used.

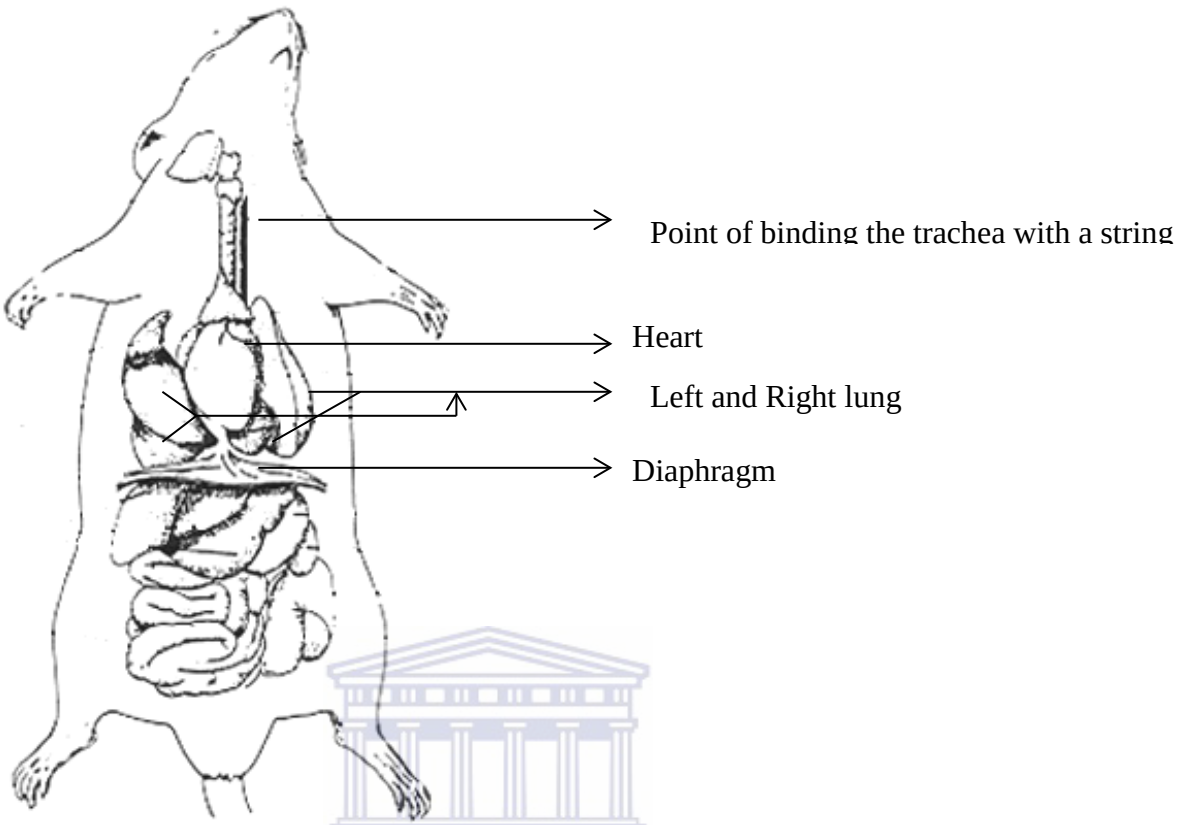


Figure 2.3.3: Rat model to illustrate the Point of binding the trachea with a string. Only the F1 offspring and the F2 generation were used.

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). Lung volumes were determined before and after the 24-hours fixation period to detect shrinkage. Because of minimal shrinkage (<2%), data were not corrected for shrinkage. The lungs were afterward placed in 10% buffered formalin where they kept for a maximum time of a week before further processing.

2.4 Processing and Embedding of Samples from F1 and F2

To remove lung tissue, rat pups were anaesthetized (90 mg Nembutal/kg body weight subcutaneously), where after the trachea was cannulated and the diaphragm punctured. The fixative (10% buffered formalin, pH 7.2) was allowed to flow into the lungs while a trans-pulmonary pressure gradient of 25 cm/H_20 fixative was maintained. After 30 minutes the lungs were ligated at the hilum and removed en bloc for morphologic and morphometric studies. The lungs were stored in buffered formalin (pH 7.2).

After the lung volumes were determined, the samples were processed using automatic tissue processor. 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. Care was taken not to cause any damage to the tissue during processing. When all the cassettes containing the lung tissue were ready, it was 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;

1	70% alcohol	2 hours
2	80% alcohol	2 hours
3	90% alcohol	2 hours
4.	100 % 1	2 hours
5	100% 2	2 hours
6	Xylene 1	2 hours
7	Xylene 2	2 hours
8	Wax bath 1	2 hours
9	Wax bath 2	2 hours

Table 1: Histological Processing of tissue for light microscopy

When the 18 hour cycle was over, the samples were embedded in paraffin wax; where after 4-µm sections were made and stained with haematoxylin and eosin

(Culling, 1974). The slides were initially examined to eliminate sections with evidence of inadequate preparation. Assessment of lung parenchymal tissue was performed in a blinded fashion on coded slides from 8 randomly selected rats from each group. The rat pups were selected from 5 litters. Five randomly selected fields from each lung were examined. Parenchymal tissue includes alveolar septa, alveolar ducts, respiratory bronchiolar tissue, and blood vessels with a diameter of <10 μm and their contents.

2.5 Morphometry and Morphology

The following parameters were used to determine the influence of maternal exposure to nicotine in tomato juice only or both nicotine and tomato juice on lung development in the offspring. For morphometry, samples were taken from the left and the right lobes of the lung. Large airways and blood vessels were avoided when determining the measurements. The mean alveolar diameter (Lm) was determined as described by Weibel and Knight (Weibel, 1963). The alveolar (V_a) and parenchymal (Uksusova and Nizovtsev) density were determined as described by Bolender and colleagues. At least 25 randomly selected, non-overlapping fields from each section were analysed (Bolender et al., 1993). Seventy five fields from each animal were counted. Alveolar wall thickness was determined as described by Bolender et al (1993).

The morphometric techniques used in this study included:

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

2.5.1 Volume Density (Va and Vt)

2.5.1.1 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.

2.5.1.2 Method

The alveolar air volume density (Va) and alveolar tissue volume density (Uksusova and Nizovtsev) 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.

2.5.2 Determination of Mean Linear Intercept (Lm)

2.5.2.1 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.

2.5.2.2 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.

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.

2.5.3 Alveolar wall thickness (Tsept)

2.5.3.1 Principle

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.

2.5.3.2 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

2.5.4 Statistical Analysis (Morphometry)

One-way ANOVA with Dunnett's posttest was performed using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com using the Tukey-Kramer test, which includes the extension by Kramer to allow for unequal sample sizes. 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.

2.6 Additional Stains

2.6.1 Introduction

The staining procedures for cell proliferation (PCNA), cell senescence (Beta galactosidase), and apoptosis were performed according to the materials and methods supplied by the supplier of the kits (Invitrogen). Once the quantification of the number of proliferating, senescent and apoptotic cells were done, the number of proliferating or senescent or apoptotic cells were expressed as cells/100 µm of alveolar wall. The quantity of senescent, proliferating, or apoptotic cells per 100 µm of alveolar wall were determined using Image J (NIH, Bethesda, MD). Pictures were taken at 400x magnification and used for determining cell numbers/100 µm of alveolar wall. Since no differences between males and females were observed at postnatal day 21, the day 21 morphometric data was excluded from the study.

Statistical analysis of differences between means was carried out by the use of the one way analysis of variance (ANOVA) for unpaired data, followed by the Student-Newman-Kuels test for pairwise comparisons. A probability level of $P < 0.05$ was designated as significant in this study

2.6.2 Staining for cell proliferation

2.6.2.1 **Principle**

PCNA (Proliferating Cell Nuclear Antigen) was previously known as cyclin. PCNA is a 36 kDa nonhistone protein found in the nucleus that plays a role in the initiation

of cell proliferation by mediating DNA polymerase. PCNA levels are elevated in the S, G2, and M phases of cell mitosis in normal and malignant tissues. PCNA expression has a broad correlation with mitotic activity and can be used as a marker for cell proliferation. PCNA has proven useful for proliferative studies of normal and neoplastic tissues both *in vivo* and *in vitro*. The PCNA staining protocol uses a biotinylated PCNA monoclonal antibody (clone PC10), which eliminates the need for a species-specific secondary antibody. Streptavidin-peroxidase is used as a signal generator, and DAB as the chromogen, to stain PCNA-containing nuclei a dark brown.

2.6.2.2 Solutions

Phosphate Buffered Saline (PBS)

- 85g sodium chloride
- 2g potassium chloride (KCl)
- 21.7 g Sodium phosphate heptahydrate ($Na_2HPO_4.7H_2O$)
- 2g potassium phosphate monohydride (KH_2PO_4)
- 1000 ml of distilled water

2.6.2.3 Method

PREPARATION

Tissue sections of 5 µm thickness were cut and placed on slides. The tissue was left to dry in a 60°C oven for 1-2 hours. Slides were deparaffinised in 2 washes of xylene

for 5 minutes each and rehydrated in a series of graded alcohol. The Proliferating Cell Nuclear Antigen (PCNA) staining protocol was performed using to the Invitrogen PCNA staining kit method (Catalogue number 93-1143).

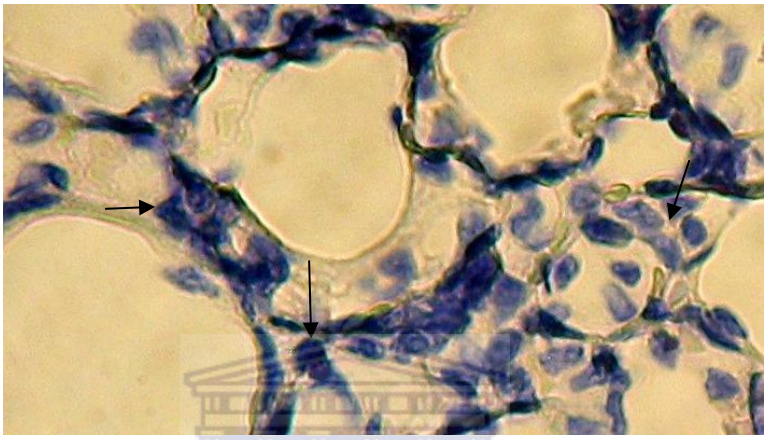


Figure 2.6.2: Arrows show proliferating cells in alveolar wall. Cells stained blue

2.6.3 β-galactosidase staining

2.6.3.1 Principle

The β-galactosidase staining for senescence was performed according to Boehringer Mannheim β-gal staining set.

The β-Galactosidase Staining Kit (Catalog #K802-250) was used for the detection of senescence in the lung tissue samples. β-galactosidase catalyzes the hydrolysis of X-Gal (5-bromo-4-mchloro-3-indolyl-b-D-galactopyranoside) to an indolyl alcohol. Subsequently, the indolyl alcohol is then oxidized to form an intense blue indigo stain. The β-Galactosidase staining kit utilizes X-gal as the substrate. The X-Gal Substrate set is designed for use with any probe labeled with *E. coli* β-galactosidase. The substrate produces a blue stain with no background color.

2.6.3.2 **Solutions**

a. 10X PBS

- NaCl 80g
- KCl 2g
- $NaHPO_4$ 14.4g
- KH_2PO_4 2.4g
- Distilled water 1000ml

b. X-gal (β – galactosidase staining) Stock Solution

- 1 mg of 5-bromo-4-chloro-3-indolyl P3-D-galactoside (X-Gal) per ml (stock = 20mg of dimethylformamide per ml)

c. X gal-Staining solution mix

- 470 µl of Staining Solution
- 5 µl of Staining Supplement
- 25 µl of 20mg/ml X-gal in DMF

2.6.3.3 Method

1	Prepare slides and deparaffinize	
2	Wash PBS X 2	5 minutes each
4	Fix tissue 0.5 ml Fixation Solution	10-15 minutes
5	Wash PBS X 2	5 minutes each
6	Add X-GAL staining solution to slides and cover	24 hours
7	Counter Stain in Nuclear Fast Red	3 minutes
8	Wash PBS X 2	5 minutes
9	Mount slide for Observation	

Table 2: De-waxing of tissue sections for microscopy

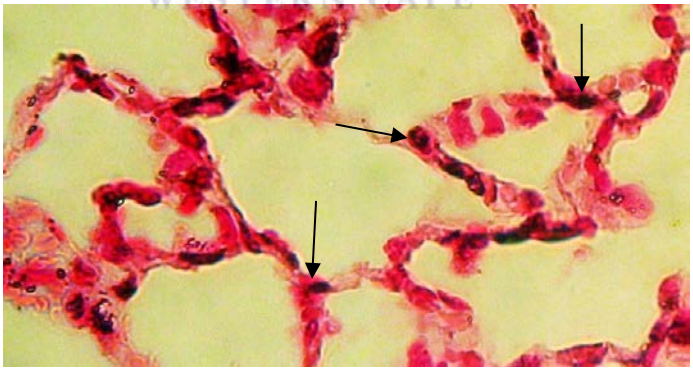


Figure 2.6.3: β–galactosidase staining of senescent cells (Brown/Black) in the alveolar walls.

2.6.4. Staining for Apoptosis-DeadEnd™ Fluorometric TUNEL

2.6.4.1 Principle

In the present study, the DeadEnd™ Fluorometric TUNEL kit (Cat. No.G3250) which evaluates apoptotic cell death in systems such as cultured cells or paraffin-embedded tissue sections was used for detection of apoptosis in the F1 generation. 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.

2.6.4.2 Method

1. Slides containing tissue sections were immersed in two changes of xylene for 5 minutes each at room temperature.
2. The tissue sections were then immersed in two changes of in absolute ethanol for 5 minutes.

3. After that, the samples were rehydrated by immersing the slides through graded ethanol washes (100%, 95%, 85%, 70%, and 50%) for 3 minutes each at room temperature.
4. The 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.
5. Following that, the samples were fixed in 4% methanol-free formaldehyde solution in PBS for 15 minutes at room temperature and then the samples were washed in two changes of PBS for 5 minutes each at room temperature.
6. The excess liquid around the tissue sections was removed 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 then incubated for 8–10 minutes at room temperature.
7. 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.
8. A final wash was then done by immersing the slides in PBS for 5 minutes at room temperature.
9. 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.
10. While the tissue samples were equilibrating, the Nucleotide Mix was thawed on ice and a sufficient rTdT incubation buffer for all samples was prepared.

11. 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.
12. 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°C inside the humidified chamber to permit the tailing reaction to occur. The chamber was covered with aluminum foil to protect it from direct light.
13. 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.
14. The reaction was then stopped the by immersing the slides in the 2X SSC in a Coplin jar for 15 minutes at room temperature.
15. The samples were washed again by immersing the slides in fresh PBS for 5 minutes at room temperature.
16. The samples were then stained using DAPI solution freshly diluted to 1ml: 10ml in PBS for 5 minutes at room temperature in the dark.
17. 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.
18. 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.

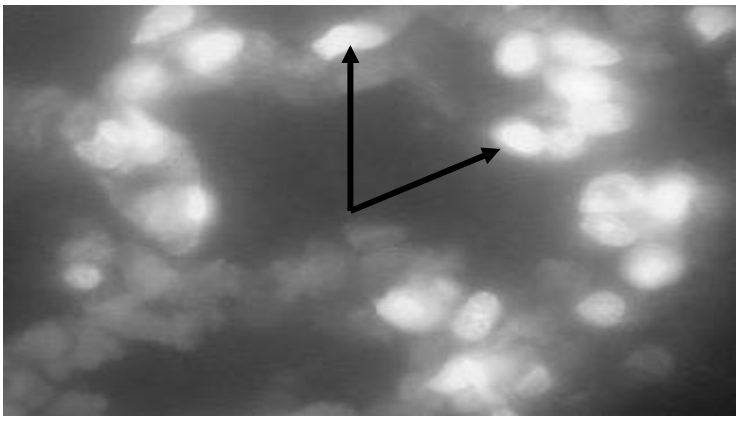


Figure 2.6.4: Apoptosis (Seidman et al.) of cells in the alveolar walls.

2.7 Statistical analysis

One-way ANOVA with Dunnett's posttest was performed using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com using the Tukey-Kramer test, which includes the extension by Kramer to allow for unequal sample sizes. 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 THREE

The effect of nicotine, tomato juice only, and of both nicotine and tomato juice on the body weight and litter size of the pregnant and lactating mothers

3.1 Introduction

Epidemiological studies show that smoking during pregnancy is one of the leading causes of death worldwide (W.H.O., 2008). While reports indicate that the prevalence of smoking during pregnancy in many countries has declined over past few decades (Surgeon-General, 2001), it is still a major public health concern (DiFranza and Dussault, 2005; Martin et al., 2003; Martin et al., 2002; Mathews, 2001). Research and medical institutions have made various attempts to promote the cessation of smoking among pregnant women and efforts have also been made to inform these mothers of the negative outcomes of tobacco smoking during pregnancy. However, the prevalence of maternal smoking during pregnancy remains high. It has been estimated that approximately 15 to 25% of women smoke during pregnancy (Coleman et al., 2004; Nelson and Taylor, 2001; Owen and Penn, 1999; Everett-Murphy et al., 2010). Literature indicates that although about 75% of these women show intentions to quit smoking during that pregnancy (Okuyemi et al., 2000; Ruggiero et al., 2000); only 20–30% actually quit the habit (Ebert and Fahy, 2007).

It has been well documented that maternal smoking during pregnancy results in interference with the development and health of the exposed offspring (Lumley et al., 2004). Maternal smoking during pregnancy is the reason for a number of unfavorable outcomes during pregnancy, such as placental abruption, spontaneous abortions, premature birth, low birth weight and an increased incidence of sudden infant death syndrome in infants born to smoking mothers (DiFranza and Lew, 1995; Hammond, 2005; Martin et al., 2002; Matthews et al., 2002; Salihu and Wilson, 2007). Furthermore, tobacco smoking during pregnancy results in a number of long-term health consequences for both the mother and the exposed offspring (Schmidt, 2004). Nicotine, the addictive substance and other toxic substances in cigarette smoke (Benowitz and Dempsey, 2004), reportedly limits the supply of oxygen and other vital nutrients to the fetus, resulting in the impairment of fetal growth and development (Crawford et al., 2008; Herrmann et al., 2008). Given that nicotine is the addictive component of tobacco, nicotine replacement therapy (NRT) is commonly recommended as a solution to assist in smoking cessation and has been endorsed by some researchers to be of assistance even to pregnant women (Benowitz and Dempsey, 2004; Okuyemi et al., 2000; Peters and Morgan, 2002; Ruiz, 2006). However, a large body of evidence continues to show that nicotine is harmful to the developing fetus and infant when maternally administered during pregnancy and lactation. Studies have shown that nicotine can cross the placenta and accumulate in the amniotic fluid and the fetal blood and alters the development of the lungs in the fetus. Nicotine may also be transported to the infant via the milk of the mother during

lactation (Luck and Nau, 1987). It is therefore conceivable that the exposure to nicotine alone may alter the in-utero environment within which the fetus develops. It has been observed that the half-life of nicotine is three to four times higher in newborns than in adults (Lambers and Clark, 1996); it is therefore plausible that the organs of the fetus are exposed to higher levels of nicotine for extended periods of time resulting in the harmful effects of nicotine on cell integrity.

The aims of this chapter are:

1. To determine effects of nicotine exposure on maternal body weight increase during pregnancy,
2. Litter size at birth of the pregnant control rats, and those that received tomato juice supplementation as well as the rats that were exposed to nicotine during gestation and lactation.
3. To establish whether tomato juice prevents any of the adverse effects of maternal nicotine exposure during gestation on growth and development of the offspring.

3.2 Results

3.2.1 Liquid intake

Figure 3.2.1 shows the liquid intake of the mothers during gestation and lactation. From the data, it can be deduced that the liquid intake of the control mothers was similar to that of the mothers exposed to nicotine, those that received tomato juice

only, as well as those that were exposed to both nicotine and tomato juice (P>0.05) during gestation (week 1 to 3) and lactation (week 4 to 6).

3.2.2 Intake of lycopene

The lycopene intake of the pregnant rats that received tomato juice only, and those that received a combination of nicotine and tomato juice, was calculated based on the lycopene content of the tomato juice (mg/ml), and the volume of the tomato juice consumed by the animals during gestation and lactation. The lycopene intake of the pregnant mothers that received tomato juice only during week one of gestation (first week of pregnancy) was 20.03±1.34 mg/g body weight/week (mg/wk), and 22.5±1.77 mg/g/wk (P>0.05) for the pregnant mothers that received a combination of nicotine and tomato juice (Table 3.2.2). At week four (beginning of lactation), the lycopene intake of the mothers that received only tomato juice was 10.67±1.4 mg/g body mg/wk, and that of the mothers that received a combination of nicotine and tomato juice, this was 10.72±1.02 mg/g body g/wk (P>0.05).

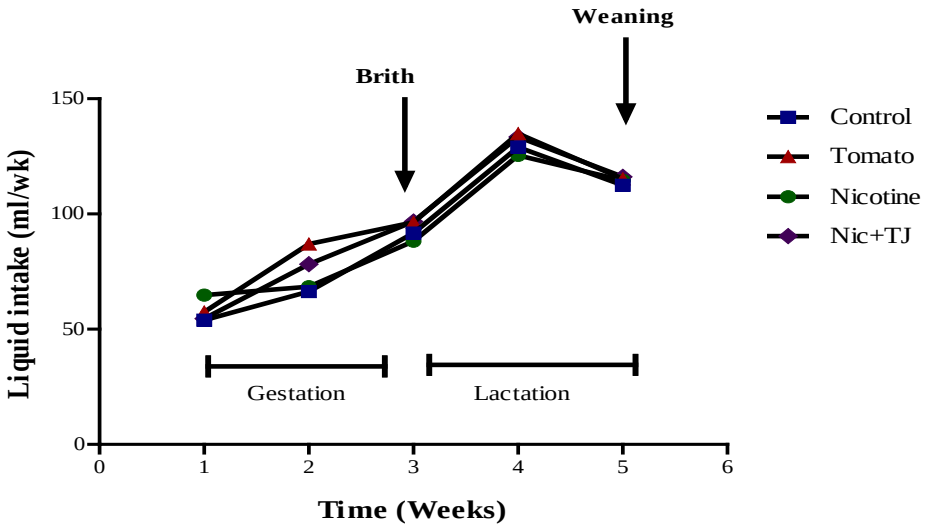


Figure 3.2.1: Liquid intake (ml/ week) of the control mothers (water), those that received tomato juice, the nicotine exposed (water), and those that received both nicotine and tomato during gestation and lactation. P-value: No differences in liquid intake within the control and experimental groups (P>0.05).

	Tomato(T)	**Tomato+ nicotine(T +N)**	**P: T vs. T+N**
Week 1	20.03±1.34	22.5±1.77	>0.05
Week 4	10.67±1.4	10.72±1.02	>0.05

Table 3.2.2: Lycopene intake per 100 g body weight per week of the rats receiving tomato juice only, and the rats receiving both tomato and nicotine.

3.2.3 Body weight increase

Figure 3.2.3 and Table 3.2.3 illustrates the weekly increase in body weight of the mothers during gestation and lactation when exposed to nicotine, tomato juice supplementation only, or exposure of the pregnant mothers to both nicotine and tomato juice. Week three marked the end of gestation. Between gestational week one and three, the weekly body weight increase of mothers that were exposed to nicotine only, increased by 54 g/week so that at gestational week three it was at 354.50±8.31

g, 32.10 % higher ($P<0.002$) than that of the control group which was 268.86±11.80 g at gestational week three and thus increased by 29.86 g/week. The body weight increase up to gestational week three of the pregnant mothers that received tomato juice only (283.71±17.87 g) and those that received a combination of nicotine and tomato juice (267.50±20.30 g) increased at a similar rate ($P>0.05$). Between gestational week one and week three, the body weight increase of the control group was not different from that of the pregnant mothers that received tomato juice only, or those that were exposed to both nicotine and tomato juice ($P>0.05$). Week six marked the end of lactation and the termination of nicotine and/or tomato juice treatment of the mothers. The weekly decrease in the BW of the mothers between gestational week four and week six was such that by week six, there was no difference between BW of the control group and that of the nicotine group or the other experimental groups ($P>0.05$).

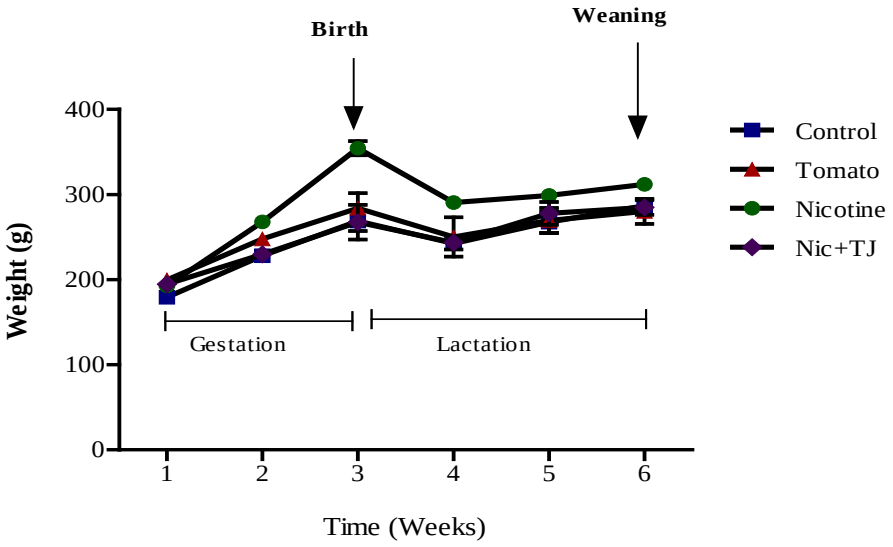


Figure 3.2.3: The effects of maternal exposure to nicotine or tomato juice, or the combination of both nicotine and tomato juice on the body weight of mothers during gestation (weeks 1 to 3) and (week 3 to week 6) lactation (Weeks 4 to 6). P-value: No differences (P>0.05) in BW within the experimental groups during gestation or lactation.

Time	Control	Tomato	Nicotine	NT	C vs. N
Week 1-3	29.86 g/ wk	28 g/ wk	54 g/ wk	24.33 g/ wk	P<0.002
Week 4-6	14.81 g/ wk	10.05 g/ wk	7.05 g/ wk	13.78 g/ wk	P>0.05

Table 3.2.3: The effects of maternal exposure to nicotine or tomato juice, or of both nicotine and tomato juice on the increase body weight per week of the mothers during gestation Weeks 1 to 3) and lactation (weeks 3 to 6). P-value: No differences (P>0.05) in BW within the experimental groups during gestation and lactation.

3.2.4 Litter size at birth.

The data in figure 3.2.4 shows that the litter size (pups/litter) at birth of the control mothers (12.86±0.59) was the same (P>0.05) as that of the mothers exposed to only nicotine (12.57±0.89), those that received tomato juice supplementation only (13.43±0.57), and those that were exposed to both tomato juice and nicotine (13.14±0.67)

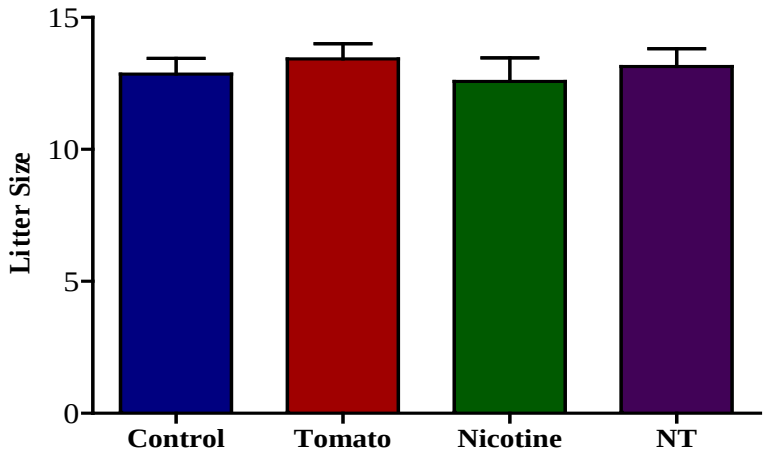


Figure 3.2.4: The effects of maternal exposure to nicotine or tomato juice, or to both nicotine and tomato juice, on the litter size at birth. P-value: No differences in litter size within the control and experimental groups (P>0.05).

3.3 Discussion

Smoking during pregnancy is the primary cause of increased prenatal morbidity and mortality (Hammoud et al., 2005; Kramer, 1987). Maternal smoking during pregnancy is one of the leading causes of a number of undesirable outcomes such as fetal growth restriction, spontaneous abortion, preterm birth, and sudden infant death syndrome (Salihu and Wilson, 2007). Maternal smoking during pregnancy also leads to intrauterine growth restriction (IUGR) (Abel, 1980b; Lieberman et al., 1994). Components of cigarette smoke such as nicotine as well as other harmful compounds limit the supply of oxygen and other vital nutrients, which results in fetal growth impairment and retardation and consequently low birth weight (Gluckman et al., 2008). Research indicate that there is a strong link between low birth-weight and fetal onset of adult disease, such as coronary heart disease, type 2 diabetes, and adiposity (Siu and Tyndale, 2007). As a result, smoking mothers are encouraged to quit the

habit. Various behavioral and pharmacotherapies are used to assist smoking mothers in quitting the habit. Pharmacotherapies include (NRT), bupropion (Faessel et al., 2009), and varenicline (Ginzel et al., 2007; Silagy et al., 2000). However, it is not advisable to be use NRT during pregnancy because of the adverse effects on the offspring (Hughes and Hughes, 1993). It is also not advisable to use the other pharmacotherapies because it shows some severe side effects in some patients (Economides and Braithwaite, 1994) and it is not known how it might affect fetal development.

It has been suggested that IUGR is due to restricted placental blood flow and thus nutrient supply to the growing fetus as a consequence of nicotine-induced vasoconstriction in the placenta (Abrams et al., 2000). If this is so, smoking or NRT will result in IUGR and lower birth weight at birth. It is also possible that the litter sizes of the nicotine exposed rats will be smaller than in the control animals. However, in this study the number of pups per litter was the same for all the groups. Furthermore, from the present study, it is clear that maternal nicotine exposure during gestation had no effect on the weight of the offspring (see chapter 4). This implies that the blood and nutrient supply to the developing fetuses was adequate to meet the demands of the growing fetuses.

Maternal weight gain during pregnancy is an essential indicator of both maternal and infant health (Abrams et al., 2000; N.R.C, 2007; W.H.O, 1995). It has been well

established in literature that gestational weight gain that is within the recommended levels, plays a vital role in reducing unfavorable outcomes during pregnancy. Several studies have shown that insufficient gestational weight gain may results in short, mid, or long term implications to maternal and pediatric health (DeVader et al., 2007; Langford et al., 2011; Strauss and Dietz, 1999). Inadequate maternal weight gain during pregnancy predisposes the infant to gestational complications and adverse outcomes such as pre-term births, low birth weight and IUGR (DeVader et al., 2007; Frederick et al., 2008; Langford et al., 2011). On the other hand, excessive gestational weight gain has also been linked to numerous complications including cesarean section delivery, hemorrhages, as well as hypertensive syndromes in pregnancy, and fetal macrosomia (Davies et al., 1976; Rantakallio and Hartikainen-Sorri, 1981; Rush, 1974). .

Reports on the influence of nicotine and smoking on maternal weight gain during pregnancy are conflicting. Some studies show that there is close relationship between maternal cigarette smoking and reduced body weight of the pregnant mother (Groff et al., 1997; Mongoven et al., 1996; Muscati et al., 1994). This is supported by other studies which showed that the weight gain of pregnant smokers tend to be less than in nonsmokers (Davies et al., 1976; Rantakallio and Hartikainen-Sorri, 1981; Rush, 1974). It has been proposed that the slower weight gain can be attributed to the fact that cigarette smoking, firstly leads to reduced appetite. From this it follows that those women who smoke during pregnancy exhibit lesser weight gain as opposed to

pregnant women who do not smoke (Benowitz, 2010). Although it is not clearly understood how smoking during pregnancy results in reduced body weight, it has been suggested that weight reduction is mediated by nicotine (Sztalryd et al., 1996).

Secondly, it has been shown that nicotine reduces lipolysis by inhibiting the activity of lipoprotein lipase, followed by a reduction of triglyceride uptake; therefore leading to a decrease in the amount of adipose tissue stored (Pardo et al., 2005). Contrary to this, other studies reported that smoking during pregnancy had no influence on the nutritional status or weight gain of pregnant mothers (Chen and Kelly, 2005; Sekhon et al., 2001a). The latter observation is supported by findings in animal studies which showed that maternal nicotine exposure during pregnancy had no influence on maternal body weight gain and body composition (Kirchengast and Hartmann, 2003). There are also studies that have indicated that smoking during pregnancy lead to higher maternal weight gain in pregnant smokers than non-smokers (Coulson et al., 1996). The reasons for the disparities in these observations are not clear.

In the present study, the exposure of the pregnant rats to nicotine, or to tomato juice or both nicotine and tomato juice, had no effect on the body weight of pregnant rats during weeks 1 and 2 of gestation. However, the BW of the nicotine exposed pregnant mothers increased faster than in the other experimental groups so that at gestational week 3, the BW of the nicotine exposed pregnant rats was significantly higher than the BW of the control rats. The higher BW of the nicotine exposed

mothers cannot be attributed to a bigger litter size. It is also not due to an increase in the amniotic fluid volume because maternal smoking, and thus by implication, nicotine intake, appear not to affect amniotic fluid volume or fetal urine output (Burn et al., 1945). It is therefore not clear why the BW of the nicotine exposed pregnant rats increased during the last week of gestation. It is that it was due to a change in control of the water balance in the body because it has been shown that cigarette smoke and nicotine stimulates the hypothalamus to release antidiuretic hormone from the posterior lobe of the pituitary gland. Although we don't have direct evidence to that effect, it is possible that nicotine in cigarette smoke is responsible for the inhibition of diuresis, because nicotine stimulates the nuclei in the hypothalamus to secrete ADH (Dalessio, 1969). The relationship between nicotine ingestion and idiopathic edema has also been documented (Finch et al., 2004). It is therefore conceivable that the continuous exposure to nicotine may result in persistent overproduction of antidiuretic hormone and a gradual accumulation of water in the body of these pregnant rats and consequently a gradual increase in body weight. Since the fluid intake by the pregnant mothers was the same as for the other groups, it could not have contributed to the increased BW of the mother during pregnancy After birth the antidiuretic effect of nicotine disappears with a consequent return of the control of water balance to normal in the bodies of these animals.. This is evident from the fact that the BW of the mothers returned to levels that matches that of the control and other experimental groups. It is also interesting to note that supplementing the diets of the nicotine exposed rats with tomato juice prevented the

increase in the body weight of the pregnant mothers that is observed at week three of gestation. The mechanism whereby supplementing the diet of the nicotine exposed pregnant rat with tomato juice prevented the anti-diuretic effect of nicotine is not known.

To conclude, maternal nicotine exposure during gestation resulted in an increase in body weight of the pregnant mothers during the last third of pregnancy. This appears to be due to an antidiuretic effect of nicotine which lasted only during pregnancy. Litter sizes were not affected by maternal nicotine exposure and was therefore not implied in the increased BW of the nicotine exposed pregnant mothers.

CHAPTER FOUR

Effects of nicotine and tomato juice on growth and lung development of the offspring

4.1 Introduction

The negative impacts of maternal smoking on the health and development of the offspring and that of smoking mothers have raised a great deal of interest through research in both medical and behavioral studies. Researchers have established the relationship between maternal smoking and low birth weight, respiratory dysfunction, delayed motor development, as well as numerous unpleasant health outcomes that manifest early during childhood and persist even in later life. In spite of the ongoing research demonstrating that cigarette smoking during pregnancy is related to unfavorable fetal, obstetrical, and developmental outcomes, reports show that approximately 15 to 20 % of pregnant women smoke during pregnancy (Bergmann et al., 2003; Andres and Day, 2000). In Cape Town, it has been estimated that 47 % of pregnant women smoke during pregnancy (Everett-Murphy et al., 2010). Tobacco smoking during pregnancy has been linked to various undesirable effects that result in, for example, alterations in lung structure as well as reduced lung function (Moshammer et al., 2006). Cigarette smoking is also associated with evidence of mild airway obstruction and slowed growth of lung function in adolescents. Adolescent girls may be more vulnerable than boys to the effects of smoking on the growth of lung function (Gold et al., 1996).

It has been shown that maternal smoking, as well as maternal inhalation of environmental tobacco smoke (ETS) has adverse effects on fetal growth. Two unfavorable birth outcomes that result in a lower birth weight include preterm delivery and intra uterine growth retardation (IUGR). Maternal smoking has consistently been illustrated to increase the risk of IUGR and to reduce the birth weight of the newborns (Jaakkola et al., 2001). Furthermore, neonates born to active smokers have smaller head circumferences, an increased risk of congenital anomalies such as oro-facial clefts (Khoury et al., 1989; Wang et al., 1997), And a reduced length and thoracic perimeters at birth (Roquer et al., 2008). This risk increases with an increase in the number of cigarettes smoked per day by the mother. Timing also affects pregnancy outcomes (Misra and Nguyen, 1999; Abel, 1980a). Women who smokes during the second halve of pregnancy have the highest risk of IUGR and low birth weight babies (Hebel et al., 1988).

Several mechanisms have been proposed to explain the tobacco smoke related effects on the fetus and neonate. Some evidence suggests that the impaired growth associated with maternal smoking is due to reduced oxygen flow to the fetus due to constriction of the blood vessels in the placenta, resulting in hypoxia and IUGR (Zigic et al., 2008; Asmussen, 1980).

Although tobacco smoke contains many different chemicals, nicotine has been implicated as the causative factor for IUGR and low birth weight of neonates (Bergen, 2006) by:

1. Suppression of maternal appetite and increased energy expenditure
2. The detrimental effect on placental structure and function
3. The detrimental effect on fetal metabolism
4. Interfering with central regulatory mechanisms.

It is suggested that the effects ascribed to nicotine is due to very high concentrations of nicotine used in some of the studies (Becker et al., 1968; Hudson and Timiras, 1972). It has been shown in some instances babies born to women using NRT, have higher birth weights than those not using NRT (Wickstrom, 2007). This means that nicotine in tobacco smoke is not responsible for the low BW of the offspring of mothers that smoke during pregnancy. On the other hand, it also displays a dual effect in that the response at high levels of nicotine differs from that at lower levels (Wickstrom, 2007). This may explain why certain studies showed a decrease in the BW of the offspring of animals that were exposed to nicotine via the placenta (Bruin et al., 2010).

Apart from having oxidant properties (Bruin et al., 2008b), nicotine also induces oxidant formation in tissue (Latha et al., 1993; Ashakumary and Vijayammal, 1996). Oxidative stress can also contribute to fetal growth restriction (Stanley et al., 2012).

This may affect fetal growth and development in mothers that smoke or use NRT. NRT is prescribed by some health professionals, but it has been shown that nicotine:

1. Have oxidant properties (Bruin et al., 2008b)
2. Reduce the anti-oxidant capacity of the mother (Aycicek et al., 2005), the aims of this study were therefore to:

a. Assess the effect of maternal nicotine exposure during gestation and lactation on the growth and development of the offspring, and

b. Determine whether any changes in growth and development due to nicotine can be prevented by restoring the oxidant/anti-oxidant capacity of the mother and fetus.

4.2 Results

4.2.1 Effects of maternal exposure to nicotine during gestation and lactation on the Body weight and growth of the male and female offspring

The data illustrated in fig 4.2.1 and table 4.2.1 show that the BW of the male and the female offspring were similar up to postnatal day 42, which means the daily increase in the BW of the male and female rats were similar between postnatal days 14 and 42. However, from postnatal day 42 (Table 4.2.1), the daily growth rate of the male rats was approximately 50% faster than that of the females so that at postnatal day 84 the BW of the males were significantly higher ($P<0.001$) than that of the females. The BW of the control male and female groups at postnatal days 42 and 84 was the same

as that of the males and females in the experimental groups (P>0.05). Fig 4.2.1b clearly shows that nicotine had no effect on the BW of the male and female offspring.

A.

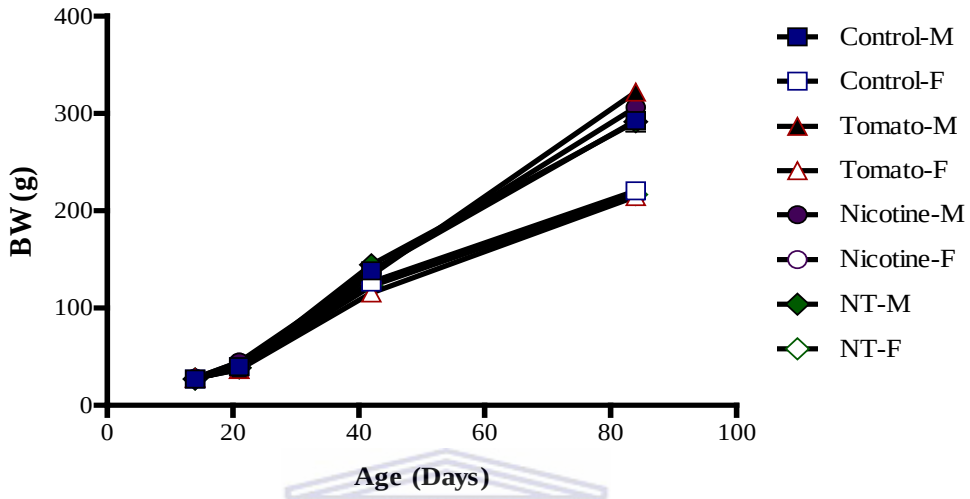


Figure 4.2.1A: The effects of maternal exposure to nicotine, tomato juice, or to both nicotine and tomato juice, on the body weight of the male and female offspring. P at postnatal days 63 and 84; Males vs. females: <0.001. No differences within experimental groups from day 14 up to day 84. (Control-M= control male; Control-F= control female; Tomato M= males received only tomato juice only; Tomato-F= Females received tomato juice only; Nicotine-M= males received nicotine only; Nicotine-F Females receiving nicotine only; NT-M= males received both nicotine and tomato juice; NT-F= Females received both nicotine and tomato juice).

B.

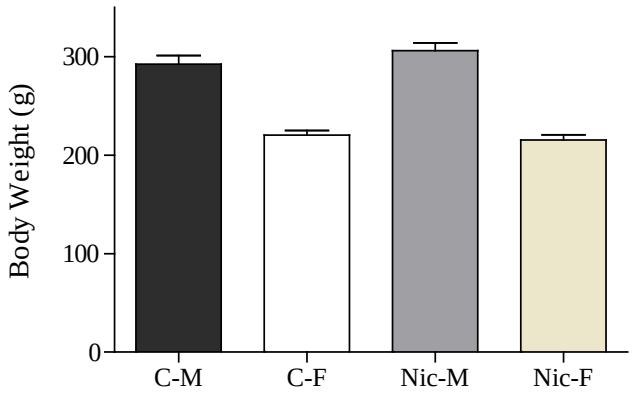


Figure 4.2.1B: The effects of maternal exposure to nicotine on the body weight of the male and female offspring at postnatal day 84. P: Significant between males and females (P<0.001). (C-M= control male; C-F= control female; Nic-M= males received nicotine only; Nic-F=Females receiving nicotine only).

Age	Gender	C (g/day)	Tomato-J (g/day)	Nic. (g/day)	N+T (g/day)
Day 14-42	**Males**	3.96	3.78	3.9	4.2
Day 14-42	**Females**	3.96	2.94	3.52	3.42
P: M vs. F		> 0.05	> 0.05	> 0.05	> 0.05
Day 42-84	**Males**	3.68	4.48	4.03	3.5
Day 42-84	**Females**	2.23	2.48	2.12	2.23
P: M vs. F		< 0.001	< 0.001	< 0.001	< 0.001

Table 4.2.1: The effects of maternal exposure to nicotine, tomato juice, both nicotine and tomato juice on the daily body weight of the male (a) and female (b) offspring between postnatal days 14 to 42, and postnatal days 42 to84. The weight of the pups is expressed in body weight increase per day (g/day) P-value: No differences within experimental groups from day 21 up to day 84 (P>0.05).

4.2.2. Effects of maternal exposure to nicotine during gestation and lactation on the chest circumference (CC) and crown rump length (CRL) of the male and female offspring

4.2.2.1 Chest circumference (CC)

As for the BW the chest circumference (CC) of the male and female animals increased at a similar rate (P>0.05) between postnatal days 14 and 42. However, between postnatal days 42 and 84, the CC of the male animals increased faster (P>0.05) than that of the females animals. At postnatal day 84, there were no differences observed (P>0.05) between the CC of the control males (150.29±1.96 mm), the males exposed to nicotine (154.67±1.84 mm), the male pups that received tomato juice only (155.61±1.83 mm), or the males exposed to both nicotine and tomato juice (148.57±2.09 mm). This implies that the CC of the control male offspring as well as those of the other experimental groups increased at the same rate between postnatal days 14 and 84.

The change in the CC of the female offspring followed the same pattern as that of the male rats. At postnatal day 84, the CC of the control female animals was 135.38±1.60 mm, and that of the females exposed to nicotine was 138.19±1.23 mm. The CC of the female rats that received tomato juice was 136.59±1.59 mm and that of the females that were exposed to both nicotine and tomato juice was 148.57±2.09 mm at postnatal day 84. No differences were observed between the CC of the control female rats and that of the females in the experimental groups between postnatal days 14 and 84.

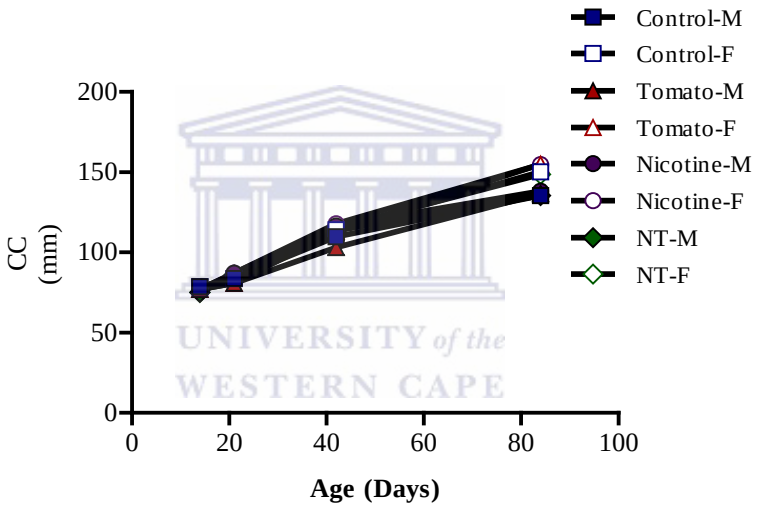


Figure 4.2.2.1: The effects of maternal exposure to nicotine, or to tomato juice, or to both nicotine and tomato juice on the chest circumference of the male and female offspring. P-value: No differences within experimental groups from day 21 up to day 84. P-value: Males vs. Females is significant (P<0.001), Nicotine.-M vs. Control.-F is not significant (P>0.05). (Control-M= control male; Control-F= control female; Tomato J-M= males received only tomato juice only; Tomato-F= Females received tomato juice only; Nicotine-M= males received nicotine only; Nicotine-F Females receiving nicotine only; NT-M= males received both nicotine and tomato juice; NT-F= Females received both nicotine and tomato juice).

4.2.2.2 Crown Rump Length (CRL)

The crown rump length (CRL) of the male animals was similar (P>0.05) to that of females between postnatal days 14 and 42. As for the CC and BW changes after

postnatal day 42, the CRL of the male animals increased faster than that of the female animals so that on postnatal day 84, the CRL of the male animals was approximately 13% higher than that of the female animals. At postnatal day 84, the CRL of the control male animals (202.43±1.48 mm) was the same (P>0.05) than that of the males exposed to nicotine (203.81±1.36 mm), as well as that of the males that received only tomato juice (205.67±1.47 mm), and those males that received both nicotine and tomato juice (203.48±1.51 mm). Similarly, the CRL of the female controls (190.11±1.07 mm) was not different (P>0.05) from that of the females that were exposed to nicotine (186.86±1.45 mm), the females that received tomato juice (183.77±1.43 mm), or those that received both nicotine and tomato juice (184.00±1.63 mm).

4.2.3 Effects of maternal exposure to nicotine during gestation and lactation on the chest circumference to body weight ratio (CC/BW) and crown rump length to body weight ratio (CC/CR) of the male and female offspring

The chest circumference to body weight ratio (CC/BW) (Fig 4.2.3.1), and the crown rump length to body weight ratio (CC/CRL) (fig 4.2.3.2) for both the males and the female pups changed with an increase in age. No difference (P>0.05) in the CC/BW or the CC/CRL ratios was seen between the control and the experimental groups at each of the age groups for both males and females. This implies that growth was proportional in all groups.

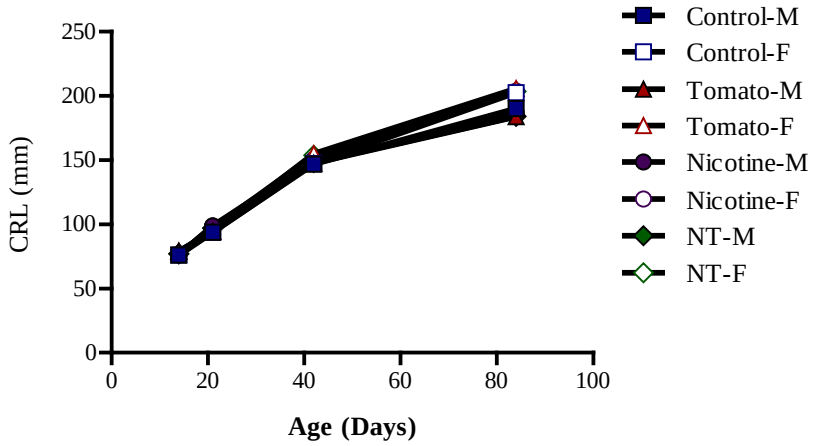


Figure 4.2.2.2: The effects of maternal exposure to nicotine, or to tomato juice, or to both nicotine and tomato juice on the crown rump length (CRL) of the male and female offspring between postnatal days 14 to 42, and postnatal days 42 to84. P-value: No differences within experimental groups between postnatal days 21 up to day 42. At postnatal day 84, P-value: Males vs. Females is significant (P<0.001), Nicotine.-M vs. Control.-F is not significant (P>0.05). (Control-M= control male; Control-F= control female; Tomato J-M= males received only tomato juice only; Tomato-F= Females received tomato juice only; Nicotine-M= males received nicotine only; Nicotine-F Females receiving nicotine only; NT-M= males received both nicotine and tomato juice; NT-F= Females received both nicotine and tomato juice).

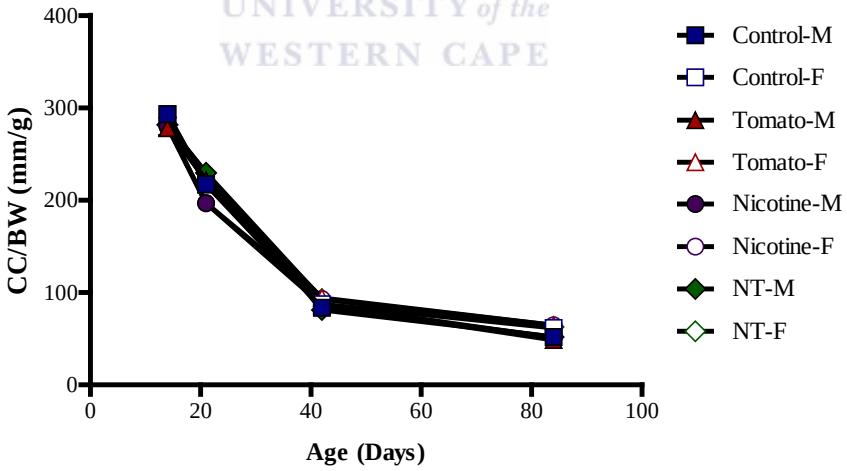


Figure 4.2.3.1: Effect of nicotine and tomato juice during pregnancy and lactation on chest circumference to body weight ratios in the females and males respectively. P-value: No differences within experimental groups (P>0.05). (Control-M= control male; Control-F= control female; Tomato-M= males received only tomato juice only; Tomato-F= Females received tomato juice only; Nicotine-M= males received nicotine only; Nicotine-F Females receiving nicotine only; NT-M= males received both nicotine and tomato juice; NT-F= Females received both nicotine and tomato juice).

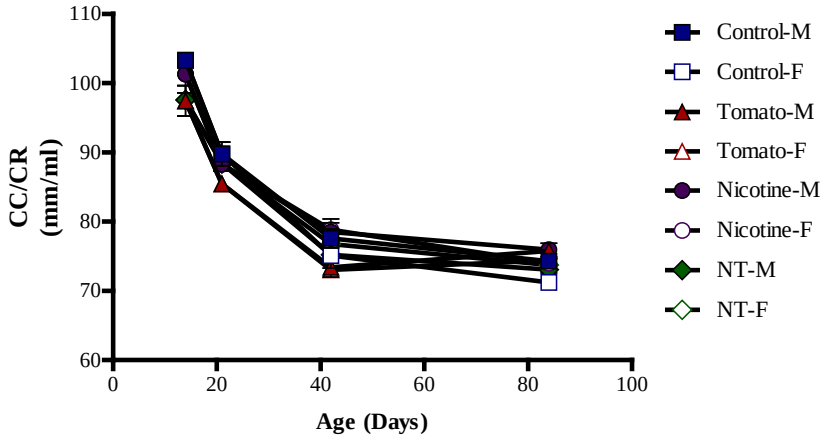


Figure 4.2.3.2: Effect of nicotine and tomato juice during pregnancy and lactation on chest circumference and crown-rump length ratios in males and females respectively. No differences within experimental groups (P>0.05) (Control-M= control male; Control-F= control female; Tomato-M= males received only tomato juice only; Tomato-F= Females received tomato juice only; Nicotine-M= males received nicotine only; Nicotine-F Females receiving nicotine only; NT-M= males received both nicotine and tomato juice; NT-F= Females received both nicotine and tomato juice).

4.2.4 Effects of maternal exposure to nicotine during gestation and lactation on lung volume (Lv) of the male and female offspring

As shown in table 4.2.4.1 and fig 4.2.4.1a, the daily increase the Lv of the male and female rat pups was the same between postnatal days 14 and 42. After postnatal day 42, the daily increase in the Lv of male animals exceeded that of the females so that by postnatal day 84 the Lv of the male animals was approximately 20 % bigger than that of the female animals (P<0.001). Between postnatal days 63 and 84, the Lv of the male offspring exposed to nicotine increased faster than that of the control males and that of the other experimental animals (P>0.001). As shown in figs 4.2.4.1a-d, the Lv of the males exposed to nicotine only (13.96 ± 0.33 ml) was about 36 % higher (P<0.001) than that of the control males (10.24 ± 0.31 ml). However, the Lv of the

males that received tomato juice only (11.24 ± 0.26 ml) and those that received both nicotine and tomato juice (10.85 ± 0.33 ml) was similar ($P>0.05$) to that of the control animals at postnatal day 84. Similarly, the Lv of the females exposed to nicotine only increased faster than that of control rats as well as that of the females in the other experimental groups ($P<0.001$) between postnatal days 63 and 84. At postnatal day 84, the Lv of the females exposed to nicotine (11.70 ± 0.37 ml) was about 27 % higher ($P<0.001$) than that of the control females (9.21 ± 0.30 ml). The Lv of the control females was similar ($P>0.05$) to that of those females exposed to tomato juice only (9.04 ± 0.25 ml) as well as those exposed to a combination of nicotine and tomato juice (8.82 ± 0.32 ml) at postnatal day 84.

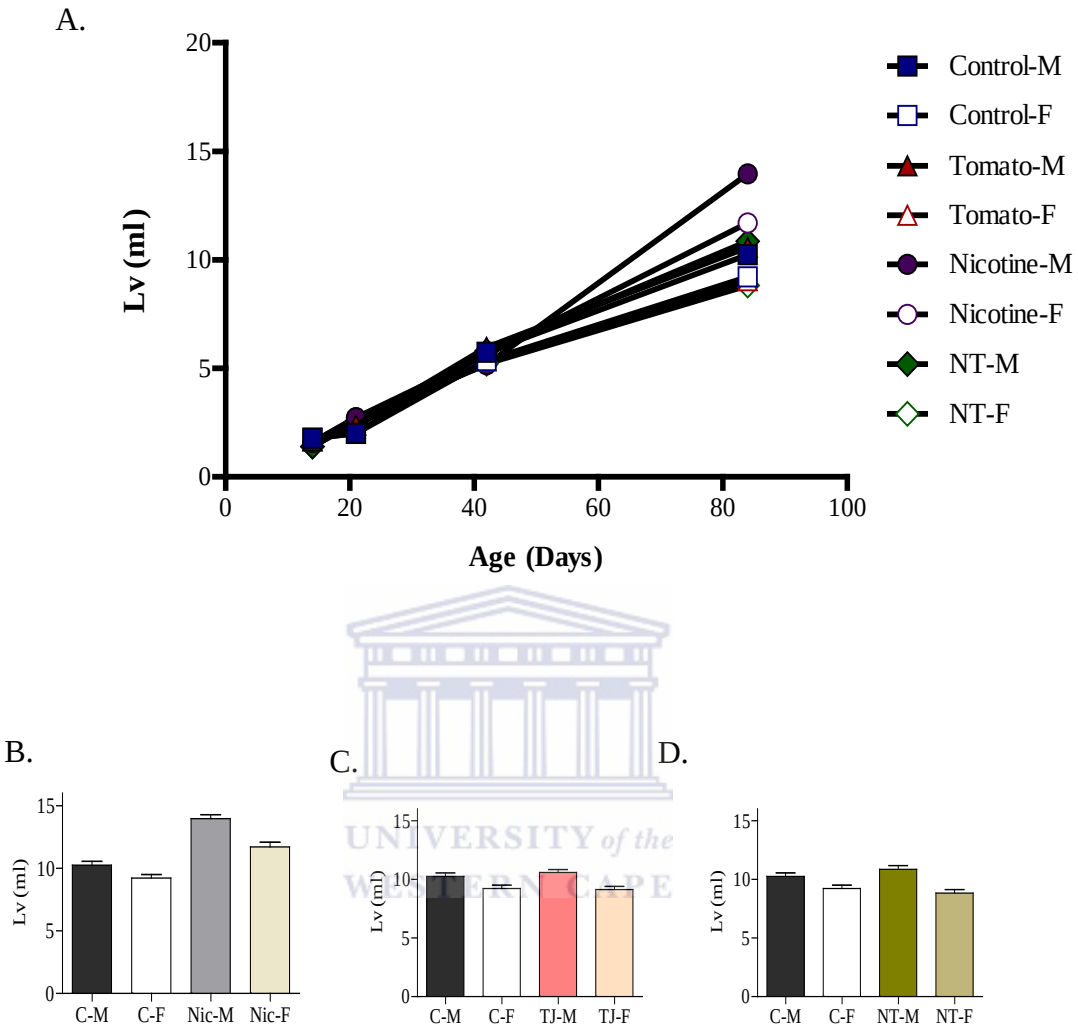


Figure 4.2.4.1A:-The effects of maternal exposure to nicotine during gestation and lactation, or to tomato juice, or to both nicotine and tomato juice on the Lung volume (Lv) of the male and female offspring. **B-D:** Illustration of the Lv of the male and female offspring at postnatal day 84 (Control-M= control male; Control-F= control female; TJ-M= males received only tomato juice only; TJ-F= Females received tomato juice only; Nic-M= males received nicotine only; Nic-F= Females receiving nicotine only; NT-M= males received both nicotine and tomato juice; NT-F= Females received both nicotine and tomato juice).

	Gender	C (ml/day)	Tomato-J (ml/day)	Nic. (ml/day)	NT (ml/day)	P: C vs. N
Day 14-42	Males	3.97	4.3	3.59	4.4	> 0.05
	Females	3.58	3.68	4.04	3.81	> 0.05
P: M vs. F		> 0.05	> 0.05	> 0.05	> 0.05	
Day 42-84	Males	4.5	5.28	8.79	5.05	< 0.001
	Females	3.85	3.78	6.07	3.61	< 0.001
P: M vs. F		< 0.001	< 0.001	< 0.001	< 0.001	

Table 4.2.4: The effects of maternal exposure to nicotine, tomato juice, and both nicotine and tomato juice on the increase on the lung volumes per day of the male and female offspring at postnatal days14, 21, 42, and 84. (C = control; Tomato J = pups that received only tomato juice only; Nic. = pups that received nicotine only; NT = pups that received both nicotine and tomato juice. The exposure to nicotine only, tomato juice only or both nicotine and tomato juice to the offspring was only via the placenta end the mother's milk. Only the mothers received nicotine or tomato juice or both nicotine and tomato juice).

4.2.5 Effects of maternal exposure to nicotine during gestation and lactation on the chest circumference/lung volume ratio (CC /Lv) and lung volume/body weight (Lv/BW) of the male and female offspring

Between postnatal days 14 and 42, the chest circumference to lung volume ratio (CC /Lv) of the male offspring was similar to that of the females of the same age (P > 0.05). The CC/Lv ratios of the control males and females were also not different (P > 0.05) from that of the experimental animals (Fig 4.2.5.1). At postnatal day 84, the CC/Lv ratios of the males and females exposed to nicotine only, was smaller than that of the control males and females as well as the males and females in the other experimental group (P < 0.001). There were no differences between the CC/Lv ratios of the control females and that of the females exposed to tomato juice only or those that received both nicotine and tomato juice (P>0.05). The faster decrease in the

CC/Lv ratios in both the male and female offspring exposed to nicotine between postnatal days 42 and 84 (Table 4.2.5) could be attributed to the faster increase in Lv observed in the male and female animals that were exposed to nicotine only.

The results demonstrated in fig 4.2.5.2a show that up to postnatal day 42 the lung volume to body weight (Lv/BW) ratios of the experimental male and female animals were not different ($P>0.05$) from the Lv/BW ratios of the control males and females. However at postnatal day 84, the Lv/BW ratios of the male and female groups that were exposed to nicotine only, were bigger ($P<0.001$) than the Lv/BW ratios of the male and female control groups. The Lv/BW ratios of the males and females exposed to nicotine only were at postnatal day 84 also higher than ($P<0.001$) those that were exposed to tomato juice only as well as those that received both nicotine and tomato juice (Fig. 4.2.5.2b-d). Contrary to the above, the Lv/BW ratios of the control males and females were similar to that of the females that received tomato juice only and those that received both nicotine and tomato juice ($P>0.05$).

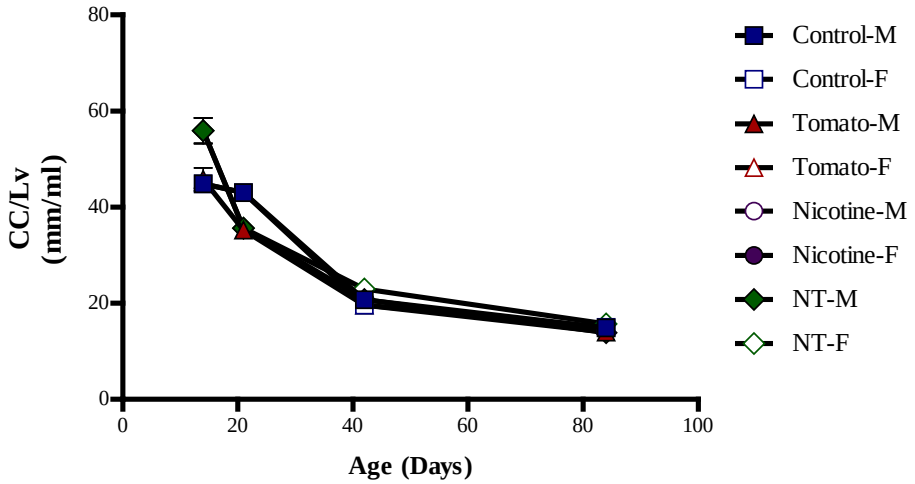


Figure 4.2.5.1: The effect of maternal nicotine exposure and intake of tomato juice on the CC/Lv ratios of the male and female rats that were exposed to nicotine via the placenta and mother's milk. (Control-M= control male; Control-F= control female; Tomato-M= males received only tomato juice only; Tomato-F= Females received tomato juice only; Nicotine-M= males received nicotine only; Nicotine-F Females receiving nicotine only; NT-M= males received both nicotine and tomato juice; NT-F= Females received both nicotine and tomato juice).

	Males (mm/ml)		**Females (mm/ml)**	
	Day 42	Day 84	Day 42	Day 84
Control	20.70±0.95	14.89±0.38	19.62±0.18	14.98±0.46
Tomato	19.62±0.81	13.96±0.30	20.82±0.86	15.23±0.46
Nicotine	21.78±1.16	11.19±0.26	23.21±1.08	12.03±0.37
N+T	20.78±1.02	13.89±0.35	23.00±1.25	15.72±0.53

Table 4.2.5: The effects of maternal exposure to nicotine, tomato juice, and the combination of both nicotine and tomato juice on CC/Lv ratios of the male and female offspring between postnatal days 14 to 42, and postnatal days 42 to 84.

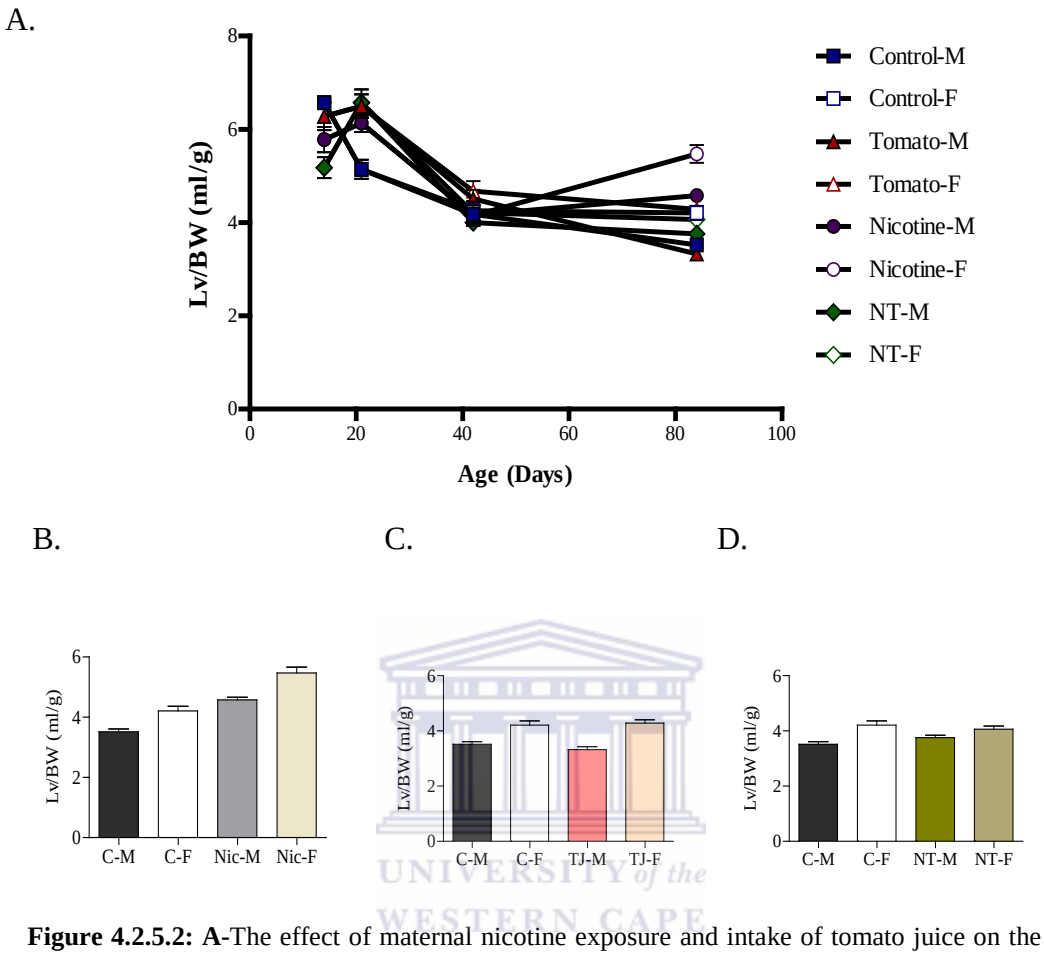


Figure 4.2.5.2: A-The effect of maternal nicotine exposure and intake of tomato juice on the Lv/BW ratios of the offspring. The offspring were exposed to nicotine via the placenta and mother's milk during gestation and lactation. **B-D:** Illustration of the Lv of the male and female offspring at postnatal day 84. P-value: C-M vs. Nic.-M is significant (P<0.001), Nic.-F vs. Nic.-F is significant (P<0.001); Males vs. Females is significant (P<0.001). (Control-M= control male; Control-F= control female; Tomato J-M= males received only tomato juice only; Tomato-F= Females received tomato juice only; Nicotine-M= males received nicotine only; Nicotine-F Females receiving nicotine only; NT-M= males received both nicotine and tomato juice; NT-F= Females received both nicotine and tomato juice).

4.3 Discussion

The objective of this part of the study was to determine the effects of maternal nicotine exposure during gestation and lactation on growth and development of the offspring. Various reports regarding the effect of maternal nicotine exposure during pregnancy and lactation on, for example body weight at birth, occur in the literature.

It is generally reported that maternal nicotine exposure causes a lower birth weight and body fat of the offspring due to higher energy expenditure under the influence of nicotine (Grove et al, 2001). Maternal nicotine exposure also slows bone lengthening in the offspring (Kortuglo et al, 2007). This means that nicotine intake via smoking or NRT during pregnancy may interfere with the growth of the fetus and offspring resulting in intrauterine growth retardation (IUGR).

Research indeed shows that cigarette smoking is associated with an increased risk of adverse postnatal health outcomes. It has been suggested that some of these adverse outcomes are a result of (IUGR (Andres and Day, 2000; England et al., 2001) because IUGR has been shown to have long-term effects on the growth (Haug et al., 2000) and development of the offspring of smoking mothers (Ashakumary and Vijayammal, 1996). One of the causes of IUGR due to maternal smoking or NRT during pregnancy (Matsubara et al., 2000; Ramsay and Reynolds, 2000) is, for example, offspring with an increased propensity for obesity, hypertension, and type 2 diabetes (Barker, 1998; Barker and Clark, 1997; Seckl, 2001). Other studies using the rat as model showed that maternal exposure to nicotine during gestation and lactation, like maternal smoking, also results in a lower BW in the offspring during infancy (Cliver et al., 1995; Kramer, 1987; Lawrence et al., 2008). It has been suggested that prenatal exposure to nicotine induces constriction of placental blood vessel causing a reduced placental blood flow. Consequently, the nutritional supply to the fetus is restricted. Maternal nicotine exposure during pregnancy could therefore

suppress fetal growth resulting in lower birth weight of the offspring (Birnbaum et al., 1994; Philipp et al., 1984).

In contrast with the above some studies have demonstrated that prenatal nicotine exposure in rats resulted in increased postnatal body weight (Gao et al., 2005; Newman et al., 1999; Somm et al., 2008) as well as an increased level of body fat. during gestation (Williams and Kanagasabai, 1984). This increase in body fat of the new born is also apparent in later life in the offspring of mothers who smoked or were exposed to nicotine during pregnancy (Gao et al., 2005; Somm et al., 2008). In the present study, maternal nicotine exposure during gestation and lactation had no effect on the outcome of the body weight, chest circumference and crown-rump length of the offspring. These results are in agreement with other studies in which it was shown that maternal nicotine exposure did not affect growth parameters such as chest circumference and crown-rump length of the offspring (Maritz et al., 2011b; Maritz and Windvogel, 2003; Pausová et al., 2003). This means that maternal nicotine exposure during gestation and lactation had no influence on growth of the offspring. This does not mean that maternal nicotine exposure during gestation and lactation had no effect at cellular level or on any of the homeostatic mechanisms of the offspring. It also does not imply that organ growth was proportional in the nicotine exposed offspring.

These contradictory findings can be ascribed to the different concentrations of nicotine used by various researchers. It has been suggested by Economides and Braithwaite (1994) that the chemical composition of cigarette smoke is more closely related to reduction in fetal growth than the number of cigarettes smoked. In smokers, the placenta appears remarkably normal. The effects of smoking could be due to one or more of the more than 4000 different chemical substances in tobacco smoke. However, apart from carbon monoxide and nicotine, little is known about the effects of other toxins. Nicotine can adversely affect uterine and placental blood flow by causing constriction of the blood vessels and thus the flow of nutrients across the placenta to the fetus. Although this explanation is offered by Economides and Braithwaite (1994), studies by Birnbaum et al. (1994) in which they used 9.6, 4.8, or 2.4 mg nicotine/ kg body weight/day , the placental weights of the rats were lower in the nicotine exposed rats. The body weights of the dams were also severely reduced. In addition the uterine and placental blood flow was reduced by more than 40%. They, however, conclude that vasoconstriction alone as a result of nicotine exposure during the last trimester of gestation does not necessarily reduce nutrient supply to the fetus and does not affect fetal growth in rats. It has been shown that only in isolated cases chronic nicotine abuse led to intra-uterine growth retardation, with no other apparent causes of placental insufficiency (Philipp et al., 1982). The stage of pregnancy when exposure occurs may thus also play a role in determining the effect of nicotine on the growth and development of the fetus.

In addition to the above, it is likely that the duration of placental vasoconstriction in this study, and consequently the time of inadequate nutrient supply were too short to have a considerable effect on growth of the offspring. In addition, the results also indicated that maternal nicotine exposure during gestation and lactation had no influence on the chest circumference (CC) and the crown rump length (CRL). This suggests that maternal nicotine exposure during gestation and lactation had no influence on the growth parameters and also on the proportional growth of the offspring. Furthermore, the half-life of nicotine in the pregnant rats is shorter because nicotine metabolism is enhanced during pregnancy (Benowitz, 2009). This implies that nicotine is removed faster from the mother's circulation and thereby further reducing the impact of nicotine on placental blood flow and transfer of nutrients to the fetus. It is therefore conceivable that the feto-placental blood supply and thus the nutrient supply were not significantly affected by nicotine. This is supported by the observation that growth of the offspring was not affected. The increase in lung volumes of the nicotine exposed pups as the animals mature up to postnatal day 42 resembled that of the controls which is a further indication that prenatal nicotine exposure had no apparent effects on the initial postnatal development of the lungs. It is therefore unlikely that lung growth, development, or maintenance of the lung in the offspring, could be as a result of poor nutrient supply to the fetus and neonate, but rather a consequence of nicotine and/or its metabolites.

Although the BW and growth parameters of the offspring was not affected by maternal nicotine exposure during gestation and lactation, the lung volumes (Lv) of the male and female rats were higher later in the life of the nicotine exposed offspring than those of the control and other experimental groups. This increase in lung volume that only became apparent at postnatal day 63 and gradually became more significant as the animals aged appears to be programmed during gestation and lactation since these animals were not exposed to nicotine after weaning at postnatal day 21. This late effect of maternal nicotine exposure during gestation and lactation is likely due to a gradual deterioration of the connective tissue framework later in the life of the offspring (Maritz and Windvogel, 2003). It is likely that this may result in an increase in the compliance of the lungs of the offspring that were exposed to nicotine via the placenta and mothers' milk.

To conclude, maternal nicotine exposure (1mg/kg body weight/day) had no effect on the growth of the male and female offspring up to postnatal day 42. The Lv of the male and female rats that were exposed to nicotine during gestation and lactation increased faster after postnatal day 42 which means that the growth was not proportional to growth of the body (e.g. Lv/BW). This faster increase in Lv of the lungs of the nicotine exposed rats was prevented by supplementing the diet of the mother with tomato juice. The increase in Lv of the nicotine exposed rats only occurred later in the life of these animals suggests that it was programmed during the

fetal and early neonatal stages to increase faster later in life. This programming was prevented by tomato juice.

CHAPTER FIVE

Effects of maternal exposure to nicotine during gestation and lactation on the Lung Morphometry and Morphology of the F1 offspring: Protective effect of tomato juice.

5.1. Introduction

Exposure to external substances in the atmosphere, such as maternal smoking during fetal and early postnatal life, may result in an altered epigenome leading to an increased propensity of the offspring to disease in later life (Anway and Skinner, 2006). Diseases resulting from tobacco smoke are amongst the primary causes of respiratory diseases and of premature death worldwide. As the main substance in tobacco accountable for the addictive nature of tobacco products, nicotine is generally accepted as a causative factor in the development of respiratory diseases (Benowitz, 2009; Park et al., 2012). Furthermore, it has also been shown that nicotine induces methylation of the epigenome (Holloway et al., 2007) and in this way influence the transfer of information to the next generation.

During the period of fetal development as well as early postnatal life, the lungs of the infant are most vulnerable to the adverse effects of foreign materials such as tobacco smoke and nicotine in tobacco smoke (Heindel, 2006; Bateson, 2007; Morgan et al.,

2005). This is because a sequence of well-coordinated developmental processes occur during this time, and if altered, the processes may be followed by disturbance in organ development and function (Skinner, 2008). Additionally, during this time of development, there is a series of critical programming events in the epigenome and transcriptome coupled with cell proliferation and organogenesis. Therefore, any alterations in the epigenome and transcriptome as a result of environmental factors will be transferred to succeeding generations (Anway and Skinner, 2006). Such programs may give rise to the development of diseases later in the life of the offspring.

Previous studies have shown that maternal nicotine exposure during gestation and lactation interfered with lung development in the offspring. These include a compromised connective tissue framework and lower alveolar numbers. The latter is accompanied by an increase in the alveolar volumes and an increase in the number of alveolar wall fenestrations (Maritz and Dolley, 1996; Maritz, 2002; Maritz et al., 2005). Human studies have shown that infants who were exposed to prenatal smoking had lower expiratory flow rates even when flow was corrected for lung size (Hanrahan et al., 1992). This denotes that maternal during pregnancy impairs in utero airway development. It is well recognized that these changes are collectively indicative of emphysema. It has also been demonstrated that gradual deterioration occurred in lungs of animals exposed to nicotine as they aged, even if they were exposed to nicotine only during lactation (Maritz and Windvogel, 2003). The changes

in lung development that are brought about by nicotine exposure via the placenta and mother's milk or during lactation only result in the failure to maintain pulmonary structural and functional integrity as the animals get older.

It has been suggested 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). Since nicotine induces oxidant formation in tissue (Bruin et al., 2008b) and also have oxidant properties (Kleinsasser et al., 2005b), it is plausible that it might, by increasing the oxidant load of the tissue, affect programming of the tissue to, for example age faster. This is likely since oxidants indeed program tissue to age prematurely (Dröge, 2003). Nicotine also depletes the antioxidant levels of tissue (Reiter et al., 2001) and in this way make the tissue more susceptible to oxidant damage.

Nicotine is also genotoxic (Kleinsasser et al., 2005b). It is therefore conceivable that nicotine in tobacco smoke, or used during NRT to assist with quitting the smoking habit, will "program" developing organs via changes in the epigenome and/or genome and in this way induce changes in respiratory system that are transferable to the next generation(s). The objectives of this chapter are therefore to:

1. Determine the effects of maternal nicotine exposure during gestation and lactation on lung development in the F1 generation.

2. Establish whether preventing the effect of nicotine on the oxidant/antioxidant capacity of the mother and offspring will also prevent its effect on the lung structure and thus respiratory health in the F1 offspring and thus in subsequent generations.

In an effort to maintain the capacity of the mother, fetus and eventually the neonate to protect itself against the effects of nicotine, tomato juice was used to supplement the diets of the mothers during gestation and lactation. The reason why tomato juice was used was because it contains lycopene, known to be a strong antioxidant. It also contains vitamin C as well as other phytonutrients that is required for the optimal efficiency of lycopene (Riso et al., 1999).

5.2 Results

5.2.1 The effect of maternal exposure to nicotine only, tomato juice only, and both nicotine and tomato juice, on tissue and air volumes of the lungs of the F1 offspring.

From fig 5.2.1.1A it is evident that the tissue volume of the lungs of all the groups increased significantly ($P<0.001$) between postnatal days 21 and 84. It was observed that the tissue volume of the control males increased linearly between postnatal days 21 and 84 from 0.71±0.03 ml at postnatal day 21 to 4.17±0.30 ml at postnatal day 84; that of the males that received tomato juice increased from 0.60±0.04 ml at postnatal day 21 to 4.03±0.08 ml at postnatal day 84, while the tissue volume of those that received both nicotine and tomato juice increased from 0.58±0.05 ml to 4.35±0.15 ml

during the same period of time. In the same manner, the V_t of the female control group increased from 0.75±0.05 ml at postnatal day 21 to 3.89±0.16 ml at postnatal day 84. The Vt of the females that received tomato juice increased from 0.60±0.04 ml to 3.41±0.02 ml between postnatal days 21 and 84, and that of those that received both nicotine and tomato juice increased from 0.58±0.05 ml to 3.55±0.11 ml. On the other hand, the V_t of the nicotine exposed male and female animals increased from 0.60±0.04 ml to 3.28±0.07 ml and 0.60±0.04 to 2.58±0.06 ml respectively between postnatal days 21 and 84. The V_t of the male and female animals exposed to nicotine was lower than that of the control males and females ($P<0.05$) at postnatal days 63 and 84. From fig 5.2.1.1A, it can also be noted that the V_t of the female nicotine rats was lower than that of the males ($P<0.05$). It is interesting to note that up to postnatal day 42, that is 3 weeks after nicotine withdrawal the V_t of the nicotine exposed rats resembled that of the other groups (figs 5.2.1.1b-d). The decrease in V_t is thus only apparent after postnatal day 42. Changes in the V_t is associated with changes in Va. This means that when V_t decrease, such as in the nicotine exposed rats at postnatal day 84, the Va of the 84-dsay-old nicotine exposed rats will increase. Figs 5.2.1.1b-d shows that the increase V_t of the male animals was similar to that of the female animals of the same age. From this data, it can deduced that tomato juice intake by the mother prevented the effect of nicotine on tissue growth in the lungs of the offspring.

The findings also indicate that a distinct increase in the Va of the control, and the experimental groups occurred between postnatal days 21 and 84. The much lower

values obtained from the nicotine treated group show the destructive effects of nicotine on the lung parenchyma that are more apparent as the animals get older. 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.

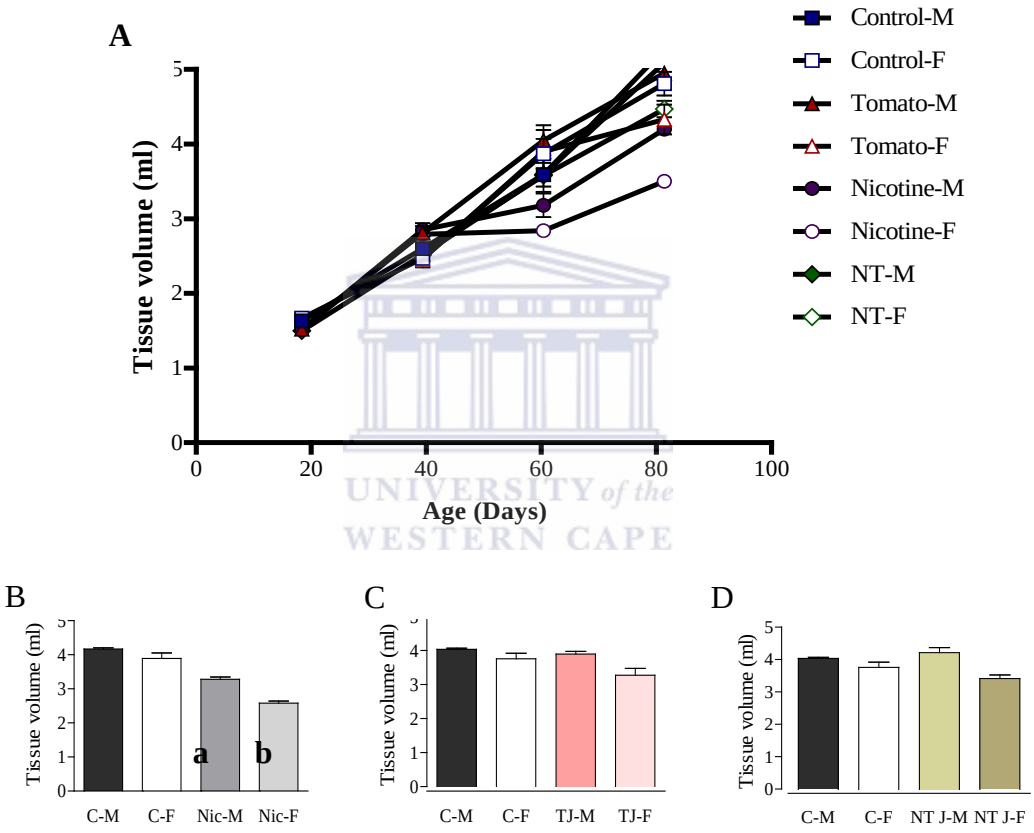


Figure 5.2.1.1.A: Effect of maternal nicotine exposure and tomato juice intake during gestation and lactation on the lung tissue volume of the offspring over time. **B-D:** Illustration of the tissue volume of the male and female offspring at postnatal day 84 where a is higher ($P<0.05$) than b. (Control-M= control male; Control-F= control female; TJ-M= males received only tomato juice only; TJ-F= Females received tomato juice only; Nic-M= males received nicotine only; Nic-F Females receiving nicotine only; NT-M= males received both nicotine and tomato juice; NT-F= Females received both nicotine and tomato juice. B-D).

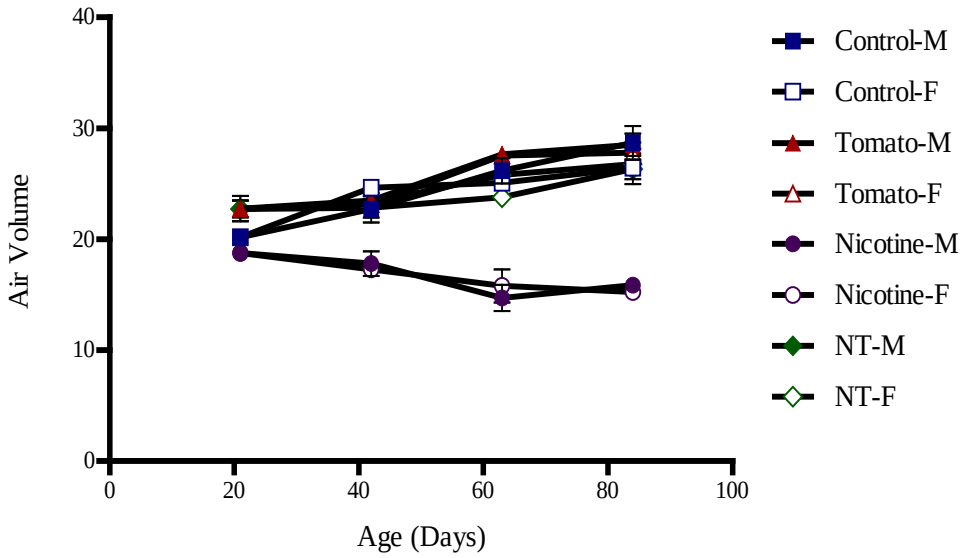


Figure 5.2.1.2: Effect of maternal nicotine exposure and tomato juice intake during gestation and lactation on the air volume of the offspring. P-value: C-M vs. Nic.-M is significant (P<0.001), Nic.-F vs. Nic.-F not significant (P>0.05); Males vs. Females not significant (P>0.05). (Control-M= control male; Control-F= control female; Tomato-M= males received only tomato juice only; Tomato-F= Females received tomato juice only; Nicotine-M= males received nicotine only; Nicotine-F Females receiving nicotine only; NT-M= males received both nicotine and tomato juice; NT-F= Females received both nicotine and tomato juice).

Age (days)	Sex	**Control**	**C vs. N**	**Tomato**	C vs. T	**Nic**	C vs. NT	**N+T**
	Males	20.19± 0.29	P<0.02	22.76± 1.13	P>0.05	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>0.05	18.77± 0.47	P>0.05	22.73± 0.75
	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
	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>0.05	15.79±1.49	P>0.05	23.77±0.09
	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

Table 5.2.1: Effect of maternal nicotine exposure and tomato juice intake during gestation and lactation on the air volume of the offspring. P-value: C-M vs. Nic.-M is significant (P<0.001), Nic.-F vs. Nic.-F not significant (P>0.05); Males vs. Females not significant (P>0.05).

5.2.2 Effects of maternal exposure to nicotine, or tomato juice only, or to both nicotine and tomato juice, on the Mean linear intercept (Lm) of the lungs of the F1 offspring.

The data presented in fig 5.2.2 shows that at postnatal day 21, the Linear Intercept (Lm) of the control male and female rats was similar to that of the experimental groups of the same age ($P>0.05$). However, between postnatal days 42 and 84, the Lm of the nicotine exposed males increased so that at postnatal day 84 it was 32 % higher ($P<0.001$) than that of the control males. Between postnatal days 21 and 84, the mean linear intercept (Lm) of the control male animals decreased from 43.77±1.57 µm to 33.65±2.17 µm, the Lm of the tomato treated group decreased from 35.23±1.63 µm to 32.13±2.16 µm, and the Lm of the group exposed to both of nicotine and tomato juice decreased from 35.23±0.75 µm to 34.18±0.56 µm ($P<0.005$). On the other hand, the Lm of the nicotine exposed males increased by 11.93 % from 36.87±0.75 µm to 47.50±2.17 µm from postnatal day 21 to postnatal day84. Between postnatal days 21 and 84, the Lm of the control females decreased from 43.77±1.57 to 32.97±0.99 µm, and that of the females exposed tomato juice only decreased from 35.23±1.63 to 32.83±0.76 µm. The Lm of the females exposed to both nicotine and tomato juice decreased from 38.90±1.47 to 33.79±0.74 µm. No differences were observed between the Lm of the female control animals (Figs 5.2.2), the females exposed to tomato juice only, and those exposed to both nicotine and tomato juice between postnatal days 21 and 84 ($P>0.05$). The Lm of the females exposed to nicotine via the placenta and mother's milk increased 10.51 % from

35.90±0.57 µm to 39.78±0.76 µm (P<0.05) from postnatal day 21 to postnatal day 84. This corresponds with that of the male rats. As a result, at postnatal day 84, the Lm of the nicotine exposed females was approximately 30 % higher (P<0.01) than that of the female control.

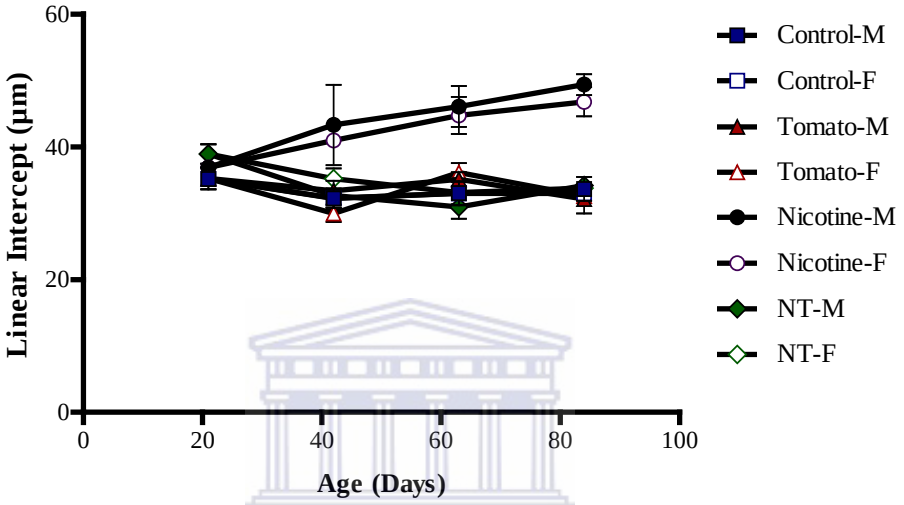


Figure. 5.2.2: Effect of maternal nicotine exposure and tomato juice intake during gestation and lactation on the Lm of the lungs of the male and female offspring. (Control-M= control male; Control-F= control female; Tomato-M= males received only tomato juice only; Tomato-F= Females received tomato juice only; Nicotine-M= males received nicotine only; Nicotine-F= Females receiving nicotine only; NT-M= males received both nicotine and tomato juice; NT-F= Females received both nicotine and tomato juice.

5.2.3 Effects of maternal exposure to nicotine, tomato juice only, or combination of nicotine and tomato juice on Alveolar wall thickness (Tsept) of the F1 offspring

Up to postnatal day 42 the Tsept (Fig 5.2.3) of the control male rats was similar to that of the males in the experimental groups (P>0.05). No differences (P>0.05) in alveolar wall thickness were observed between males and females. The increase in

the Tsept of the nicotine exposed animals only begin to show at postnatal day 63.The increase was such that at postnatal day 84, the alveolar wall thickness of the nicotine exposed males (13.92±0.85 µm) animals was 31 % higher than that of the control male animals (9.60±0.27 µm) (Fig 5.2.3).

Likewise, the alveolar wall thickness of the 84-day-old females exposed to nicotine (13.59±0.98 µm) was 37.6 % thicker than that of the control female animals (8.48±1.07 µm). At postnatal day 84 the Tsept of the male controls was the same ($P>0.05$) as those that received tomato juice only (9.99±0.27 µm), and those exposed to both nicotine and tomato juice was (9.18±0.26 µm). Furthermore, there were no differences ($P>0.05$) between the alveolar wall thickness of the control females and that of the females that received tomato juice only (8.48±0.85 µm) or those that received a combination of nicotine and tomato juice (7.86±0.30 µm). No male differences were observed between the alveolar wall thickness of the males and the females that were exposed to nicotine only between postnatal days 21 and 84 ($P>0.05$).

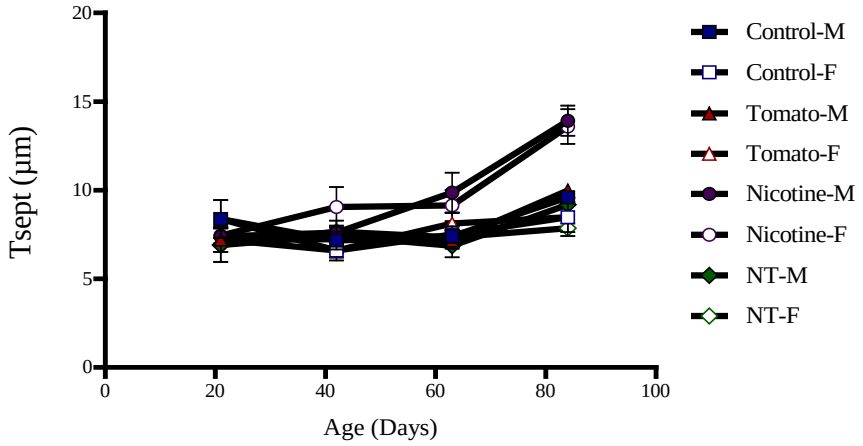


Figure 5.2.3: Effect of maternal nicotine exposure and intake of tomato juice during gestation and lactation on the alveolar wall thickness (Tsept) of the male and female offspring. (Control-M= control male; Control-F= control female; Tomato Juice-M= males received only tomato juice only; Tomato Juice-F= Females received tomato juice only; Nicotine-M= males received nicotine only; Nicotine-F= Females receiving nicotine only; NT-M= males received both nicotine and tomato juice; NT-F= Females received both nicotine and tomato juice.

5.2.4 Effects of maternal exposure to nicotine, tomato juice only, or combination of nicotine and tomato juice, on lung Morphology of the F1 offspring

Fig 5.2.4 illustrates that the lungs of the 84-day-old control animals (A) animals that received tomato juice only (B), and those that received both nicotine and tomato juice (C), have the same architecture. These results correspond with the results obtained from the morphometric measurements. Fig 5.2.4 shows that at postnatal day 84, the nicotine group (D) showed larger alveolar spaces compared to the control. This indicates that the lungs of the offspring that were exposed to nicotine during gestation and lactation show evidence of microscopic emphysema and corresponds with the morphometric data. The similarity between the controls and those rats that received both nicotine and tomato juice group suggests that tomato juice does have protective

effects on the lungs of offspring that were maternally exposed to nicotine during gestation and lactation.

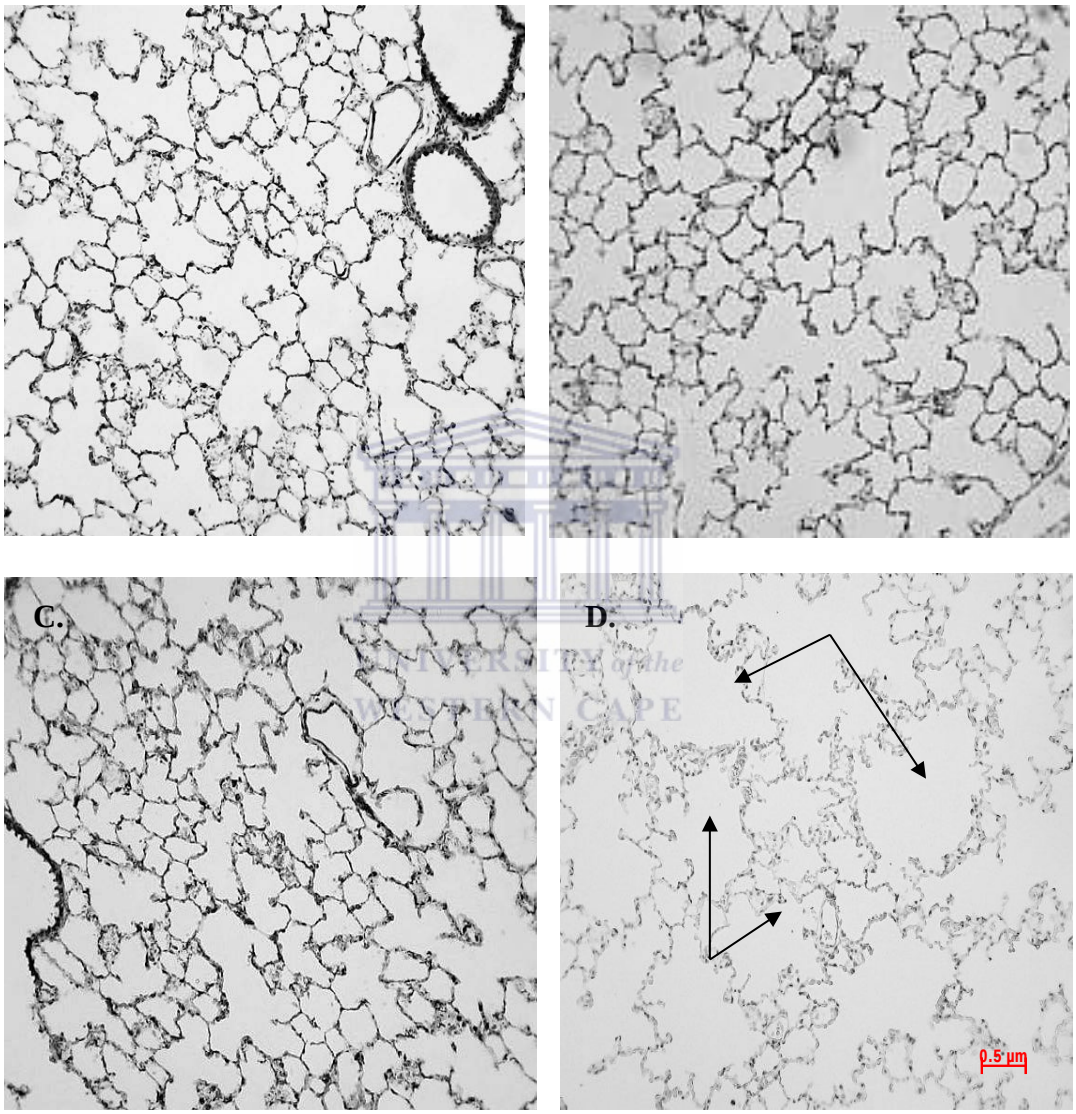


Figure.5.2.4: The effect of maternal exposure to nicotine, tomato juice and a combination of nicotine and tomato juice on lung parenchyma of the 84-day-old offspring. A. = Control; B = Tomato juice; C. = Nicotine + tomato juice; D. = Nicotine. Arrows = Emphysema. (Bar = 0.5µm). The administration of nicotine as well as the treatment of tomato juice or the combined use of nicotine and tomato juice to the offspring was only via the placenta end the mother's milk. Only the mothers received nicotine or tomato juice or both nicotine and tomato juice.

5.3 Discussion

5.3.1 Lung Structure

In this study, the female rats of the F0 generation were exposed to nicotine during gestation and lactation. Nicotine exposure commenced 24 hours after mating. The offspring (F1 generation) received nicotine only via the placenta and mother's milk. The exposure was therefore during all the phases of lung development *in utero* and the phase of rapid alveolarization which commences at postnatal day 4 in rats and lasts till postnatal day 13 (Windvogel, 2006). Nicotine exposure stopped at weaning at postnatal day 21. Exposure thus stopped at the onset of the phase of equilibrated growth because this phase takes place between postnatal days 20 and 40 in rats (Massaro and Massaro, 2007).

Lung growth, development, and maturation is determined by the oxygen demand of the body (Burri, 1974). Consequently, the surface area available for gas exchange increases as the body size increase. Increase in surface area is achieved through the formation of new alveoli (Hukkanen et al., 2001). Since the stages of lung development occurs as a series of complex coordinated events, any interference with any one of the phases during the prenatal and/or early postnatal lung growth and development will compromise lung structure and function. This is because during these phases, the lung is highly plastic and exogenous substances such as tobacco smoke or nicotine program the lung to age faster (Stick, 2000; Massaro and Massaro, 2007). In the present study it is shown that the destructive effects of nicotine occur

even after lung growth and maturation has stopped. The exposure of the developing lung to nicotine during rapid lung growth and development bring about alterations in lung structure that will compromise its function as a gas-exchanger as the animals age. This includes alveolar septal breakdown or interference with septal formation resulting in permanently fewer and larger alveoli in the lungs. As a consequence of this, there will be a permanent reduction in the total surface area available for gas exchange (Schuller et al., 2000).

During the phase of rapid alveolar formation the number of alveoli increases with an accompanying decrease in the size of the alveoli. This means that the tissue volume of the lungs will increase as it matures over time. In this study the tissue volume of the lungs of all the groups indeed increased between postnatal days 21 and 84. Between postnatal days 21 and 42, the V_t of the control male and female animals was similar to that of the experimental groups of the same age. However, between postnatal days 63 and 84, the tissue volume of the nicotine exposed male and female animals was slower than that of the control males and female animals. The alveoli of the male and females that were exposed to nicotine only were observed to be larger but fewer than that of the control group or those exposed to tomato juice or the combination of nicotine and tomato juice (Fig.5.2.4). This decrease in tissue volume of the nicotine exposed progeny can be attributed to:

1. Destruction of the alveoli, and/or
2. Slower alveolar formation.

Since the lung volumes of the nicotine exposed rats was higher at postnatal day 84 than that of the control animals of the same age due to a slower formation of alveoli and/or destruction of the alveoli it follows that the alveolar volume of the nicotine exposed rats will be higher than that of the control animals. This has indeed been supported by the higher Lm of the nicotine exposed animals. It is important to note that the decrease in tissue volume followed by an increase in Lm only became apparent after postnatal day 42. This implies that up to postnatal day 42, the tissue volume as well as alveolar volume and number of the lungs of the animals was not significantly affected by nicotine exposure during gestation and lactation. Since the formation of alveoli after postnatal day 42 is very slow, it is unlikely that a slower alveolar formation is largely contributing to the decrease in tissue volume or an increase in alveolar volume. It is however, more likely due to a gradual destruction of the lung parenchyma. If this is indeed so, it implies that gradual destruction of the alveolar walls occurred. This has indeed been shown by light microscopy that the lungs of the nicotine exposed rats developed emphysema-like lesions after postnatal day 42.

At postnatal day 84, it was observed that the tissue volume of the female animals exposed to nicotine was lower than that of the nicotine exposed male animals. It is also worth noting that tomato juice intake by the mother prevented the effect of nicotine on tissue growth in the lungs of the offspring.

The gradual decrease in the tissue volume of the rats that were exposed to nicotine can be attributed to parenchyma damage. A thinning of the alveolar walls is expected to eventually give rise to a break-down of the alveolar walls during the observed parenchymal damage. It was indeed shown in a previous study that at postnatal day 21 the alveolar walls of the nicotine exposed rats was thinner than that of control animals (Maritz and Windvogel, 2005). However, contrary to this the alveolar wall thickness of the 84-day-old nicotine exposed rats increased. The thickening of the alveolar walls of the lungs of the nicotine exposed F1 rats can be attributed to an imbalance in synthesis and degradation of the components of the alveolar walls.

The fact that the tissue volume of the nicotine exposed rats decreased despite the increase in alveolar wall thickness was probably a consequence of the faster deterioration of the lung parenchyma with the consequent loss of alveolar walls and thus parenchymal tissue. Since nicotine inhibits apoptosis, it is conceivable that this may be a contributing thickening of the alveolar septa of the lungs of rat pups that were exposed to nicotine (Maritz, 1987). Although suppression of apoptosis cannot be ruled out, it is plausible that the balance between synthesis and degradation of intercellular matrix was disturbed too with a consequent deposition of extracellular matrix components that exceeds degradation in the lungs of the nicotine exposed progeny.

It is also worth noting that the thickening of the alveolar walls was only noticeable after postnatal day 63. Given that the offspring only received nicotine via the placental blood supply and mother's milk, it not likely that these changes are due to the direct effect of nicotine or of its metabolites since the half-life of nicotine is about 90 minutes (Benowitz et al., 1982) as well as the fact that a major metabolite of nicotine, cotinine is 16 to 19 hours (Jarvis et al., 1988). It is also unlikely to be due to an oxidant-antioxidant imbalance at this relatively late stage of lung growth and development since these changes in the alveolar region of the lungs of the rats occurred 3 to 9 weeks after nicotine and its metabolites were eliminated from the lungs of the offspring. Also, these changes were noticeable at different stages of development in the lungs of the offspring. These changes are rather due to an early change to the factors that ensures that lung structure and function is preserved as the animals' age. This means that exposure of the fetuses and neonates to nicotine via the placenta and mother's milk programmed the lungs of the progeny during a phase of rapid cell proliferation and differentiation to deteriorate faster than that of the control animals. There is evidence that the changes seen in the lungs of the F1 progeny that were exposed to nicotine via the placenta and mother's milk are markers of premature lung aging. These include prolonged inhibition of glycolysis during rapid cell proliferation in the lungs of the offspring (Maritz and Burger, 1992), and accumulation of AMP (Karrasch et al., 2008). It is therefore possible that additional abnormalities may develop as the offspring gets older. This reinforces the suggestion that nicotine induced changes in the in utero environment, which in turn adversely

affect the maintenance of lung integrity as it ages. As a result of the nicotine induced programming, the lungs of the offspring become increasingly vulnerable to lung disease (Maritz et al., 2011b; Maritz and Windvogel, 2003) which will negatively impact on the function of the lung as gas-exchanger. The increase in alveolar volume and increased alveolar wall thickening are indeed signs pointing to the development of emphysema-like lesions in the lung parenchyma. These collectively are all also signs of premature lung aging (Bruin et al., 2008b).

5.3.2 Tomato juice supplementation.

In this study it was showed that supplementing the diets of the female rats during gestation and lactation with tomato juice prevented all the adverse effects of maternal nicotine exposure on the lungs of the offspring. Tomato juice is a rich source of lycopene and other antioxidants. It is conceivable that the daily tomato juice intake improved the antioxidant capacity of the mother as well as that of the offspring and thereby the ability to protect itself against foreign substances such as nicotine. Although lycopene is an important antioxidant it is likely that other antioxidants and phytonutrients in tomato juice also played a role in protecting the developing lung against the adverse effects of nicotine.

In this study tomato juice supplementation took place only during gestation and lactation; that is during the phases of lung development during which cell turnover was rapid. It was also when the animals were exposed to nicotine via the placenta and

mother's milk and thus when the oxidant release under influence of nicotine (Zhao et al., 2006) was conceivably the highest. The fact that all the adverse effects of maternal nicotine exposure during gestation and lactation was prevented by drinking tomato juice means that tomato juice, and presumably the lycopene and other phytonutrients in tomato juice, protected the lungs during the sensitive periods of lung growth and development. Human studies showed that lycopene prevent DNA damage (Neyestani et al., 2007) and cell membrane oxidation in vivo . It is thus plausible that the lycopene in tomato juice improved the protection systems of the mother and fetus to such an extent that it prevented the adverse effects of maternal nicotine intake on the lungs of the offspring.

Based on these data in this study, and directly extrapolating from rats to humans, a daily intake of about one and half glass of tomato juice, where a glass is equal to 250 ml, will supply enough lycopene, phytonutrients and other antioxidants such as vitamin C to protect the fetus and neonate in a 60 kg human female against the adverse effects of nicotine.

In conclusion, maternal nicotine exposure during gestation and lactation; that is when lung development is characterized by rapid cell proliferation and thus when the cellular DNA is most sensitive to the adverse effects of oxidants, resulted in lung structural pathology in the offspring later in life. Supplementing the diet of the mother with tomato juice during these early phases of lung development prevented

the adverse effects of nicotine on lung integrity in the long term. Whether regular intake of tomato juice during pregnancy and lactation may contribute to the protection of the lungs of the offspring and thus of respiratory health if the mother smokes would require further study.

CHAPTER SIX

Effects of maternal exposure to nicotine, tomato juice, and to both nicotine and tomato juice, on lung function in the F1 *offspring*

6.1 Introduction

The infrastructure of the lung is designed to support gas exchange function of the lung. Two of the components of this infrastructure are the internal surface area for gas exchange, and the connective tissue framework. The latter is not only important for maintaining structural stability of the lung, but also for lung compliance and recoil that is, together with the internal surface area, important for optimal gas exchange. Any damage to any one of these two components of the lung infrastructure will adversely affect gas exchange. The fact that maternal nicotine exposure during gestation and lactation resulted in the development of emphysema-like lesions relatively late in the lungs of the offspring, suggest that the connective tissue framework is compromised. This means that lung compliance will also be compromised. This also means that smoking or the use of NRT to quit smoking can have the same impact on the lungs later in the life of the offspring.

Many females indeed use nicotine replacement therapy (NRT) as a strategy to quit smoking, even during pregnancy. Nicotine readily crosses the placenta and is present in the fetal circulation and amniotic fluid at higher levels than in the maternal circulation (Aycicek et al., 2005). This implies that the mere intake of nicotine via smoking or just as NRT, changes the in utero environment within which the fetus develops. One of the changes is that it reduces the anti-oxidant capacity of the mother as well as that of the fetus or neonate (Aycicek et al., 2005). It is therefore plausible that by restoring the mother's anti-oxidant capacity, and also that of the offspring, the adverse effects of nicotine can be prevented. Tomatoes certainly assume the status of a health food if we take into consideration the large body of epidemiological evidence illustrating its role in reducing the risk for oxidant associated diseases. It is a reservoir of several different antioxidant molecules, such as ascorbic acid, vitamin E, carotenoids, flavonoids and phenolic acids. The disease-preventing potential of a food, such as tomatoes, is a consequence of a several such constituents which may show some synergistic interactions (Erdman Jr et al., 2009). This implies that giving only one micronutrient to an individual may not be as effective on its own than when given together with other micronutrients in the correct ratios. It has in fact been shown that pure lycopene is not a very effective anti-oxidant when used on its own, than when it is in the presence of other phytonutrients such as in tomatoes (Bjelakovic et al., 2008). In a study by Kasagi et al (2006) it was indeed shown that supplementing the diet of adult rats prevented nicotine induced damage to the lung parenchyma of these rats. In a more recent study it was furthermore shown that

supplementing the diet of the mother during gestation and lactation prevented the development of emphysema-like lesions in the lungs of the offspring (Maritz et al., 2011a). it is therefore hypothesized that by preventing the lowering of the anti-oxidant capacity of the nicotine exposed mothers, as well as that of the fetus and neonate by dietary supplementation of lycopene, other anti-oxidants, and phytonutrients during gestation and lactation, will prevent programming of the lungs of the offspring to develop respiratory diseases in later life.

The objectives of this chapter are thus to establish;

1. whether maternal nicotine during gestation and lactation alters the lung function of the F1 offspring by causing,

a) a decrease in the internal surface area (Sa) of the nicotine exposed rats, and

b) an increase in lung compliance (Spector and Goldberg).

2. Whether supplementing the mother's diet with tomato juice, containing lycopene, other oxidants, and associated phytonutrients, will prevent the effects of nicotine on the Sa and Static Lung Compliance (Cst). The reason why tomato juice is used instead of pure lycopene is that lycopene and the phytonutrients in tomato juice is present in the correct ratios for optimal antioxidant function.

6.2 Results

The gas exchange function of the lung is dependent on; 1) a large internal surface area (Sa) available for gas exchange, and 2) an adequate compliance to ensure air

flow into and out of the lungs (Biebuyck, 1983). If the Sa or compliance is compromised such as in emphysema, it may also compromise the gas exchange function of the lung.

6.2.1 Effects of maternal exposure to nicotine, tomato juice, and to both nicotine and tomato juice on the Internal Surface Area (Sa) of the F1 offspring.

The data illustrated in figures 6.2.1.A show that the Sa of the animals increased exponentially between postnatal days 21 and 42. At postnatal day 42, no differences were observed between the control (Fig.6.2.1B) male and the female rats ($P>0.05$) within the different groups. After postnatal day 42 the Sa of the control male rats increased faster than that of the control females so that at postnatal day 63 and 84 it was higher than that of the females. This is to be expected since the Lv of the male and female rats follows the same trend (Chapter 4).

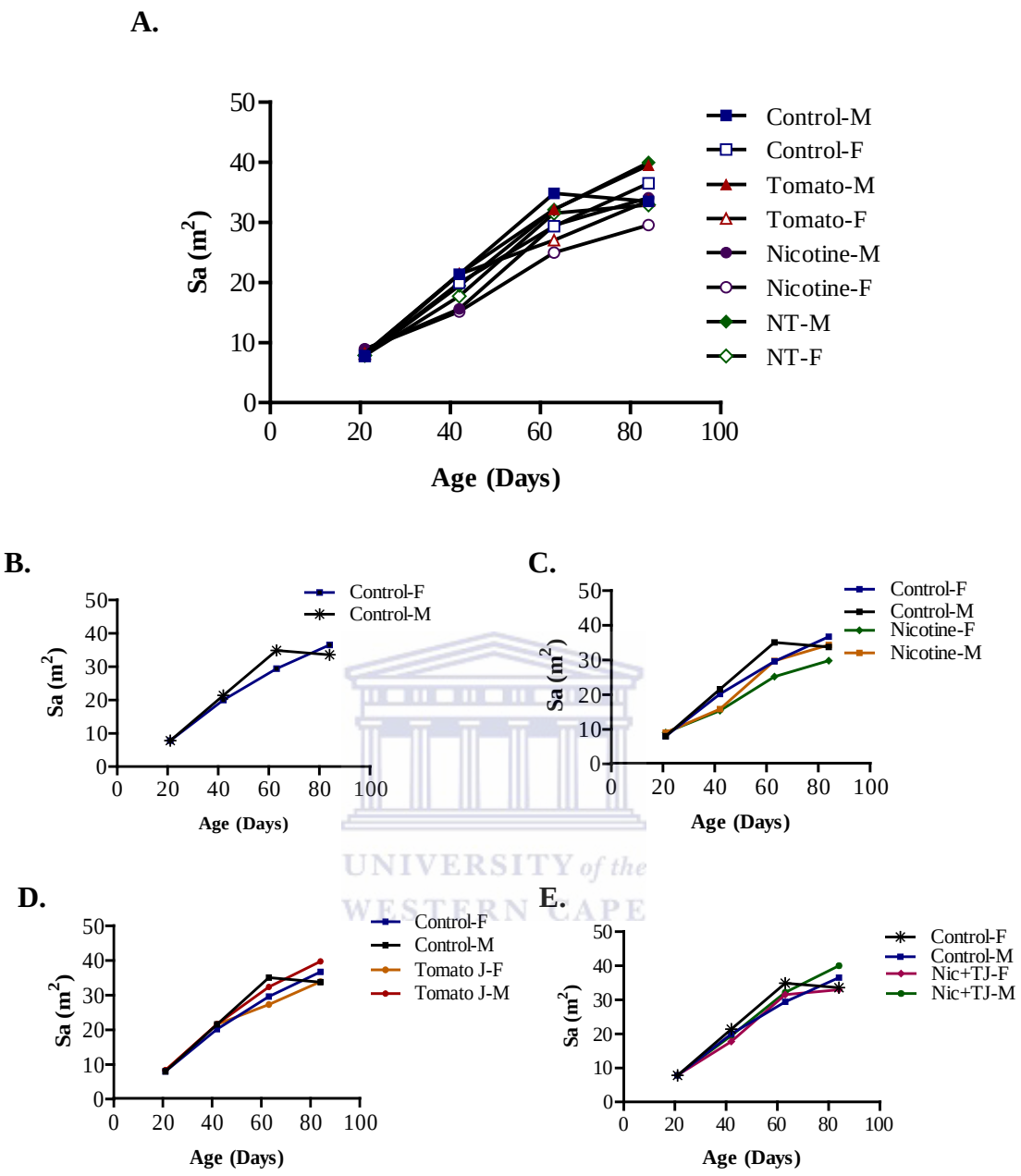


Figure 6.2.1A-E: Effect of maternal nicotine exposure and intake of tomato juice during gestation and lactation on the Internal Surface Area (Sa) of the lungs of the offspring as they aged (Control-M= control male; [Day 42, Control male and female vs. nicotine male and female P<0.001(C); Control Males vs. Females (B) P>0.05; at postnatal day 84, (C) Control female vs. Nicotine female P<0.001; Control female vs. Tomato J female P<0.02 (D); Nicotine male vs. nicotine female P<0.001; Control males and females vs. Nicotine+ tomato J male and females P>0.05 (E)].Control-F= control female; Tomato J-M= males received tomato juice only; Tomato J-F= Females received tomato juice only; Nicotine-M= males received nicotine only; Nicotine-F Females receiving nicotine only; Nic+TJ-M= males received both nicotine and tomato juice; Nic+TJ-F= Females received both nicotine and tomato juice. Only the mothers were exposed to nicotine during gestation and lactation.

The Sa of the male (15.60±0.05 m^2) and female (15.14±0.05 m^2) rats that were exposed to nicotine via the placenta and mother's milk was similar to that of the control male and female rats at postnatal day 42 (Fig. 6.2.1 C). However, despite an increase in Lv of the nicotine exposed rats after postnatal day 42 (chapter 4), the Sa of the nicotine exposed 63-day-old (29.51±0.08 m^2) and female (24.97±0.05 m^2) rats was smaller ($P<0.05$) than that of the control rats of the same age. This trend was maintained at postnatal day 84.

Maternal tomato juice consumption during pregnancy and lactation had no effect on the Sa of the male and female rats of all age groups that were not exposed to nicotine intake (Fig.6.1.1 D). However, the intake of tomato juice by those pregnant rats that were exposed to nicotine during gestation and lactation prevented the decrease in the Sa that was induced in the lungs of the male and female rats that were exposed to nicotine via the placenta and mother's milk (Fig. 6.1.1 E).

6.2.2 Effects of maternal exposure to nicotine, tomato juice, and to both nicotine and tomato juice on the Sa to BW ratio of the offspring

Between postnatal days 21 and 84, the Sa to BW ratios of the control male rats decreased from 19.58±0.54 to 11.46±0.11 cm^2/g, while that of the nicotine exposed males decreased from 19.90±0.21 cm^2/g to 11.14±0.07 cm^2/g between postnatal days 21 and 84. The Sa to BW ratios of the male animals that received tomato juice only decreased from 22.19±0.29 cm^2/g to 12.27±0.13 cm^2/g while that of the rats that

received both nicotine and tomato juice decreased from 20.37±0.54 cm^2/g to 13.72±0.11 cm^2/g between postnatal days 21 and 84 (Fig 6.2.2 A). The reason for the decrease in the Sa to BW ratios in all the groups is due to the fact that the increase in BW during this time was faster than the increase in Sa. This implies that the area available for gas exchange per unit of body weight decreased.

During the same period of time the Sa to BW ratios of the control female decreased from 19.58±0.54 cm^2/g to 16.56±0.13 cm^2/g. This corresponds with the decrease in the ratio of the male animals. During the same period of time the Sa to BW ratios of the females that received tomato juice only, decreased from 22.19±2.88 cm2/g to 15.66±0.15 cm2/g. The female offspring that were exposed to nicotine via the placenta and mother's milk decreased from 19.90±0.21 cm2/g at postnatal day 21 to 13.72±0.16 cm2/g at postnatal day 84 while that of the females that were exposed to both nicotine and tomato juice decreased from 20.37±0.27 cm2/g to 15.22±0.14 cm2/g.

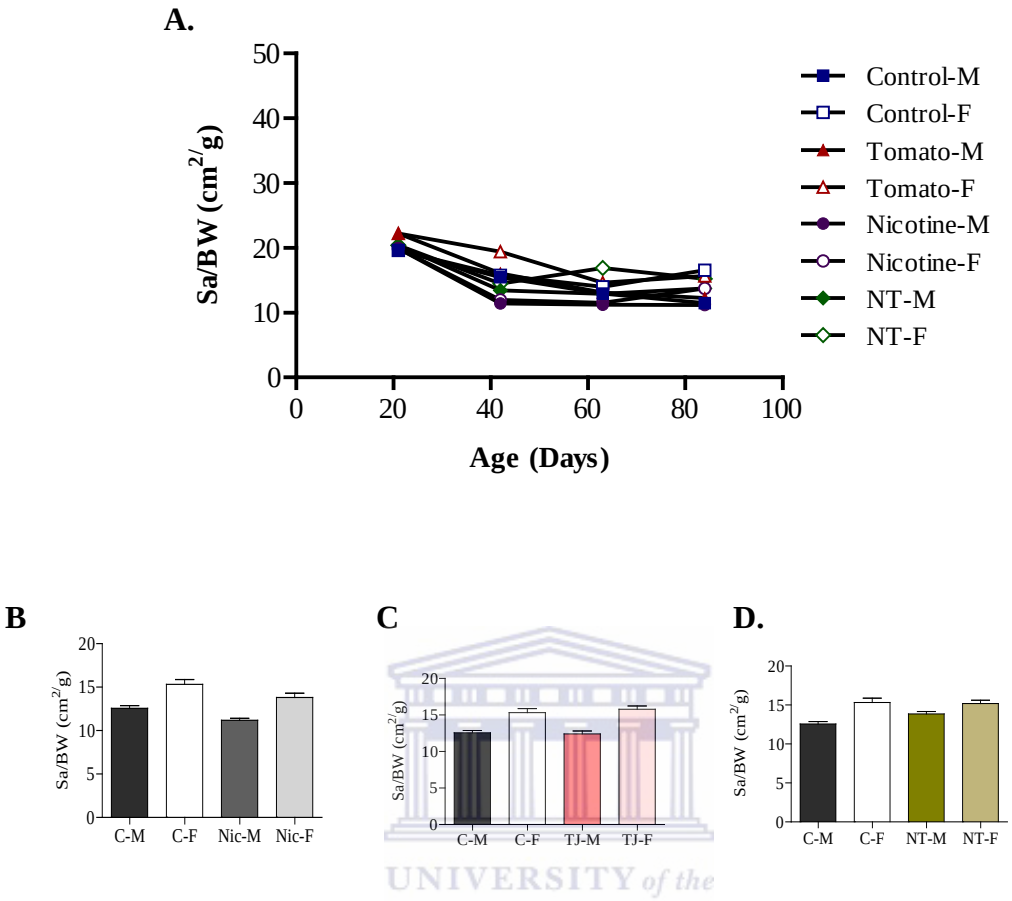


Figure 6.2.2A: Effect of maternal nicotine exposure gestation and intake of tomato juice during and lactation on the Sa/BW ratio of rat lung. A) Changes in Sa/BW over time. **B-D:** Differences at postnatal day 84. [P-value: Control vs. experimental groups: P>0.05; Males vs. Females: P < 0.01]. C-M= control male; C-F= control female; TJ-M= males received only tomato juice only; TJ-F= Females received tomato juice only; Nic-M= males received nicotine only; Nic-F Females receiving nicotine only; Nic+TJ-M= males received both nicotine and tomato juice; Nic+TJ-F= Females received both nicotine and tomato juice.

The observations of the study also indicate that at postnatal day 84, the Sa to BW ratios of the female control and nicotine exposed offspring was 22% bigger (P<0.001) than that of the male control and nicotine exposed offspring (Fig.6.2.2B). The reason for this is could be due to the fact that the body weight of the male offspring was bigger than that of the female offspring (see chapter 4). The data illustrated in figure 6.2.2B also shows that at postnatal day 84, the Sa to BW ratios of the control male

and female offspring was 12% and 10% respectively bigger (P<0.01) than that of the male and female offspring that were exposed to nicotine only. This can be attributed to the decrease in the Sa of the nicotine exposed offspring.

From Fig.6.2.2C it can be seen that at postnatal day 84, the Sa to BW ratios of the female group that received tomato juice only was about 27% higher (P<0.001) than that of the male group of the same age that were exposed to tomato juice only. This can be attributed to the smaller Sa to BW ratio of the control rats and that of all the experimental at postnatal day 84. Maternal tomato juice intake during gestation and lactation prevented the effect of maternal nicotine exposure on the Sa to BW ratios of the offspring (Fig 6.2.2 D) and can be due to the fact that tomato juice prevents an increase in Sa of nicotine exposed offspring (Fig. 6.1.1 E).

6.2.3 Effects of maternal exposure to nicotine, tomato juice, and of both nicotine and tomato juice on Static Lung Compliance (Cst)

The compliance of the lung is dependent on the integrity of the elastic tissue component of the connective tissue framework of the lung (Thibeault et al., 2000). A change in the elastic tissue will also affect lung compliance and lung recoil which will impact on the gas exchange function of the lung. In this study it was illustrated that compliance of the female lungs were consistently higher (P<0.05) than that of the males (Fig. 6.2.3 A-D). Also, the Cst of the offspring decreased (P<0.01)

progressively as a function of increasing age in all the control and experimental groups between postnatal days 21 and 84 (Fig. 6.2.3 A).

Maternal nicotine exposure during gestation and lactation had no effect on the static compliance of the lungs of the offspring up to postnatal day 42. However, at postnatal day 84 the static compliance of the male (1.83±0.03 ml/cm H_20/kg) and female (2.19±0.07 ml/cm H_20/kg) rats that were exposed to nicotine via the placenta and mother's milk was higher ($P<0.05$) than that of the control male (1.41±0.03 ml/cm H_20/kg) and female (1.68±0.06 ml/cm H_20/kg) rats (Fig 6.2.3 B).

Supplementing the diets of the pregnant and lactating mothers with tomato juice had no effect on lung compliance in male and female offspring (Fig 6.2.3 C). Furthermore, supplementing the diets of the pregnant and lactating nicotine exposed rats with tomato juice prevented the nicotine induced decrease in the lungs of the offspring (Fig. 6.2.3 D).

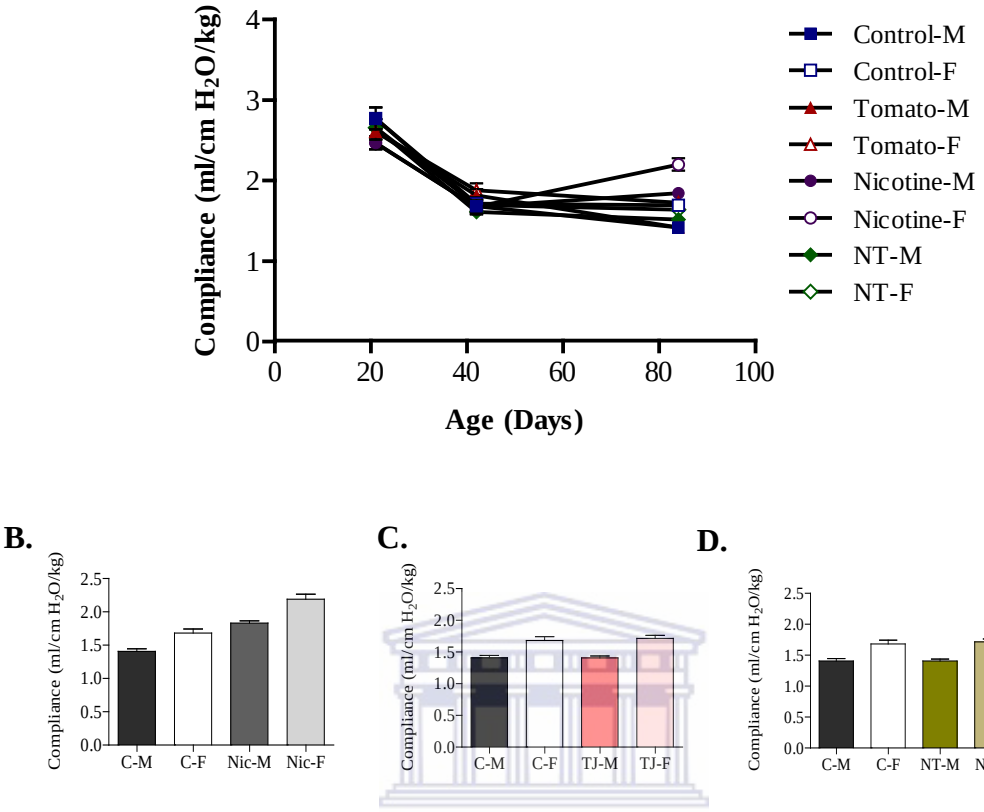


Figure 6.2.3A-D: Effect of maternal nicotine exposure and intake of tomato juice during gestation and lactation on the static compliance of the lungs of the offspring a function of time. [C-M vs. Nic.-M: (P<0.001); C-F vs. Nic.-F: (P<0.001); Males vs. Females: (P<0.01)]. C-M= control male; C-F= control female; TJ-M= males received only tomato juice only; TJ-F= Females received tomato juice only; Nic-M= males received nicotine only; Nic-F Females receiving nicotine only; Nic+TJ-M= males received both nicotine and tomato juice; Nic+TJ-F= Females received both nicotine and tomato juice. The administration of nicotine as well as the treatment of tomato juice or the combined use of nicotine and tomato juice to the offspring was only via the placenta end the mother's milk. Only the mothers received nicotine or tomato juice or both nicotine and tomato juice.

6.3 Discussion

The infrastructure of the lung is designed to ensure that gas exchange is optimal and will supply in an increase in the body's demand for oxygen. Consequently, as part of the normal growth and development of the animal, the alveolar number increase and with it the internal surface area to meet the gradual increase in the demand for energy.

A large internal surface area (Sa) is required to ensure that adequate quantities of oxygen can be absorbed during increased demand situations. In addition, a well-developed connective tissue framework is essential to ensure that lung structure as well as lung compliance is maintained. A reduction in the alveolar number with the associated decrease in the Sa will reduce the capacity of the lung to act as gas-exchanger. When the Sa is severely compromised, such as in emphysema, it may cause death in the longer term. The same is true for the connective tissue framework of the lung. The elasticity of the lung is essential for airflow into and out of the lungs. When the connective tissue framework of the lung is damaged, it will compromise lung recoil and thus increase the physiological alveolar or functional dead space in the lungs and, as a consequence, gas exchange may also be compromised. It is therefore clear that these 2 components of the lung infrastructure are essential for optimal gas exchange.

The respiratory system undergoes various anatomical, physiological and immunological changes with age. The structural changes include chest wall and thoracic spine deformities which impairs the total respiratory system compliance leading to increase work of breathing. The lung parenchyma loses its supporting structure due to loss of connective tissue and alveolar walls causing dilation of air spaces which is referred to as senile emphysema. In humans the lung matures by age 20–25 years, and thereafter aging is associated with progressive decline in lung function. The alveolar dead space increases with age (Holloway et al., 2007) due to

loss of recoil pressure and loss of internal surface area as a function of aging. It is clear that inhalation of foreign substances, such as during smoking, may damage the lungs of the smoker, or induce premature aging which may increase the propensity of the lung to respiratory disease and thereby reduce its capacity as gas exchanger. It has indeed been found that fibroblasts from the smokers with emphysema had lost their ability to divide or grow, confirming that smoking habits cause cell aging (Nyunoya et al., 2006). It has been shown that each puff of cigarette smoke contains, for example, 10^{14} oxidants (Church and Pryor, 1985b). These may impair the oxidant-antioxidant balance of the lungs and in this way induce a slow degeneration of the lung. Also, smoking changes the in utero environment within which the fetus develops, thus the program the lungs of the offspring to become more susceptible to disease (Tsuji et al., 2004a; Maritz and Windvogel, 2003; Maritz and Windvogel, 2005). It is therefore essential to protect the lungs against factors that may adversely affect lung structure and function, or that will induce premature aging of the lungs.

The results obtained in this study show that the Sa of the control male and female offspring was higher than that of the males and female offspring that were exposed to nicotine the placenta and mother's milk. This means that the gas exchange function of the nicotine exposed offspring is compromised by maternal exposure to nicotine during gestation and lactation. Since the decrease in the internal surface area only became evident 3 weeks after weaning of the offspring, it is suggested that it was programmed. This was also shown by a study done by Maritz and Windvogel (2005)

who also showed that at postnatal day 42, the Sa of the control animals was significantly higher than that of the nicotine exposed animals. Our data is, furthermore, supported by the observation that the linear intercept, and thus alveolar volume, of the nicotine exposed rats only became apparent at postnatal day 42, where after it increased as the offspring age. (See chapter 5).

The decrease in the internal surface area of the nicotine exposed offspring can be ascribed to gradual deterioration of the connective tissue framework of the nicotine exposed offspring (Maritz and Windvogel, 2005). If this is so, lung compliance will also be affected at this relatively late stage of lung growth and development of these animals.

There is a direct relationship between surface area available for gas exchange and the demand for oxygen by the body. The Sa to BW ratios indicate that there was a decrease in the ratios from between postnatal days 21 and 84 in all the animals. At postnatal day 84, the Sa to BW ratios of the control female rats were higher than that of the male animals. Since the male animals have a bigger body weight (chapter 4), it is plausible that the lower Sa to BW ratios observed in the male animals is due to a high oxygen demand (Massaro and Massaro, 2007). In addition to that, the Sa to BW ratios of the control male and female rats was higher than that of the nicotine exposed male and female offspring. Since nicotine exposure did not affect the growth parameters of the offspring, the smaller Sa to BW ratios in the nicotine exposed offspring could be attributed to the decrease in Sa in these animals.

In the present study we also showed that the compliance of the lungs of the male and female rats was the same up to postnatal day 42. However, like for lung volume, gender differences were apparent only at postnatal day 84, in that the static compliance of the female lungs was higher than that of the males of the same age. It is also apparent that the increase in the static lung compliance of the nicotine exposed offspring only begins to show at postnatal day 84. This is preceded by an increase in alveolar volume, a decreased alveolar number (Maritz and Windvogel, 2005), and the appearance of emphysema-like lesions at postnatal day 42 (chapter 5). These changes are attributed to damage to the connective tissue framework of the lung that gets gradually worse as the animal age. The relatively late appearance of the emphysema-like lesions, followed by an increase in the static compliance of the lungs of the male and female nicotine exposed offspring, supports the suggestion that it was programmed in utero. The nicotine induced changes in the internal surface area and the increase in compliance cannot be attributed to normal aging of the rats, but is rather due to the impact of maternal nicotine exposure. These changes are in all likelihood induced by oxidants that are increased by nicotine (Dröge, 2003) because supplementing the mother's diet with tomato juice rich in antioxidants prevented the adverse effects of nicotine.

6.4 Conclusion

From the data it is apparent that maternal nicotine exposure during gestation and lactation compromises the gas exchange function of the lungs of the F1 offspring.

This is prevented by supplementing the mother's diet with tomato juice. This is conceivably achieved by maintaining the oxidant-anti-oxidant ratio of the mother and of the developing fetus and neonate.

CHAPTER SEVEN

Effects of maternal exposure to nicotine, tomato juice, and of both nicotine and tomato juice on Cellular Senescence, cell Proliferation and Apoptosis in lungs of the F1 offspring

7.1 Introduction

According to the fetal onset of adult disease theory, during the period of fetal and neonatal development, the lungs are most vulnerable to changes in the environment within which it develops (Heindel, 2006; Bateson, 2007; Morgan et al., 2005). This is because a sequence of well-coordinated developmental processes occur during this time, and if interfered with, the processes may be followed by disturbance in organ development and function (Skinner, 2008). Additionally, during this time of development, there is a series of critical programming processes taking place in the epigenome and transcriptome coupled with cell proliferation as well as organogenesis. Therefore, any alterations in the epigenome and transcriptome as a result of environmental factors may program the developing lung to become more susceptible to diseases which may be transferred to succeeding generations (Anway and Skinner, 2006).

Among the theories in aging research, the oxidative stress theory of aging is the most accepted (Harman, 1956). This theory suggests that the physiological deterioration accompanied by aging is a result of a gradual increase in oxidative damage (Perez et al., 2009; Huang et al., 2000). In human studies, it was demonstrated that lung fibroblasts of patients with emphysema exhibited premature aging and a reduced proliferation rate (Müller et al., 2006) . Both premature aging and reduced rates of cell proliferation are associated with the gradual deterioration of the lung parenchyma (Naimark, 1977). It is therefore conceivable that the slow degeneration of the connective tissue framework observed in the lungs of pups exposed to nicotine, may be a consequence of premature senescence of the fibroblasts and consequently fewer cells will be capable of proliferating and thus replacement of senescent or damaged cells. Consequently the maintenance of the infrastructure will be compromised as the animal or human ages. It is well known that cigarette smoke move cells into a state of irreversible senescence (Chen, 2000), and suppresses the proliferation and migration of human lung fibroblasts and fibroblast-mediated reactions, which plays a role in the development of emphysema (Holz et al., 2004).

In the lungs, emphysema-like changes have been linked to increase in apoptosis of the alveolar epithelial cells (Yokohori et al., 2004a) and endothelial cells (Kasahara et al., 2001; Verbeken et al., 1992). In order to maintain the integrity of the alveolar structure, the cells in the alveolar wall that are lost as a result of apoptosis must be replaced by proliferation of the epithelial and endothelial cells as well as the

fibroblasts. It is known that apoptosis as well as cell proliferation is increased in the emphysematous lungs, but these two processes are not in equilibrium in these lungs and thus contributing to the gradual breakdown of lung alveolar walls with a consequent decrease in the internal surface area (Yokohori et al., 2004a). Furthermore, senescence of alveolar epithelial and endothelial cells is accelerated in patients with emphysema and may explain the abnormal cell turnover that promotes the loss of alveolar cells in emphysematous lungs (Tsuji et al., 2006). Telomere length in alveolar type II cells and endothelial cells was significantly shorter in the patients with emphysema than in the asymptomatic nonsmokers (Tsuji et al., 2006) which is in all likelihood due to the rapid proliferation of these cells in emphysematous lung and may contribute to the premature aging of cells in the alveolar walls.

The objectives of this chapter are to determine the effects maternal nicotine exposure during gestation and lactation on apoptosis, cell proliferation and cellular senescence in the lung parenchyma of the offspring as they age. This may give a better understanding of the site where nicotine act as well as the mechanism by which it induce emphysema-like lesions in the lungs of animals that were exposed to nicotine via the placenta and mother's milk.

7.2 Results

7.2.1 Cell Proliferation

Figure 7.2.1 illustrates the effects of maternal exposure to nicotine only, tomato juice only and the combination of both nicotine and tomato juice on cell proliferation in the alveolar wall of the lungs of the offsprings at postnatal day 84. The number of proliferating cells per 100 µm alveolar wall length in the control animals was at 1.430±0.060, 27.8 % higher (P<0.0.001) than the number of proliferating cells in the group exposed to nicotine only (1.120±0.030cells/100 µm alveolar wall). The number of proliferating cells in the group that received tomato juice only (1.50±0.060 cells/100 µm) and those whose mothers were exposed to nicotine and received tomato supplementation during pregnancy and lactation (1.340±0.030/100 µm alveolar wall) was the same (P>0.05) than that of the control rats. This means that supplementing the diet of the mother with tomato juice during gestation and lactation prevented the inhibition of cell proliferation in the alveolar walls.

7.2.2 Cellular Senescence

Figure 7.2.2 shows the effects of: 1) maternal nicotine exposure during gestation and lactation, and 2) those mothers whose diets were supplemented with tomato juice, and 3) the mothers that were exposed to nicotine and received tomato juice, on the number of senescent cells per 100 µm of alveolar wall of the lungs of the offspring. The number of senescent cells per 100 µm alveolar wall in the animals that were exposed to nicotine was 1.16±0.07, which was 59.48% higher (P<0.05) than that of

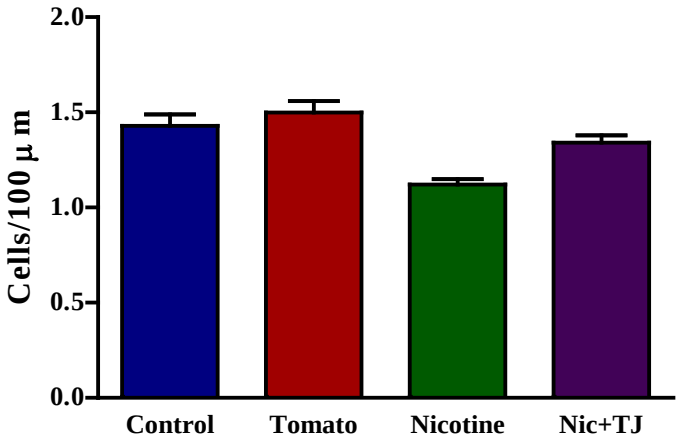


Figure 7.2.1: The effect of exposure of rats during gestation and lactation to tomato juice, nicotine and tomato juice, or to nicotine only, on cell proliferation at postnatal day 84. (Cells/100 µm alveolar wall length). (Control vs. Nicotine; P<0.001: Control vs. Tomato juice and N + T; P>0.05). The graph illustrates the actual numbers of proliferating cells in the alveolar walls. Only the mothers received nicotine or tomato juice or both nicotine and tomato juice.

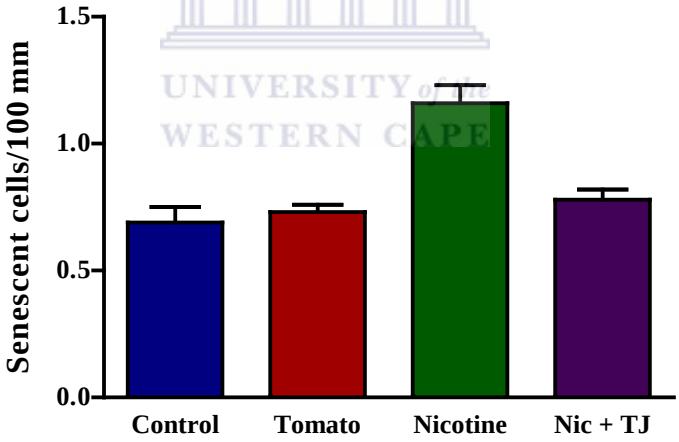


Figure 7.2.2: The effect of nicotine, tomato juice and of both nicotine and tomato juice on cellular senescence during gestation and lactation (cells/100 µm alveolar wall length). P-value: Control vs. Nicotine; P<0.001; Control vs. Tomato and Nic + TJ; P>0.05. Only the mothers received nicotine or tomato juice or both nicotine and tomato juice.

the control animals at 0.69±0.06 cell/100 µm of alveolar wall. In the group that received tomato juice, as well as those that were exposed to both nicotine and tomato juice, it was 0.73±0.03 and 0.78±0.04 cell/100 µm length of alveolar wall respectively, which was similar to that of the control (P>0.05). The data in figure 7.2.2 suggests that maternal nicotine exposure may cause premature aging in the lungs of offspring. However, the supplementing her diet with tomato juice protected the lungs against the harmful effects of nicotine on the cells of the alveolar wall.

7.2.3 Proliferating to Senescent cell ratio (P/S)

The data in Fig.7.2.3 shows that the P/S ratio of the control lungs (2.07±0.12) was about 53% higher (P<0.001) than that of the rats that were exposed nicotine only (0.97±0.10). On the other hand, no difference (P>0.05) was observed between the P/S ratio of the control animals when compared to that of the animals that were treated with tomato juice only (2.05±0.09) or those that were exposed to both nicotine and tomato juice (1.72±0.08).

7.2.4 Apoptosis

Contrary to the results obtained in figures 7.2.1 and figure 7.2.2, the data presented in figure 7.2.4 show that the number of apoptotic cells per 100 µm length of alveolar wall were the same for the lung tissue of the 84-day-old rats of all the groups (P> 0.05).

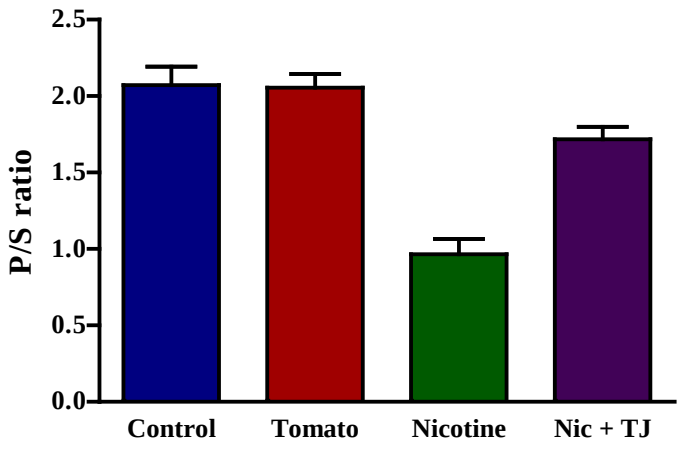


Fig.7.2.3: The effect of grand-maternal nicotine exposure during gestation and lactation on the P/S ratio (Number of proliferating cell/number of senescent cells) of the lung parenchyma of the F1 offspring at postnatal day 84. (P-value: Control vs. Nicotine; P<0.001: Control vs. Tomato juice and N + T; P >0.05).

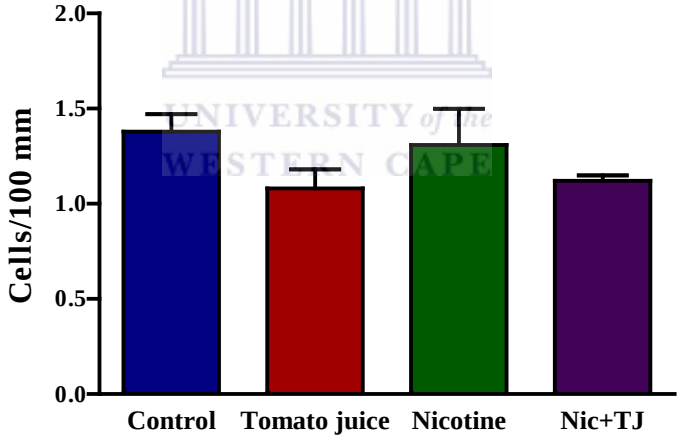


Figure 7.2.4: The effect of maternal exposure during gestation and lactation t, nicotine, or tomato juice only, nicotine and tomato juice, or to nicotine only, on apoptosis, expressed as number of apoptotic cells/100 µm alveolar wall, in the lungs of 84-day-old offspring. The administration of nicotine as well as the treatment of tomato juice or the combined use of nicotine and tomato juice to the offspring was only via the placenta end the mother's milk. Only the mothers received nicotine or tomato juice or both nicotine and tomato juice.

7.3 Discussion

The “fetal origins hypothesis” encompasses the concept of programming. This concept is well established in developmental biology and is about the idea that stimuli or insults during critical periods in early life will result in developmental adjustments that will result in permanent structural, physiological and epigenetic changes in the individual that may have health consequences later in life (Narang and Bush, 2012). An example of such a stimulus is maternal smoking or NRT during pregnancy and lactation. This changes the in utero environment within which the fetus develops and may program it to become more susceptible to disease later in life, for example emphysema or asthma (Landau, 2008a). It has indeed been shown that rat pups whose mothers were exposed to tobacco smoke during pregnancy had lung hypoplasia with fewer, but bigger saccules (Barker et al., 1989).

An important principle is that a minor change in structure and function in the lung is magnified by aging (Narang and Bush, 2012). Thus any programmed change may give rise to structural changes that are not noticed initially, but over time, as the individual becomes older, becomes progressively worse and eventually present in the form of diseases such as emphysema. Programmed changes in cellular processes such as apoptosis, proliferation, differentiation and senescence may occur which will eventually have adverse effects on the structural integrity of the lung in the longer term.

Apoptosis-proliferation Imbalance.

The cellular component of the lung parenchyma plays an important role in the normal growth and development of the lungs, as well as maintenance of lung structural and functional integrity in the longer term. This means that a dynamic but controlled process of cell death and cell replacement is necessary to ensure that the lung structure and function is maintained. Apoptosis, or programmed cell death, plays critical roles in a wide variety of physiologic processes during fetal development and in adult tissue. Lung development and maintenance requires a very dynamic process of cell replication and apoptosis (Aoshiba and Nagai, 2009), especially since the lung is exposed to many pollutants in the atmospheric air ad particularly in smokers. Although there are other hypotheses regarding the development of emphysema, such as the proteinase-anti-proteinase theory, it has been proposed that an *apoptosis-proliferation imbalance* also plays a role in emphysema (Calabrese et al., 2005). This theory states that cell proliferation does not increase at the same rate as apoptosis in severe pulmonary emphysema, and the excess of apoptosis results in the loss of cells in the alveolar walls with the consequent formation of emphysematous lesions (Calabrese et al., 2005). It was indeed shown that apoptosis and cell proliferation do not increase at the same rate (Imai et al., 2005). Observations in patients with emphysema also suggest that that an increase in apoptosis is associated with in the loss of cells in the alveolar wall (Segura-Valdez et al., 2000). Smoking has been shown to increase apoptosis and cell proliferation in smoker which contribute to the development of emphysema as they age (Banerjee et al., 2007). Although it is said

that nicotine deregulates essential biological processes like angiogenesis and apoptosis (Zeidler et al., 2007), it was clear from the present study that exposure of the offspring to nicotine via the placenta and mother's milk had no effect on apoptosis in the alveolar walls of the offspring. It is therefore unlikely that apoptosis as such could result in an apoptosis-proliferation imbalance in the alveolar walls of the nicotine exposed offspring.

Cell proliferation and cell senescence

Aoshiba and Nagai (2009) hypothesized that smoking causes alveolar apoptosis even in young individual, but it is compensated for by an increase in cellular proliferation so that no emphysematous lesions occur. On the other hand, cellular senescence is accelerated in individuals with pulmonary emphysema as a consequence of longer periods of cigarette smoke exposure. When cellular senescence occurs, cell proliferation is lost and the balance is tipped towards apoptosis and the formation of emphysematous lesions occurs over time (Aoshiba and Nagai, 2009). Since cell senescence is more prevalent as the individual becomes older the rate at which it will move into senescence will increase. Thus, an acceleration of cell senescence by, for example smoking or nicotine, will result in the appearance of emphysematous lesions earlier in the life of the individual.

The data in the present study clearly shows that maternal exposure to nicotine during gestation and lactation reduced the rate of cell proliferation in the alveolar wall of the

offspring. Furthermore, the number of senescent cells in the alveolar walls of the lungs of the offspring that were exposed to nicotine was higher than that of the control animals. This resulted in a P/S ratio in the lungs of the nicotine exposed offspring that was markedly lower than that of the control and other experimental groups. This change in the ratio in the lungs of the offspring due to maternal nicotine exposure during gestation and lactation points to a *cell proliferation-cell senescence* imbalance. These findings furthermore suggest that the maternal exposure to nicotine via the placenta and the mother's milk resulted in the premature aging of the lungs of offspring. The decrease in cell proliferation together with the increase in the number of senescent cells in the alveolar walls will compromise the ability of the lungs to replace dead and damaged cells with a consequent gradual loss of alveolar wall integrity. Since maternal nicotine exposure during gestation and lactation had no impact on the apoptosis in the alveolar walls, it means that apoptosis did not contribute to the imbalance in cell death and cell replacement in the alveolar walls of the nicotine exposed offspring and thus to alveolar wall damage. However, due to the increased numbers of senescent cells, accompanied with a decrease in cell proliferation, alveolar wall damage will occur. The consequences of this imbalance between cell damage and repair only became evident relatively late in the life of the offspring and appears to be irreversible.

From the data in this study it appears that maternal nicotine exposure during pregnancy and lactation serves as stimulus at a critical periods of lung development in

early life of the offspring that induced developmental adjustments at cellular level that contributed to a permanent structural, physiological and epigenetic changes in the offspring that will result in emphysematous changes later in life. It is also plausible that the lungs of the nicotine exposed offspring will be more susceptible to stress induced damage, such as pollution due to the imbalance in repair mechanisms.

The mechanism whereby exposure of the F1 offspring that were exposed to nicotine via the placenta and mother's milk is likely due to oxidant damage induced during the early phases of lung development. This is plausible since maternal nicotine exposure reduce the anti-oxidant capacity of the lungs of the offspring. It also increases the oxidant load of the mother and fetus (Durak et al., 2002). This increase in the oxidant load and compromised protection due to the low anti-oxidant levels will increase the susceptibility of the offspring to programming. This is supported by the observation that supplementing the nicotine exposed mother's diet with tomato juice prevented the changes in the cellular dynamic of the alveolar walls of the offspring. This is likely due to preventing the mother's anti-oxidant levels as well as that of the fetus to be maintained and to ensure the protection against the oxidant induced programming of the lungs of the offspring

7.4 Conclusion

Maternal nicotine exposure during gestation and lactation results in an increase in the number of senescent cells in the alveolar walls of the offspring as well as a decrease

in the number of proliferating cells resulting in a marked decrease in the P/S ratio. This imbalance in alveolar cell dynamics contributes to the slow deterioration of the lung alveoli and the eventual appearance of emphysema-like lesions in the lungs of the nicotine exposed offspring. This imbalance is prevented by supplementing the mother's diet with tomato juice.

CHAPTER EIGHT

The effect of grand maternal nicotine exposure during gestation and lactation on lung integrity of the F2 generation

8.1 Introduction

Epidemiological studies showed that susceptibility to respiratory disease throughout life can be programmed by environmental factors operating during foetal and early neonatal life. One of the most common environmental factors is tobacco smoke (Landau, 2008b; Lødrup Carlsen et al., 1997). Smoking is one of the greatest causes of illness and premature death in both developed and developing countries (Moore et al., 2009). It has recently been shown that tobacco exposure during the grandmother's and mother's pregnancies increase the risk of cancer in the descendants (Ortega-Garcia et al., 2010). In an effort to assist smokers to quit the habit, nicotine replacement therapy (NRT) is prescribed by health professionals in certain countries (O.M.A., 2008). Over the counter NRT is also available in several countries such as the USA, the UK, Canada and Australia, resulting in an increased use of nicotine replacement therapy to quit smoking (Shiffman and Sweeney, 2008). There are claims that using NRT is safe (Zwar and Richmond, 2006) despite the fact that several studies showed that persistent use of nicotine promote lung carcinogenesis independent of other ingredients of tobacco smoke (Dasgupta and Chellappan, 2006).

It also inhibits apoptosis(Cucina et al., 2008). Apart from the effect of nicotine, products of nicotine metabolism, such as NNK and NNN induce DNA adducts leading to the mutation of genes such as Ras, p53 and Rb, initiating cancer (Dasgupta and Chellappan, 2006; Schuller and Orloff, 1998).

Although the long-term effects of NRT use in pregnancy in humans is lacking, several studies using animals as model indicate that exposure of the foetus and neonate to nicotine via the placenta and mother's milk leads to widespread adverse effects on postnatal health. These include defective metabolic outcomes associated with obesity and type 2 diabetes (Bruin et al., 2010; Bruin et al., 2008a; Bruin et al., 2007; Bruin et al., 2008b; Holloway et al., 2005). Furthermore, Holloway et al (2006) showed that the adverse effects of nicotine on blood pressure and increased fasting insulin in response to a glucose challenge, are transferred to at least the F2 generation.

In addition, several studies showed that maternal nicotine exposure during gestation and lactation interferes with lung development and maintenance of lung structural integrity of the offspring in the long term (Maritz et al., 2011a). It is also associated with decreased lung function in the offspring (Sekhon et al., 1999; Sekhon et al., 2001b; Sekhon et al., 2002). The data suggest that maternal exposure to nicotine via tobacco smoking or NRT will increase the susceptibility of the offspring to respiratory diseases. It is also possible that NRT may result in a change in the

program that controls lung growth, maintenance and aging and consequently lung function in the offspring. It is, therefore, possible that these changes be transferred between subsequent generations. Very little evidence exists regarding the transgenerational effects of maternal and grand-maternal nicotine exposure during gestation and lactation on lung growth, development and aging. The aim of this study was therefore to establish whether changes induced in the lungs of the F1 progeny after exposure of the F0 females (grand-maternal) to nicotine during gestation and lactation, can be transferred to the F2 generation.

8.2 Results

The data displayed in figure 8.2.1E shows that the Lm of the control rats was at 37.43±1.68 µm significantly ($P<0.05$) smaller than the 52.48±3.48 µm and 52.80±3.96 µm of the CmNf and NmCf groups respectively. It was also smaller ($P<0.05$) than that of the NmNf rats at 50.47±3.41 µm. This can be attributed to the presence of emphysema-like lesions in the lungs of the animals that were descendants of rats that were exposed to nicotine via the placenta and mother's milk (Fig. 8.2.1 A to D). The gradual deterioration of the alveolar walls of those animals that were descendants of F0 females that were exposed to nicotine during gestation and lactation can partially be due to a change in the cellular characteristics of the alveolar walls of these animals compared to the controls.

A comparison of the number of proliferating cells (Fig.8.2.2) per unit length of alveolar wall of the lungs of the control rats (1.31±0.05 cells/100 µm) show that the number of proliferating cells of the NmNf (1.16±0.05 cells/100 µm) and NmCf (1.13±0.03 cells/100) groups were smaller ($P<0.05$) than that of the control animals. In contrast to the above the number of proliferating cells per 100 µm of alveolar wall length of the CmNf group (1.32±0.02 cells/100 µm) was the same as for the control animals. This means that cell proliferation in the alveolar walls of the F2 offspring of the F1 females that were exposed to nicotine via the placenta and mother's milk of the F0 mother, was not affected. The number of senescent cells per 100 µm of alveolar wall length (Fig.8.2.3) of the control lungs (0.94±0.05 senescent cells/100 µm) was much lower ($P<0.001$) than the number of senescent cells of the NmCf (1.61±0.12 senescent cells/100 µm), NmNf (1.59±0.06 senescent cells/100 µm) and, CmNf (1.62±0.12 senescent cells/100 µm) groups.

A comparison of the proliferating cell numbers with the senescent cell numbers (Fig.8.2.3) per 100 µm of alveolar wall length (P/S ratio) of the control lungs (1.47±0.10) with that of the NmCf (0.88±0.08), NmNf (0.74±0.04) and CmNf (0.70±0.03) groups, show that in the control animals the rate of cell proliferation exceeds the rate at which cells become senescence in the alveolar walls. On the other hand, the P/S ratio of the NmCf, NmNf, and CmNf groups were smaller ($P<0.001$) than that of the controls suggesting a slower proliferation rate than rate of cells becoming senescent. On the other hand, grand-maternal nicotine exposure during

pregnancy and lactation had no effect ($P>0.05$) on apoptosis of the lung parenchyma of the F2 generation (Fig. 8.2.5. The BW of the male and female F2 offspring was not affected by grand maternal nicotine exposure (Fig. 8.2.6.A1 and A2). A comparison of the data shows that the Lv of the male and female CmNf rats is the same as that for the male and female controls. However, the Lv of both the male and female NmCf and NmNf rats were larger ($P<0.05$) than that of the control male and female rats (Fig 8.2.6 B1 and B2). The Lv/BW ratios of the experimental male and female animals were significantly higher ($P<0.001$) than that of the control rats (Fig. 8.2.6 C1 and C2). This can be attributed to the higher Lv of the experimental F2 rats. The higher Lv of the NmCf and NmNf animals could be due to an increased compliance.

Furthermore, grand maternal exposure to nicotine during gestation and lactation resulted in thinner ($P<0.005$) alveolar walls (6.04 ± 0.52 µm) in the lungs of the F2 progeny (Fig. 8.2.7) than in the lungs of the control F2 rats of the same age (9.19 ± 0.73 µM). This may give rise to a change in the compliance of the lungs of the F2 offspring.

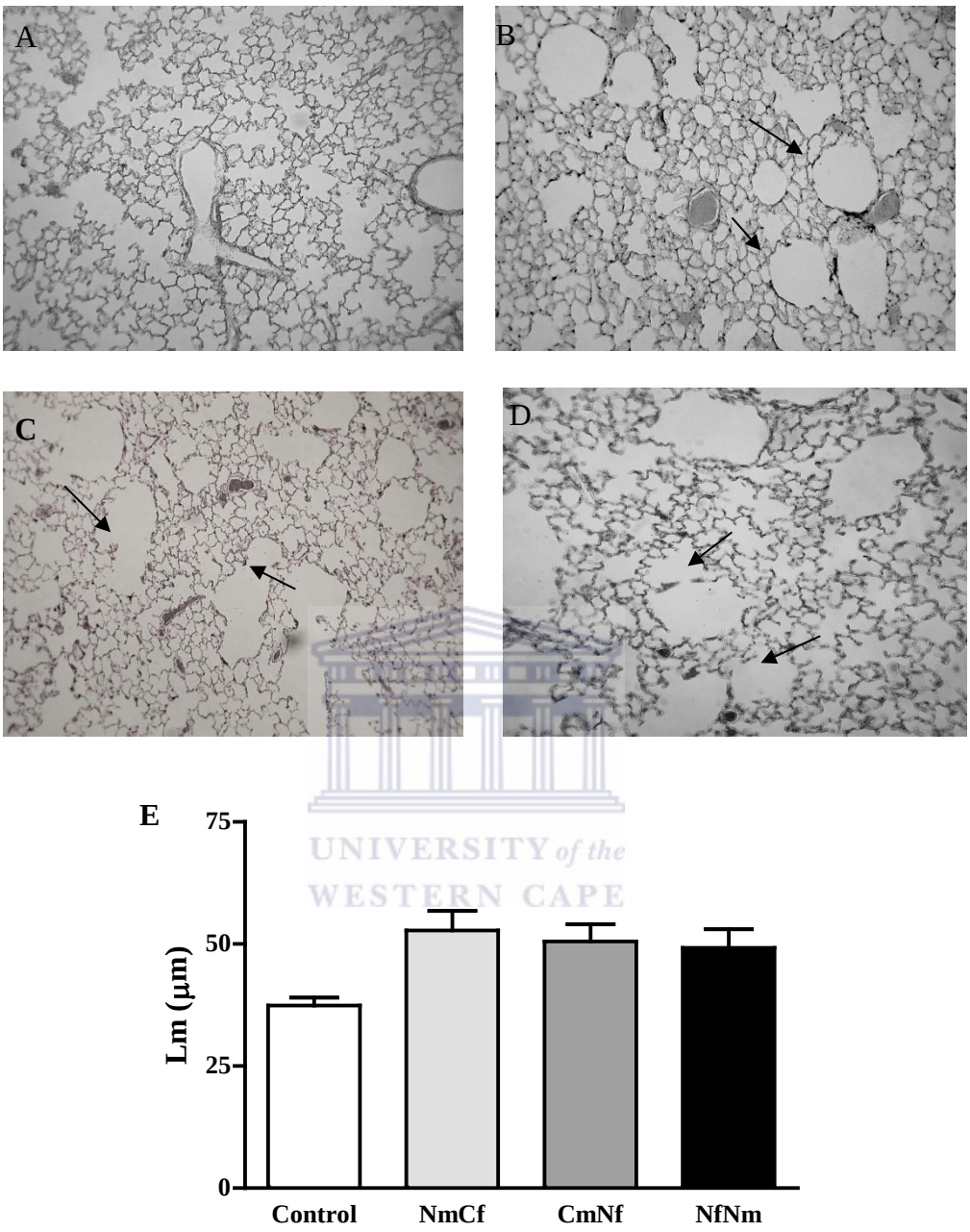


Figure 8.2.1A-E: Influence of grand maternal nicotine exposure during gestation and lactation on LM of the lungs of the F2 generation. (NmCF = Control female and nicotine exposed male; CmNf = Control male and nicotine exposed female. NmNf = both males and females exposed to nicotine. Arrows show sites of emphysema-like lesions.

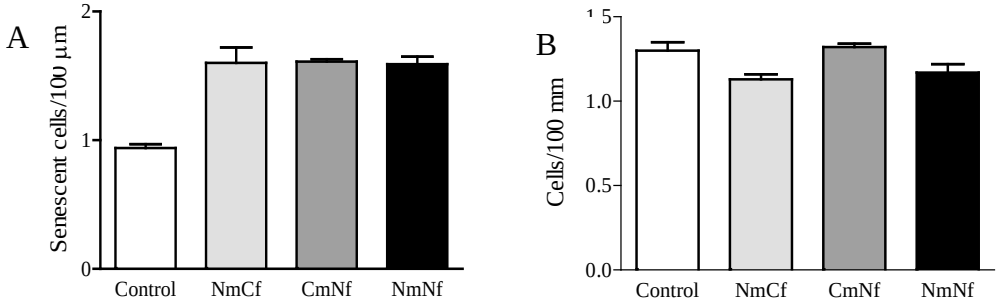


Figure 8.2.2: The effect of maternal nicotine exposure during gestation and lactation on A) cellular senescence and B) cell proliferation in the lungs of the F2 generation.

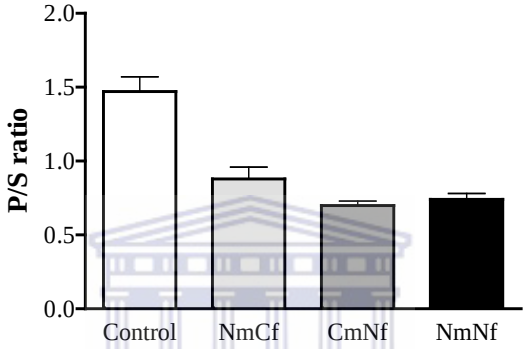


Figure 8.2.3: The effect of grand-maternal nicotine exposure during gestation and lactation on the P/S ratio of the lung parenchyma of the F2 offspring.

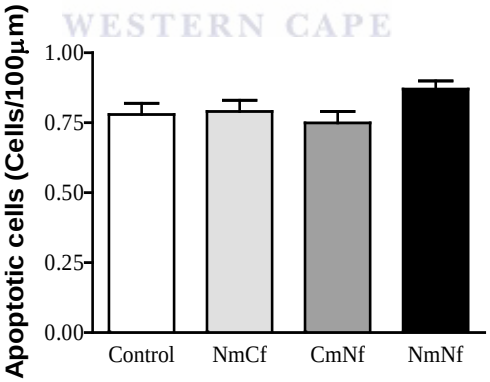


Figure 8.2.4: Effect of grand-maternal nicotine exposure during gestation and lactation on apoptosis in the lungs of the 84-day-old F2 progeny.

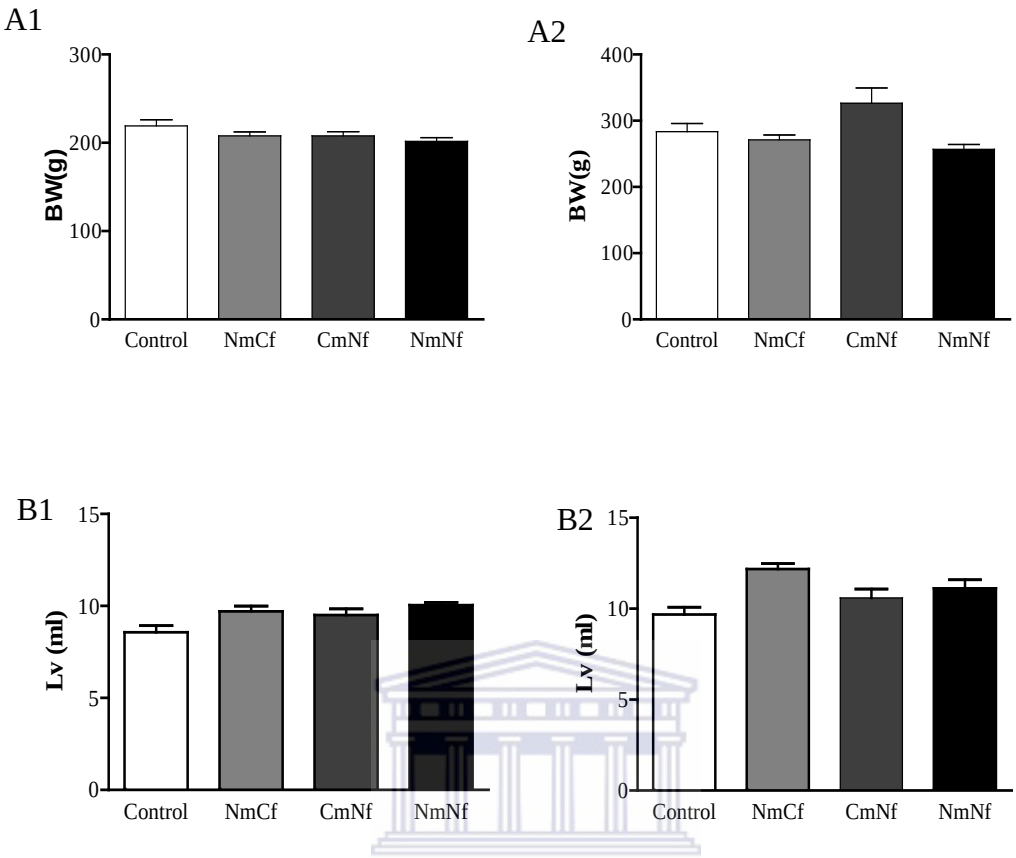


Figure 8.2.5: The effect of grand-maternal nicotine exposure during gestation and lactation on the body weight of A1) female and A2) male F2 progeny as well as the lung volumes of the B1) female and B2) male progeny.

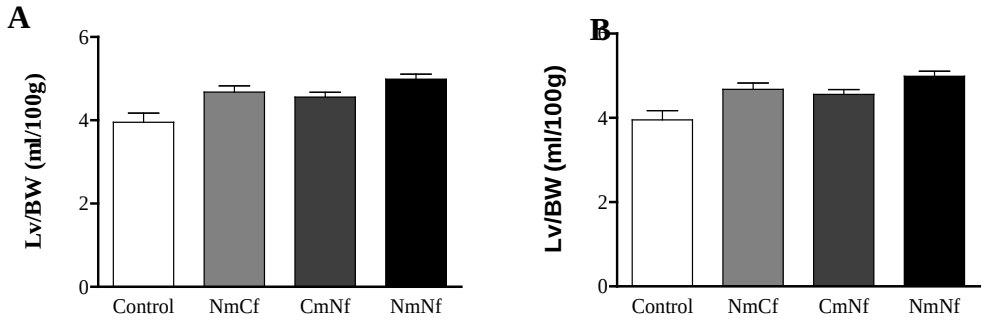


Figure 8.2.6: The effect of grand-maternal nicotine exposure on the Lv/BW ratio of the A) female and B) male F2 offspring

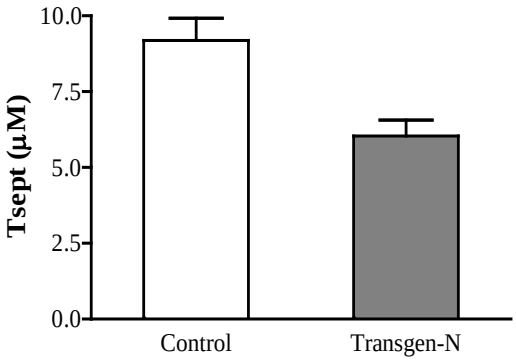


Figure 8.2.7: Effect of grand-maternal nicotine exposure during gestation and lactation on the alveolar wall thickness (Tsept) of the offspring.

The static lung compliance (Cst) of the control rats were the same as that of the CmNf male and female groups ($P>0.05$), but lower ($P<0.01$) than that of the other experimental groups. The Cst of the control males were less ($P<0.05$) than that of the females (Fig. 8.2.8)

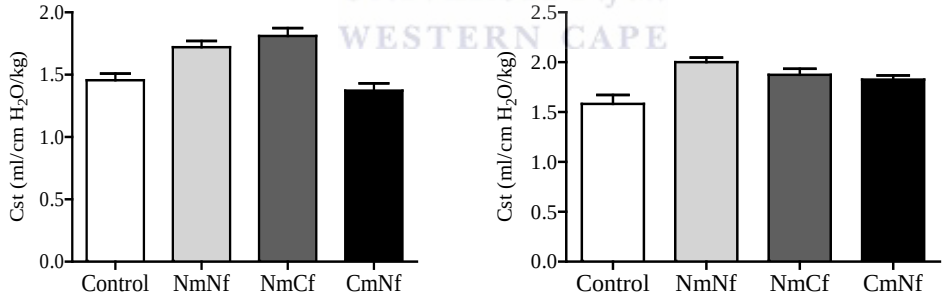


Figure 8.2.8: Effect of grand-maternal nicotine exposure during gestation and lactation on the static lung compliance (Cst) of the male and female offspring.

8.3 Discussion

Respiratory health begins in early pregnancy already. During the early phases of development the respiratory system of the foetus undergoes rapid cell division during which time it is highly susceptible to changes in the program that determines growth, development and aging (Barker, 2004). This is also the phase during which the foetus has little protection of its own against foreign substances which may enter via the mother's blood (Gebremichael et al., 1995). Nicotine, for example, freely crosses the placenta and accumulates in the developing respiratory system of the foetus (Matta et al., 2007a). Apart from being genotoxic (Aitken et al., 2009), nicotine also increases oxidative stress in the offspring (Bruin et al., 2008a). Nicotine (Aitken et al., 2009) and oxidants (Danielsen et al., 2011) are able to induce DNA damage such as strand breaks. This may result in base damage in the DNA and affect transcriptional fidelity, which may result in the production of mutant mRNA and protein in a process termed transcriptional mutagenesis. DNA repair is also impaired by nicotine (Sassen et al., 2005). Taken together, these effects of nicotine may have important implications for biologic systems such as the respiratory system. It has been established that DNA damage induced by reactive oxygen species is involved in aging and various human diseases (Marnett, 2000; Olinski et al., 2002). It is therefore plausible that nicotine and the oxidant load in smokers, as well as NRT to quit smoking, will induce DNA damage that may render the offspring more prone to premature aging and increased susceptibility to respiratory disease.

It has recently been shown that maternal (F0) nicotine exposure of rats during gestation and lactation result in the development of microscopic emphysema in the lungs of the offspring (F1 progeny) (Maritz et al., 2011a). This was first observed at postnatal day 42. Follow up studies in our laboratory showed that additional structural changes appeared in the lung parenchyma at postnatal day 84 (Maritz et al., 2011a). These changes occurred after nicotine withdrawal at postnatal day 21 of the F1 progeny. It was suggested that these changes in lung parenchymal structure after nicotine withdrawal may be due to a change in the program that controls lung growth, lung aging, and maintenance of the lung structure (Maritz et al., 2011a). The change in the program can be due to DNA strand breaks and impaired repair while the animals were exposed to nicotine via the placenta and mother's milk. It is also possible that it was due to an epigenetic mechanism because nicotine increases DNA methylation and acetylation in the foetus (Aagaard-Tillery et al., 2008; Soma et al., 2006). It is conceivable that this may have changed the program that controls lung aging and maintenance of the offspring and subsequent generations.

In the present study we demonstrated that grand-maternal nicotine exposure during gestation and lactation, in concentrations that resembles that of smokers (Zhang et al., 2011), not only resulted in the development of emphysematous lesions in the F1 generation, but also in the F2 progeny. Pulmonary emphysematous lesions appear to be a dynamic process that involves gradual loss of alveolar walls and a consequent increase in alveolar diameter. This not only involves remodelling of the extracellular

matrix, but also changes in apoptosis, cell proliferation, and cellular senescence. Apoptosis increases in emphysema and is accompanied by an increase in cell proliferation to compensate for the loss of cells from the alveolar wall (Aoshiba and Nagai, 2009). The data in this study shows that, although emphysema-like lesions occur in the lungs of the F2 animals, apoptosis of the F2 progeny was not affected by grand maternal nicotine exposure. The emphysema-like lesions in the lungs of the F2 progeny can thus not be attributed to an increase in apoptosis. Instead, the findings of this study suggest that the emphysema-like changes are accompanied by an imbalance between cell proliferation and premature cell senescence, where the rate of proliferation is evidently too slow to replace senescent cells adequately to prevent gradual breakdown of the alveolar walls. These findings cannot be attributed to the direct effect of nicotine or a nicotine-induced oxidant/antioxidant imbalance since the F2 male and female progeny were not exposed to nicotine. The mechanisms whereby nicotine induces these changes in the F1 generation can be ascribed to the genotoxic effects of nicotine (Argentin and Cicchetti, 2004) as well as the nicotine-induced oxidant/antioxidant imbalance (Danielsen et al., 2011) during the early phases of lung development while the F1 animals were still exposed to nicotine via the placenta and mother's milk. This conceivably changed the program that is involved in the development of the homeostatic mechanisms that controls lung growth, aging and maintenance. This change in the program that resulted in premature aging of the cells in the alveolar wall, and the associated reduced life-span of the cells, together with the simultaneous slower proliferation of the cells of the alveolar walls, is evidently

transferred to the F2 generation. This imbalance between cell proliferation and cell senescence, as reflected in the reduced P/S ratio of those rats that were descendants of the nicotine exposed F1 rats, can be attributed to the shorter life-span of an increased number of senescent cells in the alveolar walls. Consequently fewer viable cells are able to proliferate to replace damaged cells in the lung parenchyma. This means that the ability of the lung to repair itself is compromised because of the reduced of capacity to renew tissue. A longer term consequence of this is a gradual degradation of the lung parenchyma and the eventual development of emphysema-like lesions in the lungs. This is an important observation which shows that the use of NRT by the pregnant and lactating mother may result in the development of COPD in the offspring, not only in the F1 generation (Maritz et al., 2011a) but also in the F2 generation. This suggests that grand maternal smoking or NRT during pregnancy and lactation will give rise to generations that are more susceptible to respiratory diseases such as emphysema than those never exposed to nicotine in tobacco smoke or NRT.

It is important to note that grand-maternal nicotine exposure not only resulted in an increase in the lung volume of the 84-day-old F1 rats (Maritz et al., 2011a). It also resulted in the increase in the lung volume of the F2 rats that were born after mating of F1 nicotine exposed males with control or nicotine exposed F1 females. Contrary to this, the lung volume of the F2 rats born after mating of the nicotine exposed F1 females with control F1 males was not affected. Since the transpulmonary pressure used to determine the lung volumes was the same throughout the study, it follows that

the lungs of the rats born to F1 nicotine exposed males were more compliant than those born to nicotine exposed females. This was indeed supported by the higher compliance of the lungs of the F2 rats born to nicotine exposed F1 males than those born to nicotine exposed F1 females. The increase in compliance can be attributed to damage to the connective tissue framework of the lungs and thus the loss of support of the alveolar walls (Maritz and Dolley, 1996). The thinning of the alveolar walls can be attributed to the gradual break down of the connective tissue in the alveolar walls together with a slower replacement of the senescent cells. The premature aging and reduced cell proliferation in emphysematous lung reflects a persistent, intrinsic failure of cell replacement and maintenance of alveolar walls. Slower proliferation of lung fibroblast by translational mechanisms has indeed been shown in smokers (Vlahovic et al., 1999). It is therefore plausible that the development of the emphysema-like lesions in the lungs of the rats in the present study were due to a faster deterioration of the connective tissue framework and premature aging of the lungs of these rats.

The mechanism whereby grand maternal nicotine exposure during gestation and lactation induces premature aging, slower cell proliferation and impaired maintenance of the alveolar walls in the F2 generation is not clear. It has been shown that environmental toxins, such as nicotine, initiate DNA strand breaks in spermatozoa which eventually may lead to mutation in the embryo (Aitken et al., 2009). It is therefore possible that exposure of the foetus and neonate to nicotine may result in

defective spermatogenesis leading to impaired chromatin remodelling. This condition may increase the vulnerability of the chromatin such that the DNA becomes more susceptible to stress, particularly oxidative stress. Nicotine, due to its oxidative properties, and ability to induce oxidant formation (Bruin et al., 2008a), can therefore change the chromatin integrity and DNA damage in the male germ line (Aitken et al., 2009). Nicotine also alters oocyte structure and meiosis and adversely affects subsequent embryonic development (Rajikin et al., 2009). These changes may be transferred to the next generations and thereby result in premature senescence and increase the susceptibility of the respiratory system to disease.

It is important to note that, except for cell proliferation and lung volumes, all other changes in the lungs of the F2 descendants whose grandmothers (F0) were exposed to nicotine during gestation and lactation, was inherited from both F1 male and female progeny. However, mating of the F1 nicotine exposed females with control males had no effect on cell proliferation, or lung volume, or static compliance of the F2 offspring.

8.4 Conclusion

Grand-maternal (F0) nicotine exposure during pregnancy and lactation programmed the lungs of the F2 generation to develop emphysematous lesions due to a slower proliferation rate together with an increase in numbers of senescent cells in the alveolar walls. These data strongly suggest that NRT, as an aid to assist females to

quit smoking, is not advisable because it does not only affect the F1 progeny, but is also inherited by the F2 generation. The data suggest that the transfer of premature aging of the lungs from the F1 generation to the F2 generation is via the male and female germ cell lines.

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